In the Name of God

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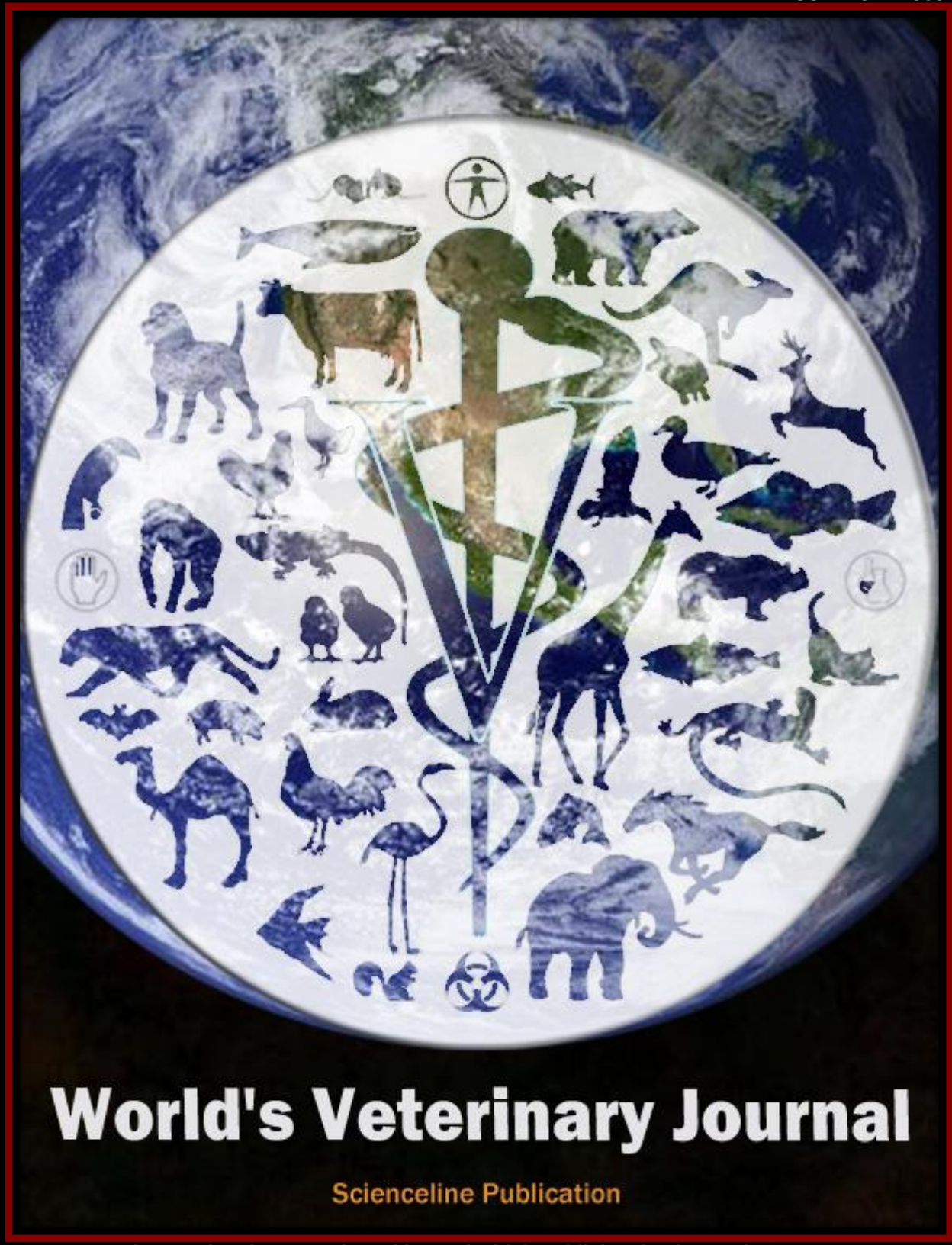

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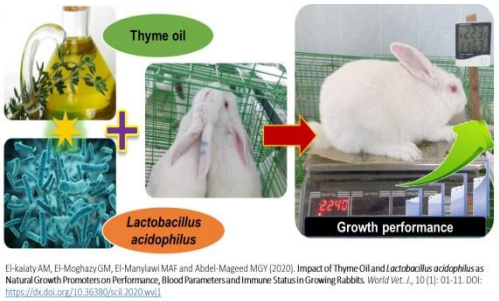
Research Paper

Impact of Thyme Oil and Lactobacillus acidophilus as Natural Growth Promoters on Performance, Blood Parameters and Immune Status in Growing Rabbits.



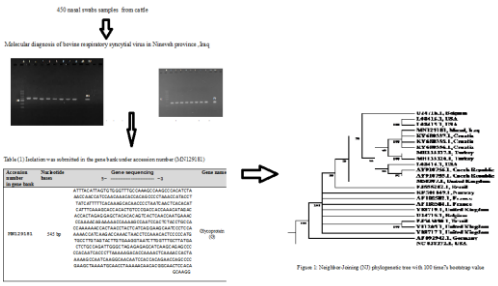
El-kaiaty AM, El-Moghazy GM, El-Manylawi MAF and Abdel-Mageed MGY.

World Vet. J. 10(1): 01-11, 2020;
pii:S232245682000001-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj1

ABSTRACT
Present study was conducted to evaluate the effect of thyme oil and *lactobacillus acidophilus* (supplement) as growth promoters in rabbit. 72 weaned V-Line male rabbits were randomly allocated into 4 equal groups. The first group (G1) was without any additives and consider as control group. The second group (G2) treated with the addition of *lactobacillus acidophilus* in drinking water in a concentration of 10^8 cfu/ml. The third group (G3) treated with the addition of thyme oil in drinking water in a concentration of 1 ml/ liter. The fourth group (G4) treated with the addition of both *lactobacillus acidophilus* and thyme oil in drinking water in a concentration of 10^8 cfu/ml plus 1ml/L, respectively. The obtained results showed that, all treatments had significant improvement effects on the measured parameters (performance characteristics, cecum characteristics, RBCs, WBCs, kidney function, trigly-cerides, total cholesterol, sheep RBC's titer, liver antioxidant markers and hormones markers) when compared to the control group. The live body weight of G3 and G4 groups were higher (2116 and 2058 g) than those found in G2 and G1 groups (1958 and 1850 g) respectively. In addition, the body weight gain of G3 and G4 groups were higher (1364 and 1307 g) than those found in G2 and G1 groups (1207 and 1100 g). Moreover, the daily weight gain of G3 and G4 groups were higher (32.49 and 31.13 g/d) than those found in G2 and G1 groups (28.74 and 26.19 g/d). In addition, feed conversion ratio of G3 and G4 groups were higher (3.41 and 3.61) than those found in G2 and G1 groups (3.66 and 4.67). While G4, G2 and G3 groups had a significant enrichment effect on the intestinal beneficial bacteria. In conclusion, in present experiment inclusion thyme oil and/or *lactobacillus acidophilus* in the drinking water that stimulated body weight gain and increased feed conversion rate, and can be used as growth promoters in rabbit nutrition successfully without notable side effects on growing rabbits. Furthermore, it showed a significant positive effect on the physiology for treatment groups G3, G4 and G2 respectively compared to the control group.
Key words: Immunity, *Lactobacillus acidophilus*, Performance, Probiotic, Rabbit, Thyme oil
[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

Molecular and Phylogenic Analysis of Bovine Respiratory Syncytial Virus in Nineveh province, Iraq.



Jassiem Hussain Kh, AL-Farwachi MI and Dhahir Hassan S.

World Vet. J. 10(1): 12-17, 2020;
pii:S232245682000002-10;
DOI: https://dx.doi.org/10.36380/scil.2020.wvj2

ABSTRACT
Bovine Respiratory Syncytial Virus (BRSV) is one of the worldwide distributed infectious agents responsible for diversified clinical disease in cattle populations which causes considerable economic loss due to its negative effects on health and production. In this study, 450 nasal swab samples were collected from cows with different ages and breeds in different areas across Nineveh province, Iraq. Molecular diagnosis using nested RT-PCR and phylogenetic analysis of the G gene were performed. The results indicated a 37.31% prevalence rate of BRSV using specific primers in the PCR technique. The local isolate was submitted in GenBank under the accession number MN129181 Mosul isolate. The phylogenetic tree of local isolates of BRSV was made using the neighbor-joining system after comparison with other GenBank data. In conclusion, phylogenetic analysis of BRSV can provide information about the viral strains present in cattle and subsequently may be useful for infection control programs.
Key words: Bovine respiratory syncytial virus, Cattle, PCR, Phylogenic analysis.

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Research Paper

Evaluation of Growth Performance, Blood Metabolites and Gene Expression Analysis in Egyptian Sheep Breeds, in Relation to Age.

Ashour G, Gad A, Fayed AK, Ashmawy NA and El- Sayed A.

Ashour G, Gad A, Fayed AK, Ashmawy NA and El- Sayed A (2020). **Evaluation of Growth Performance, Blood Metabolites and Gene Expression Analysis in Egyptian Sheep Breeds, in Relation to Age.** *World Vet. J.*, 10 (1):18-29. DOI: https://dx.doi.org/10.36380/scil.2020.wvj3

World Vet. J. 10(1): 18-29, 2020;
pii:S232245682000003-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj3

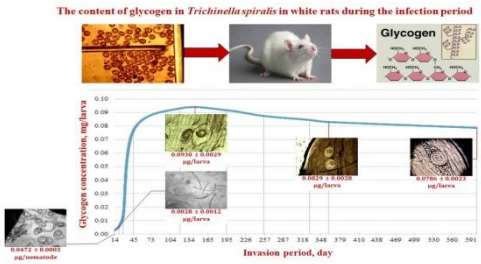
ABSTRACT
The growth performance of lambs attributes the economic viability of animals. Faster growth allows lambs to reach maturity in early age. Therefore, the aim of this study was to compare growth performance, blood metabolites and expression of IGF-1, GH, and Leptin genes in three different Egyptian sheep breeds across age. Thirty Egyptian sheep males from three breeds (Ossimi, Rahmani and Barki) were divided into three ages categorize (7 – 9, 10 – 12, and 13 - 16 months). The results showed that there was a significant increase in sheep's live body weights toward advanced ages till the second age category for all breeds, the highest values of linear body measurements were observed in Ossimi breed. There was a non-significant inverse effect of advanced age on blood glucose and total lipids levels in all sheep breeds. There wasn't any significant effect of interaction between age and breed on plasma total protein concentrations. According to age categories, Barki breed showed a significant up-regulation of GH compared to the Ossimi breed in 7-9 months age category. However, Barki breed showed a significant down-regulation of IGF-1compared to the Ossimi breed in 7-9 months. Meanwhile, Leptin expression showed significant differences in Ossimi breed between 10-12 months age category and two other age categories. We concluded that measuring of physical body measurements, blood metabolites and GH, IGF-1 and Leptin genes in early ages is a good and accurate indicator for growth performance in Egyptian sheep breeds.
Key words: Blood metabolites, Egyptian breeds, Gene expression, Growth performance, Linear body measurements

[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

The Role of Glycogen in Biological Cycle of Trichinella spiralis.

Sidor EA and Andreyanov ON.



Sidor EA and Andreyanov ON (2020). **The Role of Glycogen in Biological Cycle of *Trichinella spiralis*.** ***World Vet. J.*, 10 (1): 30-34.** DOI: https://dx.doi.org/10.36380/scil.2020.wvj4

World Vet. J. 10(1): 30-34, 2020;
pii:S232245682000004-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj4

ABSTRACT
The energy sources of *Trichinella spiralis* change in both the muscular and intestinal stages of its life in the host organism. The purpose of this study was to investigate the quantitative changes in glycogen concentration during the life cycle of *T. spiralis* in a host organism. *Trichinella spiralis* was passaged on laboratory rodents under the vivarium conditions. Sixty-nine white rats (350 g each) were infected with *T. spiralis* at a dose of 5 muscle larvae/gram of body weight. The animals were euthanized at different time periods from the start of the experiment. *Trichinella* muscle larvae were isolated by artificial fermenting meat mince in gastric juice. To determine the viability of *Trichinella* larvae, they were heated to 38 ± 2 ° C for 10 minutes their motor activity was investigated. (38 ± 2 °C). To determine the invasive properties of *T. spiralis* at different stages of its development in rats, the muscular larvae isolated from the rat muscles were used to infect laboratory mice. The invasive capacity of *T. spiralis* was assessed on day 45 post-infection. For the study of intestinal *Trichinella* larvae, laboratory rats were not fed a day before infection. Adult nematodes were isolated from the small intestine of laboratory rats at 3, 6 and 24 hours post-infection. The nematodes were counted in the Migacheva-Kotelnikov chamber in each individual sample. The concentration of glycogen in the nematodes was calculated according to the quantitative method for determining glycogen in *Trichinella* larvae. Low glycogen levels in the muscle larvae were observed on day 14 post-infection. The glycogen concentration in muscular larva was 0.0054 ± 0.0027 µg/ larva on day 21, 0.0136 ± 0.0024 µg/ larva on day 28, and 0.0771 ± 0.0025 µg/ larva on day 45 after the rats were infected. Maximum concentration of glycogen was recorded 4 months post-infection (0.0930 ± 0.0029 µg/larva). Further, the glycogen level began to decrease slowly. In the 20th month post-infection, after infection, the amount of glycogen in a *Trichinella* larva was 0.0786 ± 0.0023 µg. In the body of intestinal nematodes, 3 hours after infecting the animals, the glycogen concentration was reduced to 0.0472 ± 0.0003 µg in one nematode. The same time period later, it reached to value of 0.0272 ± 0.0002 µg. In intestinal *T. spiralis*, which remained in the small intestine of rats for 24 hours, the glycogen was not detected. The amount of glycogen at the muscle stage of *T. spiralis* development was extremely important in the first hours of the helminth's residing in the host's intestines. Energy requirements during the period when the helminth cannot obtain enough food depend on the glycogen content. When the glycogen concentration in the parasite is insufficient, the *Trichinella* larvae will lose their invasion capacity.
Key words: Bioassay test; Glycogen; Nematode; Parasitic helminth, *Trichinella spiralis*

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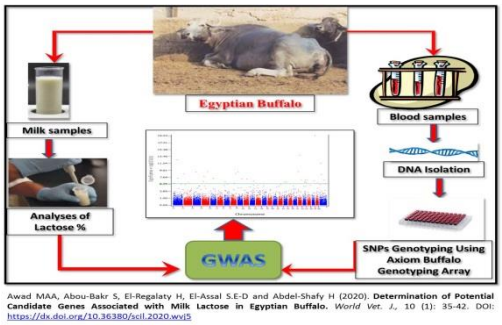
Research Paper

Determination of Potential Candidate Genes Associated with Milk Lactose in Egyptian Buffalo.

Awad MAA, Abou-Bakr S, El-Regalaty H, El-Assal S.E-D and Abdel-Shafy H.

World Vet. J. 10(1): 35-42, 2020;
pii:S232245682000005-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj5



ABSTRACT
The aim of the present genome-wide association study (GWAS) was to identify single nucleotide polymorphisms (SNPs) and candidate genes associated with lactose percentage (LP) and lactose yield (LY) in Egyptian buffalo. The phenotypic dataset included 60,318 monthly measures for LP and LY from 1481 animals. A total number of 114 animals with high and low deviated performance were selected for genotyping with Axiom Buffalo Genotyping 90K Array. Genome-wide analysis was performed using a single marker regression. The GWAS revealed 32 significant and seven suggestive SNPs for LP, however; only two suggestive SNPs were identified for LY. The identified genomic regions are overlapped with previously reported QTL in different cattle breeds. In addition, novel genomic loci were detected. The identified genomic regions harbored many candidate genes with biological roles associated with milk production traits, such as TPD52 and ZBTB10 on chromosome 15; AADAT and GALNTL6 on chromosome 3 and COL8A1 and PLOD2 on chromosome 1. Our findings provide the basis to uncover the key markers and candidate genes affecting lactose traits which facilitate the exploration of the genetic mechanisms that control lactose traits variation in Egyptian buffalo.
Key words: Candidate gene, Egyptian buffalo, Genome, Genomic loci, Lactose

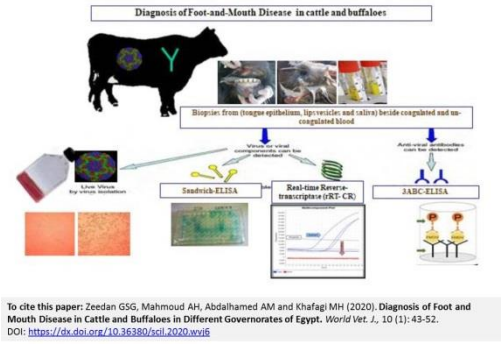
[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

Diagnosis of Foot and Mouth Disease in Cattle and Buffaloes in Different Governorates of Egypt.

Zeedan GSG, Mahmoud AH, Abdalhamed AM and Khafagi MH.

World Vet. J. 10(1): 43-52, 2020;
pii:S232245682000006-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj6



ABSTRACT
Foot and Mouth Disease (FMD) is highly contagious disease affected cloven-hoofed animals which result in substantial economic losses. The present study was aimed to detect FMDV by different serological and molecular methods in cattle and buffaloes for providing an accurate and rapid diagnosis of FMD disease. 86 samples of tongue epithelium biopsies, fluid vesicles samples and saliva, as well as 86 coagulated and uncoagulated blood samples, were collected from 64 and 22 suspected cattle and buffaloes respectively in different governorates in Egypt, during August to December 2017. Serum samples were examined by 3ABC-ELISA for differentiating between infected and non-infected animals. While tissues biopsies and un-coagulated blood samples were examined by Sandwich ELISA, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) as well as Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR). FMDV porotypes were identified by rRT-PCR in suspected cattle and buffaloes samples to FMDV serotype A, O and SAT2 and results showed that 54 samples positive for FMDV different serotypes while FMDV serotype differentiation in tissues biopsy of cattle were 18 (28.12%), 12 (18.75%), 3 (4.68 %) and 4 (6.25%). Also, the positive results of tissue samples from buffaloes examined by RT-PCR were 9 (40.09 %), 4 (6.25%), 2 (9.09 %) and 2 (9.09 %) for O, SAT2, serotype A and mixed serotypes respectively by different tests. The rRT-PCR provided an accurate and rapid laboratory diagnosis of FMDV as well as RT-PCR, and 3ABC- ELISA were given nearly the same results. Although the rRT-PCR generated results in less than 6 h and this is an important feature when definitive diagnostic results required in a short timescale during emergencies. Also, this study demonstrated the current situation of circulation FMDV type A, O, and SAT2 serotypes in cattle and buffaloes in Egypt.
Key words: 3ABC-ELISA, Buffaloes, Cattle, Foot and mouth disease, Real-time reverse transcriptase polymerase chain reaction

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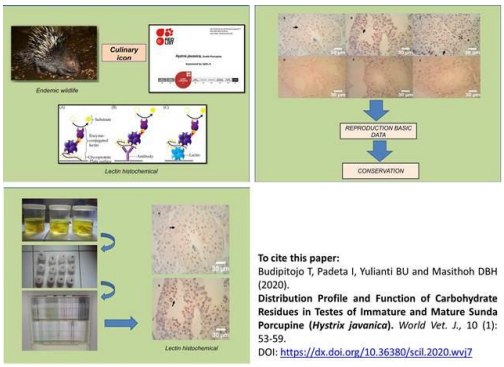
Research Paper

Distribution Profile and Function of Carbohydrate Residues in Testes of Immature and Mature Sunda Porcupine (*Hystrix javanica*).

Budipitojo T, Padeta I, Yulianti BU and Masithoh DBH.

World Vet. J. 10(1): 53-59, 2020;
pii:S232245682000007-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj7



To cite this paper:
Budipitojo T, Padeta I, Yulianti BU and Masithoh DBH (2020).
Distribution Profile and Function of Carbohydrate Residues in Testes of Immature and Mature Sunda Porcupine (*Hystrix javanica*). *World Vet. J.*, 10 (1): 53-59.
DOI: https://dx.doi.org/10.36380/scil.2020.wvj7

ABSTRACT
The population of Sunda porcupine (Hystrix javanica) declines each year since it is rarely found in nature. The present study aimed to obtain information about the distribution of carbohydrate residues contained in immature and mature of Sunda porcupine's testes and to discuss its relevant functions. This study used six testes obtained from four immature and two mature Sunda porcupine originated from Ngawi Regency, East Java Province, Indonesia. Testis tissues were stained with hematoxylin and eosin and lectin histochemistry of Lens culinaris agglutinin (LCA), Phaseolus vulgaris leucoagglutinin (PHA-L), Pisum sativum agglutinin (PSA), Sophora japonica agglutinin (SJA), and Wheat germ agglutinin (WGA). Data were analyzed with descriptive and semi-quantitative method. Lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues in the immature and mature testes with weak to very strong intensity. In the immature testes of Sunda porcupine, there was positive reactivity with PHA-L for Leydig and Sertoli cells, N-acetylgalactosamine may play an important role in the development and maturation of Leydig and Sertoli cells. Mature testes showed a strong positive reaction to the LCA, SJA, PSA, and WGA which indicated the significant roles of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues on the maturation process of early spermatid to the late spermatid. These results can be used as basic data to be implemented in the conservation efforts of Sunda porcupine.
Key words: Carbohydrate residue, Lectin, Spermatogenesis, Sunda porcupine, Testes

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Research Paper

Immunomodulatory Effect of CpG ODN-Adjuvanted Bacterin Against *Salmonella enterica* serovar Enteritidis in Broiler Chickens.

Abed M, Elhariri M, El-Helw R, Khattab MS, Setta A and Soliman R.

World Vet. J. 10(1): 60-66, 2020;
pii:S232245682000008-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj8

To cite this paper: Abed M, Elhariri M, El-Helw R, Khattab MS, Setta A and Soliman R (2020). **Immunomodulatory Effect of CpG ODN-Adjuvanted Bacterin Against *Salmonella enterica* serovar Enteritidis in Broiler Chickens.** *World Vet. J.*, 10 (1): 60-66. DOI: https://dx.doi.org/10.36380/scil.2020.wvj8

ABSTRACT
Bacterial oligodeoxynucleotide containing Cytosine Guanine motifs (CpG-ODN) has been reported to induce immunostimulatory activity against a variety of bacterial, viral, and protozoan infections in a wide range of vertebrate species. The objective of this study was to investigate the dose-dependent immunomodulatory effect of CpG ODN on *Salmonella* Enteritidis bacterin in broiler chickens. Two hundreds one-day-old broiler chicks, divided into 5 groups, were used in this study. First three groups were immunized with *Salmonella* Enteritidis bacterin adjuvanted with different doses of CpG ODN (50µg, 100µg and 200µg). The control groups included a group that was immunized with *Salmonella* Enteritidis bacterin adjuvanted with aluminum hydroxide and a non-immunized group. The intestinal colonization, cellular responses, mucosal and systemic immune responses of immunized chickens was measured at different intervals, until 42 days of age. At two weeks post-immunization, 20 chicks from each group were orally challenged by *Salmonella* Enteritidis fresh bacterial culture ($1.2x10^8$ CFU/ml). The survival rates and the pathological changes of challenged chickens in the different groups were monitored for extra 10 days. Compared to the aluminum hydroxide adjuvanted bacterin, the CpG-ODN adjuvant bacterin induced significant protection and improved survival rate of challenged chickens. Also *Salmonella* Enteritidis was not recovered from the intestinal tract of vaccinated challenged groups. There was a significant dose-dependent immunostimulatory adjuvant effect of CPG-ODN on the level of secretory IgA and the induced mucosal responses. The 200-CpG ODN group showed the highest IgA response followed by 100-CpG ODN group then the 50-CpG ODN and the aluminum hydroxide groups ($P < 0.05$). Also, cellular interactions were remarkably reduced in the liver and intestine of CpG ODN-treated chickens. No inflammatory cellular infiltrations were seen in the liver and intestine of 200-CpG ODN treated group. In conclusion, the presented findings have shown the significant immunostimulatory effect of CpG-ODN and its effect on *Salmonella* Enteritidis bacterin in controlling *Salmonella* infection in broiler chickens.
Key words: Cellular responses, CpG ODN, Mucosal immunity, *Salmonella* Enteritidis

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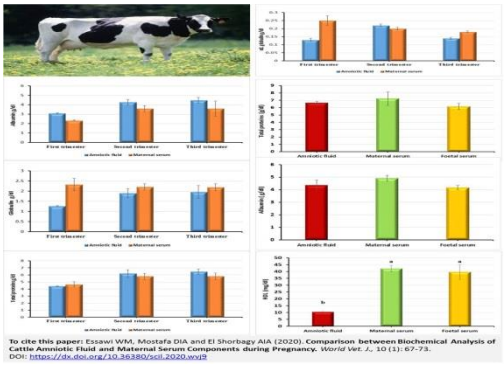
Research Paper



Comparison between Biochemical Analysis of Cattle Amniotic Fluid and Maternal Serum Components during Pregnancy.

Essawi WM, Mostafa DIA and El Shorbagy AIA.

World Vet. J. 10(1): 67-73, 2020;
pii:S232245682000009-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj9

ABSTRACT
The present study aimed to compare the biochemical components including Total Protein (TP), albumin, globulins, cholesterol, triglycerides, High and Low-Density Lipoproteins (HDL and LDL), creatinine, urea, sodium (Na), potassium (K), chloride (Cl), calcium (Ca) and inorganic phosphorus (P), of Amniotic Fluid (AF) with those of Maternal Serum (MS) during the first, second and third trimesters of pregnancy in cattle and Fetal Serum (FS) at birth. At birth AF, MS and FS were collected. Maternal blood samples and gravid uteri were collected after accidental slaughter. The actual data recorded during three trimesters according to the curved crown-anus length of the fetus. The MS concentrations of globulins, cholesterol, triglycerides, lipoproteins, creatinine, Na, K, Cl, Ca and inorganic-P were significantly higher than the AF during the first trimester. At delivery, the concentrations of cholesterol, triglycerides, and creatinine in the AF were lower than those in the MS or FS. The concentrations of Ca and inorganic-P in the FS were higher than those in the MS or AF. The levels of TP, creatinine, urea in the AF and urea in the MS increased as the gestation stages advanced. The levels of Na and Ca in the AF decreased as the gestation stage advanced while the K concentration increased. In conclusion, our results indicated an active placental transport for Ca and P. The TP, albumin, globulins, cholesterol, triglycerides, HDL and LDL, creatinine, urea, Na, K, Cl, Ca and P in AF and MS during the first, second and third trimesters of pregnancy in cattle might be changed with progressing the gestation.

Key words: Amniotic fluid, Cattle, Fetal serum, Gestation, Maternal blood

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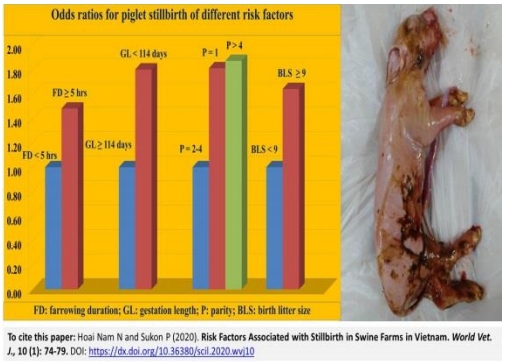
Research Paper



Risk Factors Associated with Stillbirth in Swine Farms in Vietnam.

Hoai Nam N and Sukon P.

World Vet. J. 10(1): 74-79, 2020;
pii:S232245682000010-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj10

ABSTRACT
Stillbirth in pig has been studied worldwide, but, its situation in Vietnam has never been reported. Therefore, present study aimed to investigate effects of herd, parity, gestation length, birth litter size and farrowing duration on stillbirth at sow level in swine farms in Vietnam. Data was collected from 1174 litters of 1174 Landrace x Yorkshire crossbred sows in 16 farms in the North of Vietnam. Potential risk factors for stillbirth were identified by using logistic regression. The incidence of stillbirth at sow level was 47.9%, and the stillbirth rate was 5.2%. Multivariate logistic regression showed that parity 1 (OR=1.81, 95%CI=1.24-2.63) and >4 (OR=1.87, 95% CI=1.33-2.64), a gestation length <114 days (OR=1.80, 95%CI=1.23-2.65), a birth litter size ≥9 piglets (OR=1.64, 95%CI=1.04-2.61) and a farrowing duration ≥5 hours (OR=1.48, 95%CI=1.05-2.09) were risk factors for stillbirth. This study indicated that stillbirth was common in swine farms in Vietnam. Special attention should be paid to sows at parity 1, > 4, sows with a short gestation, sows with a large birth litter size and sows with a long farrowing duration to reduce stillbirth. Since the use of highly prolific sows is increasing, stillbirth continues to be an issue to be dealt with in swine farms in Vietnam.

Key words: Farrowing; Gestation length; Litter size; Parity; Sow; Stillbirth

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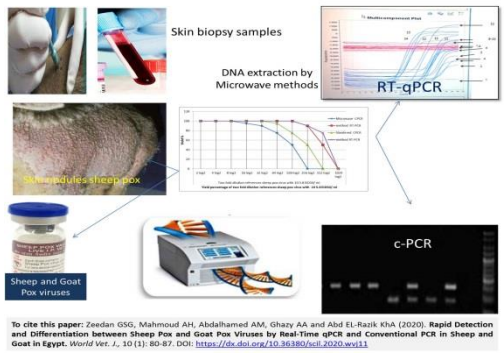
Research Paper



Rapid Detection and Differentiation between Sheep Pox and Goat Pox Viruses by Real-Time qPCR and Conventional PCR in Sheep and Goat in Egypt.

Zeedan GSG, Mahmoud AH, Abdalhamed AM, Ghazy AA and Abd EL-Razik KhA.

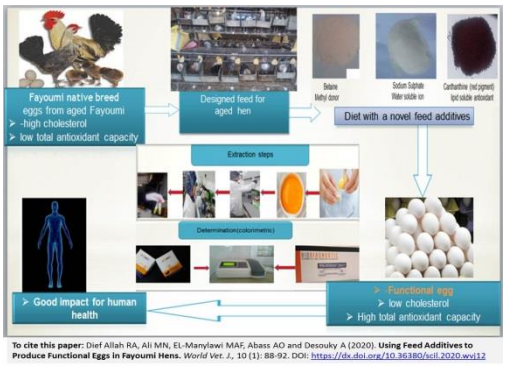
World Vet. J. 10(1): 80-87, 2020;
pii:S232245682000011-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj11

ABSTRACT
Capri Pox Virus (Ca PV) is the causative agent of important diseases in sheep and goat with severe socio-economic impact. Sheep Poxvirus (SPPV), Goat Poxvirus (GTPV) and Lumpy Skin Disease Virus (LSDV) are three members of the Capripox virus genus of Poxviridae family, which infect sheep, goats, and cattle, respectively. A rapid diagnostic assay for Ca PV by using conventional PCR RNA polymerase gene RP030 and real-time qPCR would be useful for disease surveillance, detection and differentiation of Ca PV in clinical and subclinical samples for management and treatments of outbreaks. The present study aimed to detect and identify Ca PV (SPPV and GTPV) in natural, infected scabs biopsy samples, which were collected from sheep and goats in different governorates in 2017 during outbreaks in Egypt using the conventional PCR RNA polymerase gene RP030 gene based and Real-Time qPCR fluorescent based. We collected eighty scabs from clinically affected animals (54 sheep and 26 goat) that were vaccinated in Chorio-Allantoic-Membranes (CAM) from 10-days-old embryonated-chicken eggs. The positive CAM showed pock lesions, which were observed with a thickening of the membrane after 2-3 passages post samples inoculation, and harvested positive CAMs, which were determined by Agar Gel Precipitation Test (AGPT) , Counter Immune Electrophoresis (CIE), and conventional PCR and real time qPCR were examined for the presences of Ca PVs. DNA extraction from clinical samples and positive CAM with pox lesions using DNA slandered references extraction kits compared to novel modification method (Microwave extraction). The PCR based RPO30 gene and the real-time qPCR showed 15 positive with percentage 27.77% in 54 sheep and 3 positive with percentage 12.5% in 26 goats. Although, AGPT and CIE gave lower result than molecular methods, they gave 11 and 13 positive samples from 54 sheep and in goats were 1 and 2 from 26 scab biopsy samples respectively, however they are useful for early confirmation of positive Ca PVs in low-income countries. PCR based RNA polymerase gene RP030 gene and real-time-PCR considered sensitive, rapid, and reliable methods for differentiating SPPV and GTPV from AGPT and CIE in CAM or in clinical samples without further isolation and propagation in embryonated-chicken eggs. The novel microwave method used to isolate high quality of DNA extracted from infected skin biopsy with SPPV and GPPV with no further purification steps required. It was done in 3 minutes only. The results of the current study confirmed that the suitability of the PCR-based RNA polymerase gene RP030 gene is suitable for differentiating between SPPV and GTPV; in one PCR run; without any post-processing steps.
Key words: Capripox virus, DNA extraction, Goat pox, KOH extraction method, Real-Time qPCR, RPO30, Sheep pox

[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

Using Feed Additives to Produce Functional Eggs in Fayoumi Hens.



Dief Allah RA, Ali MN, EL-Manylawi MAF, Abass AO and Desouky A.

World Vet. J. 10(1): 88-92, 2020; pii:S232245682000012-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj12

ABSTRACT
Lately human have become more apprehensive for the health and their food relationship. Egg considered cheap source of animal protein. Eggs are rich in various essential nutrients that contribute to the quality of human diet. But its cholesterol can contributes with some human serious disease. The current study examines the hypothesis that assumed addition of antioxidant such as CAX, SS, B or their mixtures to the diet can produce functional egg from Fayoumi hens at late phase of egg production. A number of 168 Fayoumi hens (46weeks of age) were randomly assigned into 8 dietary groups as follows: Basal diet alone or with CAX (6 ppm), SS (0.5 g/kg), B (1 g/kg), CAX+SS, CAX+B, SS+B, and CAX+SS+B separately. Forty eight eggs (6 per each group) were analyzed for estimating cholesterol and total antioxidant capacity. Egg of hens fed a combination of CAX+SS+B which had the best total antioxidant capacity value, while the CAX group recorded the best lowest cholesterol value compared to other groups ($P < 0.05$). It could be concluded that basal diet supplemented with CAX, SS, B alone or with mixture of them may have lowering effect on yolk total cholesterol. This could lead to produce functional eggs which have positive effects on human health and favorable for those suffering from heart syndromes.
Key words: Cholesterol, Fayoumi, Functional Egg, Total Antioxidant Capacity
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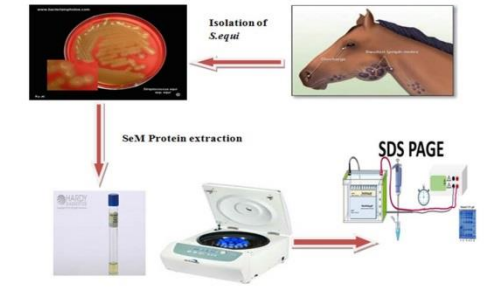
Research Paper

SDS-PAGE Profile Analysis of SeM-like Protein of *Streptococcus equi* subspecies *equi*.



Abdelmageed ShMEl, El-Shafii SEIA and El Jakee JKAH.
World Vet. J. 10(1): 93-97, 2020;
pii:S232245682000013-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj13

ABSTRACT
S. equi subspecies *equi*, causing strangles in equine, is characterized by comprising a major virulence factor called M like protein or SeM protein.

This study aimed to extract SeM protein from local *S. equi* strain in Egypt and to detect its antigenic components. After centrifugation, the native 58 kilo Dalton (kDa) SeM protein was detected both in the supernatant and sediment of the prepared extract. With modification by more centrifugation, the formed supernatants were separated and fractionated using SDS-PAGE with silver nitrate staining, which led to the appearance of a band at Molecular Weight (MW) 70.9 kDa. in SeM1, the presence of 7 bands at MW of 105, 87.8, 70.9, 61.1, 44, 37.9 and 18.4 in SeM2; 5 bands at MW 70.9, 58.9, 37.2, 29.8 and 18.3kDa in SeM3 and 4 bands at MW of 72.0, 58.6, 29.8 and 18.0 kDa in SeM4. This study suggested that a further modification of SeM extraction revealed the presence of heterogeneous complex fragments of SeM.
Key words: SeM protein, SDS-PAGE, Strangles, *Streptococcus equi* subspecies *equi*

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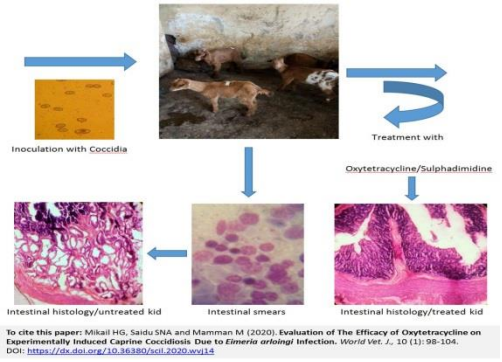
Research Paper

Evaluation of The Efficacy of Oxytetracycline on Experimentally Induced Caprine Coccidiosis Due to *Eimeria arloingi* Infection.



Mikail HG, Saidu SNA and Mamman M.

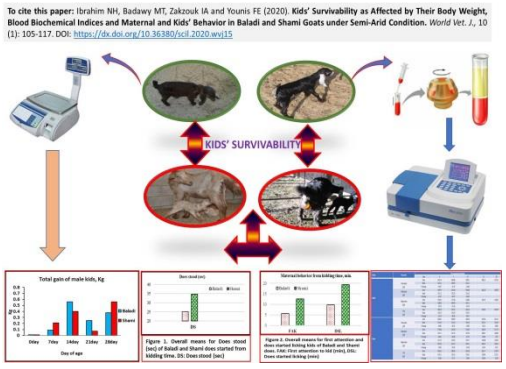
World Vet. J. 10(1): 98-104, 2020;
pii:S232245682000014-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj14

ABSTRACT
Coccidiosis is a protozoan disease caused by members of the genus *Eimeria* that affect domestic animal species. The current study was aimed at evaluating the effect of oxytetracycline administration on experimental caprine coccidiosis. Sixteen red Sokoto goat kids divided into four groups (A to D) of four goat kids each, were used for the study. Groups A, B and C were infected by oral inoculation with two ml containing 1.5 $\times 10^3$ sporulated oocysts of *Eimeria arlongi* per animal, while group D was the neutral control group. Group A was treated with 10 % oxytetracycline intramuscularly daily for five days. Group B was treated with Sulfadimidine 33.3% subcutaneously daily for five days and group C served as an infected untreated group. Fecal oocysts per gram count was conducted during the experiment. The present result showed a significant decrease ($P \leq 0.05$) in fecal oocysts load in the treated groups. Neither schizonts nor merozoites were detected in the intestinal smear of kid treated with oxytetracycline but were detected in the intestinal smear of infected untreated goat kid. Cystic degenerative changes were seen in the intestinal glandular cells of the infected untreated goat kid. Conclusively, the current finding suggests that oxytetracycline can effectively be used in treating caprine coccidiosis.
Key words: Coccidiosis, Caprine, *Eimeria arlongi*, Goat Kids, Oxytetracycline, Treatment

[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

Kids' Survivability as Affected by Their Body Weight, Blood Biochemical Indices and Maternal and Kids' Behavior in Baladi and Shami Goats under Semi-Arid Condition.



Ibrahim NH, Badawy MT, Zakzouk IA and Younis FE.

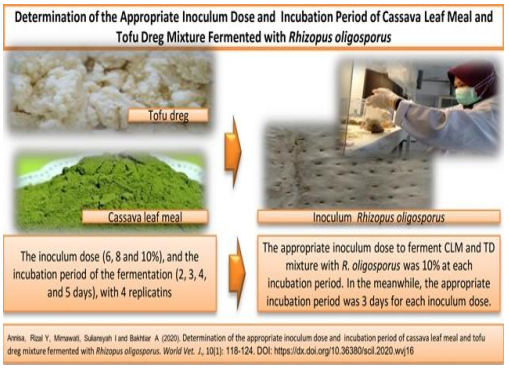
World Vet. J. 10(1): 105-117, 2020;
pii:S232245682000015-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj15

ABSTRACT
The present study was conducted to investigate the effect of body weight, blood biochemical parameters and post parturient behavioral activities of goats and their kids on kids' mortality rate in Baladi and Shami breeds during neonatal period. Twenty-five adults does of each breed (average age: 18 months old) were selected during breeding season. All female goats were estrus synchronized and naturally mated. After parturition, one hundred and one kids (39 Baladi and 62 Shami) were followed for up to 30 days of their age. The overall mean birth weights of female kids of Baladi and Shami goats were 2.47 and 2.81 Kg, respectively. For male kids, birth weights were 2.43 and 2.47 kg, respectively. There was no significant difference in average daily gain (g/day) between Baladi and Shami kids during the first 30 days of age. Male kids recorded higher mortality rate than female kids. The percent of death for male and female kids were 87.50% and 36.84% in Shami while, were 33.33% and 14.28% in Baladi respectively. However, death stopped in Baladi kids after 14 days, but continued in Shami kids to 28 days post-partum. Present data revealed that goat breed and neonatal period showed a significant effect on urea concentration and alkaline phosphatase (ALP) and insignificant effect on creatinine concentration, alanine transferase (ALT), gamma glutamyl transferase (GGT), total lipids, cholesterol and triglycerides in Baladi and Shami Kids. While aspartate transferase (AST), were not affected by goat breed and was significantly affected by both age of birth and interactions. Baladi breed showed significantly better maternal activity than Shami does as they spend lesser time to concern their newly born kids. Baladi kids had more strong behavior towards their dams when

compared to Shami ones. It was concluded that body weight, blood biochemical parameters and Maternal and kid's behavior had notable effect on kid's survivability. Our results might declare superiority of Baladi kids than Shami ones which reflected on the significant reduction of mortality rate in Baladi kids as compared to Shami ones.
Key words: Body weight, Goat, Kids behavior, Maternal behavior, Offspring survival
[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

Determination of the appropriate inoculum dose and incubation period of cassava leaf meal and tofu dreg mixture fermented with *Rhizopus oligosporus*.



Annisa, Rizal Y, Mirnawati, Suliansyah I and Bakhtiar A.

World Vet. J. 10(1): 118-124, 2020;
pii:S232245682000016-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj16

ABSTRACT
The present study was conducted to determine the appropriate inoculum dose and incubation period for the mixture of Cassava Leaf Meal (CLM) and Tofu Dreg (TD) fermented with *Rhizopus oligosporus*. This experiment was carried out in a completely randomized design in a 3 x 4 factorial treatments arrangement with 4 replications. The first factor was the inoculum dose (6, 8 and 10%), and the second factor was the incubation period of the fermentation (2, 3, 4, and 5 days). Measured variables were the changes in Dry Matter (DM), Organic Matter (OM), crude fat, Crude Fiber (CF), and Crude Protein (CP). The experimental results showed that there was no interaction between the inoculum dose and an incubation period of the fermentation in the reduction of DM, organic matter, and crude fat as well as the increase in the CP of fermented CLM and TD with *Rhizopus oligosporus*. However, the interaction was occurred between inoculum dose and incubation period in the reduction in CF. The inoculum dose significantly decreased the DM, OM, crude fat and CF and also increased the CP. The best inoculum dose effect was at 10%. The incubation period had a significant reduction in the DM, OM, crude fat, and CF and also increased the CP. The best incubation period of fermentation was at 3 days. The results indicated that the appropriate inoculum dose to ferment CLM and TD mixture with *Rhizopus oligosporus* was 10% at each incubation period. In the meanwhile, the appropriate incubation period was 3 days for each inoculum dose.
Key words: Fermentation, Inoculum dose, Incubation time, *Rhizopus oligosporus*

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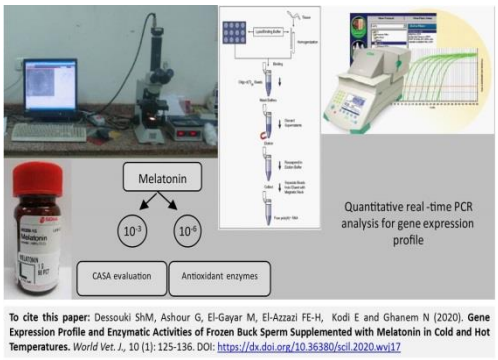
Research Paper

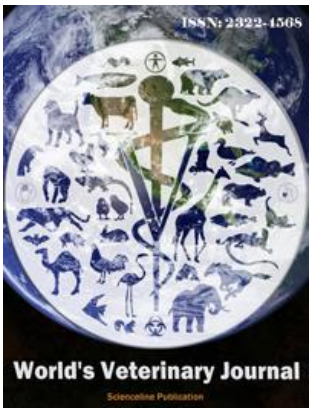
Gene Expression Profile and Enzymatic Activities of Frozen Buck Sperm Supplemented with Melatonin in Cold and Hot Temperatures.



Dessouki ShM, Ashour G, El-Gayar M, El-Azzazi FE, Kodi E and Ghanem N.

World Vet. J. 10(1): 125-136, 2020;
pii:S232245682000017-10
DOI: https://dx.doi.org/10.36380/scil.2020.wvj17

ABSTRACT
This study was carried out to improve the freezability of buck semen using two different types of cryoprotectants supplemented with melatonin as antioxidant in cold and hot temperature of breeding season. Ejaculates from four mature Egyptian baladi bucks were pooled after collection. Semen was extended with Tris-fructose-citric containing egg yolk using glycerol and dimethyl sulfoxide supplemented with two doses of melatonin (10^{-6}M and 10^{-3}M) in addition to control group. Types of motility as well as velocity, enzymatic activity and expression profile of selected genes were measured. The results revealed that the progressive motility percentage was significantly higher in samples supplemented with low dose of melatonin (10^{-6} M) compared to high dose (10^{-3}M) in glycerol (74.4 versus 64.4) and Dimethyl Sulfoxide (DMSO) based extender (35.5 versus 32.9) in cold temperature. The same trend was found in samples cryopreserved with glycerol (75.1 versus 53.5) and DMSO (32.1 versus 22) in hot temperature. The results also demonstrated that CASA parameters (VAP and VCL) were significantly increased in low compared to high melatonin dose in glycerol based extender during cold and hot temperature. The activity of total antioxidant capacity (TAC) was significantly higher in samples supplemented with low (0.49 mM/L) than high melatonin dose (0.16 mM/L) in DMSO extender. CPT2, ATP5F1A and SOD2 genes were up regulated in glycerol based extender groups in cold temperature compared to other groups of this study. On the other hand, NFE2L2 gene was up-regulated in groups cryopreserved with DMSO in hot temperature compared with all other experimental groups. Therefore, it could be concluded that the glycerol based extender in cold season supplemented with low dose of melatonin improved semen quality, antioxidant defense capacity and transcriptional profile, which may maintain the post-thaw fertilizing ability of buck semen.
Key words: Antioxidant enzymes, Bucks, Melatonin, Motility, Transcript abundance
[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]



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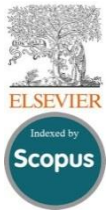

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Impact of Thyme Oil and *Lactobacillus acidophilus* as Natural Growth Promoters on Performance, Blood Parameters and Immune Status in Growing Rabbits

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ABSTRACT

Present study was conducted to evaluate the effect of thyme oil and *lactobacillus acidophilus* (supplement) as growth promoters in rabbit. 72 weaned V-Line male rabbits were randomly allocated into 4 equal groups. The first group (G1) was without any additives and consider as control group. The second group (G2) treated with the addition of *lactobacillus acidophilus* in drinking water in a concentration of 10^8 cfu/ml. The third group (G3) treated with the addition of thyme oil in drinking water in a concentration of 1 ml/ liter. The fourth group (G4) treated with the addition of both *lactobacillus acidophilus* and thyme oil in drinking water in a concentration of 10^8 cfu/ml plus 1ml/L, respectively. The obtained results showed that, all treatments had significant improvement effects on the measured parameters (performance characteristics, cecum characteristics, RBCs, WBCs, kidney function, triglycerides, total cholesterol, sheep RBC's titer, liver antioxidant markers and hormones markers) when compared to the control group. The live body weight of G3 and G4 groups were higher (2116 and 2058 g) than those found in G2 and G1 groups (1958 and 1850 g) respectively. In addition, the body weight gain of G3 and G4 groups were higher (1364 and 1307 g) than those found in G2 and G1 groups (1207 and 1100 g). Moreover, the daily weight gain of G3 and G4 groups were higher (32.49 and 31.13 g/d) than those found in G2 and G1 groups (28.74 and 26.19 g/d). In addition, feed conversion ratio of G3 and G4 groups were higher (3.41 and 3.61) than those found in G2 and G1 groups (3.66 and 4.67). While G4, G2 and G3 groups had a significant enrichment effect on the intestinal beneficial bacteria. In conclusion, in present experiment inclusion thyme oil and/or *lactobacillus acidophilus* in the drinking water that stimulated body weight gain and increased feed conversion rate, and can be used as growth promoters in rabbit nutrition successfully without notable side effects on growing rabbits. Furthermore, it showed a significant positive effect on the physiology for treatment groups G3, G4 and G2 respectively compared to the control group.

Key words: Immunity, *Lactobacillus acidophilus*, Performance, Probiotic, Rabbit, Thyme oil

INTRODUCTION

Rabbits suffer from many digestive disorders related to cecal microflora, which cause high mortality and morbidity rates (Bäuerl et al., 2014). During the first growing period, changes in feeding behavior together with immature digestive and immune systems could promote the development of potentially pathogenic microflora, which could cause digestive troubles and reduce the performance parameters. In intensive rabbit farms, antibiotics are often added to feed or water for rabbits weaning till 8 weeks of age, in order to prevent enteric diseases (Cesari et al., 2008). The European Union banned the use of sub-therapeutic levels of antibiotics to prevent disease or promote growth. The European Union has already banned antibiotics on all remaining growth promoters (Delsol et al., 2005). Therefore, the searches for alternative feed supplements have been increased extensively and considerable attention has been given to the essential herbs as replacements for antibiotics growth promoters, which should have the same beneficial effect as antimicrobial growth promoters (AGPs). The most well-known mechanism to be proposed is that AGPs have an antibacterial action that favors performance through reducing the microbial use of nutrients and improving absorption of nutrients due to thinning of the intestinal wall. Probiotics have beneficially affected the host by boosting the properties of the indigenous microbiota (Huyghebaert et al., 2011). Probiotics can be used as treatment to improve feed intake and digestion (Abd El-Hack et al., 2017). In addition, Markowiak and Śliżewska (2018) stated that probiotics maintain stimulation of intestinal microbiota and protection in the intestine, which is important to combat pathogens for stimulation of immunological response and increased production capacity. The mechanism of action of probiotics includes establishing and maintaining healthy gut microflora, improving digestion and utilization of nutrients (Alagawany et al., 2018). Probiotics has a role in competitive exclusion of harmful bacteria/pathogens, decreases pH, releases various antibacterial substances, neutralization of toxins, competition for nutrients with pathogens, reduction in ammonia production and stimulation of the immune system (Dhama et al., 2011). As feed additive, probiotics show a good impact on the rabbit performance in improving digestion, nutrient metabolism and utilization of nutrients by offering digestible proteins, vitamins, enzymes and other important

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co-factors and by decreasing the gut pH by production of lactic acids (Dhama et al., 2008). Also plant extracts have been considered as one of the important alternatives of antibiotics in animal production as Losa and Kohler (2001) reported a reduction of *Clostridium perfringens* in the intestine of poultry supplemented with a commercial preparation of essential oils in its diet. Taha et al., 2019 sated *Clostridium difficile* as an important pathogens causing diarrhea and enteritis in rabbits followed by intestinal damage and deaths. Marija et al. *(*2015) stated that essential oils obtained from plant extracts have antimicrobial and antioxidative activities. In addition, healthy and safe ingredients obtained from a variety of plant materials and have a strong antimicrobial effect. These oils are made from complex mixtures of volatile molecules that are produced by the secondary metabolism of aromatic and medicinal plants. It may perform its effect through disrupting the function of bacterial cell wall through affecting its lipopolysaccharides content leading to increase of the cell membrane permeability as reported by Faleiro (2011).

So, the aim of this study was to investigate the effect of using *Lactobacillus acidophilus*, Thyme oil and their mixture on performance and immune parameters and caecum characteristics in weaned rabbits. This additive can be perfect feeding rabbits by effect for maintain health and improve the growth of animals (Abdelnour et al., 2019; Alagawany et al., 2019).

MATERIALS AND METHODS

Ethical approval

All samples were collected as per standard procedure without giving any stress or harm to the animals and the study was conducted according to Cairo University Institutional Animal Care and Use Committee (CU- IACUC) Veterinary Medical and Agricultural Sciences Sector, Egypt under approval code # CU/II/F/34/19#.

Experimental design

A total number of 72 weaned V-Line male's rabbits, aged 30 days old with average body weight of 750 ± 32 g. were allocated randomly to four groups (18 rabbits each, which contains six replicates, 3 rabbits each replicate) and were treated as follows: The control group was without any additives in drinking water (G1). The second group treated with *Lactobacillus acidophilus* 10^8 cfu/liter of drinking water (G2). The third group treated with 1 ml of thyme oil /liter of drinking water (G3). The fourth group treated with a mixture of *Lactobacillus acidophilus* (10^8 cfu/ml) and thyme (1 ml) per liter of drinking water (G4). Thyme oil was obtained from El- Gabry for natural herbs and medicinal plants belonging to El-Giza governorate, Egypt, and was tested for its active ingredients at Organic Pollutants department, Regional Center for Food and Feed, Agricultural Research Center, Giza, Egypt. *Lactobacillus acidophilus* strain was kindly supplied by Food Safety Department, Regional Center for Food and Feed, Agricultural Research Center, Giza, Egypt. Experimental diets were formulated to meet all the nutrient requirements of growing rabbits according to (De Blas, 1998) as showed in table 1**.** From Analysis of phenolic compounds in thyme oil analyzed by GC-MS/MS:The highest active ingredients of thyme oil (Thymol, Carvacrol) and Thymol compound appeared at the retention time (9.662 min). While Carvacrol compound appeared at the retention time (10.172 min) from data illustrated in figure1.

Table 1. Composition and chemical analysis of experimental diet of growing rabbits

Ingredients	**%**
Ground barley	17.00
Wheat bran	22.50
Yellow corn	5.00
Soybean Meal (44%)	18.20
Straw	8.00
Clover hay Alfalfa	24.00
Di-calcium phosphate	1.00
Limestone	0.40
Methionine	0.20
Salt	0.40
Premix*	0.30
Molasses	3.00
Chemical analysis	
Crude protein (%)	17.88
Crude fiber (%)	14.63
Ether extract (%)	2.68
Nitrogen free extract (%)	49.21
Organic Matter (%)	80.78
Digestible energy (Kcal/Kg)	2568

* Vitamin-mineral premix provide per kg of diet vit. A. 13,340 iu; vit. D3. 2680. i.u; vit. E. 10 .i.u; vit. K, 2.68 mg; Calcium pantothenate, 10.68 mg; vit. B12, 0.022 mg; folic acid, 0.668 mg; choline chloride, 400 mg; chlortetracycline, 26.68 mg; manganese, 133.34 mg; iron, 66.68 mg; zinc, 53.34 mg ; copper, 3.2 mg, iodine, 1.86 mg; cobalt, 0.268 mg, selenium, 0.108 mg.** DE calculated according to Schneider and Flatt (1975).

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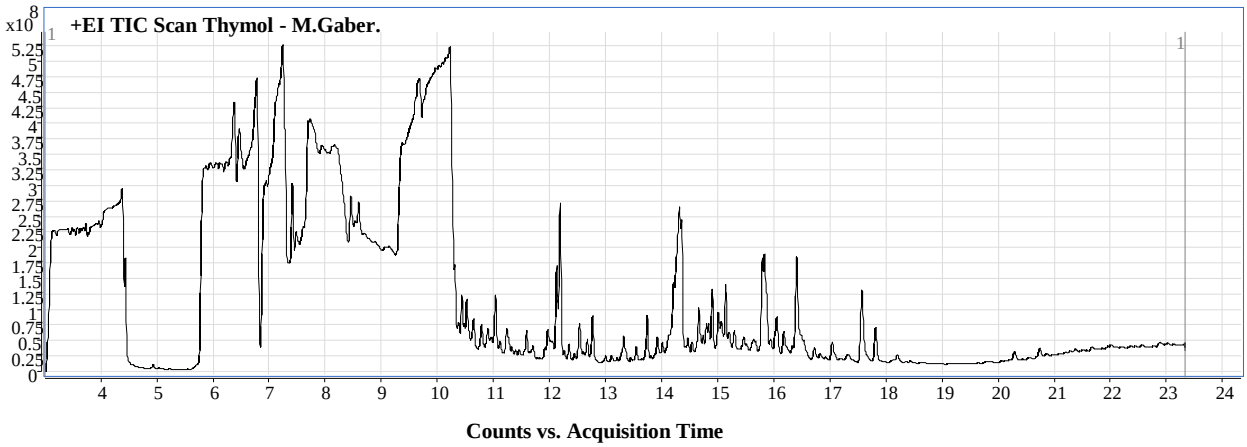

Figure 1. Chromatogram of used Thyme oil analyzed by GC-MS/MS instrument.

Determination of thyme oil

The active ingredients of thyme oil (Thymol, Carvacrol) were analyzed by GC-MS/MS according to Patricia et al. (2013). From data obtained in figure 1 it can be noticed that Thymol and Carvacrol are the most predominant ingredients in thyme oil composition as it represents about 13.71 and 23.22 % respectively to all constituents as a peak area %. These data were confirmed by the chromatogram illustrated in figure 1, which showed different intensities of the components represented by different levels of peaks. These data agreed with that reported by Karousou et al. (2005), Ortega-Nieblas et al. (2011) and Amiri (2012) who stated that Thymol and Carvacrol are the main components of thyme.

Growth performance parameters

Rabbits of all groups were kept under similar management conditions, diet and water were offered *ad libidum* during adaptation period (five days), after that diets were individually weighed for each group and feed residues were weighed daily and subtracted from the daily feed allowance to measure the actual daily feed intake (Table 1). Animals were individually weighed, weekly before offering the morning meal and continued for six weeks' period. The obtained weight was recorded as well as the consumed feed throughout the completely experimental period, which lasted for 6 weeks. The values of body weight, daily weight gain, daily feed intake, feed conversion ratio (g feed/ g body weight) were recorded.

Caecum characteristics

After slaughtering, gastrointestinal tract was individually removed from six rabbits per each group, caecum content was collected and was taken to estimate microflora count under complete hygienic measures for determination of cecal microflora. The cecal appendix fluid was collected and estimation of cecum microflora was performed without any delay *Lactobacillus acidophilus* count and *Enterococcus species* count were measured by using a selective dehydrated media de Man Rogosa and Sharpe agar (MRS); Slantz and Bartly agar from (LabM a Neogen company, United Kingdom), respectively. Also, the microbial content was determined by using a selective dehydrated media Violet Red Bile agar (VRB); Brilliant Green agar was used to estimate *E. coli* count and *Salmonella Spp* count, respectively. Which can be summarized as follows: Ten-fold serial dilutions were performed from cecum content of each sample, and then 1 ml from every dilution was inoculated into sterile petri dishes. Specific media of each targeted microorganism was poured into the inoculated petri dishes. The petri dishes were incubated as *Lactobacillus acidophilus* at 37 °C for 24 hours, *Enterococcus species* 44°C for 48 hours, *E. coli* 44°C for 24 hour and *Salmonella Spp* 37°C for 24 hours, before counting the bacteria.

Enumeration methods of *Enterococcus* species count was according to NMKL (2011), *Lactobacillus acidophilus* count was according to Soliman et al. (2015), Faecal coliform count was according to NMKL (2005) and *Salmonella* count was according to Gantois et al. (2008).

Serum biochemical parameters

Blood samples were collected after slaughtering of three rabbits per treatment into dry clean centrifuge tubes containing heparin and were centrifuged at 3000 rpm for 15 min. The plasma was collected and stored at (-20°C) to estimate total protein, albumin, globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, LDL-, HDL-cholesterol, triglycerides, creatinine and glucose. These constituents were measured using commercial kits from (DiaSya Diagnostic, Germany) according to manufacturer's instructions.

Total protein was measured using Buiret method as described by Gornall et al. (1949). Albumin reacts with bromocresol green in alkaline solution and succinate buffer (pH 4.2). The produced colored complex was measured

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at 628 nm according to the method described by Doumas et al. (1971). Globulin (g/dl) was calculated as the difference between total protein content and albumin content of plasma. The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by using the commercial kits from (DiaSya Diagnostic, Germany) according to the method that described by Reitman and Frankel (1957). Plasma alkaline phosphatase (ALP) was determined according to the modified methods of Kind and King (1954).

Total cholesterol estimation by quantitative enzymatic colorimetric determination of total cholesterol in serum or plasma according to Deeg and Ziegenhorn (1983). High density lipoprotein (HDL) was determined according to the method of Lopez-Virella et al. (1977). The plasma very low density lipoprotein cholesterol (VLDL) was estimated by the Friedewald formula (plasma triglycerides/5) as stated by Friedewald et al. (1972). The plasma low density lipoprotein cholesterol (LDL) was estimated by the Friedewald equation (LDL = Total cholesterol – High density lipoprotein cholesterol – triglycerides/5) as stated by Friedewald et al. (1972).

Triglyceride was determined in plasma according to Fossati and Prencipe (1982). Glucose was determined according to Trinder (1969). Creatinine was determined according to the method described by Bartels and Böhmer (1971). Plasma uric acid was measured according to Fawcett and Scott (1960). Serum hormones (T3, T4 and TSH) were determined by radioimmunoassay (RIA) according to Chopra et al. (1971); Wartofsky and Burman, (1982); Bolognani et al. (2001). The RBC's count analyzed by using hemocytometer according to Perkins (2009). The Hemoglobin was determined according to Van Kampen and Zillstra (1983). The Hematocrit was measured according to Bauer (1970). The WBC's count was determined by using hemocytometer according to Ewuola and Egbunike (2008). Determination of humeral immune response of Sheep Red Blood Cells (SRBC's) titer was according to the method of Van der Zijpp and Leenstra (1980).

Determination of antioxidant markers

Samples were collected from liver for each group, to determine (measure) the anti-oxidant markers. Malondialdehyde (MDA) was carried out according to the method of Mitsuru and Midori (1978). The reduced glutathione (GSH) was determined according to the method of Ellman (1959).

Statistical analysis

The data collected were statistically analyzed by General Linear Models (GLM) procedure of SAS program (SAS, 2004). During the experimental period according to the following statistical model:

$Y_{ij} = \mu + T_i + \varepsilon_{ij}$

Where:

Y_{ij} = is the j^{th} observation in the i^{th} treatment.

μ = is the overall mean,

T_i = the effect of treatment groups (i= 1, 2, 3 and 4).

ε_{ij} = is a random error.

Significant differences among means were achieved using the Duncan of multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Growth performance

Average live body weight affected by using thyme oil extract, probiotic and both as feed additives revealed that thyme had significantly the highest value of live body weight ($P<0.05$) when compared to the control group followed by the mixture between both thyme and *Lactobacillus acidophilus* then the used bacteria probiotic (Table 2). All obtained values significantly differed from each other. These results are consistent with Kritas and Morrison (2005**)** found that the beneficial effect of probiotic supplementation in broiler diet increased body weight. These results were significantly ($P<0.05$) in agreement with those obtained by de Lange et al. (2010) reported that essential oils improved the performance parameters not only by controlling enteric pathogens but also by increasing the palatability of the diet which can explain the body weight increase of rabbits in the present experiment.

As shown in table 2, all treatments significantly improved the body weight gain. The thyme group had the greatest effect followed by the mixture then the probiotic strain compared with the control. These results are consistent with Placha et al. (2013) explained that the highest weights for body weight gain achieved by thyme oil are usually associated with the protective effect by improving intestinal health. In addition, Shabaan (2012) reported that effect of *Thymus vulgaris* seeds improving the utilization of low energy broiler diet and accelerate body weight and all growth parameters in compared with control group. In addition, Chiofalo et al. (2004**)** reported that administration of probiotic strains separately and in combination was significantly improved daily weight gain and total body weight in chicken, sheep, and goat. Data obtained from table 2 showed the same trend during the experimental period on daily weight gain all over the experiment. It is clear that, at the end of the experiment, Thyme, mixture and *Lactobacillus acidophilus* had significant

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better effects on performance characteristics respectively if compared to the control group. There was a synergistic effect between essential oil and probiotics when use feed additives in poultry nutrition.

Table 2. The effect of Thyme oil and *Lactobacillus acidophilus* on performance characteristics of growing V-Line rabbits aged 72 days

Characteristics	Day old	Treatments			
		G1	G2	G3	G4
Average Live Body Weight	(Initial)30	750 ± 38.67	751 ± 28.65	751 ± 27.01	751 ± 33.35
	51	1320^{b} ± 39.72	1361^{b} ± 42.18	1506^{a} ± 30.38	1422^{ab} ± 46.77
	72	1850^{c} ± 34.49	1958^{b} ± 37.26	2116^{a} ± 42.29	2058^{ab} ± 31.02
Body Weight Gain	30-51	569^{c} ± 13.28	610^{c} ± 23.89	755^{a} ± 17.45	671^{b} ± 24.99
	52-72	530^{b} ± 17.61	597^{a} ± 20.51	609^{a} ± 16.03	636^{a} ± 12.90
	30-72	1100^{d} ± 15.09	1207^{c} ± 22.27	1364^{a} ± 17.38	1307^{b} ± 18.45
Daily Weight Gain	30-51	27.10^{c} ± 0.63	29.03^{c} ± 1.14	35.95^{a} ± 0.83	31.97^{b} ± 1.19
	52-72	25.28^{b} ± 0.83	28.45^{a} ± 0.97	29.02^{a} ± 0.76	30.28^{a} ± 0.61
	30-72	26.19^{d} ± 0.35	28.74^{c} ± 0.53	32.49^{a} ± 0.41	31.13^{b} ± 0.43
Feed Intake	30-51	105^{a} ± 0.54	86^{d} ± 0.31	94^{b} ± 0.18	91^{c} ± 0.38
	52-72	138^{a} ± 0.74	123^{d} ± 0.71	126^{c} ± 0.67	132^{b} ± 0.63
	30-72	122^{a} ± 0.71	104^{d} ± 0.31	110^{c} ± 0.23	112^{b} ± 0.44
Feed Conversion Ratio	30-51	3.92^{a} ± 0.09	3.04^{b} ± 0.11	2.65^{c} ± 0.06	2.91^{bc} ± 0.1
	52-72	5.58^{a} ± 0.18	4.40^{b} ± 0.14	4.40^{b} ± 0.1	4.41^{b} ± 0.09
	30-72	4.67^{a} ± 0.07	3.66^{b} ± 0.06	3.41^{c} ± 0.03	3.61^{b} ± 0.04

[a-d] Mean± SME in same row with different superscripts are significantly (P<0.05) different.G1 = control group; G2 = *lactobacillus acidophilus*; G3 = Thyme oil; G4= Mixture (*lactobacillus acidophilus* and Thyme oil); Significance (P<0.05); Non-Significant (P>0.05).

Data obtained from table 2 indicated the effect of the treatment at feed intake of the experimental animal during the study. All treatments significantly affected the feed intake values positively when compared to the control group the lowest consumed amount was recorded in group treated with *Lactobacillus acidophilus* followed by the group treated with Thyme. The group treated with the Mixture showed the third lowest consumed feed amount and the highest amount was recorded in the control group, through the whole duration of this study. Similar results were observed in other studies, Hertrampf (2001) reported that essential oils could be successfully used as growth promoters, by increasing the feed intake due to their aromatic characteristics in chickens. In addition, Chiofalo et al. (2004) reported that administration of probiotic strains separately and in combination was significantly improved feed intake, feed conversion rate in chicken, sheep and goat.

Data presented in table 2 showed that, the lowest FCR was recorded in group treated with thyme followed by the other two groups (*Lactobacillus acidophilus* and Mixture treated groups). While the value obtained in the control group was the highest indicated that, Thyme had the best effect on FCR followed by both *Lactobacillus acidophilus* and Mixture treated groups .This result was confirmed by those obtained by Lee et al. (2003) stated that components of essential oils, especially Carvacrol had a positive effect on FCR in broiler chickens. Also the *Lactobacillus acidophilus* were improved feed conversion for target species and benefits for the consumer through improved product quality, this agreement with those reported by Musa et al. (2009), So essential oils and *Lactobacillus acidophilus* had positive effects on FCR compared to control group in rabbit's diet.

Cecum characteristics

By studying the effect of the used treatments on caecum characteristics as an indicator of digestibility improvement, as a control approach for harmful bacteria and as a stimulant for beneficial bacteria, essential oils and probiotics had a positive effect on cecum of poultry and rabbits which caused increase performance parameters, which indicated better environment for the growth of the beneficial bacteria and unfavorable conditions for pathogenic bacteria in the cecum if compared to the control group this finding was confirmed by those reported by Bölükbaşi and Erhan (2007) found that 0.1% and 0.5% of thyme significantly reduced *E. coli* concentrations in the faeces of laying hens in treatments group compared with the control group, Also Cross et al. (2002) showed that thyme reduced the numbers of coliforms. And Simonová et al. (2010) indicated that diet enriched with some plant extracts is beneficial for the health rabbits. The antimicrobial activity in essential oils appears to be associated with phenolic compounds (Thymol and Carvacrol), these agreements with those reported by other authors (Karaman et al., 2001; Rota et al., 2008).

Mookiah et al. (2014) found *Lactobacillus* strains increased the caecal populations of *Lactobacillus* and decreased the caecal *E. coli* who stated that, essential oils and probiotics enhanced the growth of intestinal gram positive microflora and negatively affected the colonization of *salmonella* and *E.coli* due to increase of the acidity of the intertied content. Also, it was clear that all three treatments had the same positive effect on *Lactobacillus* and *Enterococcus* count (The highest number of colony forming units of the helpful bacteria was recorded in both groups which were treated with

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Lactobacillus and Mixture of Thyme and *Lactobacillus* while the cfu of the helpful bacteria was lower in the group treated with Thyme only). All treatments had significantly higher counts if compared to the count recorded in the control group. Concerning the effect of the used treatments on *salmonella* and *E.coli*, it was clear that, the bear effect was recorded in the groups treated with Thyme and mixture that showed the same significant effect followed by the effect of the added probiotic.

All treatments had a significant positive effect on the count of harmful bacteria if compared to the control group. Bölükbasi and Erhan (2007) clarified that essential oil and probiotics had stimulating effect on increasing beneficial bacteria which reduce the chances of growth and multiplication of harmful microbes that compete on the life and existence within the intestines by inhibition of harmful microbial growth. Also, *in vitro* studies have shown that essential oils to have antibacterial properties against *Escherichia coli*, *Salmonella typhimurium* that reported by Cosention et al. (1999).

Table 3. The effect of Thyme oil and *Lactobacillus acidophilus* on cecum characteristics of the growing V-Line rabbits aged 72 days

Cecum bacterial count	Treatments			
	G1	G2	G3	G4
Lactobacillus acidophilus count (log_{10} cfu/g)	$4.4^{c} \pm 0.17$	$6.92^{a} \pm 0.29$	$5.69^{b} \pm 0.29$	$6.36^{ab} \pm 0.14$
Enterococcus species count (log_{10} cfu/g)	$1.54^{c} \pm 0.22$	$3.58^{a} \pm 0.08$	$2.30^{b} \pm 0.8$	$3.22^{a} \pm 0.07$
E. coli count (log_{10} cfu/g)	$4.39^{a} \pm 0.07$	$3.49^{b} \pm 0.08$	$2.58^{c} \pm 0.29$	$2.95^{c} \pm 0.16$

[a,b,c] Mean± SME in same row with different superscripts are significantly (P<0.05) different.(log_{10} cfu/g) the count of microbial colony-forming units (CFU) has expressed as logarithmic (log_{10}) transformation per gram of cecum contentG1 = control group; G2 = lactobacillus acidophilus; G3 = Thyme oil; G4= Mixture (lactobacillus acidophilus and Thyme oil); Significance (P<0.05); Non-Significant (P>0.05).

Blood indices

Data obtained from table 4 indicated that, all treatments had no significant effect on RBC'S, WBC'S, Hemoglobin and Hematocrit from the 30th day to the 51st day of age. These results supported present findings and reported by Chen et al. (2005) reported no significant effect of addition of probiotics on blood characteristics. While data obtained from table 4 during the 2nd phase of the experiment (from 52nd to 72nd days of age), Thyme had the best effect on RBC'S count followed by *Lactobacillus acidophilus* while the mixture and the control group had lower values. All treatments showed the same significant positive effect on the count of WBC'S if compared to the control group and had no significant effect on neither hemoglobin nor hematocrits values. These results supported present findings and reported by Ezema and Eze (2012) stated that a significantly higher total WBC count in broiler rabbits fed diets supplemented with probiotics. Also Archetti et al. (2008) and Moore et al. (2015) reported that range of hemoglobin content (10.4 to 17.4 g/dl) in healthy growing rabbits.

Table 4. The effect of Thyme oil and *Lactobacillus acidophilus* on blood picture of the growing V-Line rabbits during the first and second period of experiment

Day old	Indices	Treatments			
		G1	G2	G3	G4
51	RBCs ($X10^{6}$/uL)	4.73±0.18	4.90±0.08	4.79±0.12	4.74±0.16
	Hb (g/dl)	10.96±0.30	11.58±0.09	11.23±0.26	11.03±0.25
	Ht (%)	31.06±0.63	31.95±0.35	31.73±0.65	30.68±0.59
	WBCs ($X10^{3}$/uL)	6.33±0.53	8.14±1.09	6.79±0.75	7.05±0.74
72	RBCs ($X10^{6}$/uL)	4.60^{b}±0.15	5.13^{ab}±0.45	5.94^{a}±0.29	4.80^{b}±0.18
	Hb (g/dl)	12.10±0.27	12.30±0.37	11.85±0.32	11.98±0.32
	Ht (%)	31.70±0.66	32.55±0.63	31.48±0.74	31.38±1.01
	WBCs ($X10^{3}$/uL)	4.75^{b}±0.21	7.23^{a}±0.38	7.52^{a}±0.56	7.09^{a}±0.51

Through 72 day, [a,b] Mean± SME in the same row with different superscripts are Significance (P<0.05).G1 = control group; G2 = lactobacillus acidophilus; G3 = Thyme oil; G4= Mixture (lactobacillus acidophilus and Thyme oil); Significance (P<0.05); Non-Significant (P>0.05).

Blood Plasma constituents

Studying the levels of TSH, T_3 and T_4 could clarify the immune response to the used treatments as it was clear from the data presented in table 5 that no marked significant effect was obtained on the levels of the three parameters during the first phase of the experiment (30-51 days of age). While during the second phase (52 to 72 days of age) that all treatments showed marked significant increase of TSH, T_3 and T_4 when compared to the control group. It was noticed that, the treatments which contained live microorganisms (*Lactobacillus acidophilus* and the mixture) had better effect on T3, T_4 and TSH values. This finding was similar to that obtained by Beisel (1982) that reported, nutrition plays an important role in the development and function of the immune system. In addition, Aluwong et al. (2012) indicated the effect of probiotics in thyroxin (T_4) level in broiler given feed supplemented with yeast. So, inclusion of living organisms could positively stimulate effects of TSH and consequently T_3 and T_4.

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Table 5. The effect of Thyme oil and *Lactobacillus acidophilus* on hormones indices, sheep red blood cells titer and liver antioxidant markers in growing V-Line.

Day old	Indices	Treatments			
		G1	G2	G3	G4
51	T3 (ng/dl)	178.50 ± 2.87	183 ± 7.51	185.25 ± 7.18	182 ± 9.26
	T4 (ug/dl)	3.75 ± 0.33	4.64 ± 0.37	4.48 ± 0.31	4.23 ± 0.26
	TSH (ulU/mL)	0.33 ± 0.11	0.08 ± 0.03	0.11 ± 0.03	0.16 ± 0.08
72	T3 (ng/dl)	174^{b} ± 12.53	210^{a} ± 6.17	208^{a} ± 6.68	227^{a} ± 10.63
	T4 (ug/dl)	2.81^{c} ± 0.31	3.55^{b} ± 0.17	3.90^{ab} ± 0.08	4.17^{a} ± 0.12
	TSH (ulU/mL)	0.12^{b} ± 0.03	0.29^{ab} ± 0.07	0.29^{ab} ± 0.08	0.37^{a} ± 0.08
72	Sheep RBC's titer	2.50^{c} ± 0.29	4.0^{b} ± 0	4.25^{ab} ± 0.25	5.25^{a} ± 0.63
72	Malondialdehyde (MDA) (nM/gwet. tissue)	10.98^{a} ± 0.8	9.20^{ab} ± 0.9	6.66^{c} ± 0.13	8.34^{bc} ± 0.67
	Reduced Glutathione(GSH) (mM/gwet. tissue)	2.61^{b} ± 0.21	4.05^{a} ± 0.30	3.73^{a} ± 0.29	3.77^{a} ± 0.25

[a,b,c] Mean± SME in the same row with different superscripts are significantly (P<0.05) different.G1 = control group; G2 = *lactobacillus acidophilus*; G3 = Thyme oil; G4= Mixture (*lactobacillus acidophilus* and Thyme oil); Significance (P<0.05); Non-Significant (P>0.05).

Table 6. The effect of Thyme oil and *Lactobacillus acidophilus* on some blood parameters of the growing V-Line rabbits during the first and second period of experiment

Day old	Parameters	Treatments			
		G1	G2	G3	G4
51	Total Protein (g/dl)	9.46^{a}±0.93	6.51^{b}±0.24	7.78^{ab}±0.62	7.56^{ab}±0.44
	Albumin (g/dl)	3.00±0.06	3.00±0.06	2.95±0.05	3.06±0.04
	Globulin (g/dl)	6.46^{a}±0.88	3.52^{b}±0.24	4.83^{ab}±0.67	4.50^{b}±0.44
	Triglycerides (mg/dl)	73.0±3.41	67.33±3.17	81.00±6.01	69.67±8.50
	Total cholesterol (mg/dl)	60.0^{ab}±1.54	63.33^{a}±1.78	59.50^{ab}±3.01	53.83^{b}±3.62
	HDL (mg/dl)	26.17±0.98	29.98±1.86	27.73±1.67	26.10±2.53
	VLDL (mg/dl)	14.60±0.68	13.47±0.63	16.20±1.20	13.93±1.70
	LDL (mg/dl)	19.23^{ab}±1.17	19.88^{a}±0.82	15.57^{ab}±2.83	13.80^{b}±1.55
	Glucose (mg/dl)	95.16±6.76	81.33±4.97	105.67±12.82	101.33±7.87
	Urea (mg/dl)	37.33^{a}±1.33	29.83^{b}±1.07	26.16^{b}±1.75	26.50^{b}±1.25
	Creatinine (mg/dl)	0.62±0.03	0.58±0.04	0.53±0.02	0.53±0.03
	Alkaline phosphatase(U/L)	220.16^{b}±26.42	270.33^{ab}±40.46	335.50^{a}±31.34	311.83^{ab}±23.99
	AST (U/L)	39.33±6.42	36.83±2.98	31.50±3.91	34.83±3.41
	ALT (U/L)	21.67±2.53	23.50±3.41	26.50±3.88	21.00±1.31
72	Total Protein (g/dl)	6.93^{a}±0.43	6.62^{ab}±0.25	5.70^{b}±0.32	6.65^{ab}±0.14
	Albumin (g/dl)	3.88±0.19	3.50±0.28	3.75±0.09	3.85±0.09
	Globulin (g/dl)	3.05^{a}±0.29	3.11^{a}±0.28	1.95^{b}±0.27	2.80^{a}±0.18
	Triglycerides (mg/dl)	78.16^{a}±3.86	61.16^{b}±4.48	75.33^{ab}±2.78	75.16^{ab}±6.85
	Total cholesterol (mg/dl)	62.67^{a}±1.54	53.83^{ab}±3.28	53.50^{b}±1.85	60.67^{b}±2.82
	HDL (mg/dl)	31.85±3.04	30.42±3.86	29.23±2.45	32.57±2.73
	VLDL (mg/dl)	15.63^{a}±0.77	12.23^{b}±0.89	15.06^{ab}±0.55	15.03^{ab}±1.37
	LDL (mg/dl)	15.18±3.10	11.18±1.63	9.20±2.18	13.06±2.09
	Glucose (mg/dl)	140.50±8.20	144.33±12.56	129.50±7.32	120.00±4.74
	Urea (mg/dl)	39.50^{a}±5.30	28.66^{b}±1.56	23.83^{b}±0.80	22.00^{b}±0.36
	Creatinine (mg/dl)	0.87^{a}±0.03	0.53^{b}±0.03	0.52^{b}±0.01	0.45^{b}±0.02
	Alkaline phosphatase (U/L)	132.67±15.88	142.50±21.86	113.33±4.97	130.83±13.08
	AST (U/L)	28.33±3.53	23.16±5.08	19.83±2.98	20.33±2.70
	ALT (U/L)	38.33±3.90	33.0±7.69	35.67±2.82	27.33±2.26

[a,b] Mean± SME in the same row with different superscripts are significantly (P<0.05) different.G1 = control group; G2 = *lactobacillus acidophilus*; G3 = Thyme oil; G4= Mixture (*lactobacillus acidophilus* and Thyme oil); Significance (P<0.05); Non-Significant (P>0.05).

With compared to the control group all treatments had a stimulation of immune system as represented by the positive effect on the sheep RBC'S titer that indicated the stimulation of immune system as an indicator for immunity and capability controlling pathogenic bacteria as shown in table 5. The G4 and G3 groups were higher (5.25 and 4.25; P<0.05) than those found in G2 and G1 groups (4.0 and 2.50) respectively, these data are in agreement with El-Sissi and Mohamed (2011) reported that, *Lactobacillus* improved the antibody response to NDV and IBV vaccines, as an indicator of immunity for animals.

Data obtained in table 5 indicated that all used treatment caused significant reduction of MDA levels, while the level of GSH in the seam of individuals in all treated groups shows significant increase when compared to the control group, these findings were according to with findings of Hashemipour et al. (2013) that reported Thymol improved

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antioxidant enzyme activities, and immune response. Also, Carvacrol, which play a role in raising the level of GSH, could decrease MDA which reported by Luaibi and Mousa. (2016). In addition, Kogan et al. (2008) reported that probiotics may have antioxidant activity. These results supported present findings and reported by Baratta et al. (1998) stated that thymol and carvacrol, which are the main components of thyme oil, showed strong antioxidative properties. These results showed that MDA that is one of the final products of polyunsaturated fatty acids peroxidation antioxidant activity of both probiotics and essential oils from plant exacts. In addition, GSH whose activity is to protect the organism from oxidative damage, increased as the effect of dietary probiotics and essential oils.

The table 6 showed that, all treatments caused no increase in some measured parameters which indicated no harmful negative effect on liver enzymes (ALT and AST), Glucose levels, Total protein, Albumin and Globulin. These results were agreed with those obtained with Onbasilar and Yalcin (2008) who stated that liver function tests (ALT and AST) were not affected by probiotics, Also these results disagree with those obtained with Tollba et al. (2010) that clarified the addition of aromatic herbal extract to the diets increased ($P<0.05$) total protein, albumin and globulin compared to control group under cold environmental temperature. However, except some measured parameters, it is clear from the same table 6 that, all treatments compared with the control group (G1) had significant reducing effect of kidney function parameters (Urea and Creatinine), Triglycerides and Total cholesterol as an indication of positive effect on the physiology of the experimental animals. These results were similar to the report of Shabaan (2012) who stated that broiler chicks fed the low energy diets supplemented with a mixture of 0.15% thyme and 0.15% cumin gave recorded lower values of plasma total protein, albumin and uric acid as compared to the control group. So, it is perceived both Thyme oil and *Lactobacillus acidophilus* had no harmful effect on liver function and blood protein. These data are in agreement with Lee et al. (2003) reported that, Thymol and Carvacrol have effects on growth performance and triglyceride metabolism in broiler chickens and reducing plasma triglyceride concentrations.

CONCLUSION

It was concluded that Thyme oil and *Lactobacillus acidophilus* can be used as feed additives as they had growth promoting effects and caused the improvement of performance and immunostimulant activity without notable side effects on growing rabbits. Studying the effect of Thyme oil by using (1 ml of thyme oil /liter of drinking water) has the highest significant positive effect on body weight, body weight gain, daily weight gain and feed conversion ratio followed by the mixture of thyme oil and *Lactobacillus acidophilus* by using 10^8 cfu/ml and thyme (1 ml) per liter of drinking water. Furthermore, a good positive effect on the physiology of the experimental animals compared with the control group.

DECLARATIONS

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Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article. The authors declare that they have no competing interests.

Author's contribution

Ahmed M. El-kaiaty and Gihan M. El-Moghazy they designed the experiment and laboratory analyses. Ahmed M. El-kaiaty designed article writing, revision and approval, Gihan M. El-Moghazy designed tabulation of experimental data, manuscript writing, commenting and approval, Mohamed A.F. El-Manylawi helped in statistical analysis, tabulation of experimental data and article revision; while, Mahmoud G.Y. Abdel-Mageed helped in field study, collected data, laboratory analyses, statistical analysis, manuscript writing. All authors have read and approved the final manuscript.

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Molecular and Phylogenic Analysis of Bovine Respiratory Syncytial Virus in Nineveh province, Iraq

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ABSTRACT

Bovine Respiratory Syncytial Virus (BRSV) is one of the worldwide distributed infectious agents responsible for diversified clinical disease in cattle populations which causes considerable economic loss due to its negative effects on health and production. In this study, 450 nasal swab samples were collected from cows with different ages and breeds in different areas across Nineveh province, Iraq. Molecular diagnosis using nested RT-PCR and phylogenetic analysis of the G gene were performed. The results indicated a 37.31% prevalence rate of BRSV using specific primers in the PCR technique. The local isolate was submitted in GenBank under the accession number MN129181 Mosul isolate. The phylogenetic tree of local isolates of BRSV was made using the neighbor-joining system after comparison with other GenBank data. In conclusion, phylogenetic analysis of BRSV can provide information about the viral strains present in cattle and subsequently may be useful for infection control programs.

Key words: Bovine respiratory syncytial virus, Cattle, PCR, Phylogenic analysis.

INTRODUCTION

Bovine respiratory disease has an essential economic impact on animal production and cattle industry (Hacioğlu et al., 2019). Bovine Respiratory Syncytial Virus (BRSV) belongs to the *Paramyxoviridae* family and is one of the major etiology of bovine respiratory disease displaying different severities from subclinical features to fatal outcomes (Stott et al., 1980; Easton et al., 2004; Valarcher and Taylor, 2007; Brodersen, 2010).

The BRSV is enveloped and has a negative-sense, single-stranded RNA. The genome encodes 10 major proteins including the Glycoprotein (G protein) that is responsible for the virus entry into the cell (Furze et al., 1994). The G protein has a substantial mutation rate and it is the target of investigations for molecular and phylogenetic analysis (Valarcher et al., 2000).

Shony et al. (2008) isolated the virus from calves using tissue culture in Baghdad. Recently, Hussain et al. (2019) documented the prevalence rate and risk factors of the BRSV in cattle in Nineveh province and found that 83.11 % of animals were antibody positive for BRSV. The present study is the first trial to detect and identify BRSV using nested RT-PCR in cattle in Nineveh province, Iraq.

MATERIALS AND METHODS

Ethical approval

Samples were collected as per the standard sample collection procedure without any stress or harm to the animals.

Study area and study population

The study was conducted in northern, eastern, southern and western regions of Nineveh province, Iraq from September 2017 to September 2018.In this study, 26 herds with different sizes divided into small (≤10 animals) and large herds (≥20 animals) were evaluated. From each herd, 50% of cows displaying respiratory signs were sampled.A total of 450 nasal swab samples were obtained, placed in sterile test tubes, kept in an ice bag and stored at-20 °C until analysis. The animals had no history of vaccination against BRSV. Epidemiological and demographic data (animal origin, age, sex, type of breeding, season and geographical area) were recorded (Table 1).

Laboratory analysis

RNA was extracted from nasal samples using the PrimePrep Viral RNA/DNA Extraction Kit (Genet Bio Inc., South Korea). The concentration and purity of extracted RNA were determined by using Nanophotometer™ P-Class

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(IMPLEN, Germany). The RNA concentration ranged between 54 and 72 ng. The purity of RNA was obtained by computing the ratio of A_{260} nm to A_{280} nm,the purified RNA had an A_{260}/A_{280} nm ratio of 1.9-2.1.
Nested RT-PCR was performed using One Tube RT-PCR System Script RT-PCR Premix Kit (GeNet Bio Inc., South Korea). The RT-PCR mixture consisted of 3.5 µl of PCR-Grade water, 1 µl of forward primers,1 microliter of reverse primers, 1 µl of magnesium chloride, 3.5 µl of purified RNA, and 10 µl of SuPrime Script RT-PCR Premix (2X).
Two pairs of primers B5A (5'-CCA CCC TAG CAA TGA TAA CCT TGAC-'3), B6A (5'-AAG AGA GGA TGC (T/C) TT GCT GTGG'3), B7 (5'-CATCAATCCAAAGCACCACACTGTC-'3) and B8 (5'-GCTAGTTCTGTGGTGGATTGTTGTC'-3) (Vilcek et al., 1994) were used. Reverse transcription was performed at 50 °C for 30 min, followed by the denaturation step at 95 °C for 5 min. The amplification was done in 35 cycles under the following conditions: 30s at 95 °C, 30 s at 57 °C, and 60 s at 72 °C. The reaction was completed by final elongation at 72 °C for 5 min. These steps were performed in the first and second PCR reactions. PCR products were visualized on 1.5% agarose gel stained with ethidium bromide.

Sequencing and phylogenetic analysis

Using the PrimePrep™ Gel purification kit (GeNet Bio Inc., South Korea), the RT-PCR products purified were suspended in a total volume of 25 µl of diethylpyrocarbonate-treated water. A total of eight PCR amplicons from cattle nasal swabs that were positive for BRSV were sent to the commercial company for sequencing (Macrogen, South Korea) using primers B5A, B6A, B7, and B8.

Sequences of the cDNA were analyzed by using the Bioedit program version 7.2.5 and blasted against other published BRVS sequences from the GenBank using NCBI BLAST (BLASTn) (available at http://www.ncbi.nlm.nih.gov). Sequences similarity analyses were performed using online multiple sequences alignment-CLUSTALW (GenomeNet) (available at https://www.genome.jp/tools/clustalw). Multiple sequence alignment was done employing ClustalX (NCBI) program and the phylogenetic tree was generated using the same ClustalX (NCBI) and Neighbor-joining (NJ) programs.

Table 1. Epidemiological and demographic data of animal population in the study

Factors	Category	Numbers of animals
Type of breeding	Beef cattle	191
	Dairy cattle	259
Animal origin	Native	191
	Non-native (Imported)	259
Age	≤7 months	85
	7 months -1.5 years	160
	1.5 - 4 years	175
	> 4 years	30
Sex	Male	141
	Female	309
Herd size	Small (≤ 10)	191
	Large (≥ 20)	259
Season	Fall	86
	Winter	143
	Spring	107
	Summer	156
Geographical area (Nineveh province, Iraq)	Western region	42
	Southern region	86
	Eastern region	254
	Northern region	77

RESULTS

Results of the amplification of the RNA of the BRSV isolated from 450 nasal swab samples demonstrated prevalence rate of 37.31% by using specific primers B5A and B6A of G protein gene in the first reaction (Figure 1) and specific primers B7 and B8 of G protein gene in the second reaction (Figure 2).
Finally, one sequence of BRVS was deposited in GenBank using Bankit submission tool (available at http://www.Ncbi.nlm.nih. gov/WebSub/?tool= GenBank), under the accession numberMN129181 (Table 2). In this study, the homology between variable BRSV sequences obtained from nasal swabs and GenBank database

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demonstrated that sequences were highly related (99 % identity) to sequences obtained in Croatia (KY680337.1; KY680336.1 KY680335.1), and found to have a lower identity (84%) with Brazilan sequence (FJ543090.1) (Table 3).
G gene sequences of the local isolate of BRSVwas in a major clad comprising the previously published genotypes of BRSV (KY680337.1, KY680336.1, and KY680335.1) isolated in Croatia were present in the current study (Figure 3). The phylogenetic tree of BRSV demonstrated one genetically distinguishing genotype (MN129181,Mousl,Iraq). The tree was rooted with (NC 038272.1,USA) as an outer group (Figure 3).

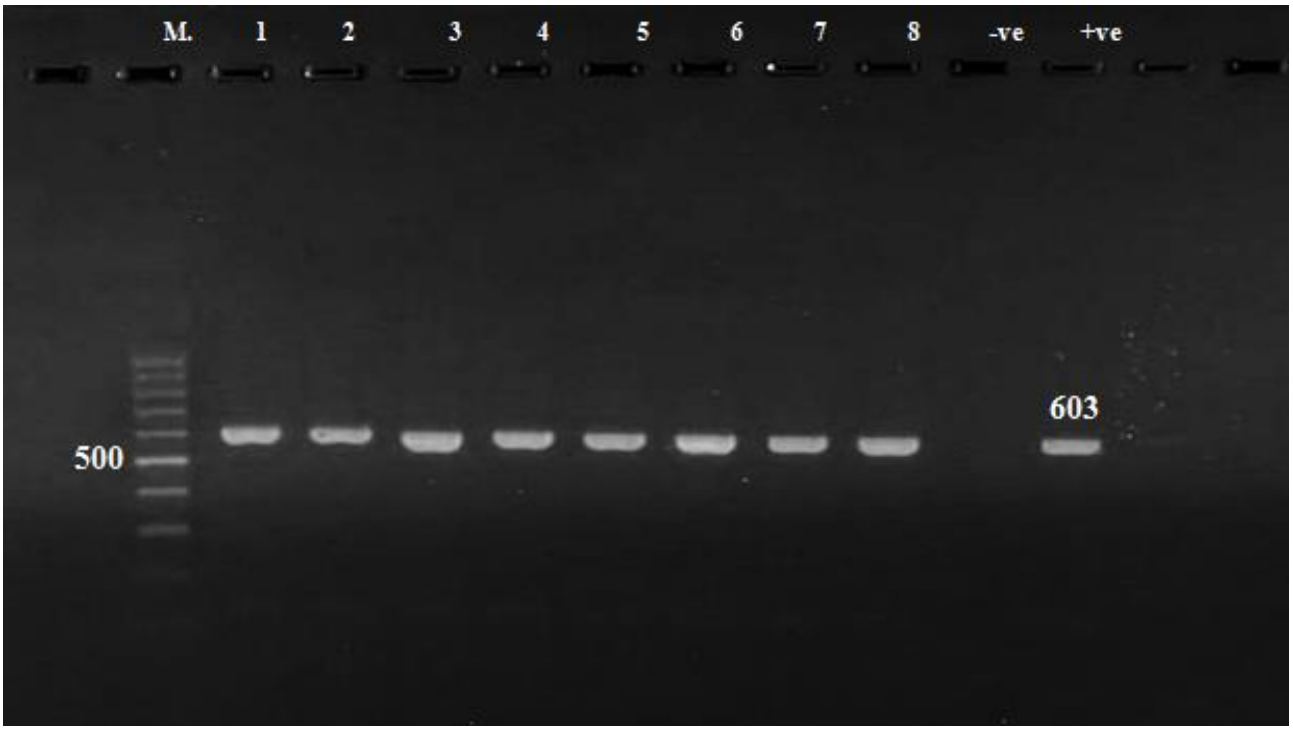

Figure 1. Gel electrophoresis of first RT-PCR reaction products using specific primers B5A and B6A of G protein gene for detection of Bovine Respiratory Syncytial Virus (BRSV) from nasal swabs of cows. lane M) Exact Mark 100-1000bp DNA ladder; Lane 1-8) positive samples 603 bp; Lane –ve) cDNA extracted from BRSV-free calf used as negative control; Lane +ve) cDNA extracted from BRSV-infected calf used as positive control.

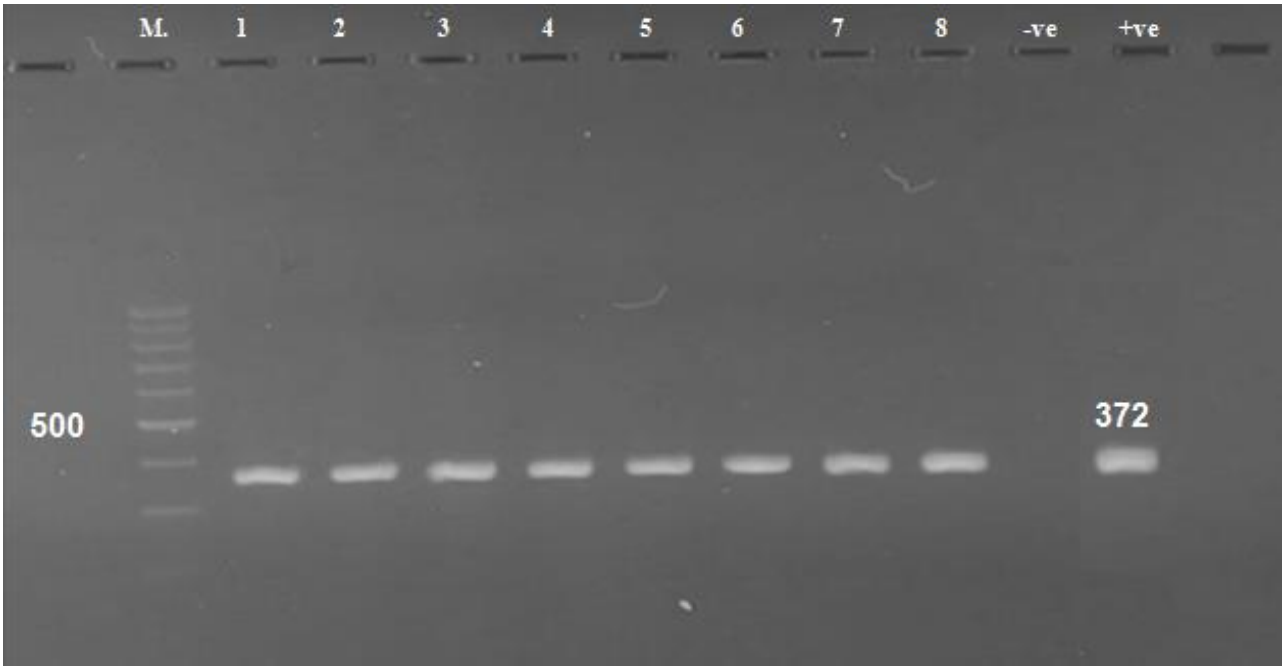

Figure 2. Gel electrophoresis ofsecond RT-PCR reaction products using specific primers B7 and B8 of G protein genefor detection of Bovine Respiratory Syncytial Virus (BRSV) from nasal swabs of cows. lane M) Exact Mark 100-1000 bp DNA ladder; Lane 1-8) positive 372 bp; Lane –ve) negative control; Lane +ve) positive control for BRSV.

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Table 2. Nucleotide sequencing of G gene of bovine respiratory syncytial virus isolated from cattle in Nineveh province, Iraq

Accession number in GenBank	Size (base pair)	Gene sequencing (5' to 3')	Gene name
MN129181	545	ATTTACATTAGTGTGGGTTTGCCAAAGCCAAGCCCACATCT AAACCAACCATCCAACAAACACCACAGCCCCTAAACCATAC CTTATCATTTTTCACAAAGCACAACCCCTAATCAACTCACAC ATCATTTCAAAGCACCACACTGTCCCGACCACCAAACATAG ACACCACTAGAGGAGCTACACACAGTCACTCAACCAATGA AACCCAAAACAGAAAAACCAAAAGCCAATCCACTCTACCT GCCACCAAAAAACCACTAACCTACTCATCAGGAAGCAATCC CTCCAAAAACCATCAAGACCAAACTAACCTCCAAACACTCC CCCATGTGCCTTGTAGTACTTGTGAAGGTAATCTTGGTTTGC TTATGACTCTGCCAGATTGGGCTAGAGAGAGCATCAAGCAG AGCCCCCACAATCACCCTTAAAAAGACACCAAAACTCAAA ACCACTAAAAAGCCAATCAAGGCAACAATCCACCACAGAA CCAGCCCCGAAGCTAAAATGCAACCTAAAAACAACACGGC AACTCCACAGCAAGG	Glycoprotein (G)

Table 3. Homology between glycoprotein (G) gene of local bovine respiratory syncytial virus obtained in this study and GenBank database using online sequence BLASTn

GenBank accession number	Country	Sequence identity	GenBank accession number	Country	Sequence identity
KY680337.1	Croatia	99%	MH133327.1	Turkey	90%
KY680336.1	Croatia	99%	MH133326.1	Turkey	90%
KY680335.1	Croatia	99%	M58307.1	United Kingdom	90%
L08414.1	USA	93%	AF188582.1	France	90%
AY910756.1	Czech Republic	93%	AF188584.1	France	90%
AY910755.1	Czech Republic	92%	Y08719.1	United Kingdom	89%
L08416.1	USA	91%	Y08717.1	United Kingdom	89%
L08415.1	USA	91%	KF501149.1	Norway	89%
U24716.1	Belgium	91%	FJ555202.1	Brazil	88%
U24715.1	Belgium	90%	Y11205.1	United Kingdom	86%
NC_038272.1	USA	90%	FJ543090.1	Brazil	84%
AF092942.1	Germany	90%			

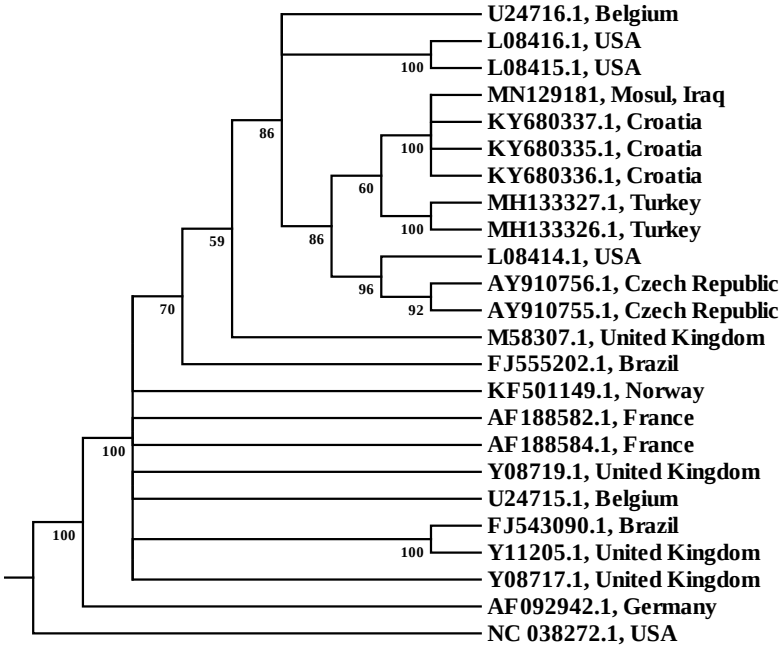

Figure 3. Phylogenetic tree of bovine respiratory syncytial virus obtained with partial sequences of G gene. The numbers on the branches indicate bootstrap supports (100 replications). GenBank accession numberMN129181 was obtained in this study.

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DISCUSSION

Diagnosis of BRSV isolated from nasal samples was determined using nested RT-PCR technique through a couple of primers target against (G) gene in nasal samples which indicate the accuracy and efficacy of the PCR technique for identification of BRSV in naturally infected cattle. This method can be used as a powerful technique in epidemiological studies, as reported in previous studies (Vilcek et al,1994; Valentova et al., 2003; Almeida et al., 2006).

The result obtained in this study demonstrated that the G gene is very conservative and can be utilized as a target sequence for the identification of BRSV through RT-PCR. This result is in agreement with Socha and Rola (2013).

This study indicated that several nucleotide sequences have a great similarity with the local isolation virus. These results are consistent with the results of other researchers as they observed a high percentage of similarity in their isolation compared to what is recorded in the GenBank (Socha, et al., 2009; Klem et al., 2014; Bertolotti et al., 2018; Krešića et al.,2018).

The phylogenetic tree for the local isolate of BRSV showed the high degree of genetic linkage between isolates, indicating the geographical location, as well as the importation of animals from different parts of the world, plays an important role in the re-distribution of genetic bonds between the isolates in different geographical regions. This is consistent with the findings of Yaegashi et al. (2005) and Hacioğlu et al. (2019).

CONCLUSION

In conclusion, the current study provides information on the molecular characterization of BRSV in Mosul, Iraq, which may be useful for future studies on the epidemiology of this infection and the selection of effective vaccines.

DECLARATIONS

Acknowledgments

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Authors' contributions

Maab Ibrahim AL-Farwachi designed the study. The collection of samples was done by Khder Jassiem Hussain and Sadam Dhahir Hassan. All samples were analyzed in a laboratory by Khder Jassiem Hussain. Maab Ibrahim AL-Farwachi and Khder Jassiem Hussain interpreted the results. Finally, after careful consideration, the manuscript was approved by all authors.

Competing interests

The authors declare that they have no competing interests.

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Evaluation of Growth Performance, Blood Metabolites and Gene Expression Analysis in Egyptian Sheep Breeds, in Relation to Age

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ABSTRACT

The growth performance of lambs attributes the economic viability of animals. Faster growth allows lambs to reach maturity in early age. Therefore, the aim of this study was to compare growth performance, blood metabolites and expression of IGF-1, GH, and Leptin genes in three different Egyptian sheep breeds across age. Thirty Egyptian sheep males from three breeds (Ossimi, Rahmani and Barki) were divided into three ages categorize (7 – 9, 10 – 12, and 13 - 16 months). The results showed that there was a significant increase in sheep's live body weights toward advanced ages till the second age category for all breeds, the highest values of linear body measurements were observed in Ossimi breed. There was a non-significant inverse effect of advanced age on blood glucose and total lipids levels in all sheep breeds. There wasn't any significant effect of interaction between age and breed on plasma total protein concentrations. According to age categories, Barki breed showed a significant up-regulation of GH compared to the Ossimi breed in 7-9 months age category. However, Barki breed showed a significant down-regulation of IGF-1compared to the Ossimi breed in 7-9 months. Meanwhile, Leptin expression showed significant differences in Ossimi breed between 10-12 months age category and two other age categories. We concluded that measuring of physical body measurements, blood metabolites and GH, IGF-1 and Leptin genes in early ages is a good and accurate indicator for growth performance in Egyptian sheep breeds.

Key words: Blood metabolites, Egyptian breeds, Gene expression, Growth performance, Linear body measurements

INTRODUCTION

In Egypt, sheep breeding forms a great part of the agricultural economy, where animals are raised mostly in rural farmers. Egyptian sheep are important animal genetic resources, they are raised basically for mutton production, while wool and milk consider as secondary products (FAO, 2017). They are the third source of red meat in Egypt after cows and buffaloes, which considered the strategic stockpile of food security (EL-Hanafy and El-Saadani, 2009, El-Malky et al.2019).

Egyptian breeds are fat-tailed that characterized by extended breeding seasons, high fertility, and well adapted to harsh environmental conditions (Galal et al., 2005; EL-Hanafy and El-Saadani, 2009 Mahrous et al., 2016, Barakat et al., 2017; El-Malky et al., 2019).The total number of Egyptian sheep breeds is raised from 5,463,169 head in 2015 to 5,697,716 head in 2017. Sheep provide 72,296 tons of meat, they contribute 6% of the total red meat produced in Egypt and 99,322 tons of fresh milk (FAO, 2017). The growth rate of lambs is an important production trait and it reflects the economic viability of animals, which has a great role in a sheep production process. It is primarily influenced by a complex system such as genetics, immune responses, physiological status, and endocrine factors, in addition to other non-genetic factors (Grochowska et al., 2017, Singh et al., 2018; Veena et al., 2018).

There are many also non-genetic factors, such as nutrition, heat temperature and management system which controlling the phenotypic expression of the growth. Identification of such factors is important for adjustment to analyze genetic parameters and better planning for herd management (Dixit et al., 2011). Gene expression analysis allows to identifying genes that are responsible for the expression of interested economic traits. It is a very useful technique in early prediction and comparing animals with different economically important characteristics like growth traits (Dunn and Ryan, 2015). The growth hormone (GH), insulin-like growth factor-1 (IGF-1) and leptin genes are candidates for growth in ovine since they play a key role in growth regulation and development (Barzehkar et al. 2009, Nazari et al., 2016; Mahrous et al., 2016).

Measuring of gene expression and blood metabolites levels is a reliable method for evaluation sheep growth performance and health status in addition to it is a useful way for judging of individual animal physiological status,

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diagnosis of metabolic disorders, clinical nutritional balances, deficit condition, treatment monitoring and prognostics (Nath et al., 2014; Beigh et al., 2018). The mortality of pre- and post-weaning lambs, lower growth rates, and lack of feed are major problems facing most sheep farmers and they are main factors which have a negative effect on total productivity in sheep husbandry and represents an economic loss (Saleem et al., 2017; Elshazly and Youngs, 2019).

So, the aim of this study was to compare growth performance, blood metabolites and expression of IGF-1, GH, and Lepton genes in three different Egyptian sheep breeds across age.

MATERIALS AND METHODS

Ethical approval

The present work has been conducted in accordance with the guidelines of the ethical committee of Faculty of Agriculture – Cairo University. The experimental fieldwork was carried out in the Agriculture Experimental Station, Faculty of Agriculture- Cairo University. Whereas gene expression and blood metabolite analyses were executed at Cairo University Research Park, Faculty of Agriculture-Egypt.

Experimental animals and diets

Thirty Egyptian sheep males from (7 Ossimi, 8 Rahmani and 15 Barki) breeds were raised from age of 7 months till 16 months. The period of rearing was divided into three age categorize (7 – 9, 10 – 12, and 13 - 16 months). The average body weight of the experimental animals within each group was (33.41 ± 2.3, 22.5 ± 1.9 and 24.9 ± 4.9 kg respectively). Sheep were fed according to (NRC, 2002) and drinking water and mineral blocks was continuously available. All animals kept under the same managerial conditions.

Growth traits measurements

Physical measurements including body weight, body length, hip height, and heart girth were measured monthly during this study. Body length was considered as the distance between the point of shoulder and pin bone (Afolayan et al., 2006). Heart girth was the circumference measurement taken behind the fore legs (Ibrahim, 2015). Hip height was measured from hips up to the end of hoof. It is a vertical distance from the highest point of the hips to the ground surface at the level of the rear legs (Afolayan et al., 2006).

Blood sampling

Blood samples were collected monthly from each individual animal in the morning before feeding. Ten ml of blood were collected from the jugular vein in a heparinized (15 ml) tube. Eight ml from each sample were used for biochemical assays and two ml were used for gene expression analysis. For biochemical assays, blood samples were centrifuged at 3000 rpm for 20 min, the supernatant of clear plasma was aspirated and placed into capped Eppendorf tubes and stored at -20ºC till use.

Biochemical assays

Glucose concentration (mg/dl) was measured colorimetrically using Glucose kit (Bio-Diagnostic Dokki, Giza, Egypt) as described by Trinder (1969). Total protein content (g/dl) was determined by using Protein Biuret Kit (Bio-Diagnostic Dokki, Giza, Egypt) as mentioned by Gornal et al. (1949) and total lipids (mg/dl) were analyzed using Total Lipids Kit (Bio-Diagnostic Dokki, Giza, Egypt) according to Zollner and Kirsch (1962). Colorimetric analysis for all biochemical assays has been done using STAT LAB SZSL0148 (ver 5.5 SPECTRUM).

Gene expression analysis

RNA isolation and cDNA synthesis

Total RNA was isolated from blood samples using QiaAamp RNA blood mini isolation kit (Qiagen, Clinilab, Cairo, Egypt) according to the manufacturer's instructions. Briefly, one ml of whole blood was mixed with five ml extraction buffer, incubated on ice for 10 min and briefly mixing by vortexing two times during incubation. Leukocytes pellet was obtained after centrifugation at 400 xg for 10 min at 4 ˚C. The collected pellet was suspended in 2 ml extraction buffer followed by vortexing and centrifugation at 400 xg for 10 min at 4 ˚C. Afterward, washing buffer 600 ul was added and mixed by vortexing. The extract was loaded onto a purification column and centrifuged to allow for the RNA to bind to the spin column. The column was washed twice with washing buffer and finally eluted with 30 µl RNase free water. For each sample, cDNA synthesis has been performed using oligo (dT) 23 primer, random primer and superscript reverse transcriptase II (Thermo Scientific, Sigma Scientific Services, Giza, Egypt). One µl of oligo (dT) 23 primer and one µl random primer were added to 10µl RNA sample and the mixture was incubated for 3 min at 70˚C and then immediately chilled on ice. Eight microlitre of the master mix containing 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl of dNTP (10 pmol/µl) and 0.3 µl of RNase inhibitor and 0.7 µl of Super Script IITM reverse transcriptase (200

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unit/µl) was added to the mixture and incubated for 90 min at 42˚C followed by heat inactivation for 15 min at 70˚C. The synthesized cDNA was stored at -20˚C for further use.

Quantitative real-time PCR analysis

Primers were designed using Primer3 software (Table 1). Quantitative analyses of cDNA samples were performed on Step One Plus™ instrument (Applied Biosystems). Independent qRT-PCR (4 animals each age group) was performed in a 20 µl reaction volume containing Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Sigma Scientific Services, Giza, Egypt), the cDNA samples and the specific forward and reverse primers. The thermal cycling parameters were set at 95°C for 3 min., 40 cycles of 95°C for 15 sec. and 60°C for 1 min. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7-second interval until the temperature reached 95°C. The comparative cycle threshold (CT) method was used to quantify expression levels as previously described (Liu, 2007). Expressions of three different transcripts (G.H, IGF-1, and Leptin) were analyzed using real-time PCR and fold changes were calculated using ΔΔCt method according to Kelly et al. (2012) Lamas et al. (2018) and Samara et al. (2008).

Table 1. Details of primers used for quantitative real-time PCR.

Gene sympol	Gene bank accession number	Primer sequences	Annealing temperature (˚C)	Reference
GH sheep	(NM_001009315)	F: 5´- GGCCCAGCAGAAATCAGACT -3´ R: 5´- CTTGAGCAGCGCATCATCAC -3	55	(Jia et al., 2014)
IGF-1	(NM_001009774.3)	F: 5´-TCGCATCTCTTCTATCTGGCCCTGT -3´ R: 5´- GCAGTACATCTCCAGCCTCCTCAGA -3	55	(Rotwein, 2017)
Leptin	(XM_027968780.1)	F: 5´- GCCTATGTGGGCATCCTTTA -3´ R: 5´- TGGAACAGGGAGGAAGACTG -3	55	(Shojaei, et al., 2011)
GAPDH	(NM_001190390.1)	F: 5´- ATCAAGTGGGGTGATGCTGG -3´ R: 5´- GGCGTGGACAGTGGTCATAA -3´	55	(Vorachek et al., 2013)

Statistical analysis

Statistical analysis of data was carried out applying SAS package (2008). All values were expressed means and standard errors (SE). The significance was measured according to Duncan Multiple Range test (1995). The statistical model was used as following: $Y_{ijk} = \mu + B_i + G_j + (B*G)_{ij} + e_{ijk}$

Where, Y is the dependent variable under study, µ is the overall mean, B and G indicate the fixed effect of breed and age category, respectively. B*G indicates the interaction between breed and age category and e is the random error assumed N. I. D. (0, δ2).The mRNA transcriptional quantity was indicated as the average ± standard error. A histogram of DCt was used to verify the conclusion that the value of DCt had a negative relationship with transcriptional quantity. The relative gene expression level was analyzed using $2^{-\Delta\Delta Ct}$ method which has been described by Liu (2007). The mean values were compared for statistical significance using Duncan's range test (Duncan, 1955). Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Growth traits

Live body weight

Data on the effect of different age categories first category (7-9 months), second category (10-12 months) and third category from (13-16 months) and sheep breeds (Ossimi, Rahmani, and Barki) on physical body measurements were calculated as (mean ± SE) in table 2. The results of this study indicated that there was a significant increase in sheep's live body weights toward advanced ages till the second age category for all breeds, followed by non-significant increasing at the third category. Meanwhile, there was a significant effect of breed between Ossimi breed and two other breeds, where live body weight for Ossimi breed was bigger (45.25 ±1.98 Kg) than two other breeds (43.89 ± 1.17 for Rahmani and 44.11 ± 1.68 for Barki), and there weren't any significant differences in live body weight between Rahmani and Barki breeds at three age categories. Meanwhile, there was significant effect of interaction between age and breed on live body weight ($P<0.05$).

Body length

The observed measures of sheep's body length showed that there was a non-significant positive trend of body length in Ossimi breed with all age categories. Furthermore, Barki and Rahmani breeds had a non-significant (somewhat positive) effect of advanced age categories. Ossimi breed had a greater value of body length (97.93 ± 4.08 cm) followed

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by Rahmani breed (95.43 ± 1.58 cm), while Barki breed had the lowest value of body length (91.50 ± 3.57 cm). Present results indicated that there wasn't any effect of sheep breed on sheep's body length at all age categories. Moreover, the effect of interaction between sheep age and breed was insignificant.

Hip (rump) height

The results of sheep's hip height, indicated that there was a positive significant effect of age and breed between Ossimi and Rahmani sheep till the second age category, while at the third age categories no significant differences of age and breed ($P<0.05$) were found between all sheep breeds. The highest value of hip height was observed in Ossimi breed followed by Barki breed and the lowest ones were measured in Rahmani. In addition to there wasn't any significant effect of interaction between age categories and breed on animal's hip height.

Heart girth

Heart girth positively increased according to age categories for all sheep breeds. It was found that Ossimi breed significantly affected ($P<0.05$) the values of heart girth comparing to two other breeds at first age category, while at second age category there was a significant difference between Ossimi sheep and Rahmani and at the third category there was significant difference between Ossimi and Barki. The largest value of heart girth was observed in Ossimi breed (85.25 ± 0.95 cm), whereas there wasn't any clear effect of breed between Rahmani and Barki breed at all age categories. However Rahmani breed has a greater value of heart girth (79.93 ± 1.70 cm) comparing to Barki breed (77.86 ± 2.02 cm). Furthermore, no significant effect of the interaction between age categories and breed was observed on sheep's heart girth.

Table 2. Physical body measurements (mean ± SE) in Egyptian sheep breeds and age categories

Breed	Age (month)	Live body weight (Kg)	Length (cm)	Hip height (cm)	Heart girth (cm)
Ossimi	**7-9**	$33.42 \pm 0.97^{b,A}$	93.50 ± 2.97	$69.83 \pm 1.11^{a,A}$	$77.83 \pm 1.87^{b,A}$
	10-12	$44.93 \pm 1.82^{a,A}$	97.57 ± 3.40	$71.43 \pm 2.36^{a,A}$	$82.29 \pm 1.49^{ab,A}$
	13-16	45.25 ± 1.98^{a}	97.93 ± 4.08	71.25 ± 1.11^{a}	$85.25 \pm 0.95^{a,A}$
Rahmani	**7-9**	$22.50 \pm 0.96^{c,B}$	93.50 ± 0.50	$61.75 \pm 2.25^{b,B}$	$66.00 \pm 1.92^{b,B}$
	10-12	$33.38 \pm 0.95^{b,B}$	91.42 ± 3.55	$64.67 \pm 0.92^{b,B}$	$72.92 \pm 2.88^{ab,B}$
	13-16	43.89 ± 1.17^{a}	95.43 ± 1.58	69.36 ± 1.01^{a}	$79.93 \pm 1.70^{a,AB}$
Barki	**7-9**	$24.90 \pm 1.48^{c,B}$	87.60 ± 4.38	$62.00 \pm 1.38^{b,B}$	$64.90 \pm 1.28^{b,B}$
	10-12	$35.90 \pm 1.30^{b,B}$	93.83 ± 2.74	$69.06 \pm 1.15^{a,A}$	$75.33 \pm 2.21^{a,AB}$
	13-16	44.11 ± 1.68^{a}	91.50 ± 3.578	70.07 ± 1.39^{a}	$77.86 \pm 2.02^{a,B}$

^{a,b,c}Significant differences ($P<0.05$) between age categories among each breed. ^{A,B,C} Significant differences ($P<0.05$) between breeds at each age category.

Blood metabolites

Glucose

The values of the blood parameters in relation to sheep age categories and breeds are presented in table 3. The results of the current study showed that there was a non-significant inverse effect of advance age and blood glucose levels in all sheep breeds. In addition to there wasn't any clear effect of sheep breed on plasma glucose concentrations. The highest values were measured in Barki breed (60.63 ± 2.74 mg/dl) and the lowest one was reviewed in Rahmani breed (50.90 ± 3.94 mg/dl). Meanwhile, there wasn't any significant effect of interaction between age and breed on plasma glucose concentrations.

Total protein

The presented data in table 3 for Ossimi, Rahmani and Barki breeds during the period from (7- 9), (10- 12) and (13- 16) months respectively showed that there were a significant inverse differences on plasma total protein between the first and third age categories for Ossimi breed, and in Rahmani breed, there were significant inverse differences between first age category and to other categories, while in Barki breed there weren't any significant differences between all age categories.The current results concluded that there weren't any significant differences on plasma total protein concentrations during the first and third age categories for all sheep breed. While during the period from 10-12 months there was a significant effect of breed between Ossimi and Rahmani. According to present results, Rahmani breed had a greater value (7.48 ± 0.28 g/dl) of total protein compared to two other breeds. Meanwhile, there wasn't any significant effect of interaction between age and breed on plasma total protein concentrations.

Total lipids

The results of this study showed that there was a non-significant negative trend of advanced age on total lipids levels in Ossimi sheep, whereas in Rahmani breed there was no clear effect of age on blood total lipids concentrations, while Barki breed showed a non-significant positive effect of age on blood total lipids concentrations. The results of this study showed that there wasn't any significant effect of sheep breed on plasma total lipids. Meanwhile, there was a significant effect of interaction between age and breed on total lipids ($P=0.0021$).

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Table 3. Blood metabolites (mean ± SE) in Egyptian sheep breeds and age categories

Breed	Age (month)	Glucose (mg/dl)	Total Protein (g/dl)	Total Lipids (mg/dl)
Ossimi	7-9	57.43 ± 6.94	7.19 ± 0.50^{a}	822.94 ± 24.40
	10-12	55.65 ± 8.47	7.01 ± 0.27^{ab,A}	820.61 ± 18.37
	13-16	50.97 ±7.41	5.88 ± 0.23^{b,B}	763.04 ± 21.02
Rahmani	7-9	50.90 ± 3.94	7.48 ± 0.28^{a}	771.63 ± 9.41
	10-12	55.67 ± 4.48	6.18 ± 0.20^{b,B}	844.89 ± 31.77
	13-16	54.75 ±3.77	6.58 ± 0.21^{b,AB}	807.07 ± 14.87
Barki	7-9	58.03 ± 3.00	6.73 ± 0.31^{a}	806.36 ± 16.10
	10-12	60.63 ± 2.74	7.06 ± 0.23^{a,A}	819.76 ± 17.32
	13-16	53.80 ±1.96	7.01 ± 0.26^{a,A}	832.70 ± 28.77

^{a,b,c} Different letters indicate significant differences (P<0.05) between ages at each breed.^{A,B,C} Different letters indicate significant differences (P<0.05) between breeds at each age category.

Gene expression analysis

Growth hormone (GH) gene

Among the age categories, the Barki breed showed a significant up-regulation of GHcompared to the Ossimi breed in the 7-9 months age category. Rahmani breed showed a significant up-regulation of GHcompared to the Ossimi breed in the 13-16 months age category. However, there were no significant differences between all sheep breeds in the analyzed age category (10-12 months) (Figure 1A). Among each breed, the Ossimi breed showed a significant down-regulation of GH expression level in the age category 13-16 months compared to the other ages. However, Rahmani and Barki breeds showed no significant differences across the different analysed age categories (Figure 2B).

Insulin-like growth factor-1 (IGF-1) gene

Among the age categories, the Barki breed showed a significant down-regulation of IGF-1compared to the Ossimi breed in the 7-9 months age category and Ossimi and Rahmani breeds in the 10-12 months age category. However, there were no significant differences between Ossimi and Rahmani breeds in the two analysed age categories (10-12 and 13-16 months) (Figure 2A). Among each breed, there were no significant differences in IGF-1 expression levels across the different age categories. However, Ossimi and Rahmani breeds tend to have a pattern of higher expression level in the age category 10-12 months but Barki breed tends to have lower expression in the 10-12 age category compared to 7-9 months age category (Figure 2B).

Leptin gene

Among the age categories, the Barki breed showed a significant up-regulation of leptincompared to the Ossimi breed in the 7-9 months age category, while Rahmani breed showed a significant down-regulation in the 10-12 months age category compared to Ossimi and Barki breeds. At the 13-16 months age category, Rahmani breed showed a significant up-regulation of leptincompared to the Ossimi breed (Figure 3A). Ossimi breed showed a significant down-regulation of Leptinexpression level in the age categories 7-9 and 13-16 months compared to the 10-12 months age category. The other two breeds didn't show any significant differences among the analysed age categories (Figure 3B).

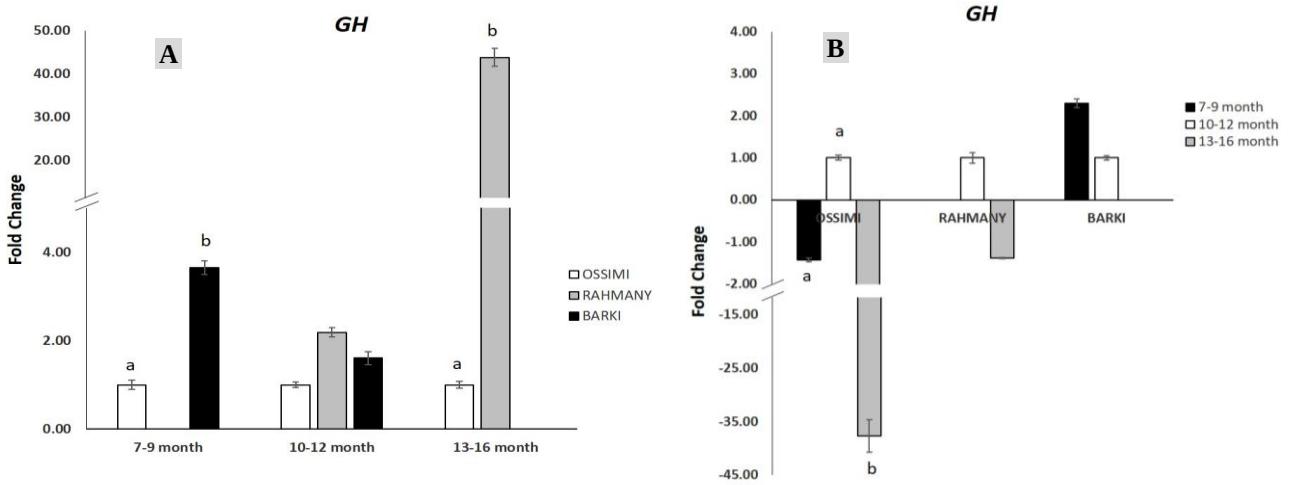

Figure 1. Expression differences in *GH* between Egyptian sheep breeds (A) or age categories among each breed (B). ^{a,b,c} Different letters indicate significant differences (P<0.05).

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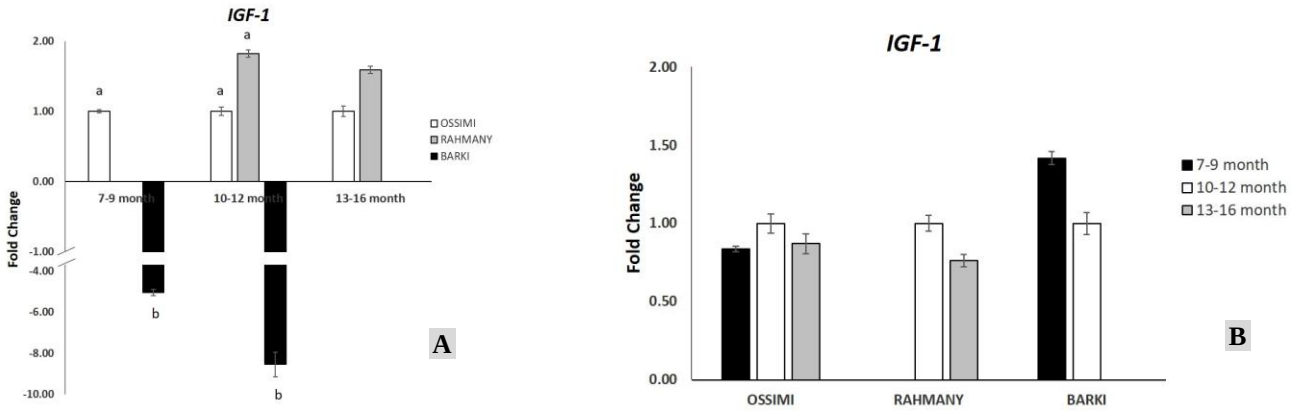

Figure 2. Expression differences in *IGF-1*between Egyptian breeds (A) or age categories among each breed (B). [a,b,c]Significant differences (P<0.05).

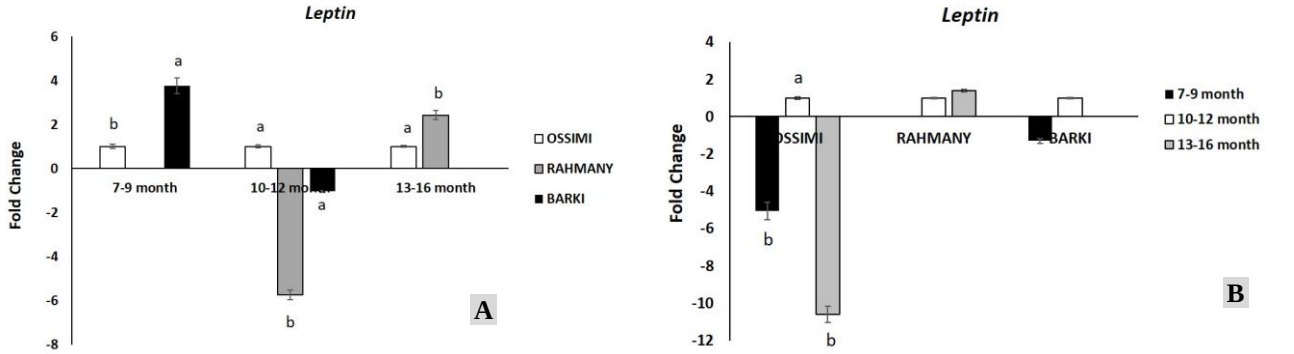

Figure 3. Differences in *Leptin* gene expression between Egyptian sheep breeds (A) or age categories among each breed (B). [a,b,c] Different letters indicate significant differences (P<0.05).

DISCUSSION

Growth traits

Measuring body weight is an accessible and necessary way to judge the value of lambs, the efficiency of raising and measure of economic profit. The identification of relationships between body weight and (breed, age, and body measurements) is very useful for selecting faster-growing animals.

Present results indicated that Ossimi sheep have the largest value of live body weight (45.25 ± 1.98 Kg) at the third age category, and there weren't any significant differences between Rahmani and Barki breeds. The results of this study are in harmony with those previously reported by Almahdy et al. (2000), Marai et al. (2009), Khalifa et al. (2013), Hassan (2017) and El-Malky et al. (2019). They mentioned that the average live body weights were ranged from (51-53 kg) adult weight for the Ossimi and Rahmani breeds, and (44 kg) for the Barki breed. Another agreement obtained by Khalifa et al. (2013) they found a positive effect of advanced age for Rahmani males live body weight. Furthermore, Marai et al. (2009) and El-Malky et al. (2019) reported that breed had a significant (P<0.01) effect on live body weight as Ossimi breed showed heavier weight than Rahmani and Barki breed.Another point of view reported by El-Tarabany et al. (2017) they mentioned that there was no difference in live body weight between Ossimi and Rahmani breeds; however, Rahmani breed had a higher body weight compared to Ossimi breed. Body length differs according to many factors such as breed, gender, yield type, and age. According to Atta and El Khidir (2004), body length and height at wither were less variable measurement for predicting sheep live body weight.

The results of body length in current study are in the agreements with results obtained by Gad (2014) and Ibrahim (2015) who found that average body length of Ossimi and Rahmani breeds was (71.5 and 72.3 cm) at 12 months, and the average of body length for Barki lambs at 9 months range from (71 to 75 cm), while, Abd-Alla (2014) reported that the average of body length for Barki sheep was (70.6 cm) at 12 months.

Similar results obtained by Abdel-Moneim (2009) found that there weren't any significant differences in body length according to advanced ages and sheep breeds. Furthermore, Afolayan et al. (2006), Mahmud et al. (2014) and Abdullah et al. (2015) found a strong positive relationship between advanced age and all body measurements for Yankasa sheep, Nigerian, and Awassi sheep breeds respectively. Also, Shirzeyli et al. (2013) reported that there wasn't

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clear effect of sheep breed in sheep's body length and the average body length for four sheep breeds was (46.28 cm for Mehrabani sheep, 52.50 cm for Shaal sheep, 50.96 cm for Macoei sheep and 54.64 cm for Zandi sheep).

The results are in the agreements with results obtained by Afolayanet al. (2006) and Mahmud et al. (2014) found a strong positive relationship between advanced age hip height for Yankasa sheep and Nigerian sheep breeds respectively. Same values reported by Musa et al. (2012) who found that the average height at the hip for Shugor sheep at 8 months of age was 72.98 cm. In addition, Mahmud et al. (2014) reported that average body height for Nigerian sheep breeds was 57.09 cm for males and 46.56 for females from birth to less than 12 months and it was 59.07 cm and 59.76 cm for males and females respectively during the age from 13- 24 months.

The results of this study are in the harmony with results mentioned by Salako (2006) he found that there are a significant differences between sheep breed in average rump height for West African Dwarf and Yankasa sheep, the hip height was 59.20 cm for West African Dwarf and it was 72.57 cm for Yankasa sheep, which indicates that the Yankasa sheep is genetically bigger than West African Dwarf the sheep. Same values reported by El-Shahat et al. (2014) reported that average heart girth for Rahmani lambs were (76.57 cm) during the period for 6 to 12 months. Also, Depisonet al. (2017) indicated that the average heart girth for high land thin-tailed sheep males.

Other results by Shehata (2013) that recorded the average heart girth for Barki lambs at 12 months (99.58 cm). Another point of view noticed by Abd-Allah et al. (2018) who reported that the average of heart girth for males and females Assaf sheep was 105.40 cm it was 101 cm respectively from 6 to 18 months. Another view reported by Abdel-Moneim (2009) and Shirzeyli et al. (2013) they found that there weren't any significant differences in heart girth with sheep breeds and advanced ages in Egyptian and Iranian sheep breeds respectively.

Using heart girth measurement to estimate the live body weight of the sheep′s would help the breeders to calculate the amount of ration to feed and dose rate for medication. To increase meat yield from this type requires genetic improvement of its live body weight. Proper and accurate measurement of this morphometric trait is requisite for achieving this objective (Musa et al., 2012).

The results of present study indicated that the highest values of linear body measurements were observed in Ossimi breed and the recorded values of Barki and Rahmani breeds were very similar between both breeds. However, Rahmani breed had greeter values of body measurement comparing to Barki breed. The highest values of linear body measurements for Ossimi sheep may be referred to as that Ossimi breed had the heaviest body weight comparing to two others breed. Abdel-Mageed and Ghanem (2013), Shehata (2013), Agamyet al. (2015) and Ahmed et al. (2017) also found positive and significant ($p<0.01$) correlation coefficients between live body weight in 12 months reported similar results between body weight and body measurements.

Blood metabolites

The results of this study are similar to results that obtained by Nour El-Dinet al. (2009), El-Shahat et al. (2014) and Shaker (2014) who reported that plasma glucose level for Awassi sheep was (54 mg/ dl) at 164 days, for Rahmani lambs was (50-55) during the period from 6 to 12 months, and for Barki ewes ranged from (39.8 to 46.93 mg/dl), respectively. While Cruz et al. (2017) reported that, plasma glucose levels for Dorper sheep were 85.4 (mg/ dl) from 15 to 30 days of age, 78.6 (mg/ dl) from 45 to 61 days of age, 65.6 (mg/ dl) from 74 to 90 days of age and 52.1(mg/ dl) from 105 to 121 days of age.

Anwaret al. (2012) reported a lower level of blood glucose compared to present results they reported that the mean values of plasma glucose for Egyptian sheep breeds were (45 for Barki, 44.15 for Rahmani and 48.01 mg/dl for cross breed Rahmani and Barki). Another point of view reported by Carloset al. (2015) they found that the values of glucose levels were not affected by the age in Morada Nova sheep during the period from 6 up to 15 months.

Many studies reported that there is an inverse relationship between lambs' age and blood glucose concentration, the higher concentration of blood glucose with early age may be due to stress of new lambs or may also be due to milk suckling as an important source of energy for newborns, as the consumption of nutrients through suckling directly interferes with the absorption and metabolism of carbohydrates (Braun et al., 2010; Abdel-Fattah et al., 2012; Abdel-Fattah et al., 2013; AL-Hadithy and Badawi 2015; Cruz et al., 2017; Rahman et al., 2018).

Similar results of breed effect obtained by El-Malkyet al. (2019) who found there wasn't a significant effect of breed on glucose levels. However, Barki breed had a higher concentration of blood glucose compared to Ossimi. Also, Stempaet al. (2016) showed that no significant differences were found between breeds (Dopper and Marino) on blood glucose. Same values of blood proteins were reviewed by Saleem et al. (2017), Rahman et al. (2018) and El-Malky et al. (2019) reported that average plasma total protein level for Saidi lambs, for Bangladesh sheep and for Ossimi and Barki was ranged from (6.07 to 7.59 g/dl) respectively. Also, Soliman (2015) and El-Bassiony (2016) mentioned that the average of blood total protein level 6.93 g/dl for Ossimi males and it was 6.80±0.13g/dl in Barki ewes. Same trend reported by Carlos et al. (2015) found that the age of the sheep significantly affected inversely ($P<0.05$) the values of blood protein during the period from 6 -12 month (7.84±0.32 g/dl) to (5.61±0.15 g/dl) at the period more than 12 months of age in Morada Nova sheep.

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The reduction in blood total protein levels in this study with advanced age may be referred to as the reduction of animal growth rate after puberty. In addition to at earlier ages, the needs of blood protein are very important to help in transportation of calcium and phosphorus and other substances in the blood by attachment to the albumin. Same results for breed effect found by Farghaly et al. (2011) who documented that there were no significant differences due to breed and sex of sheep in total protein concentration; the Ossimi and Rahmani breeds showed similar mean of plasma total protein (7.00 vs. 6.97 gm/dl) respectively and the average concentration for males and females was 6.95 and 6.99 gm/dl and also El-Malky et al. (2019) reported same trend for Ossimi and Barki.

Different results by Anwar et al. (2012) reported that Rahmani ewes showed a significant higher value of plasma total protein concentrations than both Barki and crossbred ewes, while there were no significant differences among all breeds in globulin concentration. The mean values of total protein concentrations were (7 for Barki, 7.38 for Rahmani 7.13 g/dl for cross breed Rahmani and Barki). Same values measured by Cruz et al. (2012) reported that the mean values of plasma total lipids for Santa Inês male lambs at different ages were (1048, 1091, 1149 and 1191 mg/dL) at (84, 168, 210 and 252 days), respectively. Lower levels of plasma total lipid reported by Anwaret al. (2012) reported that the mean values of plasma total lipids for Egyptian sheep breeds were 351.11 for Barki, 308.55 for Rahmani and 324.77 mg/dl for cross breed Rahmani and Barki. Meanwhile, Shaker (2014) found that the average serum total lipids in Barki ewes ranged from 254 to 303 mg/dl at 90 days. While, El-Bassiony (2016) mentioned that the averages of blood total lipids vary from 259.5±13.6 to 322.6±13.1 mg/dl in Barki ewes.

An inverse opinion reported by Anwaret al. (2012) reported that there were significant differences among breed on total lipids concentrations. Moreover, they mentioned that Barki breeds have a great value of serum total lipids and cholesterol concentrations compared with those of Rahmani and crossbred ewes. Furthermore, Rahmani ewes had lower serum total lipid concentrations than crossbred ewes.

The results of present study are different with results which reported by El-Malkyet al. (2019) which they found that plasma total lipids were higher in Ossimi than Barki (2.95 and 2.59 g/l) respectively. This might refer to the lower size of fat tail in Barki sheep compared to Ossimi sheep.

Gene expression analysis

The ovine GH is a peptide encoded by a single gene about 1.8 kb long and it contains five exons and four introns in mammals (Hajihosseinlo et al., 2013). It has been mapped to chromosome 11 in *Ovis aries.*Growth hormone (GH) gene is a promising candidate gene for farm animals' genetic selection. It has many physiological functions, such as promoting muscle and bone growth, enhancing milk production for females and also semen quality for males and development of mammary gland, regulation of fat content, it plays an important role in postnatal longitudinal growth and development, tissue growth, lactation, reproduction, as well as protein, lipid, and carbohydrate metabolism in sheep (Kumariet al.,2013; Jia et al., 2014; Mazurowski et al., 2015; Farag et al., 2016; Abdelmoneim et al., 2017).

The analyses of gene expression demonstrated different levels of expression for *GH* gene in all sheep breeds. The significant up-regulation of GH for Barki breed and Rahmani breed compared to the Ossimi breed may be related to Ossimi breed had a higher body weight compared to other breeds, therefore, Barki and Rahmani breeds need a greater concentration of growth hormone to achieve their maturity live weight.

The results indicated that the Barki breed had a significant down-regulation of IGF-1compared to the Ossimi breed in 7-9 months age category and Ossimi and Rahmani breeds in the 10-12 months age category. The result of this study is different than results reported by Su et al. (2014) who mentioned that IGF-I expression in Hu sheep increased from 2 days of birth and then continuously decreased until 3 months of age; thereafter, IGF-I expression gradually increased and reached the peak at 6 months of age, the expression of IGF-I in sheep that were 6 months of age was significant ($P<0.01$) and greater than at 1, 2, 3, and 4 months of age. The IGF1 gene plays an important purpose in the growth of multiple tissues, including mammalian muscle, somatic growth, and tissue repair (Nazari et al., 2016, Othman et al., 2016, Grochowska et al., 2017; Rotwein, 2017). IGF-1 gene is considered to be a candidate gene for predicting growth and meat quality traits in sheep genetic improvement schemes. It works to absorb glucose, decompose glycogen and increase the absorption of amino acids needed to synthesize protein and reduce protein degradation (Al Qasimi et al., 2019). Even though the IGF-I gene plays a role in the growth of an organism, it could also be directly or indirectly associated with other traits, i.e. carcass and meat quality traits (Grochowska et al., 2017).

Due to its important role in growth and reproduction traits, IGF-1 gene is considered as a candidate marker for these traits and its genetic polymorphism identification is of great interest in Egyptian small ruminant breeds (Othman et al., 2016). The leptin (LEP) gene was discovered in 1994 by Zhang et al. (1994) by positional cloning techniques. It is a promising candidate gene for voluble quantative traits in farm animals. Leptin gene has been mapped to the 5th chromosome in ovine consists of two introns and three exons that are involved in the synthesis of leptin hormone but only exons two and exons three are translated into protein (Javanmard et al., 2008). Leptin hormone, encoded by LEP gene, has significant importance in regulating various functions of growth, process and growth traits such as weaning

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weight, six-months body weight and nine-month body weight, etc. In addition to its functions, Saleem et al. (2018) stated this role in regulation of feed intake, metabolic process and meat quality traits in cattle, buffalo, goat and sheep.

The results of leptin gene expression in the current study showed that there were significant differences in the Ossimi breed between 10-12 months age category and two other age categories (Figure 3). This may be referred to Ossimi breed genetically have a greater amount of adipose tissues than Barki and Rahmani breed and also in present study Ossimi breed have the greater values of all physical body measurements.

The results of leptin gene expression in the current study (Figure 3) confirm the hormonal ones which obtained by Veena et al. (2018) they reported that there was a positive relationship between serum leptin concentration and advanced age in Bannur female sheep and the average serum leptin ranged from 1.36 to 2.35 (ng/mL). Also, Nieto et al. (2013) they reported that circulating concentrations of leptin increased from 1.31 6 0.02 to 1.78 6 0.01 ng/ml ($P<0.01$) with advance age and leptin concentrations differed with ewe breed ($P<0.05$).

The opposite results mentioned by Zarkawi and Al-Daker (2018) they reported for Syrian Awassi ewe lambs that leptin concentrations did not among different age categories from puberty up to maturity and the overall average concentration was (2.50 ± 0.84 ng/mL) from 6 to 12 months of age.

Up to current knowledge, this study is the first report discusses the gene expression analysis for G.H, IGF-1,andleptin from circulating blood cells in Egyptian sheep during the selected period from 7 months of age to 16 months of age. However there was a previous study in cattle which measured the expression levels of IGF-1 gene in circulating blood cells by Lamas et al. (2018) and other studies in human measured the expression levels of G.H gene by Kelly et al. (2012) and leptin gene by Samara et al. (2008).

CONCLUSION

Based on present research, it can be concluded that Ossimi breed had the highest values of live body weight and linear body measurements compare to Barki and Rahmani breeds the recorded values of both breeds were very similar. Gene expression analysis showed that Ossimi and Rahmani breeds tend to have a pattern of higher expression levels of GH, IGF-1 and leptin genes. So, we suggest that measuring of physical body measurements, blood metabolites and GH, IGF-1 and Leptin genes in early ages can be a good and accurate indicator of growth performance in Egyptian breed.

DECLARATIONS

Author's contributions

Gamal Ashour designed the plan of study and facilitate the experimental work, providing the experimental tools, revision of the research article, Ahmed Gad analyzed genes expression data, writing the results of gene expression analysis and revision of the research article, Ayat Alaa-El-Deen Fayed checked and approved the final form of manuscript, applied the particle part of the study and laboratory, writing of the research article and tabulation of the data, Neama Ashmawy helped in the design of the planned study, providing the experimental tools, revision of the research article, Ashraf El- Sayed helped in the designed the plan of study.

Competing interests

The authors have no competing interests, and we are with respect to this search and in agreement with each other. In addition, we have no conflict with authorship or article publication.

Consent to publish

All the authors approved and agreed to publish the manuscript

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The Role of Glycogen in Biological Cycle of *Trichinella spiralis*

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ABSTRACT

The energy sources of *Trichinella spiralis* change in both the muscular and intestinal stages of its life in the host organism. The purpose of this study was to investigate the quantitative changes in glycogen concentration during the life cycle of *T. spiralis* in a host organism. *Trichinella spiralis* was passaged on laboratory rodents under the vivarium conditions. Sixty-nine white rats (350 g each) were infected with *T. spiralis* at a dose of 5 muscle larvae/gram of body weight. The animals were euthanized at different time periods from the start of the experiment. *Trichinella* muscle larvae were isolated by artificial fermenting meat mince in gastric juice. To determine the viability of *Trichinella* larvae, they were heated to 38 ± 2 ° C for 10 minutes their motor activity was investigated. (38 ± 2 °C). To determine the invasive properties of *T. spiralis* at different stages of its development in rats, the muscular larvae isolated from the rat muscles were used to infect laboratory mice. The invasive capacity of *T. spiralis* was assessed on day 45 post-infection. For the study of intestinal *Trichinella* larvae, laboratory rats were not fed a day before infection. Adult nematodes were isolated from the small intestine of laboratory rats at 3, 6 and 24 hours post-infection. The nematodes were counted in the Migacheva-Kotelnikov chamber in each individual sample. The concentration of glycogen in the nematodes was calculated according to the quantitative method for determining glycogen in *Trichinella* larvae. Low glycogen levels in the muscle larvae were observed on day 14 post-infection. The glycogen concentration in muscular larva was 0.0054 ± 0.0027 μg/ larva on day 21, 0.0136 ± 0.0024 μg/ larva on day 28, and 0.0771 ± 0.0025 μg/ larva on day 45 after the rats were infected. Maximum concentration of glycogen was recorded 4 months post-infection (0.0930 ± 0.0029 μg/larva). Further, the glycogen level began to decrease slowly. In the 20th month post-infection, after infection, the amount of glycogen in a *Trichinella* larva was 0.0786 ± 0.0023 μg. In the body of intestinal nematodes, 3 hours after infecting the animals, the glycogen concentration was reduced to 0.0472 ± 0.0003 μg in one nematode. The same time period later, it reached to value of 0.0272 ± 0.0002 μg. In intestinal *T. spiralis*, which remained in the small intestine of rats for 24 hours, the glycogen was not detected. The amount of glycogen at the muscle stage of *T. spiralis* development was extremely important in the first hours of the helminth's residing in the host's intestines. Energy requirements during the period when the helminth cannot obtain enough food depend on the glycogen content. When the glycogen concentration in the parasite is insufficient, the *Trichinella* larvae will lose their invasion capacity.

Key words: Bioassay test; Glycogen; Nematode; Parasitic helminth, *Trichinella spiralis*

INTRODUCTION

Glycogen is known to be the main reserve polysaccharide and a key source of energy for helminths. A complex life cycle with a change of environment forces helminths to accumulate a significant amount of reserve substances at different stages of their development. One of these helminths is *Trichinella spiralis* causing trichinosis, a dangerous disease affecting animals and humans. The muscle larvae of *Trichinella spiralis* are extremely rich in glycogen, which constitutes 16% of their dry weight (Beckett and Boothroyd, 1962; Castro and Fairbairn, 1969). Many researchers used the methods of histochemical staining and electron microscopy to find the main locations of glycogen deposition at different stages of *Trichinella* biological cycle (De Nollin and Van den Bossche, 1973; Ferguson and Castro, 1973, Andreyanov et al, 2019, Rudneva et al., 2019; Sidor and Andreyanov, 2019). It was established that the total amount of glycogen increases during the maturation of the larva in muscle tissue, and then sharply decreases when larvae grow, molt and reach puberty in the intestine of the new host. In the first hours after invasion, intestinal *Trichinella* relies mainly on endogenous glycogen stores accumulated at the previous stage of development (Ferguson and Castro, 1973; Kilgore et al., 1986). Previous research established that intestinal *Trichinella* does not survive for over 12 hours *in vitro* in a cultured medium without nutrients, while muscle larvae can survive for several days (Kozar et al., 1966).

The goal of this study was to obtain data on the glycogen content in *Trichinella spiralis*, in order to understand the invasiveness level of the causative agent of trichinellosis.

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MATERIALS AND METHODS

Ethical approval

The present experimental research was conducted in compliance with health protection guidelines of experimental animals (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, ETS No.123. Strasbourg, 18/03/1986.).

Animals and study design

The experiment was conducted in the All-Russian Scientific Research Institute of Fundamental and Applied Parasitology of Animals and Plants named after K.I. Skryabin, Russia. In this study, 69 white male laboratory rats weighing 350 g each were used. Larvae of *Trichinella spiralis* were isolated from the Large White pig and passaged on laboratory rodents (white mice and Wistar rats) in the institute vivarium. The muscle larvae were released from the infected pig muscles by digestion of the pig carcass with 1% pepsin (1:1,000) and 1% hydrochloric acid (Bessonov, 1975). The Wistar rats were infected at a dose of 5 larvae/g of body weight. The rats were euthanized by cervical dislocation at specific time periods, starting on day 14 after infection and ending in month 20. *Trichinella* muscle larvae were isolated through passive peptolysis (Bessonov, 1975). The viability of *Trichinella* larvae was determined by their motor activity when they were heated to 38 ± 2 °C for 10 minutes (Skvortsova and Uspensky, 2016). In order to identify the invasive properties of the *Trichinella* larvae, they were collected at different time periods from the infected rats and used to infect laboratory mice. On the 45th day post-infection, the invasive capacity of *Trichinella* larvae was assessed based on the presence of encapsulated *Trichinella* larvae in the muscular tissue of the mice (Bessonov, 1975). Adult nematodes were isolated by dissecting the small bowel of the euthanized rats. The dissected and isolated part of the animal's intestinal tract was placed on the surface of a nylon sieve with 0.8 mm mesh size in the Baermann apparatus at 38±2°C for 6 hours. The sediment with nematodes was then rinsed 5-7 times in the physiological saline. To study adult *Trichinella spiralis* located in the small intestine of laboratory rats, the rats had not received any food for one day prior to infection. The nematodes were isolated from the small intestine of fasted rats at 3, 6, and 24 hours after the invasion.

Glycogen concentration measurement

Adult nematodes and muscular larvae of *Trichinella spiralis* in a sample (one drop of the obtained suspension) were counted using Migacheva-Kotelnikov chamber. The glycogen concentration was calculated according to the method described by Andreyanov et al. (2019) that is based on the treatment of glycogen molecules isolated from *Trichinella* with iodine reagent prepared according to the method described by Danchenko and Chirkin (2010). Briefly, 0.9 ml of 33% potassium hydroxide solution was added to the isolated *Trichinella* (1000-10,000 *Trichinella* in one drop of the suspended sedimentation). The solution was heated for 20 minutes at 100 ± 2°C and cooled in cold water to 8 ± 2 °C, then mixed with 1.3 ml of 96% ethyl alcohol and heated for 5 minutes at 100 ± 2°C. The resulting solution was cooled for 5 minutes to 8 ± 2 °C, then centrifuged for 15 minutes at 3,000 g and the supernatant was drained. The resulting precipitate was dissolved in 0.2 ml of saturated ammonium chloride solution and reheated for 5 minutes at 100 ± 2°C. The tubes containing the precipitate were cooled to 8 ± 2 °C and 0.2 ml of distilled water and 2.6 ml of the iodine reagent were added. In the control sample, 0.2 ml of saturated ammonium chloride solution, 2.6 ml of iodine reagent and 0.2 ml of distilled water were mixed simultaneously. In the resulting solutions, optical density was measured by photoelectric microcalorimeter (МКМФ-02 refractometer) with an optical path length of 5 mm at a wavelength of 425 nm. In order to measure the glycogen concentration in the samples, a calibration curve was made (the x-axis showing the glycogen content in a sample (mg); the y-axis showing the optical density values of calibration solutions). The slope tangent was 1.3. In order to determine the calibration curve, calibration glycogen solutions containing 0.01 to 0.4 mg of glycogen in a sample were prepared.

The glycogen concentration was calculated according to the following formula 1: $C = E \times k / F$

Where C is glycogen concentration (mg per 1 drop), E is the optical density of the analyzed sample, F is the factor, which is calculated as an obliquity tangent of the calibration curve, k is dilution coefficient of a sample.

RESULTS AND DISCUSSION

Glycogen level of *Trichinella* muscle larvae

The glycogen concentration was determined at different stages of isolated muscle larvae in the muscle tissue of rats. On the 14th day after the infection, *Trichinella* muscle larvae showed a low glycogen level of 0.0028 ± 0.0012 µg/larva. Larvae of this age are motile, but not invasive. Glycogen level on day 21, 28, and 45 post-infection were 0.0054 ± 0.0027, 0.0136 ± 0.0024 µg/larva, and 0.0771 ± 0.0025 µg/larva, respectively. Maximum glycogen concentration was recorded in 4 months after infection (0.0930 ± 0.0029 µg/larva). Following that, the glycogen level

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began to decrease slowly (0.0916 ± 0.0031 µg/larva at 6.5 month post-infection, 0.0844 ± 0.0027 µg/larva at 11 month post-infection, and 0.0786 ± 0.0023 µg/larva at 20 month post-infection; Figure 1).

Starting from the 21st day after the infection, the larvae were invasive, which was confirmed by the results of the bioassay test on laboratory mice. The data on the invasive capacity of the newborn *Trichinella* muscle larvae complies with the findings of Skvortsova and Uspensky (2018), who showed that the newborn larvae (15-, 16- and 17-day-old) are not invasive. By 20 month post-infection, the number of dead *Trichinella* larvae was 5% of total larvae isolated after digestion in artificial gastric juice. Starting from the 11th month after the invasion, lime salts are formed at the poles of the capsule in the muscle tissue.

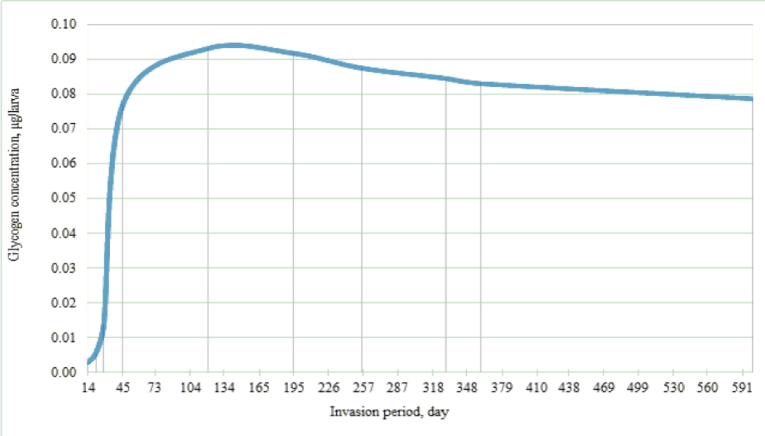

Figure 1. The content of glycogen in muscle larvae of *Trichinella spiralis* in experimentally infected rats

Adult nematodes

The rats were infected with muscle larvae that, upon reaching the intestinal tract, begin to grow, molt, differentiate into males and females and turn into adult *Trichinella spiralis*. Three hours after the infection of the fasted rats, the glycogen concentration in *Trichinella spiralis* helminths decreased to 0.0472 ± 0.0003 µg/nematode. After the same time period, the glycogen concentration reached to value of 0.0272 ± 0.0002 µg/nematode. In intestinal adult nematodes, which remained in the small intestine of fasted rats for 24 hours, glycogen was not detected (Figure 2).

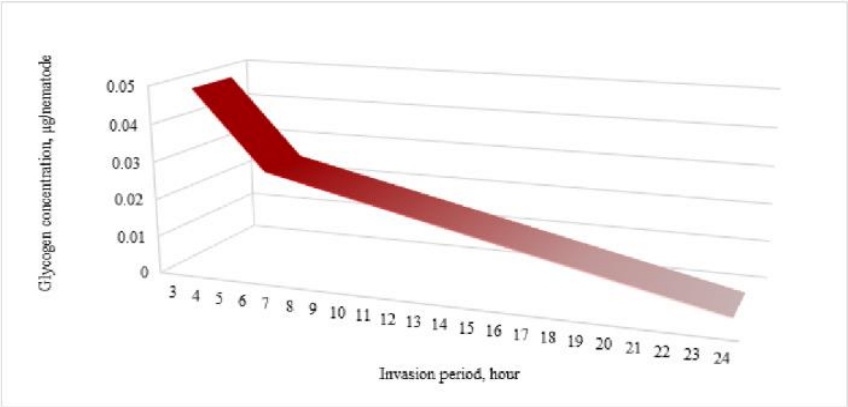

Figure 2. The content of glycogen in intestinal *Trichinella spiralis* in experimentally infected rats

Invasive capacity *of Trichinella* muscle larvae

The invasion of the larvae into the muscle fibers is marked by their intensive organogenesis and growth. Starting from the 7th day of the invasion, the larvae cause enhanced glycolysis of nearby cells and start to accumulate glucose and synthesize glycogen (Stewart, 1976; Montgomery et al., 2003; Okada et al., 2013). The *Trichinella* larvae become invasive on the 16-17th day after infection, when most larvae finish their organogenesis. Most of the *Trichinella* muscle larvae become invasive by the 19^{th}-20^{th} day after the infection. Throughout this experiment, the invasiveness of the larvae remained high, starting from the 21st day after the infection.

Wu et al. (2009) established that blood glucose levels of invasive mice in the period between the 4th and 28th days after invasion are lower than those of the non-infected ones. An increase in the glycogen content in the affected muscles was also recorded between the 8th and 24th days after infection. The researchers stated that during hypoglycemia and an increase in glycogen levels in the animals' invaded muscles, the storage of reserve polysaccharide in the larvae increase

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rapidly between the 14th and 28th days after the infection (Pereverzeva, 1966). Pereverzeva (1966) and Stewar (1976) also noted a significant increase in glycogen, by more than two times, during this period. It is noteworthy that Kilgore (1986) found that in newborn larvae of *Trichinella*, glycogen content constituted 7.8% of their dry weight, whereas, in 45-day-old muscle larvae, glycogen content was 16.1% (Castro and Fairbairn, 1969). Given that after the penetration into the muscle fibers the larvae increase in size by more than 10 times in the first 20 days of development (Castro and Fairbairn, 1969), then a significant increase in the absolute glycogen content in them is expected. From the 14th day after the invasion, newborn *Trichinella* larvae started penetrating into the muscle fibers. This is the time of the active nutrition and growth of muscular *Trichinella* larvae in the host's myocytes (Castro and Fairbairn, 1969). It is possibly due to an increase in the size of muscular *Trichinella* larvae that the glycogen concentration in them is increasing. In the present study, a small amount of glycogen in the 14-day-old *Trichinella* larvae was found. On the 28th day after the invasion, the glycogen concentration in *Trichinella* larvae increased by 4.9 times, from 0.0028 µg/larva to 0.0136 µg/larva.

Until one and a half months old, the larvae continue to actively accumulate glycogen. However, from the 45th to 134th days after infection, the intensity of storing the reserve substance is significantly reduced. Some researchers noted this phenomenon already after the 28th day of the invasion (Stewart, 1976; Wu et al., 2009). Pereverzeva (1966), using the histological method, registered the maximum glycogen content in the 6-month-old larvae. After completion of the organogenesis of muscle larvae and capsule formation, the supply of glycogen in invasive *Trichinella* larvae undergoes a sharp increase. The glycogen synthesis is likely to depend on different factors including the immune response of the host, the host species, the host gender and age, and nutrition conditions of the host. The viability of the larvae after digestion in gastric juice decreases by this time by 5%, which is probably due to capsule calcification. However, this decrease can be considered insignificant- and does not affect the invasive capacity indicators.

In previous studies, other researchers determined the glycogen content in *Trichinella* using the less specific anthrone method. They established that the glycogen concentration in the viable larvae is about 0.0784 µg/larva (De Nollin and Van den Bossche, 1973). This value corresponds with the data obtained in the current study.

The fact that the total amount of glycogen contained in the larvae increases with growth and development, and then remains at the same level for a long time, indicates that this energetic compound is essential at a later stage when the larva turns into an adult *Trichinella* in the intestines of the host (Beckett and Boothroyd, 1962).

Intestinal worms

In the first hours of development in the intestines of the host, *Trichinella spiralis* worms have a very high energy expenditure associated with the invasion into intestinal mucosa, organogenesis, preparation for reproduction and the first molting process. Kozar (1973) observed that adult nematodes of *Trichinella spiralis* do not accumulate glycogen, but use its storage formed at the larval stage of development. Due to the low permeability of the cuticle at this stage, the reserve nutrient material enters the mid-intestine and breaks up there, apparently, into oligo- and monosaccharides. After 20 hours in the host's intestines, the cuticle that its permeability had increased 8 times by that time, is responsible for supplying nutritional substances and removing metabolites from the nematodes (Ferguson and Castro, 1973). Nevertheless, the *Trichinella spiralis* nematodes are highly dependent on the previously accumulated supplies of energy substances. Ferguson and Castro (1973) measured glycogen concentration in *Trichinella spiralis* localized in the intestine of mice. The glycogen content was 0.7% of dry weight of nematode 24 hours after infection. By the 4th day, this indicator increased to 1.3% (Kilgore et al., 1986). Kilgore (1986) detected a slightly higher value for the glycogen content (4.9%) in 4-day-old nematodes. A relatively low amount of glycogen in adult worms disable them to survive *in vitro* for more than 12 hours in the absence of exogenous glucose (Kozar et al., 1966). Presence of *Trichinella spiralis* nematodes in the intestines of fasted mice for more than two days resulted in irreversible changes that led to the destruction and death of the helminths, and also after being kept in an isolated intestinal loop of a hamster, a delay in their developmental was observed (Timonov and Silakova, 1976).

CONCLUSION

The amount of glycogen stored at the muscle stage is extremely important in the first hours of the *Trichinella spiralis* development in the intestines of the host. The high energy requirements of *Trichinella spiralis* during the period when the nematodes cannot obtain enough nutrition, depend entirely on their glycogen storage. If the latter is insufficient, *Trichinella spiralis* nematodes lose their invasiveness.

DECLARATIONS

Authors' contribution

Evgenya A. Sidor contributed to data analysis and manuscript writing and performed the experimental works. Oleg N. Andreyanov was involved in the development of the methodology. Finally, all authors read and approved the final manuscript.

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Competing interests

The authors have declared no conflict of interest.

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Determination of Potential Candidate Genes Associated with Milk Lactose in Egyptian Buffalo

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ABSTRACT

The aim of the present genome-wide association study (GWAS) was to identify single nucleotide polymorphisms (SNPs) and candidate genes associated with lactose percentage (LP) and lactose yield (LY) in Egyptian buffalo. The phenotypic dataset included 60,318 monthly measures for LP and LY from 1481 animals. A total number of 114 animals with high and low deviated performance were selected for genotyping with Axiom Buffalo Genotyping 90K Array. Genome-wide analysis was performed using a single marker regression. The GWAS revealed 32 significant and seven suggestive SNPs for LP, however; only two suggestive SNPs were identified for LY. The identified genomic regions are overlapped with previously reported QTL in different cattle breeds. In addition, novel genomic loci were detected. The identified genomic regions harbored many candidate genes with biological roles associated with milk production traits, such as TPD52 and ZBTB10 on chromosome 15; AADAT and GALNTL6 on chromosome 3 and COL8A1 and PLOD2 on chromosome 1. Our findings provide the basis to uncover the key markers and candidate genes affecting lactose traits which facilitate the exploration of the genetic mechanisms that control lactose traits variation in Egyptian buffalo.

Key words: Candidate gene, Egyptian buffalo, Genome, Genomic loci, Lactose

INTRODUCTION

Buffalo milk contains higher level of total solids (fat, proteins, lactose and ash) compared to other farm animal species, with percentage of 12-25.5% (Gantner et al., 2015). The total solids of buffalo milk make it ideal for processing into dairy products due to its high quality. Lactose is the second major constituent of buffalo milk with percentage of 3.2-4.9% (Ménard et al., 2010). According to previous studies, lactose has a great impact on milk production due to its osmotic characteristic that helps to pull the water into the mammary epithelial cells (Lin et al., 2016). In dairy cattle, lactose concentration was found to be a good indicator for energy balance (Reist et al., 2002), pregnancy rate (Buckley et al., 2003), udder health (Ptak and Bieniek, 2012), immunity, and longevity (Miglior et al., 2006).

Milk production is a quantitative and complex trait; *i.e.* controlled by a large number of genes with small effects and affected by several environmental factors (Hill, 2012). This complexity makes it difficult to understand the biological and the genetic mechanisms that control the trait variation by using traditional breeding (Hill, 2012).

Recently, with the development of molecular biotechnology including sequencing of whole genome for many livestock species, it becomes possible to obtain genomic information including thousands of single nucleotide polymorphisms (SNPs) covering the whole genome. This is followed by rapid development of SNPs genotyping chips (Iso-Touru et al., 2016). The availability of SNP genotyping chips makes it possible to perform genome-wide association studies (GWAS) to identify significant genomic regions associated with the trait of interest. Therefore, GWAS increases the power to map quantitative trait loci (QTL) and defines narrower genomic regions that harbor causal genes associated with economically important traits (Bouwman et al., 2011).

In cattle, several GWAS were conducted to identify SNPs related to many of economically important traits such as body conformation (Wu et al., 2013), disease resistance (Finlay et al., 2012), growth traits (Bolormaa et al., 2011), fertility traits (Huang et al., 2010) and milk production traits (Nayeri et al., 2016). In buffalo, the commercial cattle SNP chips were used to explore genomic regions that are associated with milk production traits, since buffalo and cattle are closely related (Venturini et al., 2014). More recently, the availability of buffalo's reference genome opens the field for developing and releasing a commercial buffalo SNP chip (Axiom® Buffalo Genotyping 90K Array).

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To our knowledge, few GWAS have been performed so far using this chip. Two GWAS were conducted in each Italian (Iamartino et al., 2013 and Liu et al., 2018), Brazilian (de Camargo et al., 2015 and Gonzalez Guzman et al., 2020), and Egyptian buffalo (El-Halawany et al., 2017 and Abdel-Shafy et al., 2020); while one study was performed in each Philippine (Herrera et al., 2018) and Iranian buffalo (Mokhber et al., 2019). The results of such studies showed the benefits of GWAS to identify genomic regions associated with milk production traits and facilitate the utilization of genetic potential for improvement of Egyptian buffalo milk performance.

Therefore, the objective of this study was to perform a genome-wide association study using Axiom Buffalo Genotyping 90K Array to identify SNP markers and potential candidate genes associated with lactose percentage and lactose yield in Egyptian buffalo.

MATERIALS AND METHODS

Animals and phenotypes

Milk samples from 1481 animals (50 ml each) were monthly collected. The samples were maintained frozen at -20◦ C until performed the chemical analysis of milk constituents. Percentage of lactose was determined by Infrared Milk Analyzer (Bentley, I50®) and the automated method of infrared absorption spectrophotometry (Milk-o-Scan; Foss Electric, Hillerφd, Denmark) at Cattle Information System/Egypt (CISE) and Animal Production Research Institute (APRI) of Agricultural Research Center (ARC). Records for chemical composition of milk were checked carefully to remove abnormal phenotypic values and exclude animals that have less than three times chemical analyses per parity. After quality check, a total number of 60,318 monthly measures were remained. The average lactose percentage was 5.1±0.6. Yield of lactose was calculated by multiplying lactose percentage by milk yield at the same day. The average lactose yield was 0.44±0.15 kg/day.

Ethical approval

Sampling protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Cairo University, Egypt (approval number: CU-II-F-40-17).

Genotypic data

From the 1481 individuals, we selected the highest and lowest 114 animals for genotyping according to average daily milk yield. Blood samples were collected from the jugular vein of these animals and kept in a 15 ml Falcon tube containing 1 ml 0.5 M EDTA as an anticoagulant. The samples were immediately placed on the cooling gel in an ice box after their collection and transferred to the laboratory and kept away from the direct sunlight. The samples temporarily were stored at -20°C before DNA extraction. Genomic DNA was extracted from whole blood samples using a QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany).

Using Axiom® Buffalo Genotyping 90K array, genotyping was performed according to the standard protocol of Thermo Fisher Scientific. The signal intensity from the raw genotypic data (CEL files) were converted into genotype calls and annotated to the reference assembly of buffalo genome using Genotyping Console™ 4.2. The quality of the raw genotypes was checked using PLINK 1.9 (Chang et al., 2015). In this respect, the SNPs with unknown positions on buffalo genome were eliminated. In addition, SNPs with low call rate (missing genotype per SNP >0.15), with low minor frequency (MAF<0.01), and/or deviated markers from Hardy-Weinberg proportion (P<0.0001) were excluded. Individuals with low call rate (P<0.15) were also discarded. After applying the filtering options, the number of genotyped animals and SNPs were 113 and 64,169, respectively.

The genotyping rate for the remaining animals was 98.4%. Some pairs of SNPs may have complete linkage disequilibrium (LD), and thus would convey similar information (Anderson et al., 2010 and Laurie et al., 2010). Therefore, we excluded one of a pair of these SNPs if the LD of $r^2 > 0.5$ within a sliding window of 50 SNPs and moving 5 SNPs per set using PLINK (Chang et al., 2015). This pruning step led to reduce the SNP number to 44,985 markers.

Statistical analysis

a. Initial analysis

To determine the significant factors affecting the traits, we initially tested all available fixed effects using general linear model procedure in R program as follow: $y_{ijklmno} = \mu + MF_i + Lac_j + H_k + YS_l + AC_m + DIM_n + \varepsilon_{ijklmno}$ where, $y_{ijklmno}$ is the phenotypic observations (lactose percentage and yield); μ is the overall mean of observations, MF_i is the effect of i^{th} milking/day (i=1 to 3); Lac_j is the effect of j^{th} parity number (j=1 to 13); H_k is the effect of k^{th} herd (11 herds); YS_l is the combined effect of l^{th} year and season of calving (68 levels); AC_m is the effect of m^{th} age at calving (1,341 levels); DIM_n is the effect of n^{th} days in milk in each parity (285 levels); and $\varepsilon_{igklmno}$ is the residual error. Since all tested variables showed significant effects, we used all for the next animal model to calculate the yield deviations.

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b. Phenotype adjustment

A yield deviation is defined as a weighted average of animal's own performance adjusted for non-genetic factors. This procedure is used to initially adjust phenotypes before GWAS to reduce the residual error (VanRaden and Wiggans, 1991). In the current investigation, yield deviations are estimated for each trait as the sum of breeding value and residual for each animal. Estimated breeding values and residuals for each trait were computed by univariate animal model using BLUPF90 family (Misztal et al., 2002). Given the vector of y representing the phenotypic observations on the tested trait (lactose percentage and yield), the following univariate animal model was used: $y = X_b + Z_\alpha + W_p + \varepsilon$, where b is the vector of all fixed effects including milking frequency per day, parity number, herd, year and season of calving. Linear regressions of age at calving and the fourth order Legendre polynomials of DIM were also used. While, α and p are the vectors of random additive genetic and permanent environmental effects, respectively; and ε is the vector of random residual. X, Z and W are incidence matrices connecting observations of y to fixed, random animal, and random permanent environmental effects, respectively.

Genome-wide association analyses

A potential problem associated with population structure was adjusted with a multidimensional scaling (MDS) approach implemented in PLINK 1.9 (Chang et al., 2015). In this regards, the scaling process led to eight significant clusters representing axes of ancestry at P<0.0001. These clusters were used as covariates in the model when performing GWAS. The GWAS was performed using the linear regression model in PLINK 1.9 (Chang et al., 2015), where the adjusted lactose percentage and yield were regressed on the number of copies of the alleles using PLINK–linear option with population stratification as covariates. The results of associations were used to generate Manhattan and Q-Q plots using SNPEVG (Wang et al., 2012). To prevent false positive signals, Bonferroni correction was applied to adjust for multiple testing.

QTL and candidate genes

Since the buffalo and cattle are closely related, previously reported QTL for lactose percentage and yield were retrieved from animal QTLdb (http://www.animalgenome.org/QTLdb), release 37 (Hu et al., 2019). Candidate genes in each genomic region were extracted from the latest annotated file (na35.r2.a2) of Axiom® Buffalo Genotyping array provided by Thermo Fisher Scientific (2019).

RESULTS AND DISCUSSION

Genotypes for genome-wide scan

The Axiom Buffalo Genotyping Array used in this study featured 123,040 SNPs spanning the entire buffalo genome with an average spacing of one SNP every 34.46 kb across all loci (median spacing of 29.54 kb, a minimum distance of 0.01 kb and a maximum distance of 1.33 Mb). After quality control procedures, a total of 44,985 SNPs (36.6%) and 113 animals were remained for further analysis. This subset of SNPs covered 2,614.86 Mb of the buffalo genome with the shortest length of 42.13 Mb for chromosome 24 and longest length of 201.95 Mb for chromosome 1. The average physical distance between markers was 40.77 kb (median spacing of 31.60 kb, a minimum distance of 0.01 kb and a maximum distance of 1.56 Mb). The distribution of SNPs varied among the chromosomes, where the number of SNPs per chromosome were ranged from 1109 (chromosome 24) to 5218 (chromosome 1). While, the SNP density were ranged from 18.1 SNP/Mb (on chromosome 25) to 26.9 SNP/Mb (on chromosome 21) with an average density of 24.7 SNP/Mb. The MAF was ranged from 0.01 to 0.5 with an average of 0.29, which indicates the existence of variation in the allele frequency among the SNPs markers in current study.

Assessment of population structure for GWAS

To assess the successful correction for false positive associations resulted from population structure, the genomic inflation factor (λ) and quantile-quantile (Q-Q) plot were used (Power et al., 2016). Under null hypothesis of no association, λ should equal to one. Price et al. (2010) reported that a λ value less than 1.05 is acceptable for association studies. For Q-Q plot, it should exhibit y = x distribution under null hypothesis of no association (Power et al., 2016). In the current study, even after the correction for population structure, the λ was 1.08 and 1.28 for lactose yield (LY) and lactose percentage (LP), respectively; and the Q-Q plots showed a large deviation from the expected 1:1 relationship (Figure 1 and 2). This inflation may be due to many reasons; firstly, when a large number of loci showed a strong association with the trait (Guo et al., 2012); secondly, hidden relatedness between genotyped animals; thirdly, the low heritability and the nature of lactose traits as polygenic quantitative traits affected by many genes, each with small effects (Power et al., 2016); and fourthly, higher LD between evaluated SNPs (Abdel-Shafy et al., 2014).

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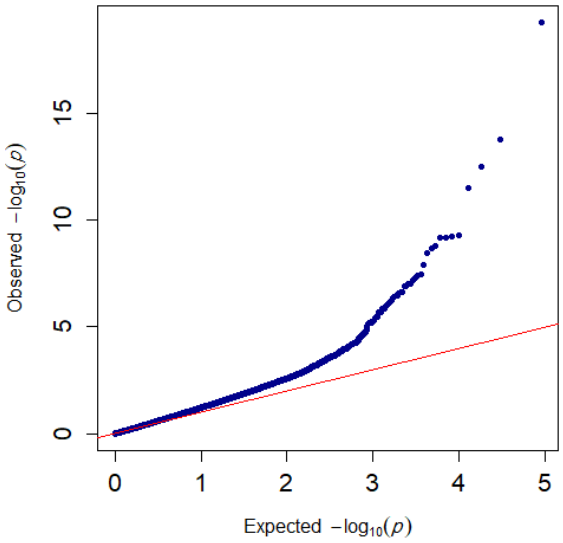

Figure 1. Quantile-Quantile plot of genome-wide association for lactose percentage in Egyptian buffalo

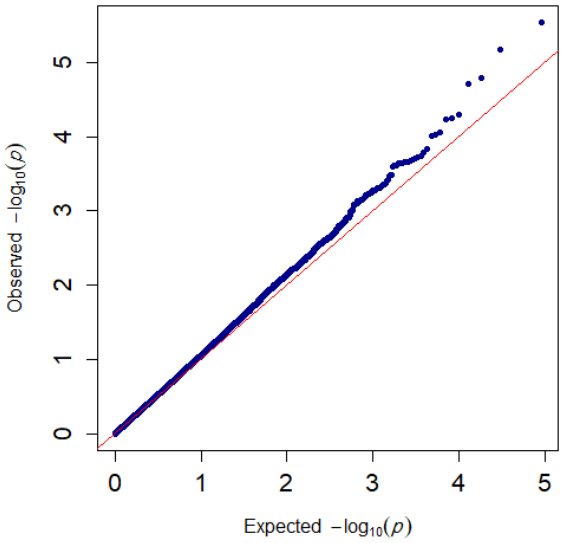

Figure 2. Quantile-Quantile plot of genome-wide association for lactose yield in Egyptian buffalo

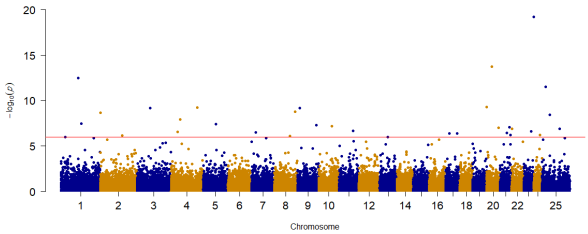

Figure 3. Manhattan plot of genome-wide association for lactose percentage in Egyptian buffalo.
The horizontal red line indicates the whole-genome significance threshold after Bonferroni correction at $\alpha = 0.05$ ($P \leq 1.11 \times 10^{-6}$)

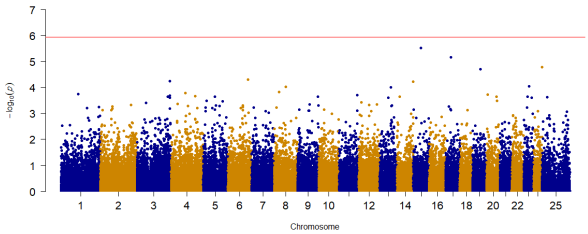

Figure 4. Manhattan plot of genome-wide association for lactose yield in Egyptian buffalo.
The horizontal red line indicates the whole-genome significance threshold after Bonferroni correction at $\alpha = 0.05$ ($P \leq 1.11 \times 10^{-6}$)

Genome-wide association for lactose percentage

Manhattan plot exhibiting P-values of genome wide scan with respect to genomic location for each trait are shown in Figure 3 and 4. Manhattan plot presents the negative logarithm of P-value for all tested SNPs across the genome (y-axis) versus its chromosomal locations (x-axis). The horizontal red line indicates the whole-genome significance threshold after Bonferroni correction at $\alpha = 0.05$ ($P \leq 1.11 \times 10^{-6}$). A strong association with the trait is represented with a small P-value, thus a negative logarithm value will have high value and the scatter plot will be at the highest peak along the y-axis in the Manhattan plot. In the current instigation, the GWAS for LP revealed 32 significant SNPs distributed on 18 chromosomes (Figure 3 and Table 1). These SNPs were occurred with MAF of 0.01 to 0.09. The most significant SNP (AX-85107786) for association with LP was located on chromosome 23 at position 50,923,849 bp with P-value of 5.90×10^{-20}. This SNP was located at 29,570 bp upstream from the ADGRA1 gene and 72,007 bp upstream from the CFAP46 gene with MAF of 0.01 and effect size of -0.429 units of LP. The effect size of minor alleles for all significant SNPs was negative, ranging from -0.861 to -0.195 units of LP, indicating their association with lower LP. In addition, seven suggestive SNPs were identified for association with LP. All these SNPs were occurred with MAF of 0.01 to 0.11. Interestingly, the effect of minor alleles for the top four suggestive SNPs on chromosomes 13, 25, 7 and 1 was negative, ranging from -0.427 to -0.142 units of LP, while the effect of minor alleles for the other three SNPs on chromosomes 2, 16 and 25 was the same and had a positive effect direction for LP (0.438 units).

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Table 1. Significant single nucleotide polymorphisms associated with lactose traits and candidate genes in Egyptian buffalo

Traits	SNP ID	Chr.	Positions (bp)	MA	MAF	β	P-value	Nearest gene	Distance (bp)	Other genes	Distance (bp)
LP	AX-85107786	23	50923849	G	0.01	-0.429	5.90E-20	ADGRA1	+29570	CFAP46	+72007
LP	AX-85075099	20	27966184	C	0.02	-0.861	1.79E-14	STRN3	Intron		
LP	AX-85088945	1	88740750	G	0.02	-0.611	3.18E-13	COL8A1	Intron		
LP	AX-85067933	25	19690758	A	0.01	-0.348	3.17E-12	SMPX	+6973	KLHL34	-58203
LP	AX-85102661	20	1344536	A	0.02	-0.615	5.27E-10	CKB	+1183	TRMT61A	+3744
LP	AX-85071608	4	135416124	C	0.01	-0.475	5.82E-10	ANK3	Intron		
LP	AX-85061010	9	11244996	G	0.01	-0.447	6.62E-10	FAM174A	+361585	CHD1	+1286215
LP	AX-85083471	3	66737950	C	0.02	-0.461	6.96E-10	AADAT	+81516	GALNTL6	+1869717
LP	AX-85086185	8	107090040	A	0.03	-0.377	1.77E-09	LOC102404258	Intron		
LP	AX-85053506	2	1531993	A	0.04	-0.279	2.26E-09	GMDS	-43447	MYLK4	+173684
LP	AX-85067145	25	41019921	T	0.06	-0.552	3.78E-09	PPP1R2C	+323714	CASK	-543346
LP	AX-85125967	4	46892040	A	0.02	-0.281	1.22E-08	ISX	+319202	LARGE1	+615445
LP	AX-85044502	1	105132869	T	0.03	-0.297	3.50E-08	GAP43	Intron		
LP	AX-85091884	5	64880712	A	0.02	-0.335	4.02E-08	BRINP3	-972845	KCTD3	+2382794
LP	AX-85093722	9	96947301	G	0.09	-0.195	5.40E-08	LOC102404549	-10377	LOC102404027	-24596
LP	AX-85105519	10	68030846	C	0.04	-0.260	6.46E-08	HS3ST5	Intron		
LP	AX-85109519	21	48673809	C	0.02	-0.467	9.30E-08	WDR82	+7122	GLYCTK	+25254
LP	AX-85098567	20	62110044	A	0.01	-0.433	9.62E-08	TTC23	Intron		
LP	AX-85055462	25	92157984	T	0.01	-0.403	1.23E-07	ZMAT1	+9638	LOC102402428	-30612
LP	AX-85121534	22	851096	A	0.01	-0.239	1.29E-07	BCL2	-43867	PHLPP1	-65403
LP	AX-85079804	11	71648876	G	0.02	-0.407	2.34E-07	DPH6	+527178	C11H15orf41	+704549
LP	AX-85088569	23	37120921	C	0.02	-0.325	2.41E-07	LOC112581570	+9890	PDZD8	-98381
LP	AX-85078270	4	33866814	C	0.04	-0.314	2.76E-07	SOX5	Intron		
LP	AX-85049384	7	22266031	T	0.02	-0.444	3.47E-07	CFAP299	Intron		
LP	AX-85049679	21	34720210	C	0.03	-0.321	3.66E-07	KBTBD8	+215342	LRIG1	+266570
LP	AX-85098463	17	58602702	A	0.06	-0.204	4.05E-07	GAB1	Intron		
LP	AX-85116698	17	19620490	A	0.04	-0.303	4.49E-07	WDR66	Intron		
LP	AX-85053490	21	53297221	C	0.01	-0.433	6.10E-07	CCR1	-613	XCR1	+64141
LP	AX-85086544	24	31016341	T	0.03	-0.319	6.43E-07	CPPED1	Intron		
LP	AX-85070682	2	114150095	T	0.09	-0.432	7.14E-07	DARS	Intron		
LP	AX-85085611	8	81186163	T	0.03	-0.397	8.74E-07	YAE1	+133461	POU6F2	-207100
LP	AX-85093634	1	22545784	A	0.01	-0.429	1.02E-06	LOC102402668	-64615	SGCZ	-102736
LP	AX-85124326*	13	40601425	T	0.11	-0.142	1.12E-06	TBC1D4	-153107	LOC112578532	-1093876
LP	AX-85078356*	25	117341243	C	0.05	-0.258	1.37E-06	CT83	-262287	LOC102398514	+377742
LP	AX-85058363*	7	75945732	G	0.02	-0.427	1.40E-06	KCNIP4	+18762	ADGRA3	-394941
LP	AX-85067191*	1	168416519	T	0.06	-0.195	1.44E-06	C1H3orf58	-980648	PLOD2	-1388647
LP	AX-85052475*	2	37072441	A	0.01	0.438	1.98E-06	CLMP	Intron		
LP	AX-85078483*	16	51213059	T	0.01	0.438	1.98E-06	LOC112578406	+30121	FOXP4	+119159
LP	AX-85070334*	25	5377908	A	0.02	0.438	1.98E-06	LOC102403725	Intron	CFAP46	+72007
LY	AX-85055593*	15	38842902	T	0.02	0.113	2.92E-06	TPD52	+114860	ZBTB10	+189227
LY	AX-85047648*	17	27161010	A	0.01	0.068	6.76E-06	ADGRD1	-197491	SFSWAP	+464689

Chr: chromosome, MA: minor allele, MAF: minor allele frequency, β: change per minor allele, LP: lactose percentage, LY: lactose yield, bp: base pair, SNP: single nucleotide polymorphisms,* Suggestive SNPs (SNPs that are close to significant threshold line based of Bonferroni correction). In the distance: "+" for upstream and "-" for downstream. Positions are given according to the latest reference assembly of buffalo genome (UOA_WB_1: GCA_003121395.1).

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The association results for LP not only provide confirmatory evidences for previously findings, but also explore a suite of novel significant SNPs that did not reported by previous association or linkage studies in buffalo and cattle populations. Among the significant SNPs that were supported by previously reported QTL in the cattle QTL database; SNP (AX-85067933) which is located on chromosome 25 at position 19,690,758 bp. This SNP is corresponding to the position of 128,877,968 bp on chromosome x in the bovine genome and close to previously detected SNP at 137.1 Mb for LP in Holstein and Jersey cattle (Benedet et al., 2019). Furthermore, the significant SNP (AX-85071608) on chromosome 4 at position 135,416,124 bp is corresponding to the position of 16,073,463 bp on BTA 28 in bovine genome. This position resides very close to previously reported QTL for LP that has been mapped using GWAS in Holstein and Jersey cattle at 16.3 Mb (Benedet et al., 2019). The three significant SNPs located on chromosome 21 between 34.72 and 53.29 Mb are corresponding to the position between 34.80 and 53.85 Mb on chromosome 22 in bovine genome. These genomic regions reside close to previously reported QTL for LP that have been mapped by linkage study in Chinese Holstein cattle at 38.8-39.0 Mb (Mao et al., 2015).

Genome-wide association for lactose yield

The GWAS for LY did not show any significant associations. However, GWAS identified two suggestive SNPs were close to the Bonferroni corrected threshold for genome wide significance ($\alpha = 0.05$, $P \leq 1.11 \times 10^{-6}$) for association with LY (Figure 4 and Table 1). The highest peak was observed on chromosome 15 for the SNP (AX-85055593) at position 38,842,902 bp (P-value of 2.92×10^{-6}). This SNP was located at 114,860 bp upstream from the TPD52 gene and 189,227 bp upstream from the ZBTB10 gene with MAF of 0.02 and effect size of 0.113 units of LY. On chromosome 17, the second suggestive SNP (AX-85107786) was located at position 50,923,849 bp with P-value of 4.00×10^{-6}, MAF of 0.01, and effect size of 0.068 units of LY. This SNP was located at 19,7491bp downstream from the ADGRD1 gene and 464,689 bp upstream from the SFSWAP gene. The minor alleles of these suggestive SNPs (AX-85055593 and AX-85107786) were favorable and associated with higher LY. These SNPs are considered novel genomic loci for LY since they were not detected in previous buffalo and cattle GWAS or linkage studies.

Identification of possible candidate genes

After conducting the association analyses to identify the top SNPs associated with studied traits and refine their positions, we used these positions to examine nearby genes in order to identify potential candidate genes affecting the relevant traits. Selection of potential candidate genes was based on previously reported QTL and the biological functions related to milk production, milk composition and immune response. For example, the candidate genes COL8A1 and PLOD2 on chromosome 1, CCR1 and LRIG1 on chromosome 21, CHD1 on chromosome 9 and ZBTB10 on chromosome 15, are associated with immune response of mammary gland, somatic cell count and mastitis resistance (Chen et al., 2015; Fang et al., 2016; Banos et al., 2017; Welderufael et al., 2018 and Szyda et al., 2019). Furthermore, the candidate gene MYLK4 on chromosome 2 was previously reported to have a significant effect on milk yield, fat percentage, fat yield and protein yield in Brazilian buffaloes (de Camargo et al., 2015). The identified region on chromosome 4 contains LARGE1 gene that is associated with daily milk yield in Egyptian buffaloes (El-Halawany et al., 2017). In addition, the candidate genes AADAT and GALNTL6 gene on chromosome 3 were previously reported to be associated with myristic saturated fatty acid content in the meat, feed efficiency and growth traits in Ireland Holstein-Friesian and Hereford cattle (Doran et al., 2014 and Seabury et al., 2017).

CONCLUSION

This is the first GWAS for lactose traits in Egyptian buffalo. Our findings provide the basis to uncover genomic regions associated with lactose traits in Egyptian buffalo. These genomic regions coincided with previously reported QTL for milk production traits in different cattle breeds, which confirm the importance of such loci for the trait variation. In addition, novel genomic loci were suggested. Future validation studies with larger sample size should be done to verify the results obtained from the current study in order to fine mapping the identified genomic loci, which may play a role to increase the rate of genetic improvement for milk production traits in Egyptian buffalo using genomic approaches.

DECLARATIONS

Authors' Contribution

MAAA collected data and samples, contributed to analyses, and wrote the manuscript. SA-B contributed to reagents and materials preparation as well as data collection. HE-R collected data and samples and contributed to analyses. SE-A contributed to reagents and materials preparation along with genotyping. HA conceived and designed the experiment, analyzed the data and contributed to the manuscript writing.

Competing interests

The authors have not declared any conflict of interests.

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Diagnosis of Foot and Mouth Disease in Cattle and Buffaloes in Different Governorates of Egypt

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ABSTRACT

Foot and Mouth Disease (FMD) is highly contagious disease affected cloven-hoofed animals which result in substantial economic losses. The present study was aimed to detect FMDV by different serological and molecular methods in cattle and buffaloes for providing an accurate and rapid diagnosis of FMD disease. 86 samples of tongue epithelium biopsies, fluid vesicles samples and saliva, as well as 86 coagulated and uncoagulated blood samples, were collected from 64 and 22 suspected cattle and buffaloes respectively in different governorates in Egypt, during August to December 2017. Serum samples were examined by 3ABC-ELISA for differentiating between infected and non-infected animals. While tissues biopsies and un-coagulated blood samples were examined by Sandwich ELISA, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) as well as Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR). FMDV porotypes were identified by rRT-PCR in suspected cattle and buffaloes samples to FMDV serotype A, O and SAT2 and results showed that 54 samples positive for FMDV different serotypes while FMDV serotype differentiation in tissues biopsy of cattle were 18 (28.12%), 12 (18.75%), 3 (4.68 %) and 4 (6.25%). Also, the positive results of tissue samples from buffaloes examined by RT-PCR were 9 (40.09 %), 4 (6.25%), 2 (9.09 %) and 2 (9.09 %) for O, SAT2, serotype A and mixed serotypes respectively by different tests. The rRT-PCR provided an accurate and rapid laboratory diagnosis of FMDV as well as RT-PCR, and 3ABC- ELISA were given nearly the same results. Although the rRT-PCR generated results in less than 6 h and this is an important feature when definitive diagnostic results required in a short timescale during emergencies. Also, this study demonstrated the current situation of circulation FMDV type A, O, and SAT2 serotypes in cattle and buffaloes in Egypt.

Key words: 3ABC-ELISA, Buffaloes, Cattle, Foot and mouth disease, Real-time reverse transcriptase polymerase chain reaction

INTRODUCTION

Foot and Mouth Disease (FMD) is an important highly contagious transboundary viral disease that affects cloven-hoofed domesticated and wild animals in Asia and Africa (Grubman and Baxt, 2004; Sobhy et al., 2018; Diab et al., 2019). FMDV is non-enveloped, spherical shape ranged from 20–30 nm in diameter, small virus with an icosahedral capsid composed of four structural proteins (VP1, VP2, VP3, and VP4), which surround a single-stranded positive-sense RNA genome is an 8.4-kilobase (Brooksby, 1958; Aktas et al., 2015; Jamal and Belsham, 2018). FMD virus belongs to genus *Aphthovirus*, family *Picornaviridae* (Knowles et al., 2012; Salam et al., 2014). All serotypes give rise to a similar disease but each serotype does not confer immunity against another (Beck and Strohmaier, 1987; Stram et al., 1995; Lloyd-Jones et al., 2017). FMD virus serotypes O, A and Asia1 are endemic or cause periodical FMD outbreaks in the Middle East and serotypes O and A cause FMD outbreaks in North Africa (Knowles et al., 2012). In Egypt from 1964 to 2005, only serotype O was recognized, but in 2006, FMDV serotype A outbreak was occurred in cattle and FMDV serotype (A) was identified as A/Egy/2006 and this strain has more than 90% nucleotide identity with A/KEN/98, A/ETH/92, and A/KEN/05, and all topotypes are closely related. It's though introduction in Egypt from East Africa was probably through imported live cattle from Ethiopia (via sea-route) (Ahmed et al., 2012; Jamal and Belsham, 2018). The dramatic upsurge FMD serotype SAT2 outbreaks occurred in cattle, water buffalo and small ruminants with severe clinical signs of FMD in Egypt in 2012 (Kandeil et al., 2013). Serotypes O, A and SAT2 have been detected in this country since 2013 (Sobhy et al., 2018).

FMD disease is the most important restraint to international trade of animal and animal's byproduct. FMD is highly infectious and can be spread by infected animals through aerosols, contact, contaminated farming equipment, vehicles, clothing or feed, and by domestic and wild predators (Salam et al., 2014). The FMDV is associated with sudden death in young calves without observable clinical signs (Yang et al., 2013; Diab et al., 2019). The accurate diagnosis of FMDV is

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important for controlling and eradication of disease in endemic countries including Egypt. It is necessary to conduct laboratory diagnosis of any suspected case of FMD to confirm the disease, includes virus isolation, genome identification techniques such as polymerase chain reaction (PCR) assays and serological tests such as the virus neutralization test, nonstructural protein (NSP)-ELISA (3ABC-ELISA) and Liquid Phase Blocking (LPB)-ELISA for screening the antibodies of FMDV serotype were applied (EL-Kholy et al., 2007; OIE, 2008; EL-Shehawy et al., 2011; FAO, 2012; El-Khabaz and Al-Hosary, 2017). The Polymerase Chain Reaction (PCR) is a quick and more accurate approach and is appropriate to be used with various types of clinical samples. The sensitivity of this method is many times higher than virus isolation which was recognized as "gold standard" in FMDV recognition (OIE, 2012). There are different methods of nucleic acid detection including real-time reverse transcription-polymerase chain reaction (rRT-PCR) which considering as one of the approaches used for detection and typing of FMDV serotypes (El Bahgy and Mustafa, 2018). The difficulties in controlling of cattle movement transboundary and also controlling this movement from neighboring countries to Egypt lead to contact with Egyptian native and hybrid herds and may provide a mechanism for spreading the FMDV. However, the roles of transboundary and animal movement in epidemiology of FMDV in Egypt have not been studied yet, so the vaccination was the only way to control the FMD disease in Egypt (Jamal and Belsham, 2018). Rapid identification of FMDV serotypes especially during outbreaks is very important in order to use the appropriate emergency vaccine and determine the origin of infection (Radostitis et al., 2007 and OIE, 2009). The present study aimed to investigate and determine FMDV serotype responsible for the reemerging outbreaks in Egypt during 2017 by serological and Real-time Reverse Transcription-Polymerase Chain Reaction (rRT-PCR) assays.

MATERIALS AND METHODS

Ethical approval

All samples were collected as per standard procedure without giving any stress or harm to the animals. The work was done according to the guidelines of the National Institutes of Health Guide (Sohair et al., 2016). All laboratory work was done at the National Research Centre and in Animals Health Institute biosafety II laboratory also, oral permission of the animal owners from the individual or private animals farms, before clinical samples from cattle were taken under the supervision of professional veterinarian according to the Egyptian general organization of veterinary services, ministry of agriculture

Study area and animals

This study was conducted in cattle and buffaloes with FMD signs in different governorates of Egypt during August to December 2017. FMD clinical signs, high fever (40°C - 41°C), vesicles, ulcerations on the gum, dorsum of plate, tongue with excessive profuse salivation, foot lesions in interdigital space with ulcerations lead to lameness, in dairy animals, vesicles and ulcerations on udder and teat. All signs ranged from sever, moderate to mild according to the immunity and health conduction of animals.

Samples collection

Total 86 samples (whole blood samples and tongue tissues biopsy samples) were collected from suspected animals (64 cattle male or female, foreign or native breed and 22 buffaloes) located at different governorates in Egypt as shown in figure 1. The biopsies from tongue epithelium, lips vesicles and saliva were collected in transport medium which was a mixture of equal amounts of sterile glycerol and phosphate buffered saline. The pH range was 7.2-7.4 with antibiotics and processed according to Callahan et al. (2002). Beside whole blood without anti-co agglutinate were collected for detection FMDV antibodies. All samples were kept on ice till reached to the lab and then prepared and stored in -20°C until used.

Serological tests

3ABC-enzyme-linked immunosorbent assay (3ABC-ELISA)

(3ABC-ELISA kit (IDEXX FMD 3ABC Bo-Ov, Spain) for detection of non-structural polyprotein of FMD antigen antibody in cattle and buffaloes sera was used and followed by the manufacturer instructions. Whole blood samples without anticoagulants were collected from suspected animals after sera separation stored at -40°C or examined after appropriate dilution by 3ABC- FMD antigen using. The 3ABC-ELISA was developed under standard laboratory conditions with all incubation steps at 37°C with gentle shaking. Plates were washed three times between incubation steps with washing buffered (phosphate-buffered saline pH 7.2 tween 0.05%). Briefly, 96-well coated with poly FMDV 3ABC nonstructural polyprotein, 50µL of the test sera samples or control sera were added at a 1: 50 dilutions (in blocking buffer) and the plate was incubated for 30 min. Control sera and the plate incubated for an additional 30min. After washing, anti-bovine horse radish peroxidase conjugate was added (50µL/well in blocking buffer) and incubated for 1h. After washing, 50µL/well of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate were added and the reaction stopped

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after 10 min at room temperature by the addition of 50µL /well of 1 mol /L H2SO4 and the absorbance of each well at 450 nm measured on an ELISA reader after blanking. Sera were tested duplicate or triplicate and the final result was expressed as the mean value. Each plate contained four replicates of the positive and negative controls. Results were presented with percentage and values ≥30% were considered positive, <20% as negative and samples between > 20% and < 30% were considered suspicious.

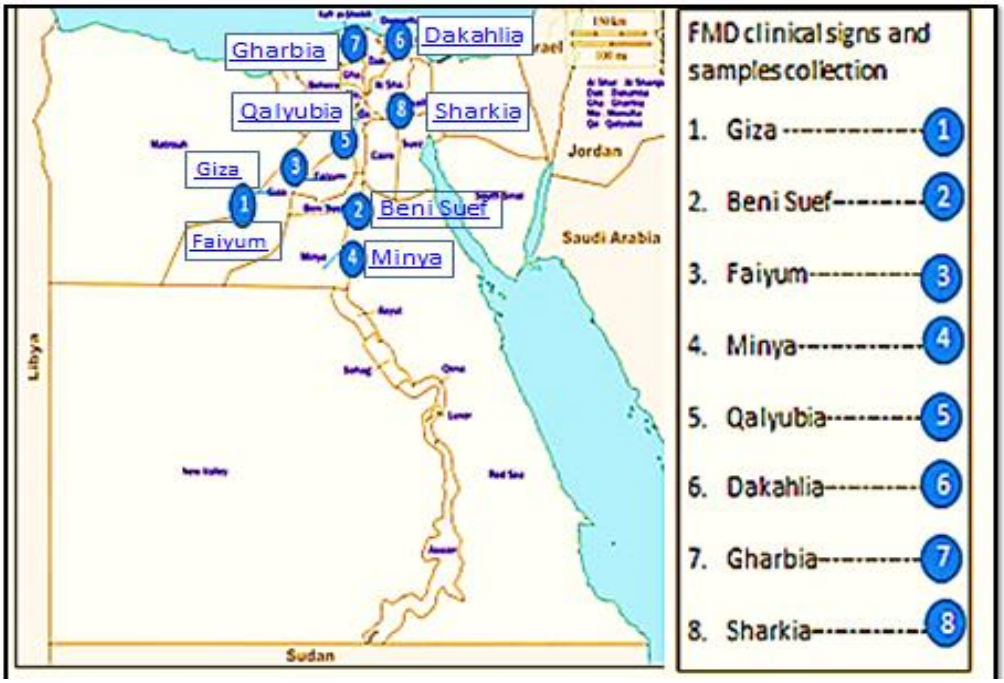

Figure 1. The geographical distribution of infected cattle's and buffaloes with foot and mouth disease clinical signs in different governorates of Egypt in 2017. Total 86 samples (whole blood samples and tongue tissues biopsy samples) were collected from suspected animals (64 cattle male or female, foreign or native breed and 22 buffaloes) located at different governorates in Egypt.

Sandwich-ELISA

Detection of FMDV serotypes presence in infected biopsy samples of cattle and buffaloes (tongue tissues biopsies, vesicles and saliva), was done by Sandwich-ELISA (Sn- ELISA), (Brescia, Italy and Pirbright, UK). Supernatants of the homogenized clinical tissue materials were tested in an in-house serotype differentiating antigen detection ELISA to detect the virus serotype involved in the outbreaks. Briefly, dilute coating sera for each serotype, as manual instruction, with 0.05 mole (mol) carbonate buffer (pH 9.6). 96-well flat-bottom plate was coated with 50 µl/well of each antiserum at 4°C overnight. The plates were washed three times washing buffer. The tested samples were added to the wells and incubated for 1 h at 37°C. The plates were washed three times with Phosphate-Buffered Saline (PBS), dispense 50µl of blocking buffer and positive and negative controls dispense 50µl of respective controls provided incubate at 37°C for 1 hour with gentle shaking followed by washed three times by washing buffer. All serum samples diluted 1:50, as suggested, with blocking buffer. 50µl of peroxidase-labeled was added to the wells and incubated for 45 min at 37°C. Afterward the plates were washed three times and 50µl of o-phenylenediamine-H2O2 substrate was added to the plates, which were then incubated in the dark for 15 min at room temperature. The reaction was stopped with 50 µl of 1.25 M H2SO4, and the optical density (OD) was measured with an ELISA reader at 492 nm.

Conventional RT-PCR

RNA extraction

The Viral RNA was extracted by RNA Mini kit (Qiagen, Germany) from blood with EDTA and homogenized tissue samples (50-100 mg) according to the manufacturer instructions.

Reverse transcription polymerase hain reaction

One-step Reverse Transcription Polymerase Chain Reaction (RT-PCR) of FMDV was carried out as described by the manufacturer's protocol to perform the reverse transcription and subsequent PCR by one-step RT-PCR (Qiagen, Germany). The primer sequences were as listed in table 1. Amplification reactions (25µL reaction mixture) were performed at the following conditions: 50°C for 30min for reverse transcription and initial denaturation at 95 °C for 15 min, then 35 cycles consisted of 94 °C for one min for denaturation followed by annealing for one min at 52 °C for serotype O and SAT2 while for serotype A, the reaction was runed at 54 °C, 55 °C, respectively. The extension was done

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at 72 °C for one min followed by one final extension step of 10 min at 72 °C. The amplicons were analyzed by 1.5% agarose gel electrophoresis.

Table 1. Oligonucleotide primers used for detection of foot and mouth disease virus different serotypes in affected cattle and buffaloes

Primer	Sequence (5´ to 3´)	Target gene	size bp
FMDV-All serotype	F 5-GCC TGG TCT TTC CAG GTC T-3 R 5-CCA GTC CCC TTC TCA GAT C-3	P1 P2	216-bp
FMDV-O	F 5 5´-AGC TTG TAC CAG GGT TTG GC-3´ R 5´- GCT GCC TAC CTC CTT CAA -3´	2B	402-bp –
FMDV- SAT	F 5-CCA CAT ACT ACT TTT GTG ACC TGG A-3 R 5-ACA GCG GCC ATG CAC GAC AG-3	ID 2B	715– 730 bp
FMDV-SAT2	F 5´-GAA GGG CCC AGG GTT GGA CTC-3 R 5´-CAC TGC TAC CACTCR GAG TG-3´	ID 2B	880 bp
FMDV-A	F 5´-TAC CAA ATT ACA CAC GGG AA-3´ R 5´-GAC ATG TCC TCC TGC ATC TG-3´	VP3 2B	863- 866 bp

Real-time reverse-transcriptase polymerase chain reaction

RNA extraction was carried out using the QI Aamp viral RNA kit (Qiagen, Germany) according to Reid et al. (2003). Primer pair (PorR/PorF) for real-time RT-PCR were synthesized by Bio Basic, Canada. Por F (5´- CCT ATG AGA ACA AGC GCA TC -3´) and Por R (5´- CAA CTT CTC CTG TAT GGT CC -3´) were derived from FMDV. RT-PCR was performed using QT SYBR Green RT-PCR Kit (Qiagen, Germany) as the manufacturer's instructions. The cycling parameters were 50 °C for 30 min and 95 °C for 15 min, then 30 cycles consisted of 94 °C for 15s, 55 °C for 30s and 72 °C for 30s. Negative control specimens were involved. Thermocycler Rotor-Gene Q (Qiagen, Germany) was used for real-time detection of FMDV by RT-PCR according to Reid et al. (2003).

Statistical analysis

The results were analyzed using the Statistical Package for Social Sciences (SPSS, version 16, Chicago, Illinois, USA). Serum and samples of tissue biopsies were calculated by the chi-square at $P< 0.05$ is considered to be statistically significant.

RESULTS AND DISCUSSION

Foot and mouth disease is one of the most destructive viral diseases for livestock animals which is endemic in Egypt and usually occurs as an outbreak because of its nature. Rapid diagnosis would play a crucial role in controlling programs (Kandeil et al., 2013; El-Khabaz and Al-Hosary, 2017). Although, the presence of a locally trivalent vaccine against FMDV serotypes A, O and SAT2, annually outbreaks are occurring in cattle and buffaloes throughout Egypt (Khodary et al., 2018; Diab et al., 2019). The FMDV strains circulated among cattle and buffalo herds in Egypt during 2013 (Abd El Wahed et al., 2013). The clinical signs of present study in cattle and buffaloes during summer 2017 were similar to FMD signs include fever 40-41 ºC, ropy salivation, vesicles and erosions in gums, dorsum of the tongue and in inter-digital spaces of cattle's and buffaloes claws as the characteristic signs of FMD and these clinical observations are agreed and recorded by many previous researches (Elhaig and Elsheery, 2014; Aktas et al., 2015; El-Khabaz and Al-Hosary, 2017). Detection of FMDV infection in cattle and buffaloes by 3ABC-ELISA were 54 out of 86 serum samples (37 and 17) positive for nonstructural protein antibody in field serum samples by 3ABC-ELISA indicated active FMDV infection respectively. Also, negative serum samples mean, no infection or animals may be vaccinated with FMDV vaccine as showed in figure1 and figure 2 and these results were agreed with (King et al., 2006; Rady et al., 2014).

Epithelial tissues contained an abundance of the FMDV which detected by Sn-ELISA for differentiation of FMDV serotypes (EL-Shehawy et al., 2011; Longjam et al., 2011; Khodary et al., 2018). Differentiation of FMDV serotypes by Sn-ELISA in tissues biopies of cattle were 12 (18.75%), 18 (28.12%), 3 (4.68 %) and 4 (6.25%) positive for A, O, SAT2 and mixed coinfection with three serotypes respectively. Also, in buffaloes, the positive precent of samples of tissue biopsy were 4 (6.25%) for serotype SAT2, 9 (40.09 %) for serotype O, 2 (9.09 %) for serotype A and 2 (9.09 %) for mixed infection with three serotypes (O, A and SAT2) as shown in table 2 and figure 3.

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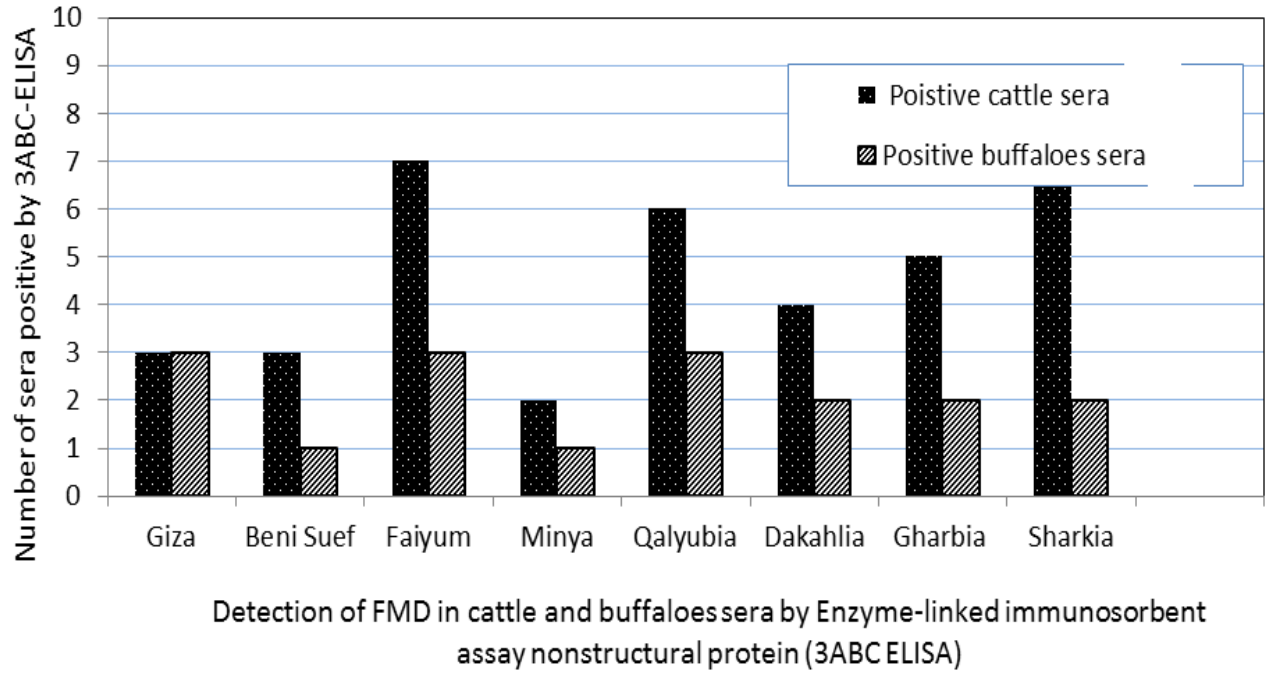

Figure 2. Detection of foot and mouth disease virus in cattle and buffaloes of different governorates of Egypt examined by Enzyme-linked immunosorbent assay nonstructural protein (3ABC ELISA)

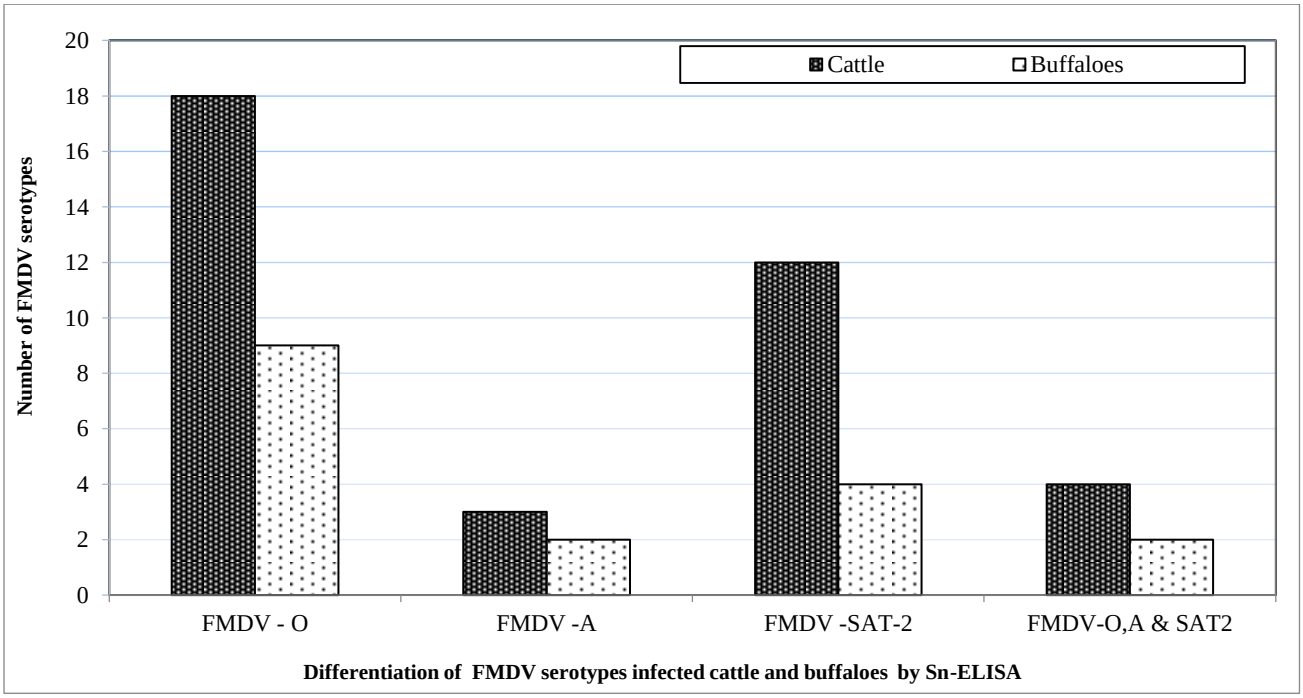

Figure 3. The differentiation of foot and mouth disease virus serotypes A, O and SAT2 in infected cattle and buffaloes at different governorates in Egypt by sandwich-ELISA. Foot and mouth disease virus serotype SAT-2: Positive results for FMDV serotype SAT-2 infected cattle and buffaloes; FMDV serotype O: Positive results for FMDV serotype O infected cattle and buffaloes; FMDV serotype A: Positive results for FMDV serotype A infected cattle and buffaloes; Mixed FMDV serotype A, O and SAT-2: Positive results for different FMDV serotype A, O; SAT-2 mixed infected cattle and buffaloes

The obtained results confirmed that the FMDV serotypes were SAT2, A, and O which circulated in Egypt yet. The serotype O is predominant FMDV serotype in Egypt as shown in table 2 and figure 3 and this result was agreed with (Domingo and Holland, 1997; Carrillo et al., 2005). The molecular identification by RT-PCR used universal primer set P1/P2 detected the FMDV regardless to the serotype, the specific band appeared at 216-bp, followed by specific primer for each serotypes O, A and SAT2 are endemic in Egypt as presented in table 1 and figure 4 this finding was agreed with many previous reports (Carrillo et al., 2005; Vallat, et al., 2017; Sobhy et al., 2018), that stated the endemic infections have been occurred more than one serotypes.

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Table 2. Detection of different foot and mouth diseases virus serotypes by sandwich-ELISA in infected cattle and buffaloes specimens in different governorates of Egypt

Location	clinical samples biopsies or sera		Sn- ELISA									
	Cattle	Buffaloes	Food and Mouth Disease Virus serotypes O (%)		Food and Mouth Disease Virus serotypes A (%)		Food and Mouth Disease Virus serotypes SAT-2 (%)		Mixed infection different serotypes		Total Positive (%)	
			Cattle	Buffaloes	Cattle	Buffaloes	Cattle	Buffaloes	Cattle	Buffaloes	Cattle	Buffaloes
Giza	8	3	2 (25)	2 (66.6)	-	-	1(12.5)	-		1(33.33)	3(37.5)	3(100)
Beni Suef	7	2	-	-	-	-	3(42.8)	1(50)		-	3(42.85)	1(50)
Faiyum	9	4	3(33.33)	2 (50)	1(11.11)	1(25)	2(22.22)	-	1(11.11)	-	7(66.66)	3(75)
Minya	6	2	1(16.66)	1(50)			1(16.66)	-		-	2(33.33)	1(50)
Qalyubia	8	4	3(37.5)	1 (25)	1(12.5)		1(12.5)	1(25)	1(12.5)	1 (25)	6(83.33)	3(50)
Dakahlia	7	2	2(28.57)	1(50)	-	1(50)	2(28.57)	-		-	4(57.14)	2(100)
Gharbia	11	3	3 (27.27%)	1(33.33)	-	-	1(0.09%)	1(33.33)	1(0.09 %)	-	5(45.45)	2(66.66)
Sharkia	8	2	4(50)	1 (50)	1(12.5)		1(12.5)	1(50)	1(12.5)	-	7(75)	2(100)
Total	64	22	18(28.12)	9(40.09)	3(4.68)	2(9.09)	12(18.75)	4 (16.66	4(6.25)	2 (9.09)	37(51.5)	17(59.09)

Sn- ELISA: Sandwich enzyme linked immunosorbent assay; FMDV: Foot and mouth disease virus

Table 3. Comparative between different diagnostic tests for detection and differentiated between Foot and Mouth Disease Virus serotypes in infected cattle and buffaloes at different governorates in Egypt in 2017.

Animals species	number of tissue biopsies collected during outbreaks	Reverse transcription polymerase chain reaction (RT-PCR)					ELISA used nonstructural protein (3ABC-ELISA)	Sandwich enzyme linked immunosorbent assay (Sn-ELISA)				Real-Time reverse transcriptase PCR (rRTPCR)				
		FMDV universal primers (U- FMDV)	FMDV SAT-2	FMDV O	FMDV A	FMDV mixed infection SAT2, O and A		FMDV SAT 2	FMDV O	FMDV A	FMDV mixed infection SAT2, O and A	FMDV universal primers (U- FMDV)	FMDV - SAT 2	FMDV -O	FMDV -A	FMDV mixed infection SAT2,O and A
Cattle	64	37	12	18	3	4	37	12	18	3	4	37	12	18	3	4
Buffaloes	22	17	4	9	2	2	17	4	9	2	2	17	4	9	2	2
Total	86	54	16	27	5	6	54	16	27	5	6	54	16	27	5	6

RT-PCR: Reverse transcription polymerase chain reaction; FMDV: Foot-and-Mouth Diseases; 3ABC-ELISA: nonstructural protein enzyme linked immunosorbent assay; Sn-ELISA: Sandwich enzyme linked immunosorbent assay; rT PCR: Real-Time reverse transcriptase Polymerase Chain Reaction; Foot and Mouth Disease Virus serotypes O, A, SAT-2; RT PCR U-FMDV: reverse transcriptase PCR used to detect FMDV universal primers; SAT-2 : FMDV serotype SAT-2; S-O: FMDV serotype O; S-A: FMDV serotype A; SAT-2, O and A: infected animals different serotypes of FMDV (Mixed infection).

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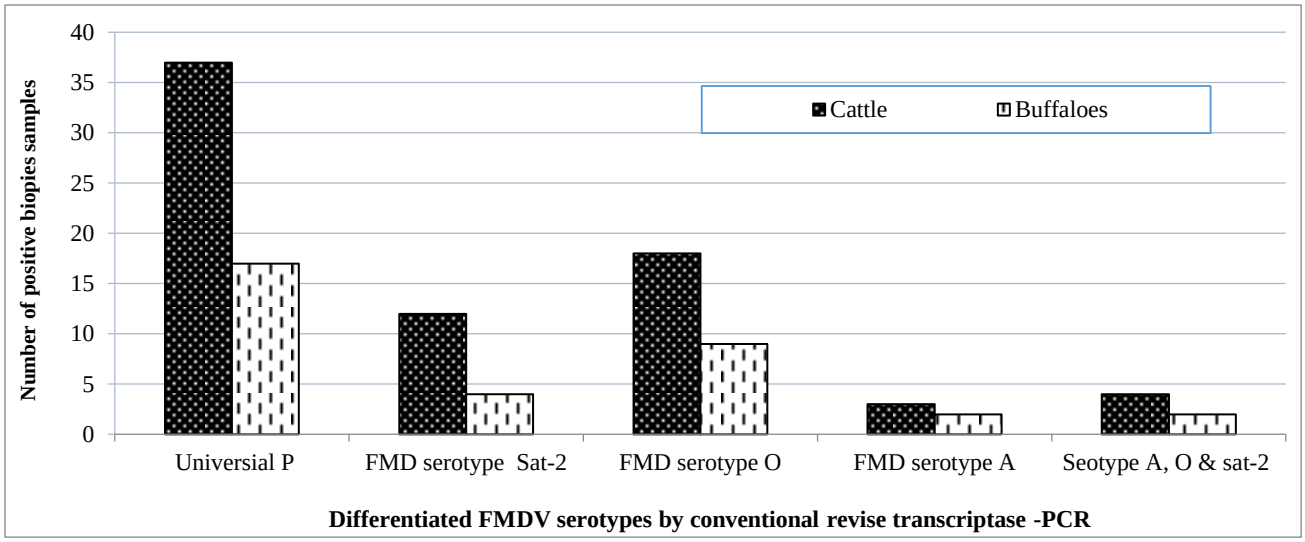

Figure 4. Detection of different foot and mouth disease virus serotypes in tissue samples of bovine by conventional revise transcriptase -PCR in different governorates of Egypt

Universal p: positive results of reverse transcription polymerase chain reaction using universal primer in specimen of cattle and buffaloes; Fserotype SAT-2: Positive results for FMDV serotype SAT-2 infected cattle and buffaloes specimens; Foot and mouth disease virus serotype O: Positive results for FMDV serotype O infected cattle and buffaloes specimens; FMDV serotype A: Positive results for FMDV serotype A infected cattle and buffaloes specimens. The examination of 86 tissue samples for FMDV from cattle and buffaloes by RT-PCR using universal primers for FMDV were 54 tissue samples positive for FMDV different serotypes but when used specific primers with RT-PCR for differentiation FMDV serotype in tissues biopsy of cattle were 18 (28.12%) , 12 (18.75%), 3 (4.68 %) and 4 (6.25%) for serotypes O, SAT2, A and mixed coinfection with three serotypes respectively. Also, the, positive results of tissue samples from buffaloes examined by RT-PCR were 9 (40.09 %), 4 (6.25%) , 2 (9.09 %) and 2 (9.09 %) for O, SAT2 , serotype A and mixed serotypes respectively, as illustrated in figure 4 and this finding was in agree with (Locher et al., 1995; Carrillo et al., 2005; Paixao et al., 2008; Shawky et al., 2013; Sobhy et al., 2014).

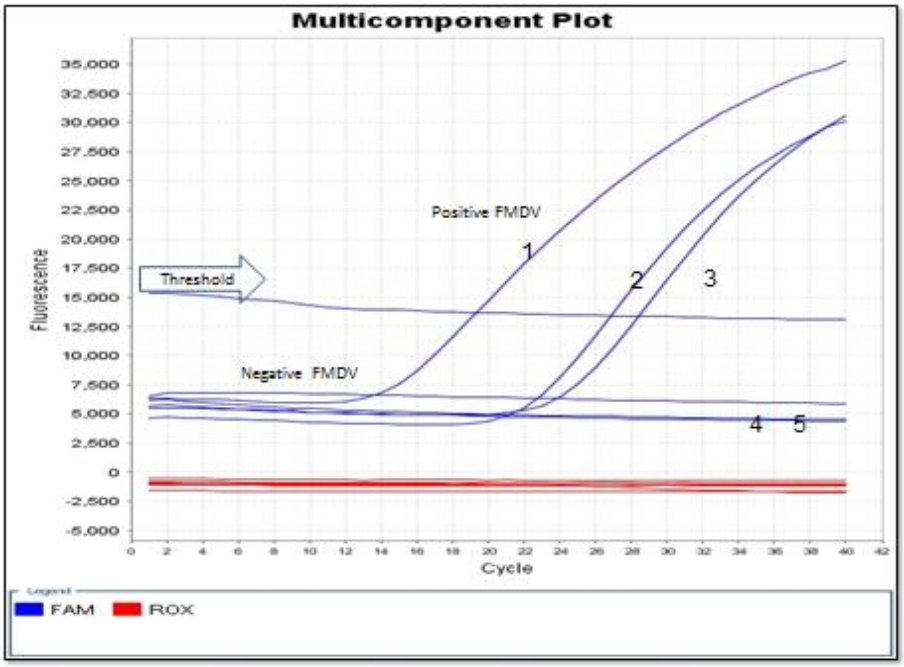

Figure 5. Tissues biopsies samples collected from cattle and buffaloes suspected to FMD in Egypt on 2017. Ct 1 : Postive control for all Foot –and Mouth Disease Virus (FMDV)serotypes using univesial primers; Ct 2,3 ,4 and 5: Tested tissues biopsies samples colected from cattle and buffaloes during FMDoutbeaks. Fam-labeled probes: FAM based probe complementary to only one of the genes and SYBR Green I as an intercalating dye; Ct: cycle threshold; ROX0: normalization 0.5 µl ROX (5x concentration) (6-Carboxyl-X-Rhodamine max – 610nm) can inhibit PCR if the concentration is too high.

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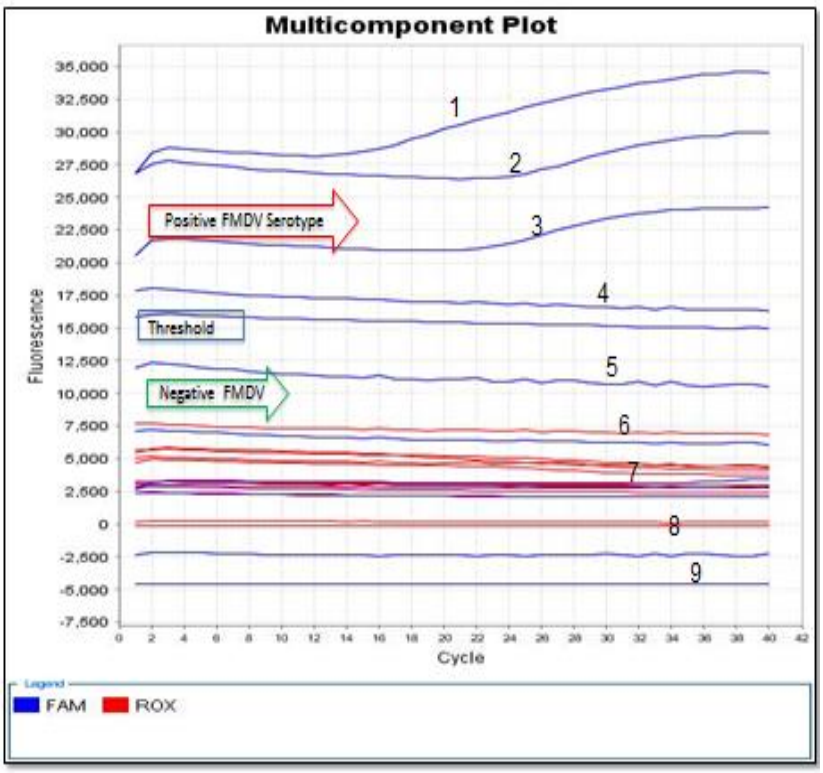

Figure 6. Differentiation of foot and mouth disease virus serotypes SAT2, O and A in tissue samples of infected cattle and buffaloes. Ct 1 positive FMDV type SAT-2. Ct 2, 3, 4 showed positive tested samples collected from tongue tissue biopsy during FMD outbreaks. While Ct 5,6,7,8and 9 showed negative results did not reach the threshold are recorded negative results or "No cycle threshold (Ct)". FMDV: Foot –and Mouth Disease Virus; FAM: based probe complementary to only one of the genes and SYBR Green I as an intercalating dye; Ct: cycle threshold; ROX: normalization 0.5 µl, ROX (5 x concentration) (6-Carboxyl-X-Rhodamine max – 610nm); ROX can inhibit PCR if the concentration is too high

Real-time reverse transcriptase Polymerase Chain Reaction (RT-PCR) detected 54 positive Foot –and Mouth Disease Virus in suspected samples of cattle and buffaloes as demonstrated in figure 5 and figure 6. FMDV positive results above the cycle threshold (Ct) and negative below the cycle threshold (Ct) are presented. Ct 1 showed postive control for all FMDV serotypes using univesial primers, Ct 2 and 3 showed positive samples for FMDV, while Ct 4 and 5 showed negative results. The fluorescence signal accumulated during amplification crosses the cycle threshold (Ct) value. A cycle threshold (Ct) value is calculated at the end of the assay. Negative results (for assays that did not reach the threshold) are recorded negative results or "No cycle threshold (Ct)".

Results of rRTPCR were indciated in less than five hours for collected tissue samples. High evident rRT-PCR results associated with samples that had CT values above the diagnostic threshold CT was sufficient for the confirmation of FMDV prevalence, this study had a beneficial result where FMD was endemic. Also, rRT-PCR able to identify and characterized different FMDV serotypes causes outbreaks (Bachanek-Bankowska et al., 2018). RRT-PCR results was similar to the result obtained by 3ABC ELISA and RT PCR. The negative results were likely to occur in cattle recovered from clinical lesions since the virus was extremely reduced with 7-10 days after the appearance of gross lesions as shown in table 3 and this result was in agreement with (Paixao et al., 2008; Lee et al., 2011). In addition, cattle and buffaloes can become carriers, and also can harbor the virus for up to three years (Locher et al., 1995; Longjam et al., 2011). Finally, prevalence of FMD in Egypt at 2017 may be related to several causes which included insufficiency of the vaccination program, improper inactivated FMDV vaccine, as well as imported cattle from Ethiopia or different Africans countries which endemic with FMDV due to the genetic mutation of the FMDV, can be dedicated that the FMD virus produces a new antigenic structure that can be escaped from the animal immune system. Also, no cross-protection between the different FMDV serotypes was indicated.

CONCLUSION

This study was proved that the FMDV serotypes O, A, and SAT2 have been occurred in cattle and buffalos in different governorates in Egypt at a period in the summer of 2017. FMDV is transboundary from neighbor countries or throughout the importation of carrier animals. the RT-RPA assay was developed for rapid and sensitive for identification of FMDV when compared to conventional RT-PCR, 3-ABC-ELISA, and Sn-ELISA.

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Competing interests

The authors declared that they have no competing interests.

Author`s contributions

Dr. GSZ, research idea, planned the study design, performed statistical analysis, and field animal's samples collection, performed serological and molecular laboratory work, and drafting the paper. Dr. AMA, sharing in the conception of the research idea, field animal's samples collection, and participated in drafting the manuscript Dr. KA, involved in samples collection, laboratory work, interpreted the data results, and helped in manuscript preparation. All authors read and approved the final manuscript.

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Distribution Profile and Function of Carbohydrate Residues in Testes of Immature and Mature Sunda Porcupine (*Hystrix javanica*)

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ABSTRACT

The population of Sunda porcupine (*Hystrix javanica*) declines each year since it is rarely found in nature. The present study aimed to obtain information about the distribution of carbohydrate residues contained in immature and mature of Sunda porcupine's testes and to discuss its relevant functions. This study used six testes obtained from four immature and two mature Sunda porcupine originated from Ngawi Regency, East Java Province, Indonesia. Testis tissues were stained with hematoxylin and eosin and lectin histochemistry of *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* leucoagglutinin (PHA-L), Pisum sativum agglutinin (PSA), Sophora japonica agglutinin (SJA), and *Wheat germ* agglutinin (WGA). Data were analyzed with descriptive and semi-quantitative method. Lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues in the immature and mature testes with weak to very strong intensity. In the immature testes of Sunda porcupine, there was positive reactivity with PHA-L for Leydig and Sertoli cells, N-acetylgalactosamine may play an important role in the development and maturation of Leydig and Sertoli cells. Mature testes showed a strong positive reaction to the LCA, SJA, PSA, and WGA which indicated the significant roles of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues on the maturation process of early spermatid to the late spermatid. These results can be used as basic data to be implemented in the conservation efforts of Sunda porcupine.

Key words: Carbohydrate residue, Lectin, Spermatogenesis, Sunda porcupine, Testes

INTRODUCTION

Hystrix javanica is an Indonesian endemic porcupine, commonly called the Sunda porcupine. Taxonomy of Sunda porcupine is: kingdom Animalia, phylum Chordata, class Mammalian, order Rodentia, family Hystricidae, genus *Hystrix* and species *H. javanica*. Based on the International Union for Conservation of Nature (IUCN), Sunda porcupine is classified in least-concern category which means that is still relatively spread, abundant and no major threat to the existence of Sunda porcupine. The Convention on International Trade in Endangered Species (CITES) which regulates the trade of endangered species of wildlife and plants, includes the Sunda porcupine in the appendix III list which means it has not been considered endangered category (Aplin, 2016).

Testes are reproductive organs that amount to one pair. The testes are in the scrotum and enveloped by tunica albuginea. The seminiferous tubules are present in the testes bounded by complex epithelium of spermatogenic cells and Sertoli cells. Spermatogenic cells produce spermatozoa (Bacha Jr and Bacha, 2012). The Sertoli cell is located in the terminal segment of the seminiferous tubule having functions as fluid transport and secretory activity as well as phagocytosis and intracytoplasmic spermatozoa degradation (Ahmed, 2005). The Sertoli cell has a pale or triangular nucleus that is a high cell extending from the basement membrane to the tubular lumen (Bacha Jr and Bacha, 2012). Interstitial tissue fills the space between seminiferous tubules with blood vessels, lymph vessels, and nerves of the testicular parenchyma (Setchell, 1986). Leydig cells are a very important source of androgens. More than 90% of the androgens in the organism are produced in testes (Ahmed, 2005).

Lectins can be defined as non-immune carbohydrate-binding proteins that can agglutinate and or form the precipitates of glycoconjugate. Glycoconjugates play a role in cell differentiation, cell maturation, cell recognition, cell adhesion, and cell interactions. The distribution of glycoconjugates in animal tissues can be investigated using histochemical lectin staining (Dias et al., 2015).

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Information on testicular biology of Sunda porcupine has not been previously reported. Limited data on the reproductive biology of Sunda porcupine was an important reason for this study. This information can be used to support Sunda porcupine breeding efforts in Indonesia. The aim of this study was to provide Information on carbohydrate residues of immature and mature testes of Sunda porcupine to determine the role of glycoconjugate in the spermatogenesis process.

MATERIALS AND METHODS

This study used the testes from the male Sunda porcupine (two adults and four immature) originated from Ngawi, East Java, Indonesia. Testes samples were trimmed by dividing three parts of the testis according to the location of epididymis which is caput, corpus, and cauda.

Hematoxylin and eosin staining

Conventional Hematoxylin and Eosin (H&E) staining, which has been used by histologists for more than 100 years, is the gold standard of histology structure (Li et al., 2018). The first stage of H&E staining was deparaffinization. The slides were dipped into the Harris Hematoxylin solution then dipped into the eosin solution. The next step was dehydration. The clearing process was done by inserting the slides into xylene. The mounting process was done by closing the tissue using glass decks and Entellan as an adhesive.

Lectin histochemical staining

The first step of lectin histochemical staining was deparaffinization with xylene and rehydration with ethanol. The slides were incubated in 3% H_2O_2 solution in methanol as endogenous peroxide inhibitor for 30 min and wash with PBS, then background sniper was applied to block non-specific proteins for 30 min. Fifteen microliter solution of *Lens culinaris* agglutinin (LCA), wheat germ agglutinin (WGA), *Sophora japonica* agglutinin (SJA), *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Pissum sativum* agglutinin (PSA) and PBS was dropped as a negative control, then incubated overnight in 4^0 C. The positive reaction of lectins was visualized by diaminobenzidine (DAB) substrate, followed by Hematoxylin Harris solution for counterstain. Then slides were dehydrated with ethanol, cleared with xylene and mounted. The semi-quantitative analysis as a parameter was based on the brown color visualized in the cells with five criteria as negative (-), weak (+), moderate (++), strong (+++), and very strong (++++).

RESULTS AND DISCUSSION

Lectins play an important role in the processes associated with the recognition and interaction of cells, protein synthesis and transport, cell division regulation, fertilization, innate immunity, etc. (De Schutter and Van Damme, 2015; Feizi and Haltiwanger, 2015). Lectins are specific to certain carbohydrate structures. Some lectins can interact only with mannose or glucose residues and others only with galactose. There are other lectins specific to fucose, sialic acid, and other monosaccharides (Kobayashi et al., 2014; Nagdas et al., 2014).

The LCA reactivities on mature Sunda porcupine testicles detected strong on the early spermatids in caput (Figure 1A), corpus (Figure 1B), and cauda (Figure 1C). LCA reactivities detected on late spermatids only in caput (Figure 1A). The LCA reactivity was not shown in spermatogonia, primary spermatocytes, early spermatids, late spermatids, Leydig cells and testicular Sertoli cells in the caput (Figure 1D), corpus (Figure1E), and cauda (Figure 1F) in immature Sunda porcupine. According to Barre et al. (2019), LCA is specific to detect alpha-D-mannose and alpha-D-glucose sugar residues. Lectin histochemical staining of LCA in mature male Sunda porcupine detected in early spermatid and late spermatid shows that alpha-D-mannose and alpha-D-glucose sugar residues are needed at the early stages until the final stage of spermatid differentiation. While, lectin histochemical staining of LCA not detected in immature testicles indicates that the immature Sunda porcupine testicles did not require alpha-D-mannose and alpha-D-glucose sugar residues. Research on mice (Lee and Damjanov, 1984) showed different results in which LCA reacted positively to Sertoli cells, spermatogonia, spermatocytes, spermatozoa, and Leydig cells.

The PHA-L reactivity on mature Sunda porcupine was detected weak to testicular spermatogonia in corpus (Figure 2B), and negative in caput (Figure 2A) and caudal (Figure 2C). In late spermatid, PHA-L reactivities were weak in corpus (Figure 2D). Reactivity of PHA-L in Leydig cells was weak in caput (Figure 2A) and corpus (Figure 2C). Immature Sunda porcupine, PHA-L in Leydig cells showed positive reactivity in caput (Figure 2D), and weak reactivity in corpus (Figure 2E). Sertoli cells showed weak PHA-L reactivity in caput and corpus. According to Zhang et al. (2014), PHA-L bound N-acetylgalactosamine. Lectinhistochemical staining PHA-L detected in Leydig cells, spermatogonia, and late spermatids on mature and immature Sunda porcupine shows that N-acetylgalactosamine sugar residues are required in the process of differentiation and maturation of these cells. According to Arya and Vanha-perttula (1985), the need for glycoconjugate in small portions of Sertoli cells is thought to be closely related to the

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phagocytic ability and the process of forming residual bodies in the final stages. According to Ahmed (2005), Leydig cells are an important source of androgen hormones and 90% of the androgen hormones in the animal body are produced by the testes. N-acetylgalactosamine sugar residue is required for optimal development of Leydig cell so that Leydig cells can produce testosterone. PHA-L staining in mice (Lee and Damjanov, 1985) showed that PHA-L reacted positively to spermatogonia, spermatocytes, spermatids, and spermatozoa.

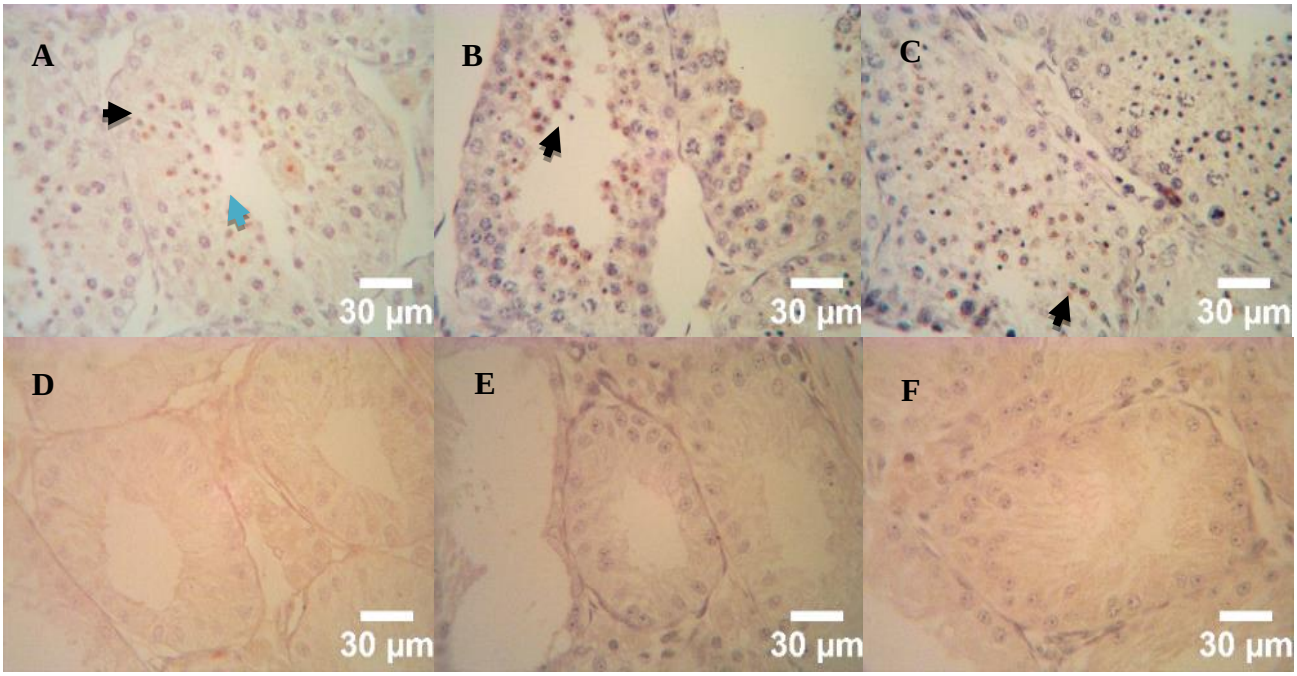

Figure 1. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with LCA). **A:** positive LCA reactivity in the early spermatid (black arrow) and the late spermatid (blue arrow) in caput of the mature Sunda porcupine testicle. **B:** LCA reactivity in the early spermatid (black arrow) in corpus of the mature Sunda porcupine testicle. **C:** LCA reactivity in the early spermatid (black arrow) in cauda of the mature Sunda porcupine testicle. Negative LCA-reactivity in caput (**D**), corpus (**E**) and cauda (**F**) regions of immature Sunda porcupine's testicle.

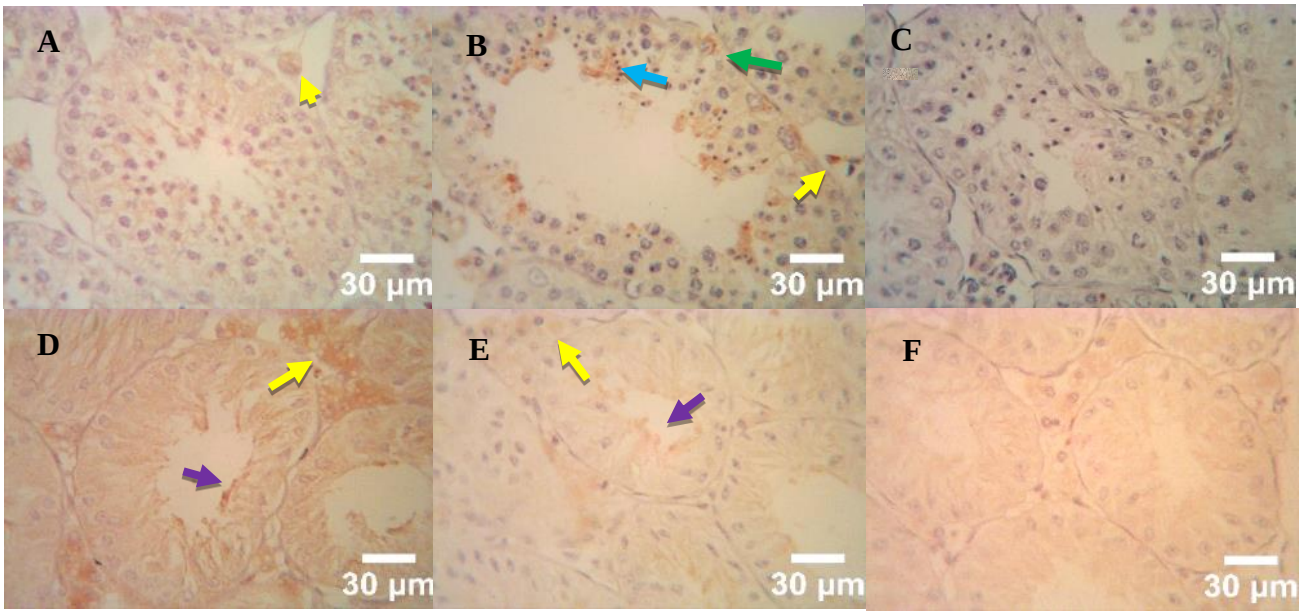

Figure 2. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with PHA-L). **A:** positive lectin reactivity in Leydig cells (yellow arrow) of testicle of mature Sunda porcupine in caput region. **B:** positive lectin reactivity in spermatogonia (green arrow), late spermatid (blue arrow) and Leydig cell (yellow arrow) of mature Sunda porcupine in corpus region. **C:** negative lectin reactivity in caudal region of mature Sunda porcupine testicle. **D:** PHA-L reactivities detected in Leydig cells (yellow arrow) and Sertoli cell cytoplasm (purple arrow) of immature Sunda porcupine testicle. **E:** an immature Sunda porcupine testicle in the corpus show positive PHA-L reactivities in the Leydig (yellow arrow) and Sertoli cell cytoplasm (purple arrow). **F:** the immature Sunda porcupine testicle in cauda showing negative PHA-L reactivity.

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Lectin histochemical staining with PSA for primary spermatocytes of mature Sunda porcupine showed week reactivity in cauda region (Figure 3C). PSA reactivity was detected moderate in primary spermatocyte, early spermatid in caput region (Figure 9A) and weak in corpus (Figure 3B) and cauda (Figure 3C). PSA reactivity was detected moderate in late spermatid in caput (Figure 3A), and weak in cauda (Figure 3C). Lectin histochemical staining with PSA of testis of immature Sunda porcupine showed weak reactivity in Leydig cells in caput (Figure 3D). According to Zhang et al. (2014), lectin histochemical PSA binds to mannose. PSA reacted positively to primary spermatocytes, early spermatids, and late spermatids of mature Sunda porcupine, and reacted positively to Leydig cells of immature Sunda porcupine. According to Novelina et al. (2010), glycoconjugate plays an important role in various metabolic processes of the body such as regeneration, cell differentiation, adhesion, and intercellular communication as well as other functional processes. Cells that react positively to PSA indicate that the cell requires a residual sugar mannose in its development. According to Wahyuni et al. (2016), the detectable glycoconjugate in spermatids indicates the importance of glycoconjugate in spermatogenesis, especially in spermiogenesis.

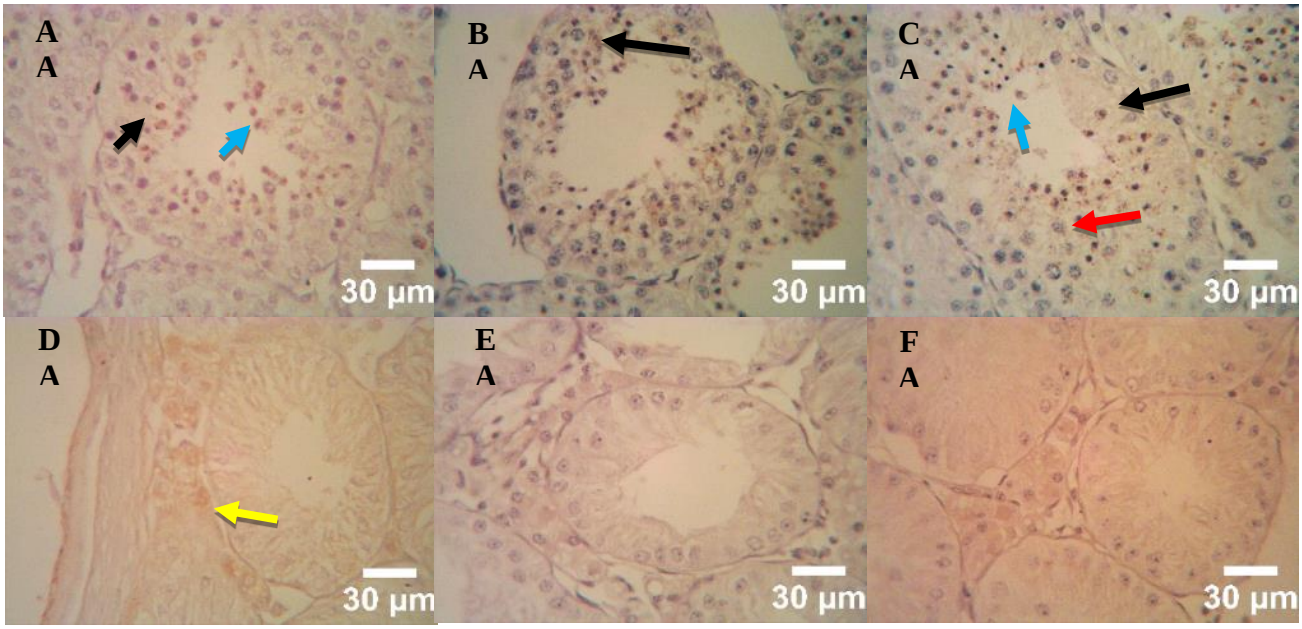

Figure 3. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with PSA). **A:** the testicles of mature Sunda porcupine in caput region showing PSA reactivities in the early spermatid (black arrow) and late spermatid (blue arrow). **B:** mature Sunda porcupine testicle in the corpus showing PSA reactivities in early spermatid (black arrow). **C:** testis of mature Sunda porcupine in caudal part showing PSA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow) and the late spermatid (blue arrow). **D:** the immature Sunda porcupine testicle in caput showing positive PSA-reactivity in Leydig cell (yellow arrow), whereas the negative reactivity in corpus (**E**) and cauda (**F**)

Histochemical lectin staining with SJA in early spermatids of mature Sunda porcupine showed strong reactivity in cauda (Figure 4C), moderately positive reactions in caput (Figure 4A), and weak reactions in corpus (Figure 4B). The late spermatid showed moderate SJA reactivity in caput (Figure 4A). The immature Sunda porcupine's Leydig cells showed weak SJA reactivity in caput (Figure 4D). According to Zhang et al. (2014), SJA binds N-acetylgalactosamine. SJA showed positive reactions in the early spermatids and late spermatids of mature Sunda porcupine, and immature Sunda porcupine's Leydig cells. Cells that react positively to SJA show that the cell requires N-acetylgalactosamine for its development. The positive pattern was found from the early stage to the late stage of the spermatid. Although in the final stages, the intensity of the positive reaction decreased, the positive affinity pattern of Sunda porcupine was similar to that of the horse (Verini-Supplizi et al., 2000).

Lectin histochemical staining of mature Sunda porcupine showed that WGA reacted positively strong in primary spermatocytes, early spermatids and late spermatids in caput (Figure 5A) and cauda (Figure 5C), whereas in corpus (Figure 5B) was positively weak. In immature Sunda porcupine, WGA reacted positively weak on Leydig cells in the corpus (Figure 5E). According to Wahyuni et al. (2016), WGA binds with N-acetylglucosamine sugar that is necessary for differentiation process of spermatocyte and spermatid cell. WGA reactivity in rats was detected in spermatogonia, spermatocytes, spermatozoa, Sertoli cells, Leydig cells, and lamina propria (Lee and Damjanov, 1985; Shegedin et al., 2017). In Syrian hamster, WGA reactivities were detected in spermatogonia (Hernández et al.2018).

Lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine residues (Murakami et al., 2014; Belicky and Tkac, 2015) in the immature and mature testes.

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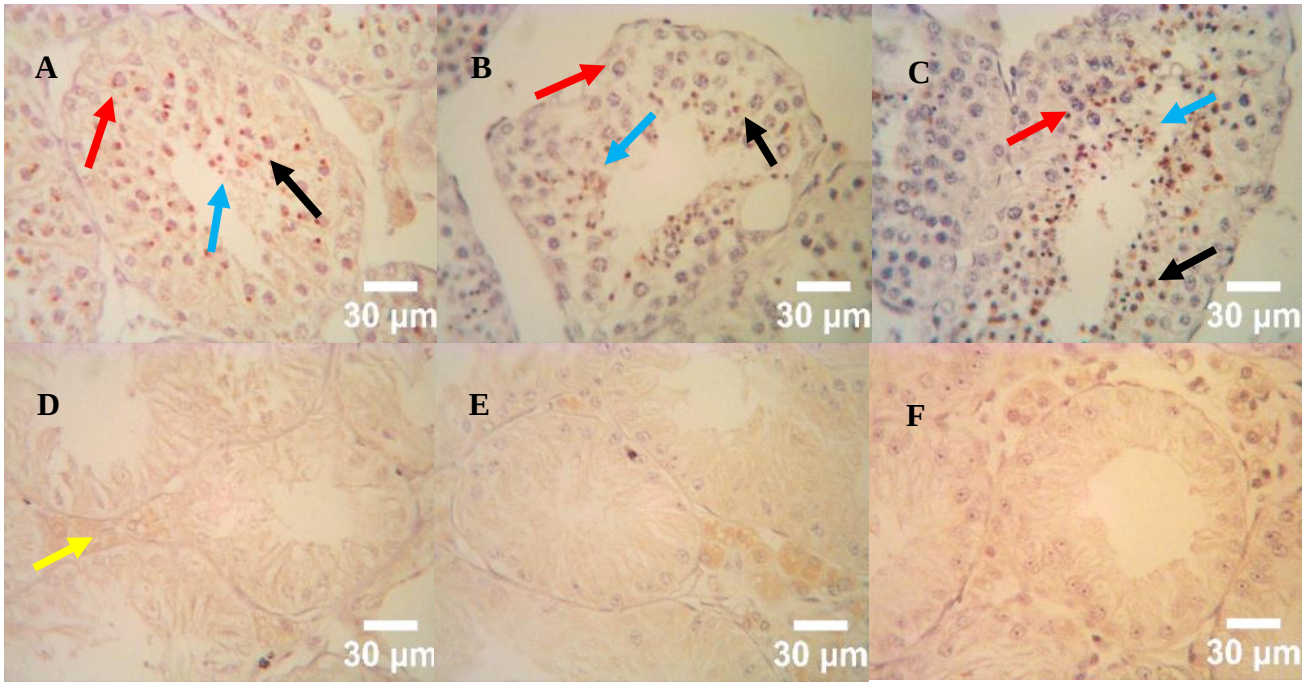

Figure 4. Testicular micrographs of *Hystrix javanica* (lectin histochemical staining with SJA). **A:** testicles of mature Sunda porcupine in caput region showing SJA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow) and the late spermatid (blue arrow). **B:** mature Sunda porcupine testicle in the corpus show SJA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow). **C:** testicle of mature Sunda porcupine in caudal regions show SJA reactivities in primary spermatocyte (red arrow), early spermatid (black arrow). **D:** immature testicle in caput show SJA reactivities in Leydig cells (yellow arrow), while show negative SJA-reactivity in corpus (**E**) and cauda (**F**).

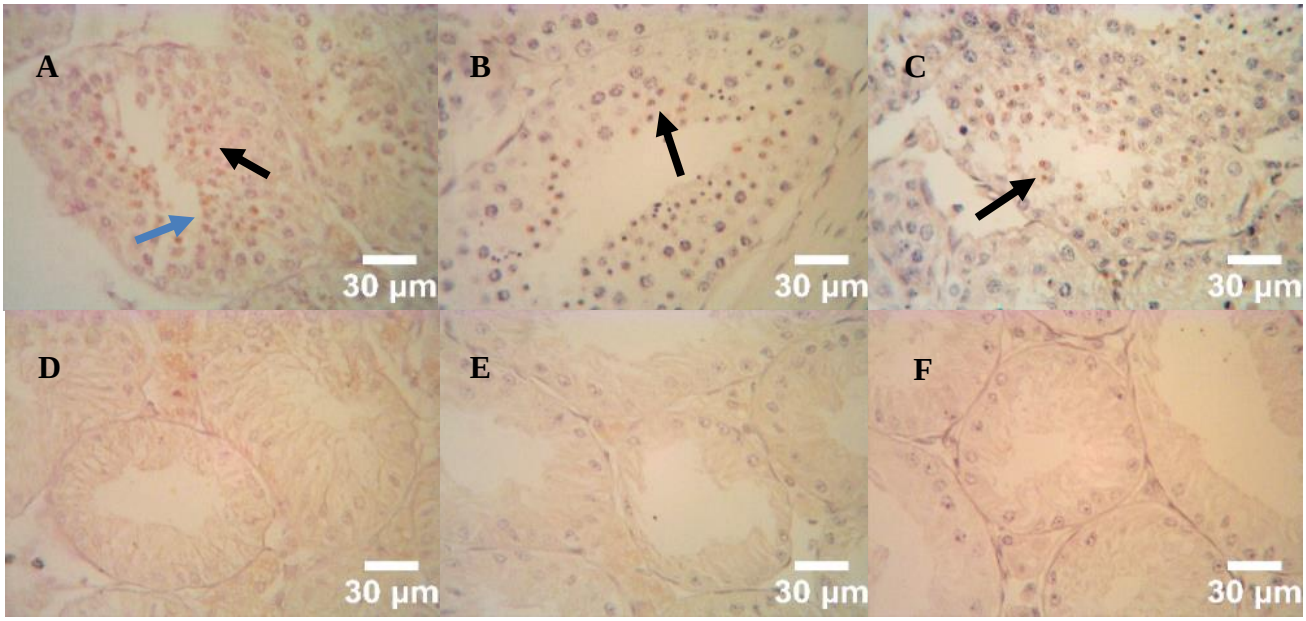

Figure 5. Testicular micrographs of *Hystrix javanica* (lectinhistochemical staining with WGA). The mature Sunda porcupine testicles in caput (**A**) and corpus (**B**) show positive WGA reactivities in early spermatid (black arrow) and the late spermatid (blue arrow). **C:** the testicle of Sunda porcupine in caudal regions show positive WGA reactivity in spermatids (black arrows). The immature Sunda porcupine in caput part (**D)** and cauda (**F**) show negative WGA reactivity, while in corpus region. **E:** show positive reactivity in the Leydig cells.

CONCLUSION

In conclusion, the present study showed that lectin histochemical staining with LCA, PHA-L, SJA, PSA, and WGA indicated the presence of alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and N-acetylglucosamine sugar residues in the immature and mature testes of Sunda porcupine with weak to very strong intensity. In the immature testes, N-acetylgalactosamine may involve in the development and maturation of Leydig and Sertoli cells, whereas in the mature testes, alpha-D-mannose and alpha-D-glucose, N-acetylgalactosamine, mannose, and

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N-acetylglucosamine residues play important roles in the maturation process of early spermatid to the late spermatid.

DECLARATIONS

Authors' contribution

Teguh Budipitojo developed the concepts and designed the experiments, analyzed and interpreted the data. Irma Padeta collected tissue samples and fixed them in Bouin's solution, processed tissues for paraffin-embedded method. Beninda Ulima Yulianti developed the concepts and designed the experiments, visualized lectin reactivity and wrote the manuscript. Dian Bekti Hadi Masithoh wrote the manuscript and analyzed data. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

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Immunomodulatory Effect of CpG ODN-Adjuvanted Bacterin against *Salmonella enterica* serovar Enteritidis in Broiler Chickens

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ABSTRACT

Bacterial oligodeoxynucleotide containing Cytosine Guanine motifs (CpG-ODN) has been reported to induce immunostimulatory activity against a variety of bacterial, viral, and protozoan infections in a wide range of vertebrate species. The objective of this study was to investigate the dose-dependent immunomodulatory effect of CpG ODN on *Salmonella* Enteritidis bacterin in broiler chickens. Two hundreds one-day-old broiler chicks, divided into 5 groups, were used in this study. First three groups were immunized with *Salmonella* Enteritidis bacterin adjuvanted with different doses of CpG ODN (50µg, 100µg and 200µg). The control groups included a group that was immunized with *Salmonella* Enteritidis bacterin adjuvanted with aluminum hydroxide and a non-immunized group. The intestinal colonization, cellular responses, mucosal and systemic immune responses of immunized chickens was measured at different intervals, until 42 days of age. At two weeks post-immunization, 20 chicks from each group were orally challenged by *Salmonella* Enteritidis fresh bacterial culture ($1.2x10^8$ CFU/ml). The survival rates and the pathological changes of challenged chickens in the different groups were monitored for extra 10 days. Compared to the aluminum hydroxide adjuvanted bacterin, the CpG-ODN adjuvant bacterin induced significant protection and improved survival rate of challenged chickens. Also *Salmonella* Enteritidis was not recovered from the intestinal tract of vaccinated challenged groups. There was a significant dose-dependent immunostimulatory adjuvant effect of CPG-ODN on the level of secretory IgA and the induced mucosal responses. The 200-CpG ODN group showed the highest IgA response followed by 100-CpG ODN group then the 50-CpG ODN and the aluminum hydroxide groups ($P < 0.05$). Also, cellular interactions were remarkably reduced in the liver and intestine of CpG ODN-treated chickens. No inflammatory cellular infiltrations were seen in the liver and intestine of 200-CpG ODN treated group. In conclusion, the presented findings have shown the significant immunostimulatory effect of CpG-ODN and its effect on *Salmonella* Enteritidis bacterin in controlling *Salmonella* infection in broiler chickens.

Keywords: Cellular responses, CpG ODN, Mucosal immunity, *Salmonella* Enteritidis

INTRODUCTION

Salmonella enterica subspecies enterica is a leading cause of global food-borne zoonosis. Poultry and poultry-derived food remain the main source of infection with non-host adapted serovars, including *Salmonella* Enteritidis and *Salmonella* Typhimurium (Burr et al., 2005; Much et al., 2007; Stephens et al., 2007), hence they are good colonizers of the chicken caeca without inducing apparent clinical manifestations (Barrow, 2007). Controlling such kind of silent infection to reduce potential human transmission remains of global interest.

Host-pathogen interaction is a dynamic process which modulates the host immune response to infection (Janeway and Medzhitov, 2002). The host innate immune system recognizes pathogens via a wide range of recognition components called pathogen recognition receptors (PRRs) which recognize pathogen conserved motifs called pathogen associated molecular patterns (PAMPs) that are expressed by the invading infectious microbes, including pathogen cell-surface components, such as bacterial LPS and or pathogen nucleic acid, including single- and double-stranded RNA and CpG DNA (Janeway and Medzhitov, 2002).

Bacterial DNA is a potent stimulator of innate immune responses which is reasoned by the presence of unmethylated CpG dinucleotide (Krieg, 2002). Indeed, it has been shown that bacterial DNA and synthetic oligodeoxynucleotide containing unmethylated CpG-dinucleotides (CpG-ODN) stimulate host responses during infections. Previous literatures have demonstrated that bacterial DNA and CpG-ODN components induce nitric oxide and oxygen production and intracellular killing of *Salmonella* in avian macrophages and heterophils (Xie et al., 2003; He et al., 2007). In mammals, the CpG-ODN motifs are recognized by Toll-like receptor (TLR-9) leading to stimulation of several types of innate and acquired immune cells, including phagocytes, lymphocytes and polymorph nuclear cells

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(Ribeiro and Schijns, 2010; Tovey and Lallemand, 2010). This stimulation leads to activation of Th-1 cellular immune responses via induction of proinflammatory cytokines and chemokines, including interleukins (IL-1, IL-6, IL-18) and interferon (IFN-δ) (Ribeiro and Schijns, 2010). Unlike mammals, chickens respond to CpG-ODN through TLR-21 and TLR-15 (Keestra et al., 2010; Ciraci and Lamont, 2011). The present study investigated the dose-dependent effect of CpG-ODN as a vaccine candidate and its effect on intestinal colonization, local and humoral immune responses and cellular interactions in chickens in response to infection with *Salmonella* Enteritidis.

MATERIALS AND METHODS

Bacterial strain

Field isolates of *Salmonella* Enteritidis recovered from 25-day old broilers with, Diarrhea, cloacitis, hepatitis, and splenitis were obtained from the Biotechnology Centre for Research & Services (CBRS), Faculty of Veterinary Medicine, Cairo University, Egypt. Aliquots of the bacteria were stored at –70°C in a 50% brain heart infusion broth (BHI; Oxoid) supplemented with 25% (w/v) glycerol. This bacterial strain was fully identified using bacteriological and molecular methods.

Preparation of *Salmonella* Enteritidis bacterial cultures

Two to three colonies of *Salmonella* Enteritidis bacteria were added to 200 ml of Luria Broth (LB) in a 1000 ml Erlenmeyer flask. To obtain *Salmonella* Enteritidis in the stationary phase, the cultures were grown at 37°C for 16-18 hours with shaking at 200 rpm/min. The stationary phase cultures contained approximately 1×10^9 CFU/ml of *Salmonella* Enteritidis was used for the preparation of bacterin. Preparation *Salmonella* Enteritidis challenge culture include growing of *Salmonella* Enteritidis in the logarithmic growth phase where a 1:1000 dilution of the stationary phase culture was added to 100 ml of Luria Broth in a 500-ml Erlenmeyer flask and incubated at 37oC for three hours with shaking at 200 rpm. After shaking, the culture contained approximately 1×10^9 CFU per ml. This culture was further diluted with saline to the concentration of bacteria required for the challenge experiments. Serial dilutions were plated in duplicate on Tryptose soy agar plates and incubated for 18-24 hours at 37°C. Following incubation, bacterial colonies on the plates were counted to validate the challenge dose.

Ethical approval

All procedures involving animals were done according to a protocol approved by the Faculty of Veterinary Medicine, Cairo University Committee on Animal Care and Ethics in accordance with research regulations. All dead chickens were humanly handled.

Animal model development

Chickens were randomly allocated to five groups (forty chicks per group) and housed. All groups were given free access to water and commercial starter broiler rations. The photoperiod was set at 24 hours daily for the first three days and 16 hours per day for the remaining seven days. Room temperature was maintained at 30-32 °C for the first week and 28-30 °C for the second week.

Experimental birds

A total of 200 one-day-old chickens (Cobb 500) were kept in closed pens at a maximum initial density of 15 birds per m^2 according to the breed company instructions. Wood shavings were used as litter. The birds had access to commercial diet and water ad libitum. Ambient temperature and ventilation were regulated in keeping with standard breeding practices.

Experimental design and *Salmonella* infection

Two hundred chickens were randomly distributed into five groups (n=40). At the second day of age, chickens in the first four groups were immunized with S. Enteritidis bacterin. The chicks in group one were immunized with S. Enteritidis bacterin adjuvanted with Aluminium hydroxide (AHG). Chickens in group two were immunized with S. Enteritidis bacterin adjuvanted with 50 µg ODN-DNA (50-ODNG). Chickens in group three were immunized with S. Enteritidis bacterin adjuvanted with100 µg ODN-DNA (100-ODNG) and chickens in groups four were immunized with S. Enteritidis bacterin adjuvanted with 200 µg ODN-DNA (200-ODNG). Chickens in group five were injected with phosphate buffered saline (PBS) and kept as negative control group (NCG). Two weeks post immunization 20 chicks from each group were challenged orally with 100 µl of *Salmonella* Enteritidis strain containing $1.2\text{x}10^8$ CFU/ml of stationary phase culture of *Salmonella* Enteritidis. From the remaining chickens in all groups sera and intestinal samples were collected at 18, 35 and 42 days of age for determination of humoral and mucosal immune responses.

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Effect of different adjuvants in *Salmonella* Enteritidis bacterin on the level of intestinal secretory immunoglobulin A (SIgA)

The concentration of SIgA was evaluated in all experimented groups at 18, 35 and 42 days of age by sandwich enzyme-linked immunosorbent assay (NOVA, Beijing, China).

Measurement of *Salmonella* Enteritidis-specific immunoglobulins titer in the immunized chickens

Blood samples were collected from 3 chickens/group from all 5 groups. Serum was separated and stored at -80°C till examined. *Salmonella* Enteritidis-specific immunoglobulins were determined using a commercial ELISA kit (IDEXX *Salmonella* Enteritidis Ab X2 Test Kit; IDEXX Laboratories, Westbrook, Maine, USA). Serum samples with S/P ratios of less or equal to 0.2 should be considered negative. Samples with S/P ratios greater than 0.2 was considered as positive.

Histopathology

Liver and intestinal tissue samples were collected at 35 days of age from the chickens (three chickens per group) representing all treated chicken groups examined at necropsy were fixed in 10% neutral buffered formalin, trimmed, routinely processed, paraffin embedded, sectioned at 4 mm thickness, and stained with hematoxylin and eosin (H&E) for histopathological examination (Setta et al., 2012a).

Statistical analysis

Data were analysed using one-way ANOVA test using Graph Pad software. Differences between groups were considered significant if $P < 0.05$.

RESULTS

Effect of immunization with CpG ODN adjuvanted *Salmonella* Enteritidis bacterin on survival rate of *Salmonella* Enteritidis challenged chickens

The survival rate of challenged chickens was recorded in differently treated chicks for 10 days following infection with *Salmonella* Enteritidis (Table 1). The CpG-ODN adjuvanted *Salmonella* Enteritidis bacterin had significantly improved the survival rate of *Salmonella* Enteritidis-infected chickens (75-85%) when compared to aluminum hydroxide adjuvanted *Salmonella* Enteritidis bacterin control group (65%). Interestingly, the survival rate of birds immunized with *Salmonella* Enteritidis bacterin adjuvanted with 200µg of CpG-ODN induced higher survival rate (85%) compared to the other tested groups.

Effect of CpG-ODN adjuvanted *Salmonella* Enteritidis bacterin on intestinal colonization of *Salmonella* Enteritidis in immunized chickens

Table 2 presents the rates of recovery of *Salmonella* Enteritidis from vaccinated challenged chickens groups. Triplicate samples were taken from chickens of each group at 42 day of age. Chicken groups immunized with CpG-ODN adjuvanted *Salmonella* Enteritidis bacterin were able to clear *Salmonella* Enteritidis colonization from the gut. *Salmonella* Enteritidis bacteria were not detected in all tested groups. In the control group, however, only three positive samples by 4.27 $\log_{10}$ colony forming unit/gram (CFU/g) were recorded.

Mucosal immune response and SIgA production in chicken immunized by differently adjuvanted *Salmonella* Enteritidis bacterin

The concentration of SIgA was measured using ELISA in all experimented chicken groups at 18, 35 and 42 days of age. The results presented in table 3 showed that the highest SIgA concentration was recorded in chicken groups which immunized *Salmonella* Enteritidis bacterin adjuvanted with CpG ODN and aluminum hydroxide (AHG) as compared with the non-immunized control group (NCG). A dose dependent effect of CpG ODN on induction of SIgA was recorded. In comparison with the aluminum hydroxide adjuvant, the CpG ODN appeared to have strong immune stimulatory activity on SIgA response in all tested time points. The 200-ODNG group showed the highest response followed by 100-ODNG group then the 50-ODNG and the AHG groups ($P < 0.05$).

Effect of adjuvant type and concentration on the humoral immune responses to *Salmonella* Enteritidis bacterin

Salmonella Enteritidis-specific antibodies in blood of differently immunized chickens were measured using ELISA (Table 4). No statistical differences were seen between the different groups although numerical differences were observed in the tested groups compared to the non-vaccinated controls. The CpG-ODN appeared to have strong immune stimulatory activity on *Salmonella*-specific antibodies response in comparison with AHG in all tested ages. The 200-ODNG group showed the best humoral immune response followed by the 100-ODNG group then finally 50-ODNG and AHG group.

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Cellular responses following *Salmonella* Enteritidis infection in immunized chickens

Avian-*Salmonella* interactions were reported at the cellular level in intestinal and liver tissues at 35 days of age using H & E histopathological staining (Figure 1). Comparable to serological results, histopathogical examination of liver and intestinal tissue samples taken from chickens in the 200-ODNG groups showed no marked cellular changes. Nonetheless, tissue samples from AHG have shown mild to moderate tissue reaction, including inflammatory mononuclear cell infiltration as a result of *Salmonella* interaction at the cellular level.

Table 1. Effect of CpG adjuvant in *Salmonella* Enteritidis bacterin on the survival rate in different groups of broiler chickens challenged with *Salmonella* Enteritidis

Days post-challenge	Non Vaccinated group		Groups vaccinated with *Salmonella* Enteritidis bacterin adjuvanted with							
			AHG		50-ODNG		100-ODNG		200-ODNG	
	No.	%	No.	%	No.	%	No.	%	No.	%
1st	19/20	95	19/20	95	17/20	85	19/20	95	19/20	95
2nd	19/20	95	19/20	95	17/20	85	19/20	95	19/20	95
3rd	17/20	85	19/20	95	17/20	85	19/20	95	19/20	95
4th	17/20	85	17/20	85	17/20	85	19/20	95	19/20	95
5th	15/20	75	17/20	85	17/20	85	19/20	95	19/20	95
6th	14/20	70	17/20	85	17/20	85	17/20	85	19/20	95
7th	13/20	65	15/20	75	15/20	75	17/20	85	19/20	95
8th	13/20	65	15/20	75	15/20	75	17/20	85	19/20	95
9th	13/20	65	15/20	75	15/20	75	17/20	85	17/20	85
10th	13/20	65	15/20	75	15/20	75	14/20	70	17/20	85

AHG: Aluminium hydroxide group, 50-ODNG: 50 µg ODN-DNA group, 100-ODNG: 100 µg ODN-DNA group, 200-ODNG: 200 µg ODN-DNA group.

Table 2. Effect of CpG-ODN adjuvant in *Salmonella* Enteritidis bacterin on the intestinal colonization of *Salmonella* Enteritidis in broiler chickens of different groups

Chicken Groups Immunized with *S.* Enteritidis Bacterin adjuvanted with:	No. of Samples	*Salmonella* positive	Log CFU/g
AHG	3	0	0
50-ODNG	3	0	0
100-ODNG	3	0	0
200-ODNG	3	0	0
Non immunized	3	3	4.27±0.02

Results are represented as mean log CFU /g ± standard deviation (n=3). AHG: Aluminium hydroxide group, 50-ODNG: 50 µg ODN-DNA group, 100-ODNG: 100 µg ODN-DNA group, 200-ODNG: 200 µg ODN-DNA group

Table 3. Effect of CpG-ODN adjuvant in broiler chickens immunized with *Salmonella* Enteritidis bacterin on the intestinal secretory immunoglobulin (SIgA) concentration

Age	Non Immunized Control	Dose of CpG-ODN Adjuvant in *Salmonella* Enteritidis bacterin			
		AHG	50-ODNG	100-ODNG	200-ODNG
18 days	(5.31 ±0.30)	(6.73 ±0.06)*	(16.12 ± 0.25)*	(20.12 ± 0.29)*	(27.12 ± 0.29)*
35 days	(3.12 ±0.15)	(5.73 ±0.25)*	(18.49 ±0.40)*	(21.49±0.42)*	(29.49 ± 0.22)*
42 days	(1.31 ±0.10)	(6.97±0.058)*	(20.90 ± 0.08)*	(23.50 ± 0.28)*	(35.6 ± 0.13)*

The results are represented as mean ± standard deviation (n=3). * indicate significant differences from non-immunized controls ($P < 0.05$). AHG: Aluminium hydroxide group, 50-ODNG: 50 µg ODN-DNA group, 100-ODNG: 100 µg ODN-DNA group, 200-ODNG: 200 µg ODN-DNA group.

Table 4. Detection of *Salmonella* Enteritidis-specific immunoglobulin in serum of broiler chickens immunized *Salmonella* Enteritidis bacterin adjuvanted with different concentrations of CpG-ODN adjuvant.

Group	OD Values		
	18 days	35 days	42 days
200-ODNG	0.657	0.867	1.0181
100-ODNG	0.432	0.543	0.889
50-ODNG	0.321	0.435	0.768
AHG	0.203	0.321	0.601
NCTG	0.421	0.871	1.567

Results are expressed in ELISA optical density values (OD). AHG: Aluminium hydroxide group, 50-ODNG: 50 µg ODN-DNA group, 100-ODNG: 100 µg ODN-DNA group, 200-ODNG: 200 µg ODN-DNA group

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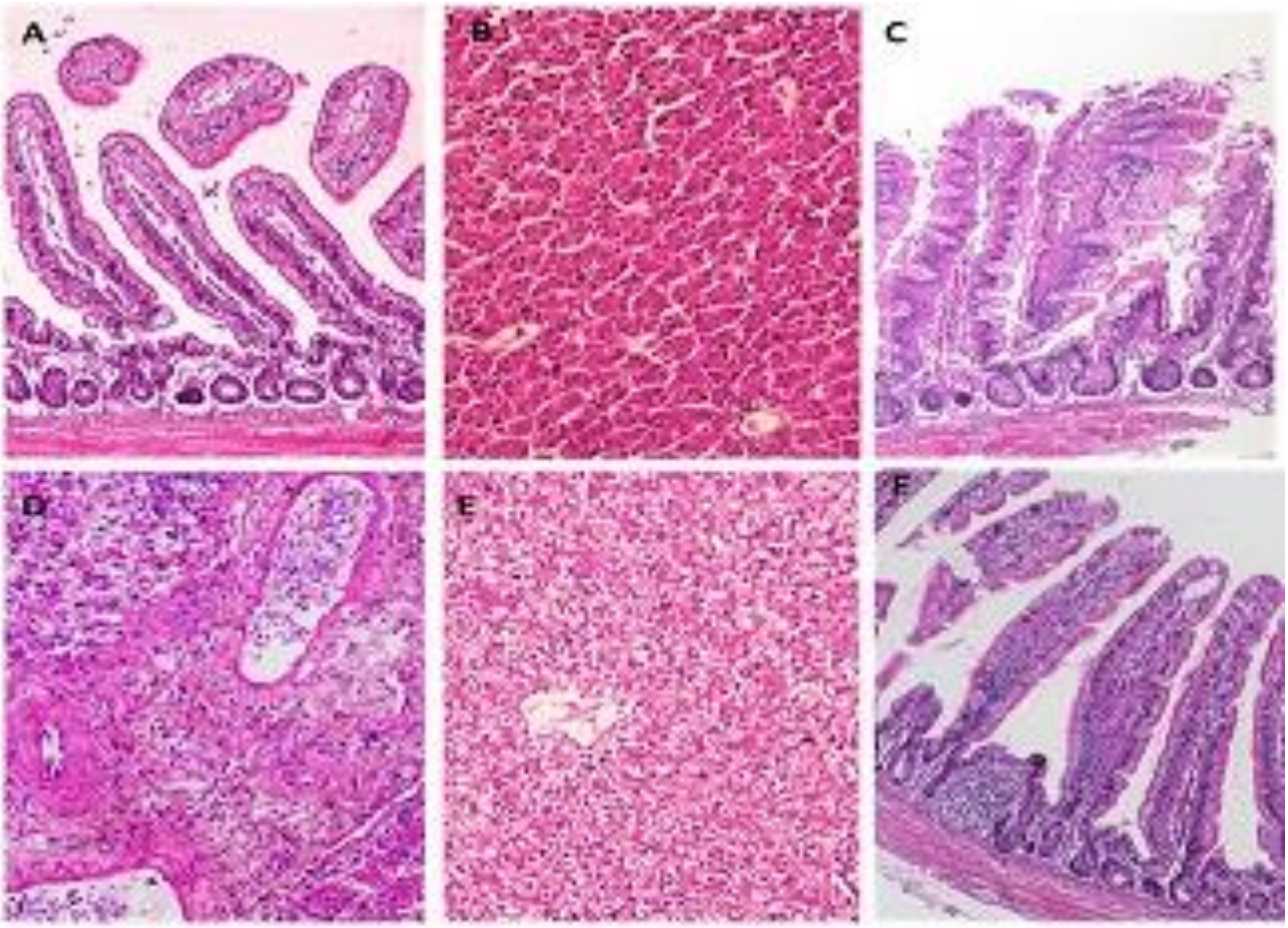

Figure 1. Histopathological images (H&E staining) from intestinal and liver tissues of broiler chickens at 35 days of age in CpG ODN inoculated and non-inoculated groups. **A**: Intestine of chicken in 200-ODNG group showing apparently normal histological structure (X100). **B**: Liver of chicken in 200-ODNG group showing apparently normal histological structure (X200).**C**: Intestine of chicken in non-vaccinated group (NVG) showing severe epithelial and goblet cells hyperplasia (X100). **D**: Liver of chicken in N NVG showing increased portal fibrous connective tissue with few mononuclear cells infiltration and vacuolation of adjacent hepatocytes (X200). **E**: Liver of chicken in chickens immunized showing mild to moderate vacuolated hepatocytes (X200). **F**: Intestine of chicken in AHG adjuvant group showing focal mononuclear cells infiltration and slight goblet cell hyperplasia (X100).

DISCUSSION

The current study provides further insights on the interaction of *Salmonella* with avian host and highlights the role CpG-ODN in immunity against these leading food-borne zoonotic bacteria. In this study, the CpG-ODN adjuvanted *Salmonella* Enteritidis bacterin had significantly improved the survival rate of *Salmonella* Enteritidis-infected chickens. Moreover and unlike the negative control group, chicken groups immunized with CpG-ODN adjuvanted *Salmonella* Enteritidis bacterin were able to clear *Salmonella* Enteritidis colonization from the intestine. In this study, the lack of *Salmonella* recovery from the intestinal tract of challenged immunized chickens could be reasoned to the bactericidal activity of CpG DNA. Indeed, previous literature have shown that CpG-DNA possess an antimicrobial activity inducing microbial killing by stimulation of nitric oxide and oxygen production from defense cells that limit the systemic spread of *Salmonella* (Xie et al., 2003; He et al., 2007).

Data from this study have shown a dose dependent effect of CpG ODN on induction of SIgA. The CpG ODN appeared to have strong immune stimulatory activity on SIgA response in all tested time points with the 200-ODNG group showed the highest response followed by 100-ODNG group then the 50-ODNG and the AHG groups ($P < 0.05$). This finding is comparable with previously published researches, which strongly support that CpG ODN stimulates host defense mechanisms against pathogens, involving both innate and acquired immune responses (Krieg, 2002). The obtained results have shown that CpG-ODN induces a significant increase in the mucosal immune responses, which could play a major role in containing *Salmonella* infection in the gut, via Th2 allergic pathway rather than Th1 inflammatory immune responses. These results could be influenced by the *Salmonella* strain, age and dose of challenge, age of sampling or concentration of CpG-ODN.

In the present study, cellular responses were recorded in intestinal and liver tissues at 35 days of age using H & E histopathological staining. Contrary to serological results, tissue samples from AHG have shown mild to moderate tissue reaction while liver and intestinal tissue samples taken from chickens in the 200-ODNG group showed no apparent cellular changes. These findings contradict previous researches which demonstrate, in vitro, the immune-modulatory

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activity of CpG ODN through modulation of neutrophil apoptosis and reactive oxygen production with further activation inflammatory responses via IL-1B and nitric oxide production in stimulated avian macrophages (Sanjaya et al., 2017; Golenkina et al., 2019). This could be reasoned by other contributing factors, including the age of sampling and *Salmonella* strain used. Indeed, *Salmonella* Enteritidis infection upregulates mRNA gene expression of proinflammtory cytokines and chemokines, including CXCLi1, CXCLi2, iNOS and IL-6 in the caecal tonsils and HD11 macrophages (Setta et al., 2012a and 2012b). However, data from this paper has shown that the administration of CpG ODN motifs were able to enhance chicken survival and inhibit intestinal colonization following oral exposure to *Salmonella* Enteritidis, with stimulation of mucosal SIgA in a dose-dependent manor. Thus, it appeared that the CpG ODN-induced local immune response has played an important role in the mucosal responses, again pointing out the possible role for Th2 responses. Further studies might be needed to investigate the allergic immune responses to CpG ODN in vivo in chicken model.

CONCLUSION

This study stating an interesting data since *Salmonella* continues to present a major threat to poultry industry as well as of public health burden with poultry as the main source of infection. The present paper provides an update about the mucosal and cellular responses of chickens to *Salmonella* Enteritidis following administration of Toll-like receptor (TLR)-21-agonist oligodeoxynucleotide containing Cytosine Guanine motifs CpG DNA. In response to CpG DNA administration, chickens were able to clear *Salmonella* from enteric sites. This research study also shows that CpG DNA modulates immune responses to *Salmonella* as it induces significant immune stimulatory activity demonstrated by increased secretory IgA production and remarked reduction in cellular interactions. Further studies on the gene expression of immune markers representing Th1/Th2 paradigm in response to CpG ODN are perhaps needed.

DECLARATIONS

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Competing interests
The authors declare that they have no competing interests.

Author`s contributions
All authors participated in making the design, support with sampling, interpretation of results and writing the paper. Mohamed Abed and Mahmoud Elhariri did the experiments, Ahmed Setta, Mahmoud Elhariri and Rafik Soliman designed the experiment and analysed the results. Mohamed Abed, Mahmoud Elhariri, Rehab El-Helw, Marwa S. Khattab, Ahmed Setta and Rafik Soliman wrote and revised the manuscript.

Competing interests
The authors declare that there is no conflict of interest.

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Comparison between Biochemical Analysis of Cattle Amniotic Fluid and Maternal Serum Components during Pregnancy

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ABSTRACT

The present study aimed to compare the biochemical components including Total Protein (TP), albumin, globulins, cholesterol, triglycerides, High and Low-Density Lipoproteins (HDL and LDL), creatinine, urea, sodium (Na), potassium (K), chloride (Cl), calcium (Ca) and inorganic phosphorus (P), of Amniotic Fluid (AF) with those of Maternal Serum (MS) during the first, second and third trimesters of pregnancy in cattle and Fetal Serum (FS) at birth. At birth AF, MS and FS were collected. Maternal blood samples and gravid uteri were collected after accidental slaughter. The actual data recorded during three trimesters according to the curved crown-anus length of the fetus. The MS concentrations of globulins, cholesterol, triglycerides, lipoproteins, creatinine, Na, K, Cl, Ca and inorganic-P were significantly higher than the AF during the first trimester. At delivery, the concentrations of cholesterol, triglycerides, and creatinine in the AF were lower than those in the MS or FS. The concentrations of Ca and inorganic-P in the FS were higher than those in the MS or AF. The levels of TP, creatinine, urea in the AF and urea in the MS increased as the gestation stages advanced. The levels of Na and Ca in the AF decreased as the gestation stage advanced while the K concentration increased. In conclusion, our results indicated an active placental transport for Ca and P. The TP, albumin, globulins, cholesterol, triglycerides, HDL and LDL, creatinine, urea, Na, K, Cl, Ca and P in AF and MS during the first, second and third trimesters of pregnancy in cattle might be changed with progressing the gestation.

Keywords: Amniotic fluid, Cattle, Fetal serum, Gestation, Maternal blood

INTRODUCTION

The amnion formation occurs on days 13-14 of pregnancy in cattle, and then, the Amniotic Fluid (AF) fills the amniotic sac (Robert, 1986). Fetal membranes are extra-embryonic in nature (Minazaki et al., 2008). Amniotic fluid (AF), the protective liquid contained in the amniotic sac, is essential for fetal development and growth during gestation (Underwood et al., 2005, Fitzsimmons and Bajaj, 2019). AF is formed partly or entirely by the amnion, secretion from the respiratory tract, buccal cavity, nasal cavity, and embryonic skin before keratinization occurs (Moore 1982, Brace 1994, Hammer et al., 1997). AF accumulates early and subsequently diminishes as the embryo itself grows and this occurs in all species of mammals (Adolph, 1967). The fixed exchange of water and fluid component between the fetal compartments and the mother circulation by the fetoplacental unit shows the changes in the physical, chemical, and biochemical constituents of fetal fluids (Aidasani et al., 1993).

The fetal fluids are important for the handling of the fetal waste products and protect the fetus from the mechanical shock that has developed throughout pregnancy (Amle et al., 1992); they prevent adherence between fetal skin and the amniotic membrane (Williams et al., 1993); and during the expulsive stage they lubricated and widened the birth canal (Asbury and Blonc, 1993); they allow fetal development and movement inside the uterus (Zanella et al., 2014); and also inhibit bacterial and fungal growth (Zare-Bidaki et al., 2017). Biochemical analysis of AF is significantly important for the evaluation of fetal metabolism and pathological conditions during gestation (Prestes et al., 2001).

The purpose of the present study was to evaluate the biochemical components of AF in relation to maternal blood serum during the first, second and third trimesters in addition to those of Fetal Serum (FS) at birth to support our assumption. Cattle AF was a simple MS or FS dialysate, and the fetus played an important role in the final biochemical composition of the amniotic fluid during pregnancy.

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MATERIAL AND METHODS

Ethical approval

This experiment was conducted according to the rules of the Research Ethics Committee of the Veterinary faculty of Aswan University in Egypt.

Sampling

A field survey planned on using data collected from different abattoirs located in Darwa, Aswan, Egypt during the period between 2018 and 2019. Maternal serum and amniotic fluid were harvested from 40 emergency slaughtered animals at different stages of gestation. The age of these animals varied from 2 and 12 years old and the breeding history was unknown. 20 animals were enrolled during normal labor.

Maternal serum

Maternal blood samples were obtained via jugular venipuncture and collected in sterile glass tubes. The blood samples were allowed to clot, and centrifuged at 3000 round per minute (rpm) for 15 minutes; the serum was separated and stored at 20°C for further analysis.

Amniotic fluid

The gravid uteri were removed immediately after slaughter to collect the AF. The gravid uteri were incised through greater curvature with a sharp scalpel to locate the fetal sacs, and then carefully separated from the endometrium and slowly enclosed outside the horn. The AF was collected by puncturing the amniotic sac and 10 ml of AF was aspirated from the amniotic sac using 10 ml disposable syringes, or amniotic fluid samples were obtained transcervically during delivery. The aspirated fluid was stored in labelled tubes and frozen at -20°C until biochemical analysis.

Fetal serum

Fetal blood was obtained from the umbilical vessels. The blood samples were allowed to clot, and centrifuged at 3000 rpm for 15 minute; the serum was separated and stored at -20°C for further analysis.

Fetal age and detection of the gestation period

The fetuses were expelled from enclosing membranes and the fetal ages were determined by applying the age estimation formula, X= 2.5(y+21), presented by Richardson et al. (1990), where X equaled to developmental age in days and y was the crown-anus length in centimeters. The cattle presented different stages of pregnancy and were divided into first, second and third trimesters of pregnancy.

Biochemical studies

Serum urea and creatinine levels were determined calorimetrically using diagnostic kits according to Tietz et al. (1995). The serum Ca level was determined according to Connell (2012). The inorganic phosphorus (inorganic P) level in the serum content was determined according to Berti et al. (1988). The Na and potassium (K) levels in the serum were estimated with a flame-photometer according to Bauer (1982) and serum chloride (Cl) level was determined according to Chirife and Resnik (1984). The Total Protein (TP) content was determined according to Henry et al. (1974). Albumin content was determined according to Doumas and Biggs (1972). The serum globulin level was calculated by subtracting the albumin level obtained from the TP content. Immunoglobulin electrophoresis was performed as described by Henry et al. (1974). The total serum cholesterol was measured according to Mamoru et al. (1977). The serum triglyceride concentrations were measured according to Izzo et al. (1981). The serum High-Density Lipoprotein Cholesterol (HDL-C) and Low-Density Lipoprotein Cholesterol (LDL-C) levels were estimated according to Nauck et al. (2002) and Friedewald et al. (1972) respectively.

Statistical analysis

The data obtained were statistically analyzed by F-test according to Tamhane and Dunlop (2000) using the computer program MSTAT-C. Means values in the same row with different letters are statistically significant and the highest values are represented with the letter (a). Statistical significance was declared at the $p \leq 0.05$ level and the data are presented as the mean ± SE.

RESULTS

The TP, albumin, globulin, α_1 globulin, α_2 globulin, β globulin, γ globulin, cholesterol, triglycerides, HDL, LDL, some metabolites such as urea and creatinine, monovalent cations (Na and K) and monovalent anion (Cl), as well as Ca and phosphorus levels of the MS and AF are given in tables 1 and 2.

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Table 1. Lipids and proteins levels in amniotic fluid and maternal serum of pregnant cattle slaughtered in Darwa and Aswan abattoirs, of Egypt during first, second and third trimesters of gestation.

Parameter	Type of fluid	Trimester		
		First (n=20)	Second (n=10)	Third (n=10)
Total proteins, (g/dl)	Amniotic fluid	4.37 ± 0.08^{b}	6.16 ± 0.56^{a}	6.44 ± 0.39^{a}
	Maternal serum	4.66 ± 0.35^{b}	5.80 ± 0.44^{a}	5.81 ± 0.46^{a}
Albumin,(g/dl)	Amniotic fluid	3.1 ± 0.07^{b}	4.26 ± 0.28^{a}	4.48 ± 0.31^{a}
	Maternal serum	2.33 ± 0.06^{c}	3.59 ± 0.3^{a}	3.6 ± 0.81^{a}
Globulin, (g/dl)	Amniotic fluid	1.27 ± 0.02^{c}	1.9 ± 0.23^{b}	1.96 ± 0.32^{b}
	Maternal serum	2.33 ± 0.3^{a}	2.21 ± 0.15^{a}	2.2 ± 0.17^{a}
α1 globulin,(g/dl)	Amniotic fluid	0.13 ± 0.01^{c}	0.22 ± 0.009^{ab}	0.14 ± 0.006^{c}
	Maternal serum	0.25 ± 0.03^{a}	0.20 ± 0.009^{b}	0.18 ± 0.003^{bc}
α2 globulin,(mg/dl)	Amniotic fluid	0.28 ± 0.03^{c}	0.22 ± 0.08^{c}	0.32 ± 0.02^{c}
	Maternal serum	1.45 ± 0.19^{a}	1.17 ± 0.009^{a}	0.87 ± 0.06^{b}
β globulin,(g/dl)	Amniotic fluid	0.42 ± 0.04^{d}	1.89 ± 0.18^{b}	0.45 ± 0.029^{d}
	Maternal serum	2.9 ± 0.44^{a}	1.6 ± 0.057^{bc}	1.11 ± 0.015^{c}
γ globulin,(g/dl)	Amniotic fluid	0.42 ± 0.03^{e}	1.84 ± 0.12^{c}	0.55 ± 0.06^{e}
	Maternal serum	2.71 ± 0.12^{a}	2.12 ± 0.06^{b}	1.68 ± 0.08^{d}
Cholesterol,(mg/dl)	Amniotic fluid	17.32 ± 0.56^{d}	16.93 ± 0.97^{d}	16.08 ± 0.79^{d}
	Maternal serum	182 ± 6.47^{a}	136 ± 2.7^{b}	124.57 ± 5.85^{c}
Triglycerides,(mg/dl)	Amniotic fluid	20.41 ± 3.22^{c}	22.3 ± 4.3^{c}	18.7 ± 0.91^{c}
	Maternal serum	103.34 ± 4.1^{a}	95.83 ± 1.09^{a}	77.2 ± 6.1^{b}
HDL, (mg/dl)	Amniotic fluid	28.1 ± 2.7^{b}	25.38 ± 2.07^{b}	21.42 ± 0.6^{b}
	Maternal serum	49.24 ± 2.17^{a}	47.17 ± 1.92^{a}	43.72 ± 2.18^{a}
LDL,(mg/dl)	Amniotic fluid	5.67 ± 0.23^{d}	5.24 ± 0.42^{d}	5.13 ± 0.5^{d}
	Maternal serum	54.4 ± 1.69^{c}	62.33 ± 2.85^{b}	69.67 ± 0.96^{a}

Data are expressed as mean values ± Standard error; the number of studied samples in each trimester of gestation are shown in parentheses. Values with different superscripts are significantly different at $p \leq 0.05$. n = the number of studied samples, HDL= High Density Lipoprotein, LDL= Low Density Lipoprotein

Table 2. The levels of metabolites and ions in amniotic fluid and maternal serum during first, second and third trimesters of gestation in cattle slaughtered in Darwa and Aswan abattoirs of Egypt.

Parameters	Type of fluid	Trimesters		
		First (n = 20)	Second (n = 20)	Third (n = 20)
Creatinine (mg/dl	Amniotic fluid	1.06 ± 0.17^{b}	1.26 ± 0.13^{ab}	1.62 ± 0.14^{a}
	Maternal serum	1.63 ± 0.4^{a}	1.70 ± 0.05^{a}	1.77 ± 0.06^{a}
Urea (mg/dl)	Amniotic fluid	22.87 ± 1.27^{c}	24.37±2.01^{c}	26.3 ± 2.06^{ab}
	Maternal serum	24.11 ± 0.58^{c}	27 ± 1.5^{ab}	28.37 ± 1.68^{a}
Sodium (mEq/L)	Amniotic fluid	111.36 ± 1.43^{b}	107.3 ± 3.7^{b}	86.2 ± 5.47^{c}
	Maternal serum	135.8 ± 1.9^{a}	133.27 ± 0.93^{a}	131.33 ± 4.67^{a}
Potassium (mEq/L)	Amniotic fluid	1.76 ± 0.29^{c}	2.06 ± 0.17^{c}	2.7 ± 0.06^{b}
	Maternal serum	3.84 ± 0.12^{a}	3.5 ± 0.06^{a}	3.57 ± 0.22^{a}
Chloride (mEq/L)	Amniotic fluid	78.55 ± 2.45^{b}	80.29 ± 0.09^{b}	83.2 ± 0.97^{b}
	Maternal serum	95.81 ± 0.79^{a}	94.27 ± 0.38^{a}	93.33 ± 0.88^{a}
Calcium (mg/dl)	Amniotic fluid	7.23 ± 0.3^{b}	6.69 ± 0.22^{b}	6.13 ± 0.23^{c}
	Maternal serum	9.22 ± 0.71^{a}	9.24 ± 0.15^{a}	8.82 ± 0.18^{a}
Inorganic phosphorus(mg/dl)	Amniotic fluid	3.22 ± 0.21^{b}	3.9 ± 0.19^{ab}	4.04 ± 0.09^{ab}
	Maternal serum	5.16 ± 0.08^{a}	5.05 ± 0.11^{a}	4.77 ± 0.14^{a}

Data are expressed as mean values ± Standard error; the number of studied samples in each trimester of gestation is shown in parentheses. Values with different superscripts are significantly different at $p \leq 0.05$. *n* = the number of studied samples

Proteinogram

The AF proteinogram showed a significant ($p \leq 0.05$) increase in TP content in the 2nd and 3rd trimesters compared to the MS. The TP, α_2 globulin and γ globulin levels increased as the gestation increased. The MS TP content was not significantly higher than the AF TP content during the first trimester. The albumin level increased significantly in the first trimesters in the AF compared to the MS. The globulin, α_1 globulin, α_2 globulin, β globulin and γ globulin levels were significantly lower in the AF than in the MS.

Cholesterol, triglycerides, HDL and LDL

The cholesterol, triglycerides, HDL and LDL levels in the AF were not significantly different among the three trimesters of gestation but remained low compared to those in the MS throughout the pregnancy. The mean cholesterol, triglycerides, and HDL values in the MS were decreased significantly ($p \leq 0.05$) as the gestation stage advanced, but the LDL level increased with gestation progressed.

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Creatinine

The AF creatinine increased with gestation stage. The AF creatinine level was lower than the MS creatinine level in the 1st trimester. The MS creatinine level did not change significantly ($p \leq 0.05$) throughout the pregnancy.

Urea

The concentration of urea in the AF gradually increased during gestation. The urea concentration in the AF was significantly lower than that in the MS in the 2nd trimester of gestation.

Sodium (Na)

The AF Na concentration decreased significantly ($p \leq 0.05$) as the pregnancy progressed and was lower than that in the MS, but the MS Na concentration did not change significantly during pregnancy.

Potassium (K)

The AF K level increased significantly ($p \leq 0.05$) with increasing the gestational stage and remained in lower concentrations than the MS K levels. However, the MS K levels did not differ significantly ($p \leq 0.05$) during the three trimesters of pregnancy.

Chloride (Cl)

The concentrations of Cl were significantly higher in MS than in the AF.

Calcium (Ca)

The AF showed a significant ($p \leq 0.05$) decrease in Ca concentration as the gestation stage advanced while there was no significant difference in Ca levels in the MS. The AF Ca levels were consistently lower than the MS Ca levels throughout the pregnancy.

Inorganic P

The concentrations of inorganic Pin the AF increased insignificantly ($p \leq 0.05$) as the pregnancy progressed. The MS inorganic P level did not change significantly throughout the pregnancy. At delivery state, the levels of all the examined biochemical components were determined simultaneously in the AF, MS and FS samples (Table 3). The mean concentrations of TP, albumin and globulin in the matched samples were insignificantly ($p \leq 0.05$) different among the AF, MS and FS, but the α_1 globulin, α_2 globulin, β globulin and γ globulin levels in AF were significantly ($p \leq 0.05$) lower than those in the MS or FS. The concentrations of cholesterol and triglycerides were significantly ($p \leq 0.05$) lower in the AF than in the MS or FS. The creatinine level in the AF was significantly lower than that in the MS and FS. The urea level was not significantly different in the matching samples; amniotic fluid (AF) and maternal serum (MS) samples collected during three trimesters of gestation in cattle. The concentrations of Na, K and Cl in the AF were not significantly higher than those in the MS and FS, and concentrations of other electrolytes including Ca and inorganic P were significantly lower in the AF and the MS than in the FS.

Table3. The levels of biochemical components in amniotic fluid, maternal serum and fetal serum at birth time of cattles slaughtered in Darwa and Aswan abattoirs of Egypt

Parameter	Amniotic fluid (n=20)	Maternal serum (n=20)	Fetal serum (n=20)
Total proteins (g/dl)	6.64 ± 0.21	7.22 ± 0.94	6.15 ± 0.43
Albumin (g/dl)	4.37 ± 0.41	4.91 ± 0.23	4.17 ± 0.17
Globulin (g/dl)	2.27 ± 0.14	2.31 ± 0.31	1.98 ± 0.31
α1 globulin (g/dl)	0.05 ± 0.02^{c}	0.19 ± 0.003^{b}	0.21 ± 0.02^{a}
α2 globulin (g/dl	0.093 ±0.04^{c}	1.22 ± 0.07^{a}	1.17 ± 0.12^{b}
β globulin (g/dl)	0.17 ± 0.07^{c}	1.5 ± 0.057^{a}	1.81 ± 0.23^{a}
γ globulin (g/dl)	0.22 ± 0.08^{c}	1.99 ± 0.05^{a}	1.02 ± 0.12^{b}
Cholesterol (mg/dl)	15.67 ± 0.67^{c}	103.5 ± 4.8^{a}	85.87 ± 4.5^{b}
Triglycerides (mg/dl)	18.32 ± 2.67^{b}	76.05 ± 4.8^{a}	75.57 ± 4.27^{a}
HDL (mg/dl)	10.43 ± 0.29^{b}	42.17 ± 2.35^{a}	39.62 ± 5.32^{a}
LDL (mg/dl)	5.82 ± 0.23^{c}	73.67 ± 2.9^{a}	34.39 ± 2.49^{b}
Creatinine (mg/dl)	1.75 ± 0.11^{b}	1.85 ± 0.07^{ab}	2.27 ± 0.28^{a}
Urea (mg/dl)	26.96 ± 2.28	28.47 ± 2.3	29.83 ± 2.08
Sodium (mEq/L)	125 ± 1.53	121.7 ± 4.36	117.00 ± 2.31
Potassium (mEq/L)	2.99 ± 0.05	2.85 ± 0.12	2.8 ± 0.02
Chloride (mg/dl)	91.12 ± 0.68	89.09 ± 1.37	88.29 ± 0.41
Calcium (mg/dl)	8.43 ± 0.22^{b}	8.77 ± 0.14^{b}	10.54 ± 0.25^{a}
Inorganic phosphorus (mg/dl)	4.64 ± 0.32^{b}	4.38 ± 0.17^{b}	7.35 ± 0.11^{a}

Data are expressed as mean values ± Standard error; rows with different letters (a, b, c) are significantly different at $p \leq 0.05$. The number of studied samples is shown in parentheses. n = the number of studied samples, HDL= High Density Lipoprotein, LDL= Low Density Lipoprotein.

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DISCUSSION

The developing fetus is surrounded by AF. The AF volume and composition are affected by fetal urination, drinking and fetal membrane permeability. Hormones such as prolactin and cortisol, may play an important role by affecting membrane permeability (Wintour et al., 1986).

The Na concentrations in the AF decreased in the three trimesters studied compared to those in the MS. In ovine, a similar result was recorded by Prestes et al. (2001) who found that the minerals act on fetal kidneys, increasing the K and decreasing the Na concentration in fetal urine. At the end of pregnancy, the fetal kidney reabsorbed 85% to 95% of Na ion from the filtrate load. So, the hypo-tonicity of fetal urine compared to plasma, indicating the efficacy of the collecting duct (Robillard et al., 1988). The results of the present study were concurred with high Na reabsorption of and a relatively low AF concentration in the third trimester of gestation, as mentioned by Brenner (1990). According to Wintour et al. (1986) a classic Na pump could be responsible and the alteration in the relative permeability for Na and K might affect the transport. The K content increased in the AF as gestation progressed. These results incompetence with the maturation of distal and collecting tubules, that are responsible for K regulation by the fetal kidney (Satlin, 1991), confirming observations reported by Benzie et al. (1974). There was an insignificant decrease in Cl concentration as pregnancy progressed, and the concentration was significantly lower in the AF than in the MS. According to Mellor and Slatter (1971), fetal orosomucoid secretion could be a source of Cl (found in the AF).

The Ca content in the AF decreased as gestation advanced. This was contrariwise for the phosphorus content in AF. Ca and phosphorus are important for the development of the fetal skeleton. Thus, it would be expected that the fetus excreted very little into the AF when preserving these elements. Wales and Murdoch (1973) reported that the Ca concentration in the ovine fetus AF decreased very slightly between 31st and 44th day of pregnancy.

At delivery, there were no available references to compare the results of the present study with it. Comparing the biochemical components and electrolytes levels in AF, MS and FS in the cattle, had been found that first the levels of organic substances in the AF, including TP, cholesterol, and triglycerides were lower than in MS and FS; second insignificant change in the concentrations of metabolites including urea and creatinine in the AF and MS or FS except significant increase in the FS urea than in the AF, and third the levels of Na, K and Cl in the AF were almost the same as in the MS and FS and the levels of Ca and P in the FS were significantly higher than those in the AF and MS. These data suggested that the electrolytes in the AF might be derived from MS but the fetus was the main source of other organic components and metabolites present in the AF. The present study revealed that the Ca and P levels in fetal serum were higher than in MS, which could be caused by active transport of Ca and P from pregnant cattle to the fetus through the placenta and increased absorption in the fetus. The presence of metabolites, including urea and creatinine in the AF, represented the excretion of urine from the fetus (Anderer and Schinder, 1975). The content of TP in the AF and FS were similar in the present study, which could suggest that AF played a role in fetal development and that both electrolytes and proteins found in the AF were required to balance the osmotic pressure between AF and blood, as reported by Tong et al. (2009).

CONCLUSION

Cattle AF was a simple MS or FS dialysate, and the fetus played an important role in the final biochemical composition of the amniotic fluid during pregnancy. The concentration of cholesterol, triglycerides, TP, creatinine, urea, Na, K, Cl, Ca and P in cattle AF and MS might change as the pregnancy progressed. Studying the changes in the biochemical composition of the AF during the development of pregnancy was of great value, because it explained the mechanisms of AF formation and determined the physiological function of AF during the development of the fetus. In addition, this knowledge was of utmost importance in order to understanding fetal metabolism and pregnancy associated abnormalities. Further research is needed to better correlate changes in the AF biochemical composition changes with fetal organ development and maturation, and to investigate pregnancy-associated abnormalities.

DECLARATION

Competing interests

The authors declare that there is no conflict of interest in the present work.This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sector.The protocol was approved accordance with National Regulations on Institutional Animal Care and Use Ethical Committee and animal welfare.

Consent to publish

All authors have given their consent before entering the study.

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Authors' contributions

Walaa M. Essawi detected the stage of pregnancy, collected the samples and participated in the preparation of the manuscript, Doaa I.A. Mostafa and Amal I.A. El Shorbagy performed the biochemical investigations, data analysis and prepared the manuscript (writing and revision). All authors approved the final version of manuscript before publication.

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Risk Factors Associated with Stillbirth in Swine Farms in Vietnam

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ABSTRACT

Stillbirth in pig has been studied worldwide, but, its situation in Vietnam has never been reported. Therefore, present study aimed to investigate effects of herd, parity, gestation length, birth litter size and farrowing duration on stillbirth at sow level in swine farms in Vietnam. Data was collected from 1174 litters of 1174 Landrace x Yorkshire crossbred sows in 16 farms in the North of Vietnam. Potential risk factors for stillbirth were identified by using logistic regression. The incidence of stillbirth at sow level was 47.9%, and the stillbirth rate was 5.2%. Multivariate logistic regression showed that parity 1 (OR=1.81, 95%CI=1.24-2.63) and >4 (OR=1.87, 95% CI=1.33-2.64), a gestation length <114 days (OR=1.80, 95%CI=1.23-2.65), a birth litter size ≥9 piglets (OR=1.64, 95%CI=1.04-2.61) and a farrowing duration ≥5 hours (OR=1.48, 95%CI=1.05-2.09) were risk factors for stillbirth. This study indicated that stillbirth was common in swine farms in Vietnam. Special attention should be paid to sows at parity 1, > 4, sows with a short gestation, sows with a large birth litter size and sows with a long farrowing duration to reduce stillbirth. Since the use of highly prolific sows is increasing, stillbirth continues to be an issue to be dealt with in swine farms in Vietnam.

Keywords: Farrowing; Gestation length; Litter size; Parity; Sow; Stillbirth

INTRODUCTION

In recent decades, pig production has focused on increasing birth litter size (Koketsu et al., 2017). However, large litter size positively correlates with long farrowing duration and increases stillbirth rate (Fahmy and Friend, 1981; Borges et al., 2005; Vanderhaeghe et al., 2010). Stillborn piglets can be classified as non-fresh, prepartum, intrapartum and postpartum in which the third type accounts for 66.8% (Leenhouwers et al., 2003). Intrapartum stillborn piglets are attributable to various factors including maternal and piglet traits as well as management and pharmacological interventions (Leenhouwers et al., 1999; Cozler et al., 2002; Lucia et al., 2002; Borges et al., 2005; van Dijk et al., 2005; Canario et al., 2006; Mota-Rojas et al., 2006; Vanderhaeghe et al., 2010).

Currently, no studies on factors associated with stillbirth in swine farms in Vietnam are available. Therefore, the present study aimed to evaluate the effect of herd, parity, gestation length, litter size and farrowing duration on stillbirth in pig farms in Vietnam.

MATERIALS AND METHODS

Ethical approval

This observational study was performed according to all ethics and animal rights of Vietnam National University of Agriculture, Vietnam.

Animals

This observational study was conducted from August 2016 to November 2017; included 1174 sows in 16 swine farms from 6 provinces in the North of Vietnam. All farms had a breeding herd of 200-700 sows. During gestation, sows were housed in individual crates sized 57 cm width x 220 cm length. Approximately a week before estimated farrowing date, animals were moved to individual farrowing crates sized 180cm width x 220 cm length. A 60 x 220 mm slatted floor in the farrowing crates was assigned for each sow.

During the first 84 days of gestation, sows were daily fed 1.8-2.5 kg of feed containing 13% of crude protein and a metabolizable energy of 2900 Kcalo/kg (Hi-Gro 566, Charoen Pokphand, Vietnam). At the last trimester, sows daily

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received 3.0-3.5 kg of feed containing 17% crude protein and a metabolizable engery of 3100 Kcalo/kg (Hi-Gro 567S, Charoen Pokphan, Vietnam). At parturition, no feed was available to sows. Water was provided *ad libitum* through a bite nipple system.

Data and definition

Before parturition, parity number was recorded, and classified as parity 1, parity 2-4 and parity >4. At farrowing, birth litter size, gestation length, stillbirth and farrowing duration were recorded. Stillborn piglets were defined as those died before being expulsed and had no signs of decay (Vanderhaeghe et al., 2010). The incidence of stillbirth at sow level was the proportion of litters with stillborn piglet(s) to all litters. Stillborn rate was the proportion of all stillborn piglets to total born piglets of all litters. Birth litter size was defined as number of piglets born/litter (number of live born piglets and stillborn piglets). Birth litter size was allocated into 2 categories, i.e. <9 and ≥ 9 piglets/litter. Gestation length was the interval between the date of the first artificial insemination and the date of farrowing, and was divided into 2 categories (< 114 and ≥ 114 days). Farrowing duration was defined as the interval between the first and the last piglet expulsion, and was classified as < 5 and ≥ 5 hours. During farrowing, all sows were injected with oxytocin, the number of administrations, the dose, and the time of injection, however, were not recorded.

Statistical analysis

All statistical analyses were conducted in SPSS program (SPSS Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp). Descriptive statistics for parity, gestation length, birth litter size, farrowing duration and stillbirth were generated from all available data of 1174 sows (Table 1). Due to missing data of 363 sows, full records of 811 sows were included in risk analyses. The incidence of stillbirth at sow level among herds was compared by means of the Chi-square test of association. Potential risk factors for stillbirth were herd (1-16), parity number (parity 1, parity 2-4 and parity > 4), gestation length (< 114 and ≥ 114 days), birth litter size (< 9 and ≥ 9 piglets/litter) and farrowing duration (< 5 and ≥ 5 hours). The binary dependent variable was defined as presence of at least one stillborn piglet or absence of stillborn piglet(s) in a given litter (Vanderhaeghe et al., 2010).

Univariate logistic regression was used to analyze effects of individual potential risk factors on stillbirth. Risk factors conferring a significant value at P < 0.15 by Wald's test (2-tailed) were used for calculation of Pearson's correlation and variance inflation factor (VIF) to detect multicollinearity. Only risk factors that had low Pearson's correlation (r < 0.3) and low VIF (VIF < 5) were used to build multivariate logistic regression models. Based on biological plausibility and correlations, 4 risk factors including parity number, birth litter size, gestation length and farrowing duration were enrolled in the multivariate analysis. Multivariate models were built by enrolling the backward stepwise elimination of non-significant factors (P > 0.1). The predictability of the multivariate models was measured by Hosmer-Lemeshow goodness of-fit tests.

Table 1. Descriptive statistics for reproductive parameters of 1174 sows in 16 commercial swine herds in Vietnam

Characteristics	Number of sows	Mean±SD/Percentage	Range
Parity	1174	3.5±2.2	1-13
Gestation length (days)	1020	114.8±1.6	105-126
Farrowing duration (hour)	957	3.6±1.8	1.0-9.5
Birth litter size	1174	12.4±3.0	3-25
Incidence of stillbirth at sow level (%)	1174	47.9 (562/1174)	-
Stillbirth rate (%)	1174	5.2 (756/14542)	-

SD: standard deviation

RESULTS

In the Table 1, totally 47.9% of the litter had at least one stillborn piglet, and 5.2% (756/14542) of piglets were stillborn. Gestation length, birth litter size and farrowing duration were 114.8 ± 1.6 days, 12.4 ± 3.0 and 3.6 ± 1.8 hours, respectively. The incidence of stillbirth at sow level did not significantly differ among 16 herds (37.5% - 60.8%, P = 0.35). Table 2 shows the results for univariate analysis of individual factors for stillbirth at sow level. Herd had no effect on the stillbirth. By contrast, parity, birth litter size, gestation length and farrowing duration all associated with stillbirth. Among risk factors for stillbirth, gestation length had a very low association with parity (r = -0.099) and farrowing duration (r = -0.082). There was no association among other factors. Besides, VIF for parity, birth litter size, gestation length and farrowing duration were 1.010, 1.004, 1.017 and 1.011, respectively. Therefore, all significant factors derived from univariate analysis were retained for the multivariate analysis. Table 3 shows the final multivariate logistic regression. Sows at parity 1 and parity > 4 had respective 1.81(P = 0.002) and 1.87 (P <0.001) times higher odds for stillborn piglets compared with sows at parity 2-4. A birth litter size ≥ 9 significantly increased the risk for stillbirth

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compared with a birth litter size < 9 (OR = 1.64, P = 0.035). Sows with a gestation length < 114 days had 1.80 times higher odds for stillbirth compared with sows with a gestation length ≥ 114 days (P < 0.001). Sows with a farrowing duration ≥ 5 hours had significant higher odds for stillbirth compared with sows with a farrowing duration < 5 hours (OR = 1.48, P = 0.026). The multivariate model conferred 56.7% "percentage correct". Hosmer Lemeshow test showed that the model had a good fit between the observed and expected incidence of stillbirth at sow level (P = 0.899).

Table 2. Results of univariate logistic regression for association between potential risk factors and stillbirth in 16 Vietnamese swine farms (2016-2017).

Characteristics	Incidence of stillbirth at sow level (%)[a]	OR[b] (95% CI)[c]	P value
Herd	-	-	0.364
Farrowing duration < 5 hrs	48.8 (308/631)	Reference	
Farrowing duration ≥ 5 hrs	60.0 (108/180)	1.57 (1.12-2.20)	0.008
Gestation length ≥ 114 days	48.4 (324/669)	Reference	
Gestation length < 114 days	64.8 (92/142)	1.96 (1.35-2.85)	<0.001
Parity 2-4	44.4 (202/455)	Reference	
Parity 1	59.0 (92/156)	1.80 (1.25-2.60)	0.002
Parity > 4	61.0 (122/200)	1.96 (1.40-2.75)	<0.001
Birth litter size < 9	43.2 (38/88)	Reference	
Birth litter size ≥ 9	52.3 (378/723)	1.60 (1.02-2.51)	0.04

[a]Sows with stillborn piglet(s) in each group/total sows in each group. [b]OR =odds ratio. [c]CI =confidence interval

Table 3. Results of multivariate logistic regression for association between potential risk factors and stillbirth in 16 Vietnamese swine farms (2016-2017).

Characteristics	OR[a] (95% CI)[b]	P value
Farrowing duration < 5 hrs	Reference	
Farrowing duration ≥ 5 hrs	1.48 (1.05-2.09)	0.026
Gestation length ≥ 114 days	Reference	
Gestation length < 114 days	1.80 (1.23-2.65)	<0.001
Parity 2-4	Reference	
Parity 1	1.81 (1.24-2.63)	0.002
Parity > 4	1.87 (1.33-2.64)	<0.001
Birth litter size < 9	Reference	
Birth litter size ≥ 9	1.64 (1.04-2.61)	0.035

[a]OR=odds ratio; [b]CI = confidence interval

DISCUSSION

Studies on risk factors for stillbirth in the pig have been carried out in various countries with numerous results (Vanderhaeghe et al., 2013; Koketsu et a., 2017; Rangstrup-Christensen et al., 2017). However, the present study is the first to determine risk factors for stillbirth in swine farms in Vietnam. The incidence of stillbirth at sow level in this study (47.9%) is higher in comparison with those in Brazil (27.8% to 33.1%) (Lucia et al., 2002; Borges et al., 2005), it is, however, similar to that in Belgium (48%) (Vanderhaeghe et al., 2010) and in Denmark (44%) (Rangstrup-Christensen et al., 2017). The stillbirth rate in this study (5.2%), is within the range of reported results (4.1%-7.5%) (Lucia et al., 2002; Borges et al., 2005; Vanderhaeghe et al., 2010; Rangstrup-Christensen et al., 2017; Bhattarai et al., 2019a; 2019b).

Unfavorable association between farrowing duration and stillbirth has been previously substantiated (Van Dijk et al., 2005; Canario et al., 2006; Baxter et al., 2009). Borges et al. (2005) reported that sows with a farrowing duration >3 hours had 2.0 times higher odds for stillbirth when compared with sows with a farrowing duration ≤ 3 hours. By contrast, Lucia et al. (2002) found that a farrowing longer than 4 hours did not increase the risk for stillbirth. In this study, a farrowing lasting 5 hours was considered normal (Bjorkman et al., 2018), and when a farrowing was considered "prolonged" it increased the risk for stillbirth. During the farrowing process, uterine contraction reduces the blood flow to the placenta and may even break fetal umbilical cords resulting in asphyxia in piglets (Mota-Rojas et al., 2006). Therefore, prolonged farrowing predisposes piglets to increased unfavorable condition, and consequently, to higher risk for stillbirth (Alonso-Spilsbury et al., 2005).

A reverse association between gestation length and stillbirth and stillbirth rate in pig has been widely substantiated (Zaleski and Hacker, 1993; Leenhouwers et al., 1999; Sasaki and Koketsu, 2007; Rydhmer et al., 2008). In another study, a non-existent association between gestation length and stillbirth was suggested to be due to a narrow variation of gestation length (Canario et al., 2006). Gestation length of the pig is widely known to be 114 days. In the present study, 17.5% sows had early parturition (<114 days) (Table 2). At the time close to term, the lung of swine fetus develops in an

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almost exponential rate, and its development is only fully completed by about 114 days of gestation (Olson, 1979; Kirwood, 2015). Therefore, piglets born before 114 days of gestation might have immature lungs that increased their risk for stillbirth (Rydhmer et al., 2008).

In the present study, sows at parity 1 and parity >4 had a higher risk for stillbirth in comparison with sows at parity 2-4 (Table 3). The result partly agrees with that reported by Borges et al. (2005). In that study, sows at parity > 5 had 1.6 times higher odds for stillbirth compared with sows at parity 2-5, sows at parity 1, however, had a lower odds for stillbirth in comparison with sows at parity 2-5 (OR=0.7). Lucia et al. (2002) reported that the incidence of stillbirth at parity ≥ 4 was 2.2 times higher than that in the parity 2-3. In the present study, the incidence of stillbirth at parity 2, 3, 4 was very close together, i.e. 43.5%, 44.8% and 45.5%, respectively. The effect pattern of parity on stillbirth in this study was somewhat similar to that reported by Vanderhaeghe et al. (2010). In that study, incidence of stillbirth in sows at parity 2 was lower than that in sows at parity 1, 3-6 and >6 (9.7 % vs 26.4 %, 44.7 % and 19.1 %, respectively). The association between stillbirth and higher parities has been suggested to be due to long farrowing durations and large litter sizes in older parities (Borges et al., 2005; Almond et al., 2006). However, it is not a perfect explanation to the association between parity and stillbirth in this study since the proportions of sows at parity 1, 2-4 and > 4 had a farrowing duration ≥ 5 hours were not significantly different (21.2%, 21.5% and 24.5%). Furthermore, the percentage of sows at parity 2 - 4 and > 4 had a litter size ≥ 9 was very the same (89.9% and 90.0%) and did not differ from that in parity 1 (85.9%). The result of this study may be partly explained by that the proportion of sows at parity 2 - 4 had a gestation length < 114 days tended to be lower than that of sows at parity 1 and > 4 (14.3% vs 19.9% and 23.0%, respectively, data not shown in the table).

The effect of litter size on the stillbirth found in this study was in agreement with many previous results (Zaleski and Hacker, 1993; Leenhouwers et al., 1999; Knol et al., 2002; Lucia et al., 2002; Borges et al., 2005; Vanderhaeghe et al., 2010). This effect may link to the association between birth litter size and farrowing duration and piglet birth weight. Birth litter size positively correlates with farrowing duration (Van Dijk et al., 2005). Such association also existed in the present study since a higher percentage of sows with a birth litter size ≥ 9 had a farrowing duration ≥ 5 hours when compared with sows with a birth litter size < 9 (23.2% vs 14.8%). A long farrowing may predispose piglets to hypoxia which has been showed to increase the stillbirth of piglets (Borges et al., 2005; Van Dijk et al., 2005; Canario et al., 2006). Furthermore, birth litter size negatively correlates with average birth weight of piglets (Mungate, 1999; Bergstrom et al., 2009). In other word, a larger birth litter size has a lower average birth weight (Akdag et al., 2009). Lighter piglets have lower hemoglobin level in comparison with heavier piglets (Zaleski and Hacker, 1993). During the farrowing process, piglets actively moved through the pelvic canal (Taverne and van der Weijden, 2008). Therefore, it may be hypothesized that heavier piglets participate in farrowing process more efficiently so they had a lower risk for stillbirth (Canario et al., 2006).

As an initial study, the present work has some limitations. Firstly, this study did not differentiate prepartum from intrapartum stillbirth. In the present study, the definition of stillbirth may overestimate the intrapartum stillbirth because piglets died shortly before the onset of parturition may be wrongly defined as intrapartum stillbirth. Secondly, infectious stillbirth, implying most cases of prepartum stillbirth (Sprecher et al., 1974; Borges et al., 2005), was not ruled out in this study. However, the failure of differentiation of stillbirth types should not cause any serious problems in this study because prepartum stillbirth was reported to account for only about 5% of all cases (Leenhouwers et al., 2003). Thirdly, although oxytocin was used in all studied sows during farrowing its doses, administration routes and time of application were not recorded. Intravenous and intravulval routes may cause more intrapartum stillbirth in comparison with intramuscular route (Mota-Rojas et al., 2006). Also, the use of oxytocin with different doses and/or at different birth order results in different stillbirth rates (Mota-Rojas et al., 2007a; 2007b). Therefore, a detailed record of oxytocin use during parturition will help evaluate the effect of oxytocin on the stillbirth in the swine farms in Vietnam where oxytocin is used ubiquitously and abusively.

CONCLUSION

The present study indicated that stillbirth in pig was common in swine farms in Vietnam. Parity 1 and > 4, gestation length <114 days, birth liter size ≥ 9 and farrowing duration ≥ 5 hours all increased stillbirth. It suggested that, to reduce the stillbirth, all sows, especially those with increased risk should be monitored carefully during farrowing.

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Competing interests

The authors declare that there is no conflict of interests.

Author's contribution

Authors contributed equally to this work and checked the final edition of the article for publication in the World's Veterinary Journal.

Consent to publish

Authors give consent for information concerning the article to be published in the World's Veterinary Journal.

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Rapid Detection and Differentiation between Sheep Pox and Goat Pox Viruses by Real-Time qPCR and Conventional PCR in Sheep and Goat in Egypt

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ABSTRACT

Capri Pox Virus (Ca PV) is the causative agent of important diseases in sheep and goat with severe socio-economic impact. Sheep Poxvirus (SPPV), Goat Poxvirus (GTPV) and Lumpy Skin Disease Virus (LSDV) are three members of the Capripox virus genus of Poxviridae family, which infect sheep, goats, and cattle, respectively. A rapid diagnostic assay for Ca PV by using conventional PCR RNA polymerase gene RP030 and real-time qPCR would be useful for disease surveillance, detection and differentiation of Ca PV in clinical and subclinical samples for management and treatments of outbreaks. The present study aimed to detect and identify Ca PV (SPPV and GTPV) in natural, infected scabs biopsy samples, which were collected from sheep and goats in different governorates in 2017 during outbreaks in Egypt using the conventional PCR RNA polymerase gene RP030 gene based and Real-Time qPCR fluorescent based. We collected eighty scabs from clinically affected animals (54 sheep and 26 goat) that were vaccinated in Chorio-Allantoic-Membranes (CAM) from 10-days-old embryonated-chicken eggs. The positive CAM showed pock lesions, which were observed with a thickening of the membrane after 2-3 passages post samples inoculation, and harvested positive CAMs, which were determined by Agar Gel Precipitation Test (AGPT) , Counter Immune Electrophoresis (CIE), and conventional PCR and real time qPCR were examined for the presences of Ca PVs. DNA extraction from clinical samples and positive CAM with pox lesions using DNA slandered references extraction kits compared to novel modification method (Microwave extraction). The PCR based RPO30 gene and the real-time qPCR showed 15 positive with percentage 27.77% in 54 sheep and 3 positive with percentage 12.5% in 26 goats. Although, AGPT and CIE gave lower result than molecular methods, they gave 11 and 13 positive samples from 54 sheep and in goats were 1 and 2 from 26 scab biopsy samples respectively, however they are useful for early confirmation of positive Ca PVs in low-income countries. PCR based RNA polymerase gene RP030 gene and real-time-PCR considered sensitive, rapid, and reliable methods for differentiating SPPV and GTPV from AGPT and CIE in CAM or in clinical samples without further isolation and propagation in embryonated-chicken eggs. The novel microwave method used to isolate high quality of DNA extracted from infected skin biopsy with SPPV and GPPV with no further purification steps required. It was done in 3 minutes only. The results of the current study confirmed that the suitability of the PCR-based RNA polymerase gene RP030 gene is suitable for differentiating between SPPV and GTPV; in one PCR run; without any post-processing steps.

Key words: Capripox virus, DNA extraction, Goat pox, KOH extraction method, Real-Time qPCR, RPO30, Sheep pox

INTRODUCTION

Sheep pox and goat pox are contagious viral diseases of small ruminants. In endemic areas the affected animals showed mild clinical symptoms, but were fatal to newly introduced animals (Lamien et al., 2011). The current criterion used for classification of CaPVs based on animal species from which the virus was isolated, LSDV from cattle, GTPV from goats and SPPV from sheep, respectively (Santhamani et al., 2013). The Capri poxvirus was endemic to the Middle East, including Egypt (Manjunathareddy et al., 2017; OIE, 2017; Lafar et al., 2020). SPPV and GTPV are spread directly or indirectly through aerosols and/or close contact with infected animals (Zangana and Abdullah, 2013; Manjunathareddy et al., 2017;Abd-Elfatah et al., 2019). Pox disease characterized by fever, appearance of papules, pustules, and scab in checks, lips, nostrils, medial part of the thigh and under the tail usually remitted and cured within 5-6 weeks (Sharma et al., 2018). The isolation of Ca PV on CAM from ECE is considered to be a preliminary diagnostic test by developing characteristic pock lesions CAM (Bhanuprakash et al., 2010; Sharma, 2019). Interestingly, the virus was host specific, even sheep and goats were reared together. The SPPV only infected sheep and GTPV only infected goats (Gelaye et al., 2013). Electron microscopy (EM) cannot differentiate Ca PV infection from the affected sheep and goat based on morphological characterization in infected tissue biopsy samples. PCR was considered a rapid, sensitive technique for the detection and differentiation of SPPV from GTPV (Gelaye et al., 2013 ; Zeedan et al., 2019). Several suitable molecular assays have been developed for the detection of CaPVs (Zeedan et al., 2014; Abdallah et al., 2018). Most of

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these tests were restricted to detect only one viral species (Tassew et al., 2018; Gelaye and Lamien, 2019). RPO 30 gene-based PCR depended on the presence of RPO30 gene encoding the 30 kilo Dalton (kDa) RNA polymerase subunit in Ca PVs (Assefa, 2017). The present study aimed to detect and differentiate sheep pox virus from goat pox virus infected small ruminants during the outbreak of pox disease in 2017 by using conventional PCR RPO30 gene based and real-time qPCR.

MATERIALS AND METHODS

Ethical approval

The research was ethically conducted and approved by the Medical Research Ethical Committee Research, the National Statement on Ethical Conduct in Human and animals Research at National Research Centre in Egypt under registration number 19149 and the International Animal Ethics Committee in Egypt (Fahmy and Gaafar, 2016) and in accordance with local laws and regulations.

Sample collection

A natural sheep pox disease, which usually shows clinical symptoms in sheep and goat flocks which had not vaccinated with SPPV in the past, was recorded in different governorates (Beni-suef, El-Fayoum, Giza, Monifia, Sharkia and El-Menia) in Egypt in 2017. Clinical signs in sporadic cases of sheep and goat related to skin lesions such as papules, nodules and scab's formation on an area free of wool and hair, which led to a suspected infection with pox disease. Eighty skin biopsy samples from crusted scabs lesions were collected from 80 affected sheep and goat and were stored at -40ºC until use.

Preparation biopsy samples

According to 3, 15 (OIE, 2017) 10% suspension of suspicious tissue samples (papules and scabs) prepared in Phosphate Buffer Saline (PBS) containing antibiotic (penicillin (100 U/ml), streptomycin (100 µg/ml), neomycin (2.5mg/ml) and nystatin (50 U/ml)). The samples were ground with sterile sand in a mortar. The homogenized suspension was frozen–thawed three times and then partially clarified by centrifuging at 5000 rpm for 15 minutes to remove tissue depressed and then stored at -40ºC till used.

Virus propagated in embryonated chicken eggs

Approximately 10-12 specific pathogen-free production (SPF) of embryonate chicken egg (ECE) were purchased from Specific pathogen-free production (SPF) Kaom-Oshan Company, El-Fayoum Egypt. The Chorio-Allantoic Membrane (CAM) protocol for inoculation of the virus for virus isolation in CAM, described in (Gelaye and Lamien, 2019) by using the artificial air sac route. Briefly- Embryos of 10 to 12 days were candled for embryos viability. An area was marked and disinfected approximately 1/4 inch below and parallel to the base of the air cell, then at this point a hole was drilled, and another hole was drilled directly on the top of the air cell. The embryo was placed horizontally with the hole facing up. Holding the embryo in the same position and using a rubber bulb, air was drawn out of the air cell by placing the bulb over the hole at the top of the embryo. This negative pressure created the artificial air cell by pulling down the CAM. A fine needle was inserted into air sac about 1/8 inch and 0.1 ml inoculum released. The embryo was placed horizontally for 24 hours then returned to upright position. The holes in the inoculated eggs were sealed with molten wax and incubated for five days at 37°C in an egg incubator. After 5-6 days of inoculation, eggs were harvested from the embryo. Both putative SPPV and GTPV were inoculated on CAM and identified by PCR and RT-PCR.

Counterimmunoelectrophoresis

An improvement on the precipitation method was described with the development by Page et al. (2015). The antigen and their specific antibody move through the gel and can be accelerate by applying an electrical current, and precipitation occurs within a few hours (Aguilar-Torres et al 1976)

Agar gel precipitation test

This method was performed with minor modifications to the methods of Zeedan et al. (2015). 1.5 gram agarose (Difco) and 1.5 gram glycine were added to 100 ml distal water containing 0.85 gram sodium chloride. The mixture was boiled in water bath to dissolve the agarose and left at room temperature until 45°C was reached, and then poured in 5 cm diameter Petri dishes in diameter to obtain an agar thickness of 2 mm. The plates were allowed to solidify at room temperature. After the agarose had solidified of in Petri dishes, 7 wells with a diameter of 3 mm in were made by using metal cutter. The central well was filled with (Positive control SPPV or GTPV) and 4 peripheral wells were filled with tested serum samples. The upper and lower peripheral wells received positive and negative serum as controls. Then incubated at room temperature in a humidity chamber, and checked every 12, 24, 48 and 72 hours until lines of precipitation were detected.

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DNA extraction

Microwave irradiation

First, DNA was extracted from the collected CAMs with pock lesions and from clinically collected scabs biopsy samples by using the QIAamp DNA extraction kit (QIAGEN). The three SOPs, fresh whole blood, heparinized blood samples in heparinized tubes, small amounts of skin biopsy samples in 30 µl PBS. Ten µl of blood were transferred into 0.5 ml tubes adjusted microwave at 800 W for 2 minutes until precipitated and condensed droplets were visible on and retrievable from the tube walls. One µl of the clear DNA was taken from the walls or from lid of the tube according to Melendez (2016). Alternatively, for enduring storage, 30 µl of sterile phosphate buffer saline (PBS) were added to the irradiated sample. In particular, smaller tubes can break and be destroyed by air expansion and thus carry the risk of contamination (Seesui et al., 2018; Yuan et al., 2019)

Polymerase chain reaction

The polymerase chain reaction **(**PCR) protocol described by Kumar et al. (2016) and Assefa et al. (2018), which was based on the RPO30 gene to differentiate GTPV from SPPV, was used. The test was carried out in a 25 µl capacity PCR tube.

Table 1. Preparation of PCR reaction tube component

Component	Volume/Reaction
Maxima Green PCR Master Mix (2x premix)	12.5 µl
Nuclease free water	8.5 µl
Forward primer (20 pmol) RNA Pol F 5'-TCTATGTCTTGATATGTGGTGGTAG-3'	1 µl
Reverse primer (20 pmol) RNA Pol 5'-AGTGATTAGGTGGTGTATTATTTTCC-3'	1 µl
Samples extracted DNA included + ve and – ve control	2 µl
Total	25 µl

12.5 µl= Green PCR Master (Gendirex, Inc, USA), 1ul µl of each primer (20 pmol/µl), 2 µl of extracted DNA, and completed with nuclease free water up to 25 ul. All running PCR included positive control (Positive Reference Pox virus from Biotechnology Department animal health institute, Giza, Egypt. Negative control used nuclease-free water.

Table 2. PCR amplification conditions using RNA polymerase gene primers PO30

PCR amplification conditions						
Gene	**Primary denaturation**	**Main Cycle for amplification**				**Final extension**
		Secondary denaturation	**Annealing**	**Extension**	**No. of cycles**	
Sheep pox RNA polymerase PO30	95	95	55	72	40	72
	4min	30sec	30sec	30sec		5 sec

The amplification conditions were initial denaturation, followed by 40 cycles of denaturation, annealing and extension and final extension at 72°C for 5 min in a thermocycler (Biorad).

Agarose gel electrophoreses

It was performed with modification by (Zeedan et al., 2019). 10 µl PCR product samples were mixed with amplified reference pox positive control with loaded dye solution and loaded in 2 % agarose gel in TAE (Tris/ Acetate/ EDTA) buffer containing 0.5 ul of ethidium bromide , 100 bp DNA-marker ladder (Gendirect). Separate the products at 100 volts for 60 minutes and visualize using a UV Transilluminator according to (Sambrook et al., 1989).

Real-time-qPCR assay to detect sheep and goat pox virus

Real-Time qPCR was used to prepare a reaction mixture according to the manufacturer's guidelines. To detect and differentiate SPPV dtec-qPCR target species were used as a mono-dose of ready prepared dtec-qPCR (contains a dehydrated mixture of specific primers and labeled probe, dNTPs, BSA, polymerase and buffer at optimal concentrations, 96 dtec-qPCR 96 reaction). While, GTPV was used to target dtec-qPCR-mix species (contains a mixture of specific forward/reverse primers and probe, lyophilized at optimal concentration after synthesis, 100 dtec-qPCR 96 reaction) with probe and DNA template for internal control of fluorogenic minor groove-binding TaqMan probe (5′ CAATGGGTAAAAGATTTCTA 3′; labeled with 6-carboxyfluorescein and a no fluorescent quencher) (200 nm) were included in each reaction mixture. Sample template (2.5 µl) was added to the reaction mixture in a 25-µl reaction tube. The cycling conditions consisted of an initial denaturation at 95°C for 120 seconds, followed by 45 amplification cycles (95°C for 2 s and 60°C for 60 s). The assay was run with a Bio-Rad Real Time Thermocycler. Positive and negative controls were included with each set of reaction mixtures.

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Statistical analysis

Statistical analysis of performance of the SPPV and GTPV real-time PCR assay was compared to CIE and/or conventional PCR agarose gel based on the detection of Ca PV infected sheep and goats in clinical and scab biopsy samples of the percentage and accurate Fisher's test at 95% based on the confidence interval ($p \leq 0.05$) and used the Statistics Package for the Social Sciences (SPSS, version 16, Chicago, USA).

RESULTS

Harvested positive CAMs with characteristic pock lesions for the passage of sheep pox virus were characterized by the opaque thickening and edema of the membrane, and hemorrhage was also observed in CAM. The preliminary positive resulted from sheep and goat scabs biopsies on the CAM of ECE were 13 out from 56 from sheep and 2 out from goat samples. While the negative sample showed no pathological changes, CAM with SPPV and GTPV after 5 days of inoculation, as shown in table 3, also showed pathological changes after the 3rd passage in ECE. Positive pock lesion samples were prepared for antigen detection, characterization and DNA extraction by a new modified microwave extraction method compared to a standard extraction method. DNA extraction was achieved from various sources, including supernatant from collected clinical samples and positive CAM with pock lesion samples. Microwave tubes 0.5 ml containing 10 µl of supernatant within a microwave led to boiling and partial desiccation of the sample and to the formation of vapour, tissue-free condensed vapour contained the nucleic acid on the micro-tube walls and appropriate templates for further lid processing both in conventional standard PCR and in Real Time PCR assays.

Table 3. Detection of sheep and goat pox viruses by pock lesion on CAM of ECE and PCR based on PRO30 from skin biopsy samples from small ruminants in different governorates (Beni-suef, El-Fayoum, Giza, Monifia, Sharkia and El-Menia) in Egypt during sheep and goat pox outbreak on 2017.

Small ruminants	Egg inoculation CAM of ECE 10 days old % +ve	Confirmation positive CAM by PCR based on PRO30 % +ve		Results confirmed by PCR
		C-PCR	Real-Time -PCR	
Sheep scab biopsy samples	13/54 (24.07 %)	13/13 (100 %)	13/13 (100 %)	SPPV
Goat scab biopsy samples	2/26 (7.69 %)	2/2 (100%)	2/2 (100%)	GTPV
Control positive SPPV	1/1 (100 %)	1/1 (100 %)	1/1 (100 %)	SPPV + ve C
Control negative PBS PH 7.2	0/1 (0.00 %)	0/1 (0.00 %)	0/1 (0.00 %)	PBS -ve C

% +ve = percent of positive samples

Analytical sensitivity

The sensitivity of the cell Culture PCR (C –PCR) and real-time qPCR assays used for the microwave extraction method was determined on the basis of serial two fold dilution /100 µl of positive SPPV at titer 10 5.5 EID50/ ml. DNA was extracted from all dilutions applying standard extraction method (Qi amp Qigean) and the microwave extraction method was detected at a dilution of 1/128 to 1/512.

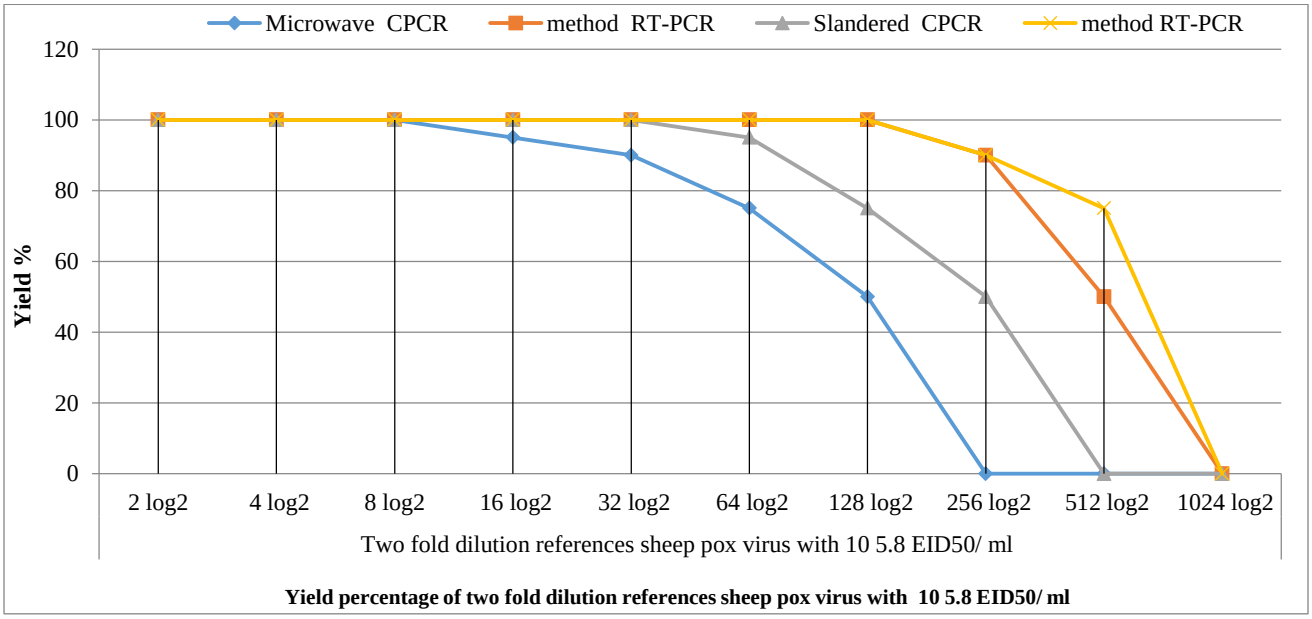

Figure 1. Different extraction methods used to SPPV DNA amplification by conventional PCR and Real-Time PCR

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Table 4 showed that the sensitivity of CIE compared to AGPT was determined by screening 80 sheep and goats scabs biopsy samples collected from naturally infected animals during the sheep pox outbreak in 2017 for the presence of precipitins. While only 12 of the 80 samples were positive by the AGPT, the CIE gave positive 15 out of 80 sheep and goat samples, as shown in table 4. The CIE was better than the AGPT for detection of SPPV and GTPV in skin lesions of sheep and goats. Examination of the scab biopsies of sheep and goat using RPO30 gene based PCR showed that 18 out of 80 samples were positive (15 sheep and 3 goats). The control positive of sheep and goat pox was included. The appropriate sheep pox DNA fragment is 151 bp and for GTPV is 172 bp using RPO30 gene-based PCR were obtained. The results revealed no cross infection in any of the tested animal samples (Table 4 and figure 2).

Table 4. Comparison of different diagnostic methods for the detection of sheep pox virus (SPV) and goat pox virus (GTPV) in different governorate in Egypt on 2017

Small ruminants	No. of scabs biopsy samples	AGPT			CIE			C PCR			Real Time PCR		
		+ve	-ve	% +ve	+v	-ve	% +v	+v	-ve	% +v	+v	-ve	% +ve
Sheep	54	11	43	20.37	13	41	24.07	15	39	27.77	15	39	27.77
Goat	26	1	25	3.8	2	24	7.69	3	21	11.53	3	21	11.53
Total	80	12	68	15	15	65	18.75	18	60	22.5	18	60	22.5

% +ve = percent of positive samples

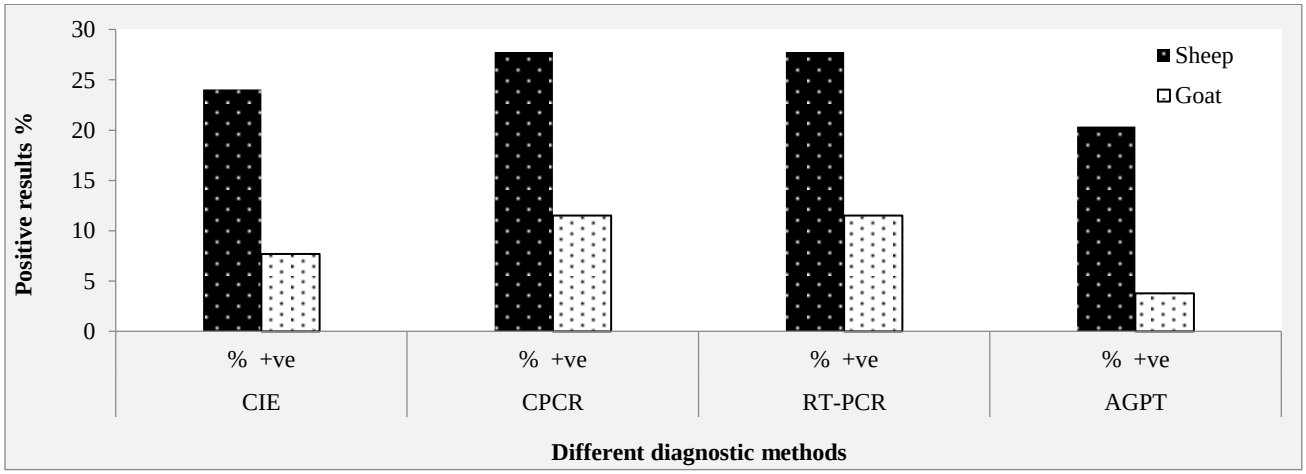
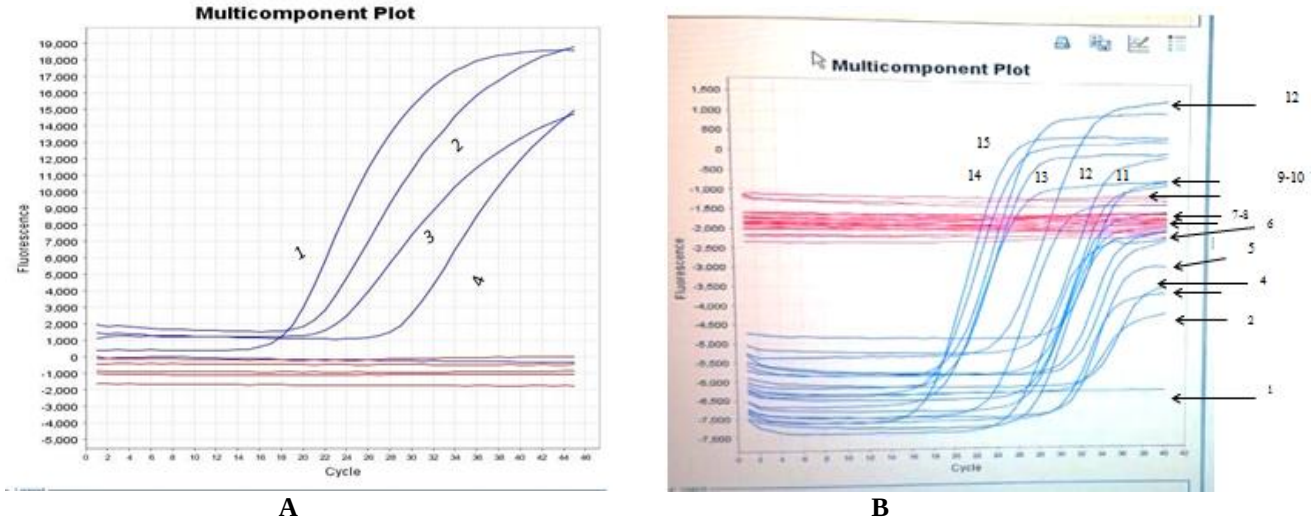


Figure 2. Positive percentage results for scab biopsy samples of suspected sheep and goat infected with Pox virus, which were examined using different diagnostic methods. % +ve CIE= Positive percent of counter immune electrophoresis, % +ve c-PCR = Positive percent of conventional PCR, % +ve RT-PCR= Positive percent of Real-Time PCR, % +ve AGPT= Positive percent of agar gel precipitation test. % +ve = percent of positive samples



A **B**

Figure 3. A) Amplification plot showed a positive control, and Ct2, Ct1 and Ct3 are positive tested samples for DNA extracted from a biopsy skin lesion. Ct 4 showed positive DNA extracted from infected CAM with pathogenic pock lesion for SPPV. Ct = DNA-free negative control (graphic generated by ES Equant). **3B)** Amplification plot showed negative results (Ct from 1 to 8) and showed negative DNA extracted from sheep and goat skin lesion. Ct 9 to12 showed positive results for GTP positive CAM with pathogenic pock lesion from GTPV. Ct 12 to15 showed positive results for SPPV positive CAM with pathogenic pock lesion of SPPV

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DISCUSSION

Laboratories had encountered problems with the use of commercial extraction kits that results from interference tissues for DNA extraction. Direct DNA amplification by whole blood or tissue biopsy and cell culture was very difficult to achieve. Cheap and simple methods had been described in which heat denaturation or chemical lysis by KOH, an Eiken boiling-spin method and a water bath or thermoblock, could be used for DNA extraction in field and laboratory applications (Melendez, 2016; Seesui et al., 2018). The present study showed an optimized microwave extraction method as an alternative to slandered commercial DNA extraction used for conventional and Real-Time PCR to detect and differentiate between SPPV and GTPV in clinical samples collected during the 2017 Egyptian outbreak (Table 3 and figure 2). The microwave extraction method was tested for sensitivity by repeated serial dilutions using commercially available kit as shown in figure 1. The DNA was successfully extracted from scabs tissue biopsy samples in less than 3 minutes and no additional further chemicals were required for isolation or purification. Our study demonstrates that DNA was extracted from blood samples, tissue scabs biopsy samples and CAM tissue samples (Kumar et al., 2016; Assefa, 2017). SPPV and GTPV could not be distinguished by serological assays due to close antigenic relationships (Zeedan et al., 2014; Shehbaz and Hassan, 2017). Due to the low sensitivity and specificity, the serological tests were replaced by PCR. The diagnosis of sheep and goat pox virus was often described clinically only as sheep pox or goat pox, respectively followed by virus isolation on cell cultures with further confirmation by PCR (Sambrook et al., 1989; Al-Shabebi et al., 2014;Fentie et al., 2017). The classification of Ca PVs based on the animal species from which the virus was isolated suggested that the Ca PVs were strictly host-specific and these results concurred with (Shehbaz and Hassan, 2017; Abd-Elfatah et al., 2019). In 2017, sheep and goat in different governorates of Egypt showed different clinical signs such as increasing body temperature, nasal lacrimation discharges and scabs on head, face, nostrils, oral and lips, as well as multiple nodules on medial aspect of thigh and under the tail similar to finding the outbreak of sheep pox recorded by (Mahmoud and Khafagi, 2016; Atalla and Alzuheir, 2019). The present study showed the PCR was more useful than conventional methods as isolation and AGPT and it was a perfect tool for viral identification and differentiation of Capripox based on RPO 132 gene, and these findings were similar to outbreak of SPPV (Mahmoud and Khafagi, 2016; Atalla and Alzuheir, 2019). A comparison between AGPT and CIE revealed deviations in the results of the two diagnostic techniques which sheep (11 and 13/ 56) and goats (1 and 2/26) presented from the previous results. The CIE method was more sensitive than AGPT diagnostic methods for detection of SPPV and GTP. For the differentiation of SPPV and GTPV by PCR based RPO30 genes, which had a 21-nucleotide deletion in the 5' end in SPPV and were not present in GTPV. The present study was showed that SPPVs and GTPVs could be detect and differentiate by PCR and Real-Time PCR and that the screening of samples was possible in a short time compared to time required to isolate viruses in ECE. Examination of 80 samples with conventional PCR, identified a total 18 positive samples from (15 sheep and 3 goats), as shown in table 2 and figure 2. RPO30 gene of SPPV had a 21-nucleotide deletion at the 5' end compared to GTPV, the amplicon size of SPPV was 152 bp, while the amplicon size of GTPV was 172 bp according to Cohen et al. (1971), Yan et al. (2012), Page et al. (2015), Zeedan et al. (2015), and Yang et al. (2019). The present study revealed that the RPO30 gene based PCR and real-time qPCR were successfully detected and differentiated sheep pox and goat pox in field clinical samples in sheep and goat as shown in table 4 and in figure 3 A and B according to (Bhanuprakash et al., 2011; Gelaye et al., 2013). Real-Time PCR and RPO30 gene-based PCR were accurate and rapid detection of SPPV and GTPV. The present result provided meaningful results for the identification and differentiating of SPPV from GTPV in clinical biopsy samples collected during outbreak sheep and goat in Egypt in 2017 without isolation of virus requirement.

CONCLUSION

The Real-Time and conventional PCR based PRO30 gene had been successfully used to differentiate and identify SPPV and GTPV from clinical samples of infected small ruminants without the need for further testing or confirmation. The nucleic acid extraction by novel modified microwave method enabled isolating DNA from scab biopsy samples and CAM positive samples, with the highest quality DNA being isolated in less than five minutes, and offers cheap fast extraction methods.

DECLARATIONS

Authors' Contribution

Gamil SG Zeedan, Ayman H. Mahmoud and Abeer M. Abdalhamed found research idea, planned the study design, performed data, and samples collection, performed laboratory works such as PCR, Real time PCR application and drafted the manuscript. Alaa A Ghazy and Khaled A Abd EL-Razik sharing in the conception of the research idea, sharing laboratory work, provided some reagents and materials and helped in manuscript preparation

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Competing interests

The authors declared that they have no competing interests.

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Using Feed Additives to Produce Functional Eggs in Fayoumi Hens

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ABSTRACT

Lately human have become more apprehensive for the health and their food relationship. Egg considered cheap source of animal protein. Eggs are rich in various essential nutrients that contribute to the quality of human diet. But its cholesterol can contributes with some human serious disease. The current study examines the hypothesis that assumed addition of antioxidant such as CAX, SS, B or their mixtures to the diet can produce functional egg from Fayoumi hens at late phase of egg production. A number of 168 Fayoumi hens (46weeks of age) were randomly assigned into 8 dietary groups as follows: Basal diet alone or with CAX (6 ppm), SS (0.5 g/kg), B (1 g/kg), CAX+SS, CAX+B, SS+B, and CAX+SS+B separately. Forty eight eggs (6 per each group) were analyzed for estimating cholesterol and total antioxidant capacity. Egg of hens fed a combination of CAX+SS+B which had the best total antioxidant capacity value, while the CAX group recorded the best lowest cholesterol value compared to other groups ($P < 0.05$). It could be concluded that basal diet supplemented with CAX, SS, B alone or with mixture of them may have lowering effect on yolk total cholesterol. This could lead to produce functional eggs which have positive effects on human health and favorable for those suffering from heart syndromes.

Key words: Cholesterol, Fayoumi, Functional Egg, Total Antioxidant Capacity

INTRODUCTION

The native poultry breeds are very highly important for rural economy in developing countries, they are phase of equiponderant agriculture suit that have spirited part in the rural family's food as animal protein exporter (Padhi, 2016). Fayoumi chicken is originally an ancient native breed from the Egyptian cities. Egg considered good quality and cheap source of animal protein. Healthy nutrition has an essential role in reducing the cardiac disease (Khan et al., 2017). Eggs are rich in various essential nutrients that contribute to the quality of human diet. The yolk contains high ranges of both cholesterols and unsaturated fatty acids (Huang et al., 2019). Egg cholesterol is a major dietary cholesterol source (Ylilauri et al., 2017). A high serum cholesterol concentration is a known risk factor for dementia (Deckers et al., 2015), cardiovascular diseases (Nelson, 2013) and Alzheimer disease (Altman and Rutledge, 2010). Many researches focused on functional food, lately Sireesha and Prasanna (2019) made a brief overview about the poultry meat and eggs as functional food.

Hen age and nutrition influenced on the cholesterol values of chicken eggs. Betaine (Trimethylglycine) most Common sources of B are Sugar beet and their by-products (Eklund et al., 2005), naturally occurring component, which is widely distributed in many plants and animal tissues. It plays several roles in body one of the hopeful medications for improving the physiological systems protection mechanisms against oxidative stress (Ganesan et al., 2010). it classfied as anti-oxidant, where it's the metabolism (Zhang et al., 2016), enhanced lipase activity (Zou et al., 1998), it is referred to as 'carcass modifier' (Ratriyanto et al., 2009), promote small intestine osmoregulation (Kettunen et al., 2001). Dietary betaine straight utilized as methyl group donor (Kidd et al., 1997). Canthaxanthin (CAX) a red-orange carotenoid naturally occurring pigment is present in bacteria, algae and some fungi (Esatbeyoglu and Rimbach, 2017), is commonly added to the diets of poultry. One of feed additive used to resist oxidative stress caused by numerous factors including but not fixed to high temperatures (Ma et al., 2005). Sulphate (SS) has an encouraging effect on laying hens production at late period of age (Ali et al., 2007, 2012, 2018). Sulfate groups can be identified as antioxidant (Huang et al., 2005). Several researchers have examined antioxidant properties of tested material as feed additives.

Therefore, the objective of this research was to study the influences of B, SS and CAX alone or as a combination supplemented to the diet on egg cholesterol and total antioxidant of aged Fayoumi laying hens.

MATERIALS AND METHODS

Ethical approval

The present experiment was carried out at EL-Azab Poultry Research Station, EL-Fayoum Governorate, Egypt

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during the period of 8th January to 8th April, 2018. The chemical analyses were performed in the Laboratories of Poultry Cellular and Molecular Physiology, Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt. The experimental protocols were approved and carried out according to the regulation and guidelines set by Cairo University Ethics Committee for the Care and Use of Experimental Animals in Education and Scientific.

Treatment groups

One hundred and sixty-eight Fayoumi hens, 42-weeks-age, were selected and housed individually in single cages. Birds were randomly distributed into 8 treatment groups (18 hens per group), where all groups had almost the same averages of body weight and egg production rate, at the beginning of the experiment. The experimental basal diets were calculated to meet the requirements recommended from the MAD (1996) as given in table 1. Birds from 46-54 weeks of age, in each treatment group were assigned to one of the following dietary supplementations: Control without supplementation, CAX (6 ppm), SS (5 g/kg), B (1 g/kg), CAX+SS, CAX+B, SS+B, and CAX+SS+B. Artificial light was used to provide 16 hours daily photoperiod and the water was available *ad libitum*, while feed was restricted at 100 g/hen/day. Eggs were collected and recorded every day.

Table 1. Composition and calculated analysis of the experimental basal diets used in the feeding trial

Ingredients	**kg**
Yellow corn	647.5
Soya bean 44%	219.0
Wheat bran	29.0
Limestone	85.0
Salt	3.0
Premix*	4.0
Mono calcium Phosphate	12.1
DL methionine	0. 50
Total	1000
Calculated analysis**	
CP (%)	15.15
ME Kcal/kg	2696.7
Crude fiber (%)	3.10
Crude fat (%)	2.91
Calcium (%)	3.48
Available phosphorus (%)	0.35
Lysine (%)	0.76
Methionine (%)	0.33
Methionine + Cysteine (%)	0.58

*The premix (Vit. & Min.) was added at a rate of 4 kg per ton of diet and supplied the following per kg of diet (as mg or gm or I.U. per kg of diet): Vit. A 15000000 I.U., Vit. D3 4000000 I.U., Vit. E 80000 mg, Vit. K3 4000 mg, Vit. B1 2200 mg, Vit. B2 12000 mg, Vit. B6 5500 mg, Vit. B12 20 mg, Niacin 40000 mg, Biotin 300 mg, Pantothenic acid 20000 mg, Folic acid 1500 mg, choline chloride 1000 gm, Mnganese 100 gm, copper 10 gm, Se 0.3 gm, Iodine 2 gm, iron 60 gm and Zinc 80 gm. ** DL methionine: essential amino acid; CP: Crude protein; ME: Metabolizable energy. ***According to feed composition tables for animal and poultry feedstuffs used in Egypt (2001).

Egg biochemical assay

Total number of 48 eggs (6 eggs from each group) were collected (at the end of 54 weeks of age) to determine the egg quality traits. After measuring egg quality and yolks separated from albumen and then analyzed to measure egg yolk TAOC and total cholesterol were determined calorimetrically by using commercial diagnostic kits and spectrophotometer (model, GBC906 AA), following the same steps as described by manufactures. Samples from the broken were extracted according to the method of Folch et al. (1957).

Statistical analysis

Data were analyzed using one-way analysis of variance. The statistical analysis was computed using the General Linear Models (GLM) procedure in SAS program (SAS Institute Inc., 2011). The significant differences among 8 treatment groups (Control, CAX, SS, B, CAX+SS, CAX+B, SS+B and CAX+SS+B) for all parameters were separated by Duncan's Multiple Range test. The significance level was set at $P < 0.05$. Results are expressed as Least square means LSM ± SEM.

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RESULTS AND DISCUSSION

Effects of dietary supplementation eggs cholesterol and total antioxidant concentration in the yolk Fayoumi laying hens at late phase of egg production are shown in table 2. In respect of yolk cholesterol concentration, the highest level with significantly different recorded by hen fed control group (210.86 mg/100g). However, the lowest significant value recorded in CAX group (89.5 8mg /100g). Hen age influenced on the cholesterol values of chicken eggs (Zemkova et al., 2007). Jiang and Sim (1991) found an increasing cholesterol level with age (mg/egg), also Shafey et al. (1998) found a positive correlation between the cholesterol concentration (mg/g yolk) and the hen's age. Sulphate alone or in combination with CAX significantly reduced egg yolk cholesterol and these results agree with those find by Ali et al. (2012). These finding may be due to the effect of CAX and SS components on lipid metabolism. From these results, it could be concluded that CAX may have lowering effect on total cholesterol in the yolk. This could lead to produce enriched eggs that are healthier for human consumption and beneficial for those suffering from heart diseases. Regarding B effect data showed that B group recorded (134.52 versus 210.86) mg/100g this may be due to epigenetic effect of B as methyl donor alter methylation profiles of chicken lipoprotein lipase (LPL) gene, moreover it reduces mRNA level of lipogenesis genes and on promoter CpG methylation of fatty acid synthase (*FAS*) gene in laying hens (Xing et al., 2009 and 2012). Effect of B may be extending to progeny through egg. Idriss et al. (2018) indicated that feeding B to the hens modifies hypothalamic expression of genes complicated in cholesterol metabolism and brain tasks in F1 cockerels through modification of promoter DNA methylation. Hu et al. (2015) indicated that epigenetic mechanisms including DNA and histone methylations can regulate hepatic cholesterol metabolism in chicks by *in ovo* injection of B. Data in table 2 indicated that egg total antioxidant capacity of Laying hen eggs fed diet supplemented by B +CAX+ SS group was superior over all other groups were contain considerable amount of total antioxidants. This superiority was significantly highest over most groups (except those supplemented with CAX or SS. On the other hand, the hen fed control group holed the lowest value with insignificantly difference with those fed B with both CAX and SS groups. This may be attributed to the complementary action or synergism between additives. Each of feed additive alone had positive effect on egg yolk TOAC this approved by Surai (2012) reported that TAOC of egg is influenced by maternal diet antioxidant content. Also results agree with those obtained by Johnson (2013) who reported that antioxidant properties of CAX are apparent in both eggs and chicks of hens supplemented with CAX. Putting in mind that CAX is deposited into the yolk of the egg when it supplemented to broiler breeder hens (Surai et al., 2003). The same trend was found by Zhang et al. (2011) enrichment egg yolk with CAX was associated with a significant improvement of TOAC. This better anti oxidative status of egg yolk might be important for the development of the embryo; rather, egg nutrients act as the 'enhancer' of antioxidant defense against range of diseases. Sulphate alone or in combination with CAX significantly increases TOAC and these results agree with those found by (Ali et al., 2012). Considerable amount of B identifies in Chicken eggs (Zeisel et al., 2003), it could work as anti-oxidant (Zhang et al., 2016). Egg rich in antioxidants and low cholesterol may play a critical role in human health also lower risk Chronic and endemic diseases.

Table 2. Effects of dietary treatment on yolk characteristics of Fayoumi layers at late phase of egg production

Treatments	Yolk cholesterol (mg/100g)	Yolk total antioxidant capacity
Control diet	210.86^{a}	0.404^{c}
CAX	89.59^{c}	0.507^{ab}
Na_2SO_4	134.52^{abc}	0.501^{ab}
Betaine	116.67^{bc}	0.409^{bc}
Betaine+CAX	169.94^{ab}	0.404^{c}
Betaine+Na_2SO_4	175.59^{ab}	0.409^{bc}
CAX+Na_2SO_4	147.32^{abc}	0.411^{bc}
Betaine+(CAX+ Na_2SO_4)	176.19^{ab}	0.544^{a}
±SEM	24.49	0.031
P value	0.0001	0.0001

[a,b,c,d;] Means within a column with different superscripts differ significantly (P<0.0001). For each word or item that are required to explanation. *P < 0.0001. CAX: Canthaxanthin[2]Sodium Sulphate; SEM: Pooled Stander Error Means.

CONCLUSION

It can be demonstrated that supplementation of betaine, canthaxanthin and sodium sulphate or their mixtures in Fayoumi laying hens diets reduce the cholesterol composition and improve total antioxidant capacity of egg yolk and consequently produce healthier egg from Fayoumi aged hen.

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DECLARATIONS

Authors' Contribution

Randa A. Dief Allah was responsible for data collection, data analysis, and manuscript writing. M. N. Ali designed the study, drafted and revised the manuscript. M. A. F. EL-Manylawi responsible for scientific material collection, shared in drafted and revised the manuscript. Ahmed O. Abass was responsible for laboratory analysis. A. Desouky shared in samples collection and interpretation of data**.** All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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SDS-PAGE Profile Analysis of SeM-like Protein of *Streptococcus equi* subspecies *equi*

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ABSTRACT

S. equi subspecies *equi*, causing strangles in equine, is characterized by comprising a major virulence factor called M like protein or SeM protein. This study aimed to extract SeM protein from local *S. equi* strain in Egypt and to detect its antigenic components. After centrifugation, the native 58 kilo Dalton (kDa) SeM protein was detected both in the supernatant and sediment of the prepared extract. With modification by more centrifugation, the formed supernatants were separated and fractionated using SDS-PAGE with silver nitrate staining, which led to the appearance of a band at Molecular Weight (MW) 70.9 kDa. in SeM1, the presence of 7 bands at MW of 105, 87.8, 70.9, 61.1, 44, 37.9 and 18.4 in SeM2; 5 bands at MW 70.9, 58.9, 37.2, 29.8 and 18.3kDa in SeM3 and 4 bands at MW of 72.0, 58.6, 29.8 and 18.0 kDa in SeM4. This study suggested that a further modification of SeM extraction revealed the presence of heterogeneous complex fragments of SeM.

Key words: SeM protein, SDS-PAGE, Strangles, *Streptococcus equi* subspecies *equi*

INTRODUCTION

Streptococcus equi subspecies *equi* is the causative agent of an important, frequently diagnosed infectious disease in equine, which is known as strangles (Kasuya et al., 2019). Although Jordanus Ruffus reported strangles disease in 1251 for the first time, it is still considered as one of the most frequently detected diseases in horses in the world (Ikhuoso et al., 2019). Strangles disease is featured by the abscesses of the head and neck lymph nodes (Robinson et al., 2018). Detection and differentiation of *S. equi* subtypes could typically be accomplished using traditional culture, Polymerase Chain Reaction (PCR), matrix assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI–TOF MS), and SeM sequencing (Tartor et al., 2019). *S. equi* virulence and immunogenicity were referred to several components, including M-protein (Taylor and Wilson, 2006). 58 kDa SeM protein was detected and identified as one of the main virulence factors of *Streptococcus equi* subspecies *equi* and also considered as one of its protective antigens. Opsonogenic action of SeM is restricted to *S. equi*, but not to *S. zooepidemicus*, while the opsonogenic action of SzPSe is restricted for *S. zooepidemicus*, but not to *S. equi* (Timoney et al., 1997). M-proteins or M-like proteins were considered as cell surface proteins that bind fibrinogen and possess antiphagocytic activity (Staali et al., 2003). Resistance to phagocytosis and killing required the presence of both capsule and SeM, with the antiphagocytic property of SeM being significantly decreased in the absence of capsule (Timoney et al., 2014).

Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE), the first step of immunoblotting, involved the electrophoretic separation of proteins on the gel (Kalagiri et al., 2020). Protein complex mixtures can be separated using electrophoresis, which can be also used for protein purification for other applications. Polyacrylamide gel electrophoresis involves an electrical field that induced the migration of protein through pores in a polyacrylamide gel matrix.

The protein migration rate was determined by pore size and protein charge, size, and shape. Continuous SDS-PAGE is considered as a non-complicated method, in which the same buffer could be used for both the gel and electrode solutions (Gallagher, 2006). SDS-PAGE is a reliable method for peptide mapping for the peptide fragments separation. The comparison of the separation patterns reveals the proteins structural relationship. The peptides moderate separation could be completed by this technique, which is uncomplicated, cheap and without any special equipment (Judd, 2009). The present study aimed to produce SeM-like protein and to analyze its components using SDS-PAGE.

MATERIALS AND METHODS

The isolation

A locally isolated *S. equi* subspecies *equi* was obtained from Equine Bacterial Disease Lab (Animal Health Research Institute, Egypt). It was cultured on blood agar and incubated for 24 hours at 37ºC with 10% Co_2 (Boyle et al., 2018). The isolate, which showed β-hemolysis was subjected to Lancefield grouping using the Mastastrep Kit (Biomerieux) as identified as Group C and biochemical identification using API20 Strep (Biomerieux) (Ferris et al., 2017).

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Acid Extraction of SeM-like protein of *Streptococcus equi* subspecies *equi*

According to Woolcock (1974) with some modification, *S. equi* local strain was grown on Todd-Hewitt broth (Oxoid), supplemented with glucose and incubated for 24 hours at 37°C. The culture was centrifuged at 3000 round per Minute (rpm) /5 minutes for cells collection, washed twice with normal saline, followed by a wash with Distilled Water (DW) and two further washings in phosphate buffer saline (PBS), pH 7.0. Finally, the cells were suspended in saline; the pH was adjusted to 2.4 with 1Normality (N) HCl, and heated to 95° C. in a boiling water bath for 12 minutes. The suspension was cooled and neutralized with 1N NaOH and centrifuged at 3000 rpm /5 minutes. The bacterial sediment was re-suspended in saline, and the extraction procedure was repeated. The combined supernatants were next treated with 6N HCl to give a flocculent precipitate at pH 2.0. This acidified material was kept refrigerated for 2 hours and centrifuged at 3000 rpm / 10 minutes. The deposit was re-dissolved in 0.1M PBS, pH 8.0. The ribonuclease enzyme was added at a final concentration of 0.001mg/ml. Two chloroform drops were added, and the solution was dialyzed against 30 volumes of 0.01M PBS, pH 8.0. Ribonuclease digestion continued for 5 hours at 37° C., followed by overnight dialysis at 4° C. The ammonium sulfate fractionation of the dialyzed fluid was performed at 4°C. The solution was saturated to 30% and centrifuged at 3000 rpm /10 minutes. The resulting supernatant was then saturated to 60%, and the solution was mechanically stirred at 4°C for 30 minutes. The obtained pellet after centrifugation was solubilized in 10ml of DW and dialyzed against PBS for 18 hours at 4°C. The modification was applied by further centrifugation (3000 rpm /10 minutes) for both the supernatant and sediment of the SeM acid extract, and the resulting supernatants of both centrifugation were also analyzed, with four samples being analyzed electrophoretically, including SeM acid extract sediment (SeM1), SeM acid extract supernatant (SeM2), supernatant from centrifuged SeM1 (SeM3), and supernatant from centrifuged SeM 2 (SeM 4).

Detection of SeM protein concentration

The required reagents consisted of solution A, which contained 2% sodium carbonate in 0.10 N NaOH, solution B, which contained 1 % sodium tartrate, solution C, 0.5% copper sulfate, solution D, which was freshly prepared from 48 ml of solution A, 1 ml solution B, and 1 ml solution C Folin- ciocalteau reagent (Fischer scientific, Co., USA). Bovine Serum Albumin (BSA, Sigma- Aldrich Co., USA) was used to prepare a standard protein curve in the range of 0.625 to 10 mg/ml. According to Lowry et al. (1951), ten µl of the samples were diluted to a final volume of 0.2 ml in DW. The different dilutions of BSA were mixed in the same amounts of the samples as standard. 0.2 ml of DW was used as a blank. 1 ml of solution D was added to all test tubes, mixed well and left at room temperature for 10 minutes. 0.1 ml of diluted folin reagent (V/V with DW) was added to each tube followed by rapid vortex, and incubated in a dark place for 30 minutes at room temperature. The standard and the samples were read against the blank at 750 nm wavelength using a spectrophotometer. The standard curve was blotted using the diluted standard protein solutions. The unknown protein concentration was calculated from the curve slope.

Electrophoresis analysis

Four samples of prepared SeM-like protein were analyzed electrophoretically by using SDS-PAGE including SeM acid extract sediment (SeM1), SeM acid extract supernatant (SeM2), supernatant of centrifuged SeM1 (SeM3), and supernatant of centrifuged SeM 2 (SeM 4) according to the method of Laemmli (1970).

After the gel sandwich was assembled, the separating gel was carefully poured to minimize trapped air bubbles. One cm layer of M-butanol was added to keep the gel surface flat. The gel was allowed to polymerize, before pouring the stacking gel where the comb was inserted obliquely. Following the gel polymerization, it was placed in electrophoresis chamber filled with running buffer and the comb was carefully removed to avoid damaging well ears and trapped air bubbles. The four samples (70 µg/ well each), were diluted at 1:4 ratio using the sample buffer and immersed in a boiling water bath for 4 minutes to ensure denaturation of the protein. The pre-stained protein ladder; wide range 10-250 kDa, and the treated samples were loaded into the wells. A voltage of 140V and 80 mA was applied until the sample buffer bromophenol blue staining had reached the gel bottom.

After separation, 50% methanol was used for gel fixation, which was then stained using silver stain according to Wray et al. (1981). Where the gel was picked and transferred into a small container and soaked in 50% methanol /10% acetic acid for at least 1 hour and methanol/acetic acid changed for 2-3 times. The gel was then rinsed with water, which must be changed at least 3 times. Solution A prepared from 0.8 g silver nitrate in 4ml DW. Solution B prepared from 21 ml 0.36% NaOH with 1.4ml of 14.8 molarity (M) ammonium hydroxide; and solution C prepared by adding Solution A to solution B drop-wise with constant vigorous stirring so that allowed a brown precipitate to get clear; then water was added up to 100ml (solution C had to be used within 15 minutes). The gel was placed in a clean container and stained in Solution C for 15 minutes with constant gentle stirring. It was then rinsed twice in deionized water and soaked 2 minutes with gentle stirring. The gel was then removed into a clean container and washed in freshly prepared solution D (0.5ml of 1% citric acid mixed with 50 µl of 38% formaldehyde, then water added up to 100ml) so that the bands within 10

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minutes appeared. The staining was stopped by rinsing in 1% acetic acid. The gel was finally washed in water for at least 1 hour, at least 3 changes of water.

The gel was imaged and analyzed on the Molecular Imager Gel Doc^{TM} XR System (Bio-Rad, USA). The relative Molecular Weights (MW) of separated proteins were calculated based on the MW standards of pre-stained protein ladder, which were electrophoresed on the same gel.

RESULTS

SeM protein concentration

The SeM protein concentration was detected both in the sediment and in the supernatants of the prepared extract. The sediment concentration of SeM protein was about 2.358mg/ml. While the supernatant concentration of the SeM protein was 0.364mg/ml.

SDS-PAGE of extracted partially purified SeM protein

Electrophoresis of the extracted sediment from SeM 1 in lane 1 revealed the appearance of a band at MW 70.9 kDa (figures 1 and 2), While the electrophoresis of SeM 2 in lane 2 revealed the presence of 7 bands at MW of 105, 87.8, 70.9, 61.1, 44, 37.9 and 18.4 kDa (figure 1 and figure 3). Five bands were detected in Lane 4 which represent SeM3 (figure 1 and figure 4) at MW 70.9, 58.9, 37.2, 29.8 and 18.3 kDa, and SeM 4 showed four bands in lane 5 at MW of 72.0, 58.6, 29.8 and 18.0 kDa (figures 1 and 5).

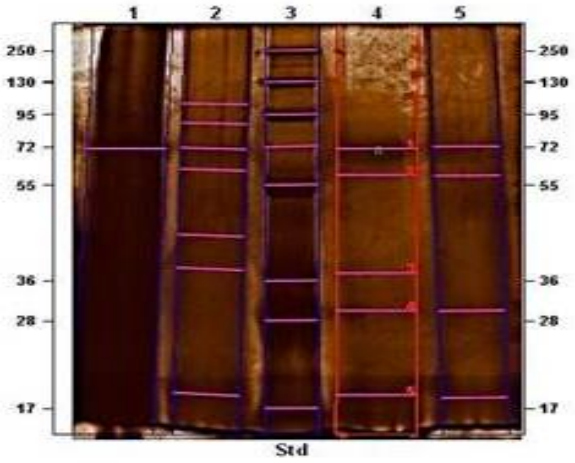

Figure 1. SDS-PAGE of four samples. Lane 1= SeM1; Lane 2= SeM 2; Lane 3= ladder; Lane 4= SeM 3 and Lane 5= SeM4

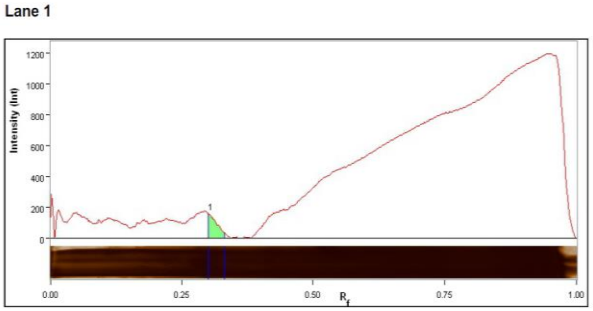

Figure 2. SDS-PAGE of SeM 1 showed one band

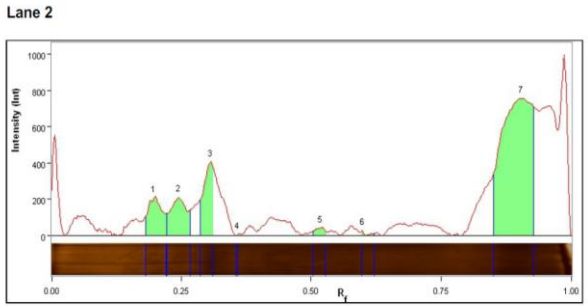

Figure 3. SDS-PAGE of SeM2 showed seven bands

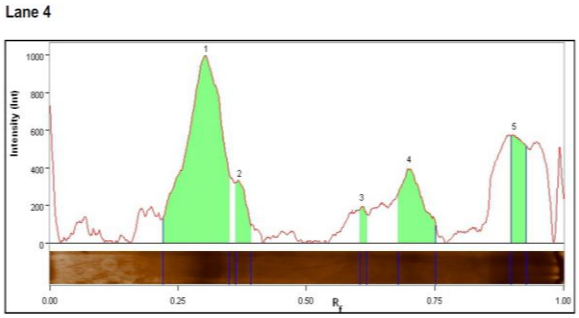

Figure 4. SDS-PAGE of SeM3 showed five bands

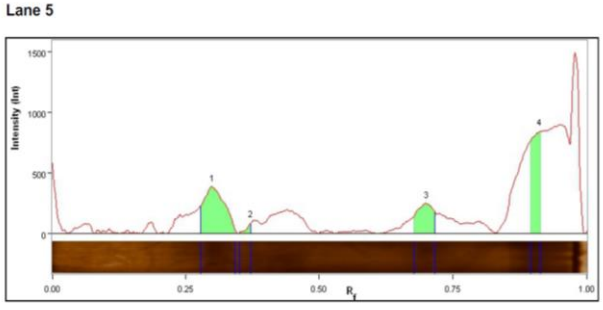

Figure 5. SDS-PAGE of SeM4 showed four bands

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DISCUSSION

Streptococcus equi subspecies *equi* causes strangles, a highly contagious disease of the nasopharynx and draining lymph nodes of members of the family Equidae. *S. equi* is a *Streptococcus* from Lancefield group C (Boyle et al., 2018).

M proteins were first described for the Lancefield group A streptococci, and subsequently have been found on groups C and G streptococci (Jacks-Weis et al., 1982; Bisno et al., 1996). Thirty-three percent of horses investigated by Delph et al. (2018) had SeM antibody titers greater or equal to 1:12800, eight weeks after infection with *S. equi*. Also, Boyle et al. (2017) reported that Timoney and others (Sweeney et al., 2005) have suggested that horses with high SeM-specific serum antibody titers (≥ 3200) may be suitable equine vaccinated for *S. equi* with the attenuated-live intranasal *S. equi* vaccine.

In the present study, electrophoresis of the extracted sediment of SeM1 in lane 1 revealed the appearance of one band at MW 70.9 kDa (figure1 and figure2), lane 2 revealed the presence of 7 bands at MW of 105, 87.8, 70.9, 61.1, 44, 37.9 and 18.4 kDa (figure1 and figure3), lane 4 showed five bands at MW 70.9, 58.9, 37.2, 29.8 and 18.3 kDa (figure1 and figure 4), and lane 5 showed four bands at MW of 72.0, 58.6, 29.8 and 18.0 kDa (figure 1 and figure 5).

Frequent centrifugation of supernatants (SeM2, SeM3 and SeM4) revealed the presence of four proteins with a MW of 58.9, 37.2, 29.8 and 18.3 kDa, while SeM was a protein of 58-kDa M-like protein which was considered as major virulence factor and protective antigen with antiphagocytic properties (Timoney et al., 1997). Timoney and Trachman (1985) also found that M-like protein was analogous to group A streptococci M-protein molecules, which is structurally quite complex with functionally heterogeneous regions, including 29 and 37 kDa fragments that carry determinants that induce serum bactericidal antibodies. No protein with a MW 41 and 46 kDa was present in the present analysis, while Galan and Timoney (1985) found that 41 and 46 kDa fragments were responsible for the stimulation of the mucosal immune response and the independence of the mucosal and systemic immune response to *S. equi* clearly proved in horses.

In the present study, a fractionation of protein with a MW of 18.3kDa was also obtained this result was reported by Groschup et al. (1990), who detected bands at 70, 54, 42, 40, 31-28,17-15 and less than 15kDa in *S. equi* by using SDS-PAGE and Coomassie blue staining.

CONCLUSION

Streptococcus equi subspecies *equi* is an important equine pathogen. Further modification of SeM extraction revealed the presence of heterogeneous complex fragments of SeM. This multiple centrifugations modification procedure resulted in more purified antigen. There is a wide variety of SeM protein among *S. equi* isolates. The SDS profile analysis of SeM protein among *S. equi* isolates is recommended as a diagnostic test for strangles. Further studies are still required so that the modified preparation of SeM can be used for immunization.

DECLARATIONS

Competing interests

The authors declared that they have no competing interests.

Consent to publish

All the authors were aware of the fact and agreed to be so named. The data of this study did not partially published elsewhere.

Author`s contributions

Shaimaa M.E. Abdelmageed collected the data, participated in the design of the work protocol, performed the laboratory work, and was involved in the preparation of the manuscript. Soumaya S.A. El-Shafii found the research idea, shared in the performed data and designed the work protocol. J. El Jakee participated in the design of the work protocol, contributed to the manuscript review and interpreted the results. All authors were involved in the preparation of the manuscript and had read and approved the final manuscript.

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Evaluation of The Efficacy of Oxytetracycline on Experimentally Induced Caprine Coccidiosis Due to *Eimeria arloingi* Infection

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ABSTRACT

Coccidiosis is a protozoan disease caused by members of the genus *Eimeria* that affect domestic animal species. The current study was aimed at evaluating the effect of oxytetracycline administration on experimental caprine coccidiosis. Sixteen red Sokoto goat kids divided into four groups (A to D) of four goat kids each, were used for the study. Groups A, B and C were infected by oral inoculation with two ml containing 1.5×10^3 sporulated oocysts of *Eimeria arlongi* per animal, while group D was the neutral control group. Group A was treated with 10 % oxytetracycline intramuscularly daily for five days. Group B was treated with Sulfadimidine 33.3% subcutaneously daily for five days and group C served as an infected untreated group. Fecal oocysts per gram count was conducted during the experiment. The present result showed a significant decrease ($P \leq 0.05$) in fecal oocysts load in the treated groups. Neither schizonts nor merozoites were detected in the intestinal smear of kid treated with oxytetracycline but were detected in the intestinal smear of infected untreated goat kid. Cystic degenerative changes were seen in the intestinal glandular cells of the infected untreated goat kid. Conclusively, the current finding suggests that oxytetracycline can effectively be used in treating caprine coccidiosis.

Keywords: Coccidiosis, Caprine, *Eimeria arlongi*, Goat Kids, Oxytetracycline, Treatment

INTRODUCTION

Coccidiosis is a protozoan disease caused by members of the genus *Eimeria* that affect domestic animal species (Geremew, 2018). It is also a disease that causes adverse effects on the general health of various domestic animals. Acute invasion and destruction of the intestinal mucosa, diarrhea, fever, anorexia, emaciation, weight loss and sometimes death are characteristics of the disease (Geremew, 2018). In goats, coccidiosis is an enteric parasitic disease caused by multiple protozoan parasite species of the genus *Eimeria* (Engidaw et al., 2015). Goats production in recent years is faced with major constraints of high kids' mortalities due to the disease condition (Engidaw et al., 2015). Invasion and destruction of the hosts' intestinal mucosal cells by coccidia could lead to electrolyte loss, poor absorption of nutrients and anemia (Engidaw et al., 2015). Diarrhea is one of the most common clinical symptoms of infection and affected animals can show a rough hair coat, weakness and poor weight gain (Geremew, 2018).

The disease can be diagnosed based on clinical findings such as diarrhea, dehydration and progressive emaciation, and the presence of large numbers of oocysts in the feces (Engidaw et al., 2015) along with a demonstration of the organisms in intestinal mucosal smear during post mortem. Anticoccidial drug therapy either orally or parenterally is the main treatment of coccidiosis (Papich, 2018). The largest serving anticoccidial agents in small animals and poultry are Sulfonamide drugs (Papich, 2018). They include Sulfadimethoxine, Sulfaquinoxaline, and Sulfamerazine, Sulfachloropyrazine, etc (Papich, 2018). Coccidian parasites are developing resistance to common anticoccidial drugs (Papich, 2018). Oxytetracycline has a broad spectrum of activity against protozoan parasites, but there is little or no literature regarding its proven efficacy scientifically against coccidian parasites (Papich, 2018). It was therefore imperative to investigate the effectiveness of oxytetracycline scientifically against coccidian parasites in small ruminants. The aim of the present study was to evaluate the effects of oxytetracycline administration on experimental coccidiosis in goat kids.

MATERIALS AND METHODS

Experimental animals

Sixteen kids aged between 4 to 10 weeks were purchased from a market in Zaria Local Government Area of Kaduna state, Nigeria. The animals were screened for the presence of coccidian parasites/Eimeria oocysts using simple

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flotation technique (Geremew, 2018). The kids were allowed to acclimatize for seven days and were fed with maize bran, beans, hay and water ad libitum during the experimental period.

Ethical approval

The experiment was conducted according to the University of Abuja, Nigeria, Ethical Committee, on Animal Use (UAECAU/2018), along with ethical permission from the Ahmadu Bello University Zaria, Nigeria, committee, on animal use and care (ABUCAUC) with approval number: (ABUCAUC/2020/48).

Isolation and identification of coccidia oocysts

Animals were diagnosed positively infected using simple flotation technique and light microscopy method of detecting oocysts of *Eimeria* species (Geremew, 2018). Six grams of feces were collected and filtered through a sieve covered with folded gauze from animals positively infected with *Eimeria* oocysts (Rakhshandehroo et al., 2013). The material filtered was centrifuged for 10 minutes at 250 g. The supernatant was then removed. Oocysts sporulation was achieved by using 2.5% (w/v) potassium dichromate solution as described by Rakhshandehroo et al. (2014). Identification of the oocysts specie was done as reported by Rakhshandehroo et al. (2014).

Experimental design

The sixteen kids were divided into four groups (A to D) of four animals each. Groups A, B and C were infected by oral inoculation with two ml containing 1.5×10^3 sporulated oocysts per animal, while group D remained as uninfected untreated neutral control group. Group A kids were treated with 10% oxytetracycline (OCECURE®, India) intramuscularly daily for five days at the dose rate of 10 mg/kg. Group B kids were treated with the common anticoccidial agent, sulfadimidine 33.3% (Shanghai Gongyi, China) subcutaneously daily for five days at the dose rate of 30 mg/kg and Group C served as infected untreated negative control group. All treatments were initiated on the day after the oocysts were first noticed in the infected kids' feces by microscopy.

Determination of fecal oocysts load

Fecal samples were collected from each kid in all the groups and examined for the presence of oocysts, using the sedimentation-floatation technique in a sucrose saturated solution (El-Ashram and Suo, 2017). Oocysts Per Gram (OPG) of feces counts were conducted using the McMaster technique (Kheirandish et al., 2014). Calculation of OPG was performed on days 5, 7, 9, and 14 Days' Post Inoculation (DPI) and thereafter on daily basis during the treatment regimen on days 16, 17, 18, 19 and 20 and on days 21 to 27, the post-treatment days, up to the end of the experiment on day 28 to establish decrease or absence of Eimeria oocysts in the feces of the experimental animals.

Intestinal smears and histopathological evaluations

At the end of the experiment, a kid from each group was sacrificed humanely, intestinal mucosal smears were taken and stained with Giemsa stain and examined under oil immersion micoscopy for viewing the schizonts and merozoites at × 1000 magnification. For histopathological evaluation, 2 cm^2 tissue pieces from the caeca and posterior region of the small intestine were collected and fixed in 10% buffered formalin solution. Multiple transverse slices were embedded in paraffin wax. Sections were cut at 5µm, and all were stained with hematoxylin-eosin (Gelberg, 2012).

Data management and analysis

Data obtained from the study were presented in tables, figures and plates. Results were presented as mean plus/minus standard error of the mean (Mean ± SEM). The data were also analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc with the aid of SPSS statistical software (version 4.0). Values ≤ 0.05 were considered statistically significant.

RESULTS

Oocysts counts in the experimentally infected kids

Coccidia oocysts were detected in the feces of the kids inoculated with *E. arloingi* (Figure 1). A significant decrease ($P\leq 0.05$) was recorded on days 18 and 19 on fecal OPG counts in the groups treated with oxytetracycline and sulfadimidine when compared with the infected untreated group (Figure 2), as well as on days 22 to 28 (Figure 3). Treatment with oxytetracycline eliminated the coccidian oocysts on day 19 in one of the kids in group A, sulfadimidine treatment eliminated the coccidian oocysts on day 22 in one of the kids in group B, while fecal oocyst per gram counts of 14,440 (OPG) was recorded in one of the infected untreated kids in group C on day 28, which was the last day of the experiment (Figure 4). Neither schizonts nor merozoites were detected in the intestinal smear of kid treated with oxytetracycline on day 28. However, at the end of the experiment (day 28), schizonts were detected in the intestinal

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smear of kid treated with sulfadimidine and both schizonts and merozoites were detected in the intestinal smear of the infected untreated kid (Table 1 and figure 5).

Effect of treatment with oxytetracycline on the intestinal histopathology of kids experimentally infected with *E. arloingi*

There was no histopathological lesion seen in the group treated with oxytetracycline (Figure 6). Cystic degenerative changes were seen in intestinal glandular cells of the infected untreated group (Figure 7). Moreover, no histopathological lesion was seen in the uninfected untreated group (Figure 8).

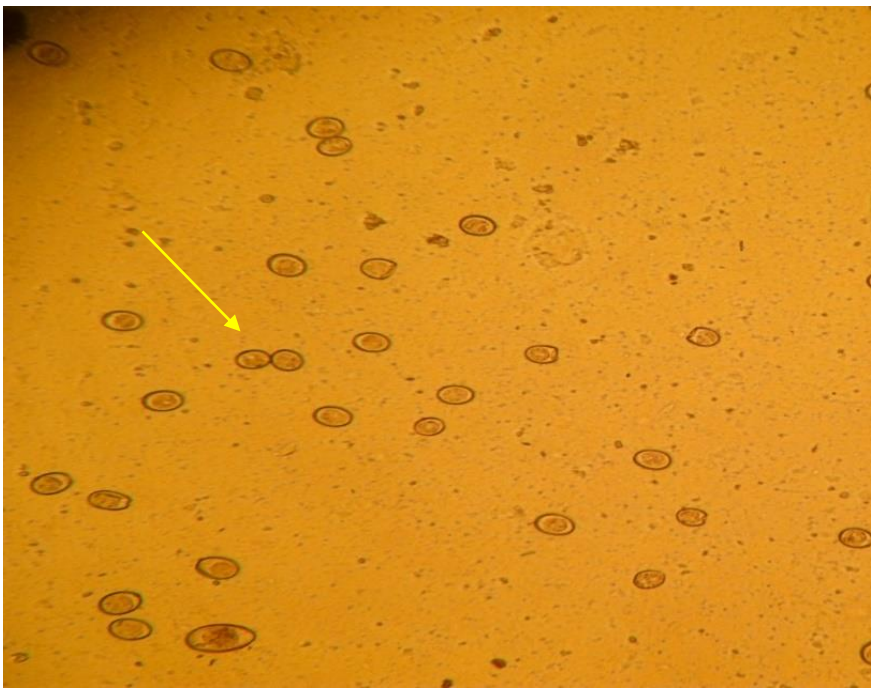
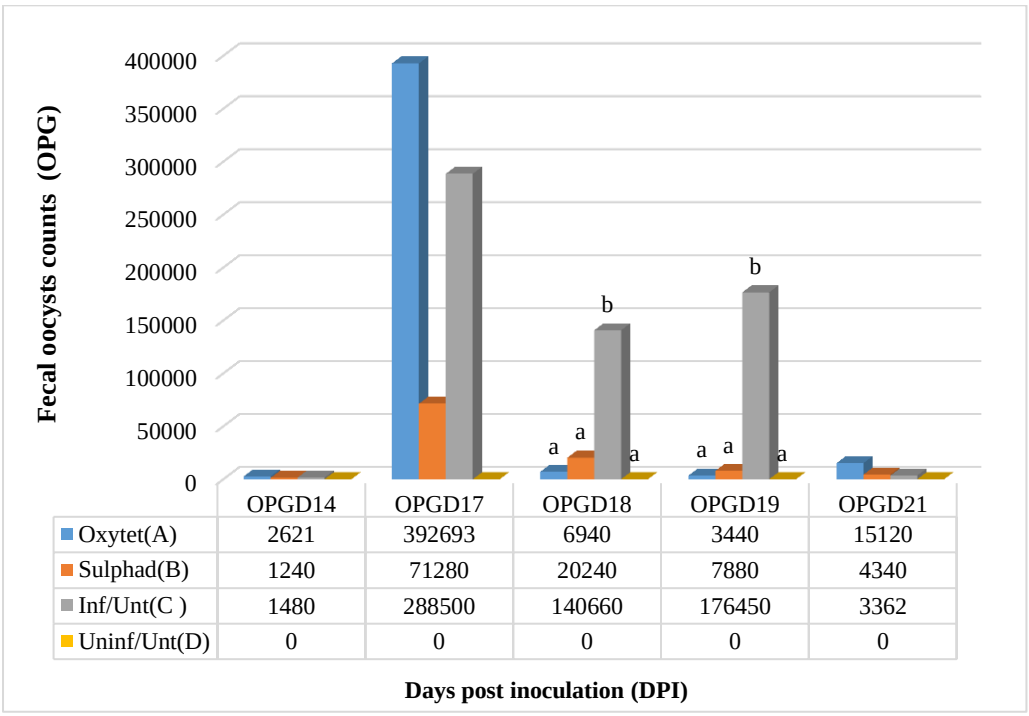


Figure 1. Microscopic view of oocysts (arrow) from fecal sample of experimentally infected kid with *Eimeria arloingi* oocysts in Ahmadu Bello University (A.B.U.) Zaria, Nigeria on June 2018 (× 100 magnification)



	OPGD14	OPGD17	OPGD18	OPGD19	OPGD21
Oxytet(A)	2621	392693	6940	3440	15120
Sulphad(B)	1240	71280	20240	7880	4340
Inf/Unt(C)	1480	288500	140660	176450	3362
Uninf/Unt(D)	0	0	0	0	0

Figure 2. Effect of oxytetracycline treatment on fecal oocysts count in experimental caprine coccidiosis of kids infected with *Eimeria arloingi* oocysts, 14 - 21 days' post inoculation in A.B.U. Zaria, Nigeria on June 2018. Data are presented as mean ± SEM. Group A: Infected and treated with 10%; oxytetracycline; Group B: Infected and treated with sulphadimidine; Group C: Infected and untreated; Group D: Uninfected and untreated; **a-b**: Significant difference ($P \leq 0.05$) between group C and groups A, B and D; OPG: Oocysts per gram; DPI: Days post inoculation; D: Day; Oxytet: Oxytetracycline; Sulphad : Sulphadimidine

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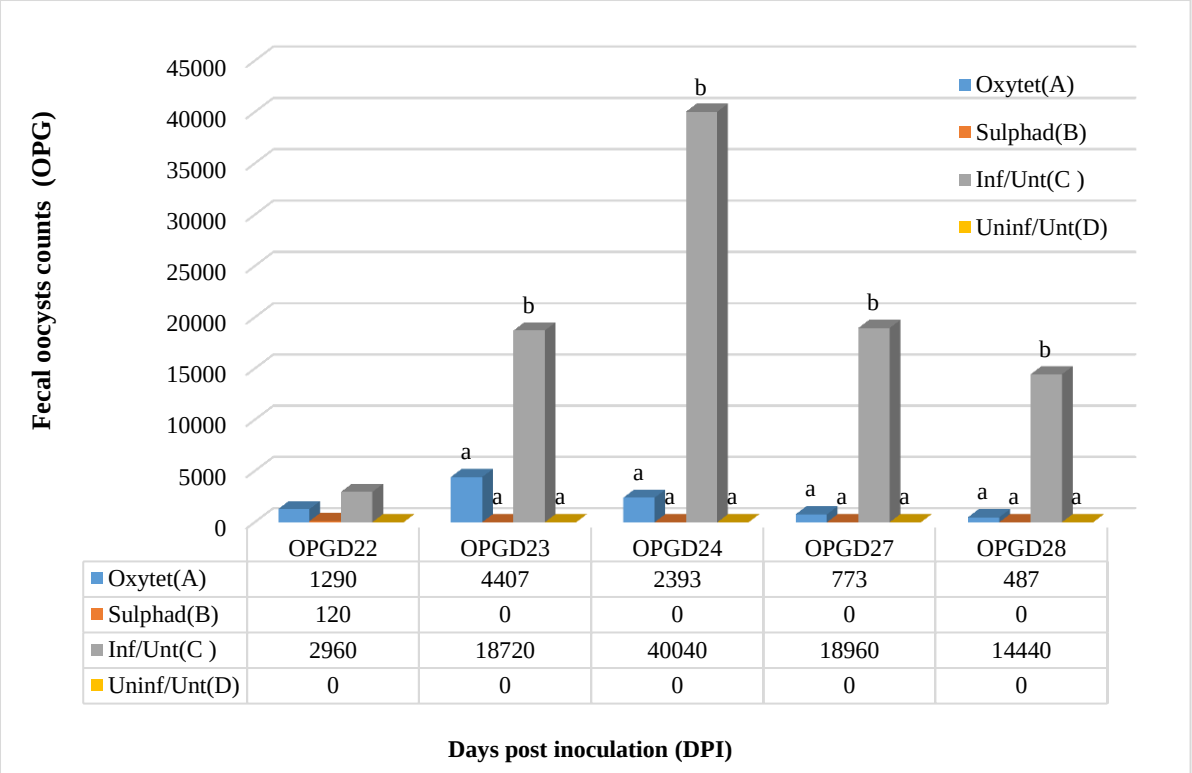
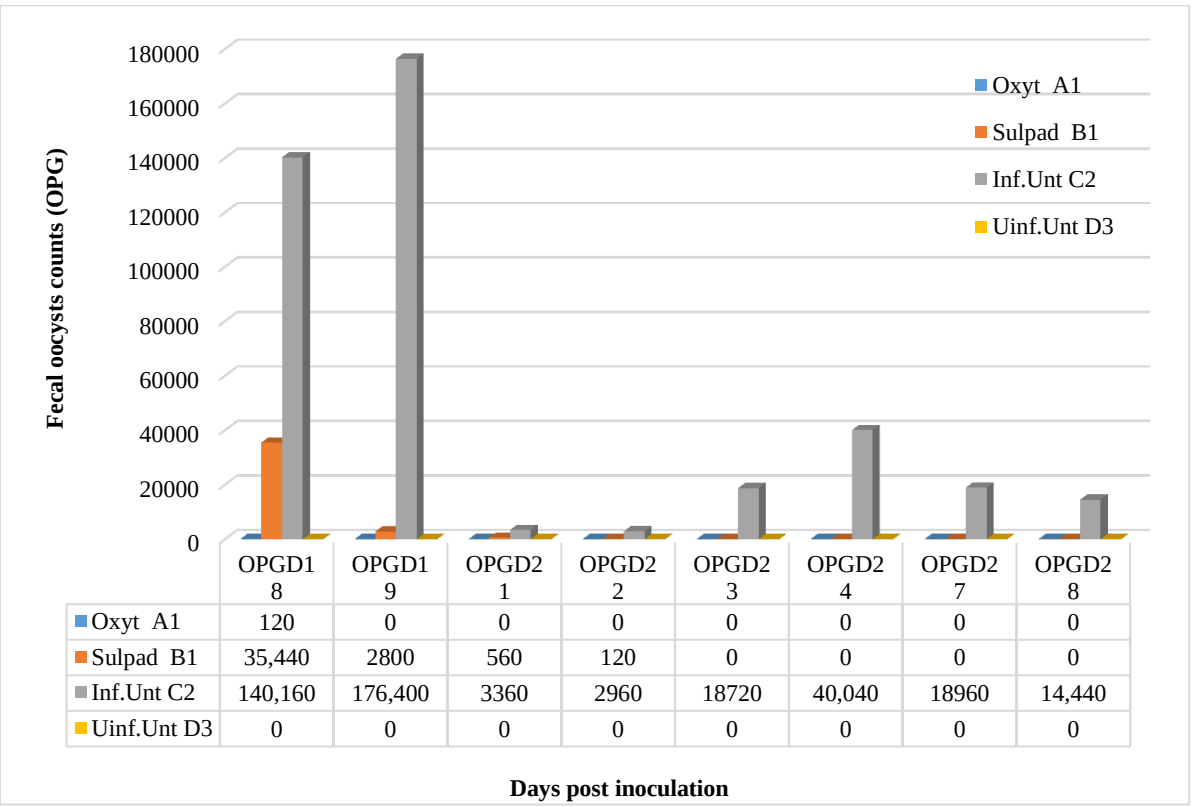


	OPGD22	OPGD23	OPGD24	OPGD27	OPGD28
Oxytet(A)	1290	4407	2393	773	487
Sulphad(B)	120	0	0	0	0
Inf/Unt(C)	2960	18720	40040	18960	14440
Uninf/Unt(D)	0	0	0	0	0

Figure 3. Effect of oxytetracycline treatment on fecal oocysts count in experimental caprine coccidiosis of kids infected with *Eimeria arloingi* oocysts, 22 - 28 days' post inoculation in A.B.U. Zaria, Nigeria on June 2018. Data are presented as mean ± SEM**; a-b**: Significant difference (P≤ 0.05) between group C and groups A, B and D; Group A: Infected and treated with 10 % oxytetracycline; Group; B: Infected and treated with sulphadimidine; Group C: Infected and untreated; Group D: Uninfected and untreated; OPG: Oocysts per gram; DPI: Days post inoculation; D: Day; Oxytet: Oxytetracycline; Sulphad: Sulphadimidine



	OPGD18	OPGD19	OPGD21	OPGD22	OPGD23	OPGD24	OPGD27	OPGD28
Oxyt A1	120	0	0	0	0	0	0	0
Sulpad B1	35,440	2800	560	120	0	0	0	0
Inf.Unt C2	140,160	176,400	3360	2960	18720	40,040	18960	14,440
Uinf.Unt D3	0	0	0	0	0	0	0	0

Figure 4. Effect of oxytetracycline treatment on fecal oocysts count in experimental caprine coccidiosis of kids per group infected with *Eimeria arloingi* oocysts, 18 – 28 days' post inoculation in A.B.U. Zaria, Nigeria on June 2018. Group A: Infected and treated with 10 % oxytetracycline; Group B: Infected and treated with sulphadimidine; Group C: Infected and untreated; Group D: Uninfected and untreated; OPG: Oocysts per gram; DPI: Days post inoculation; D: Day; Oxytet: Oxytetracycline; Sulphad: Sulphadimidine

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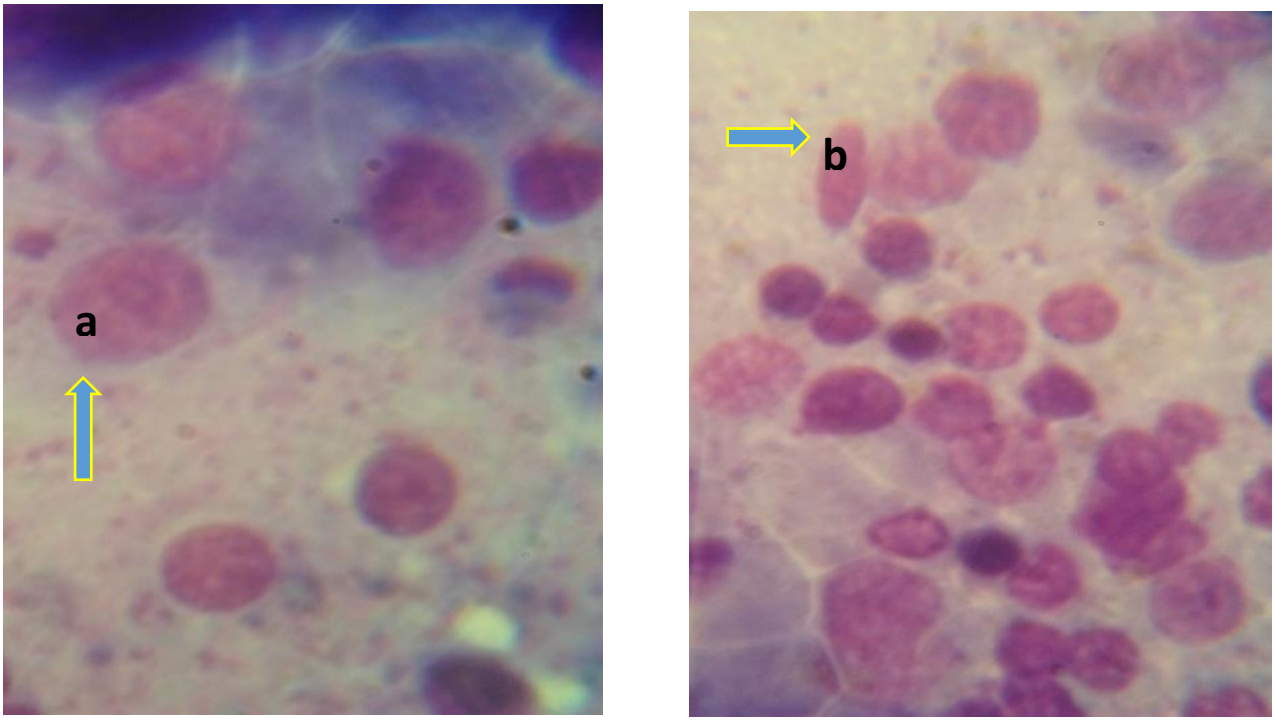

Figure 5. Microscopic view of schizonts (**a**) and merozoites (**b**) (Oil immersion × 1000 magnification) in the intestinal smear of infected untreated kid in A.B.U. Zaria, Nigeria on June 2018.

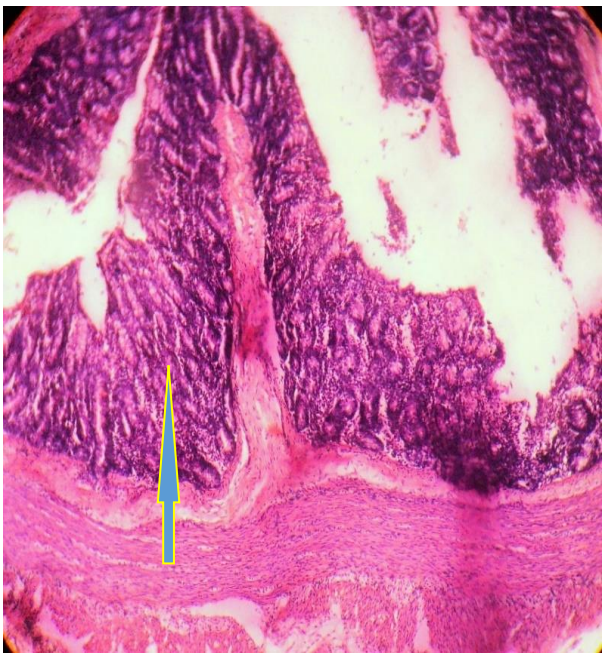

Figure 6. Histopathological findings from the intestine of a kid infected experimentally with *Eimeria arloingi* oocysts and treated with 10% oxytetracycline in A.B.U. Zaria, Nigeria on June 2018 (group A) showing intact glandular cells (arrow) H and E × 200

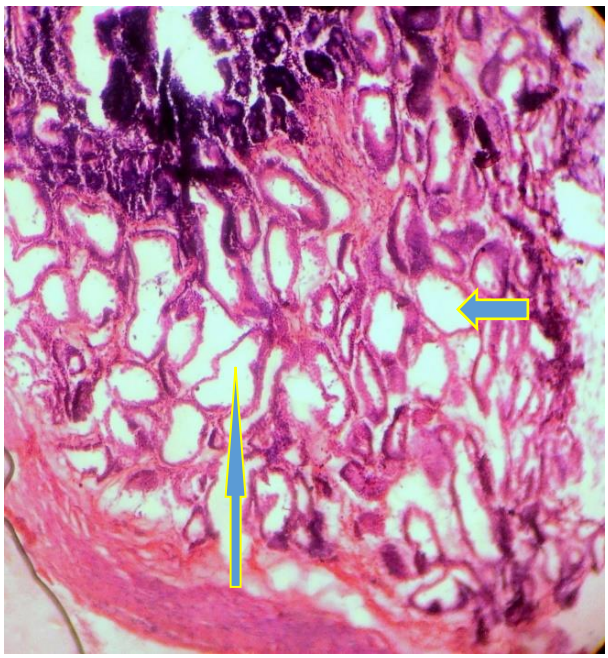

Figure 7. Histopathological findings from the intestine of kid experimentally infected with *Eimeria arloingi* oocysts but not treated in A.B.U. Zaria, Nigeria on June 2018 showing cystic degenerative changes (arrow) H and E × 200

Table 1. Findings of the kids' intestinal smear after experimental infection with *Eimeria arloingi* oocysts and treatment with antibiotics in A.B.U. Zaria, Nigeria on June 2018

Experimental groups	Animals sacrificed	Intestinal regions	
		Caecum	Ileum
A (10 % Oxytetracycline)	A1	-ve	-ve
B (Sulphadimidine)	B1	Schizonts	-ve
C (Infected untreated)	C2	Schizonts Merozoites	Schizonts
D (Uninfected untreated)	D4	-ve	-ve

-ve: Absence of schizont or merozoite

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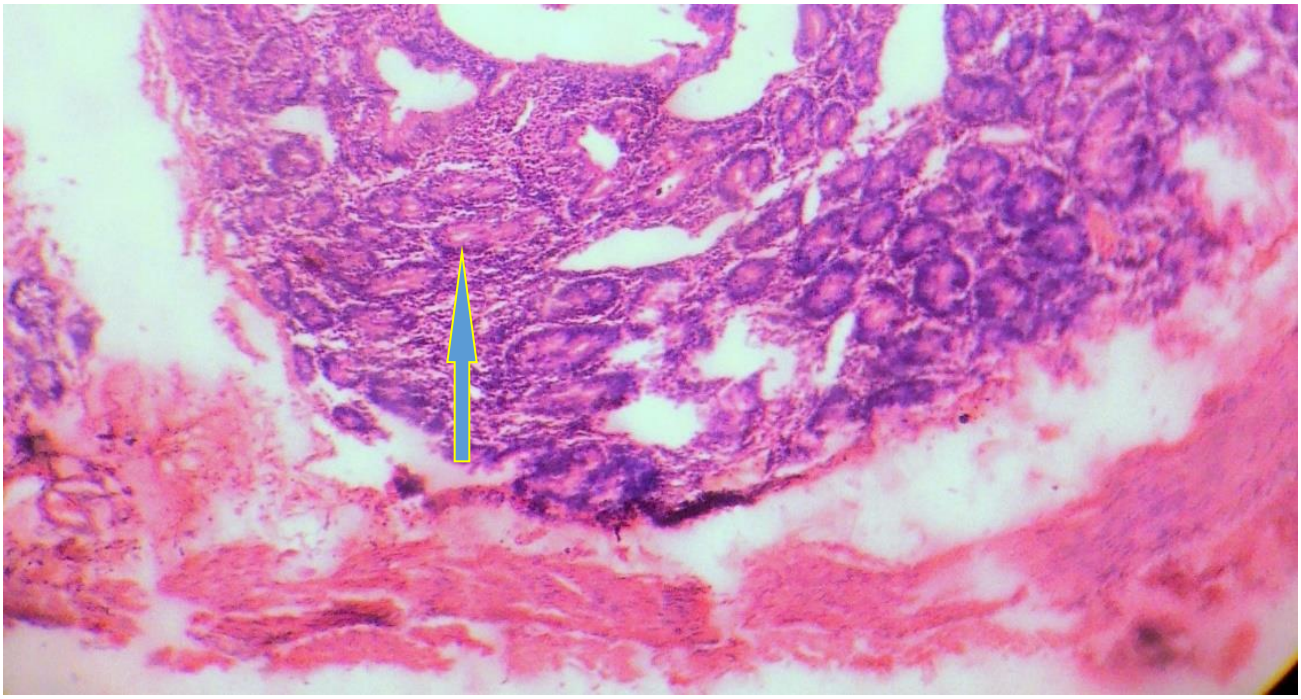

Figure 8. Histopathological findings from the intestine of uninfected untreated kid in A.B.U. Zaria, Nigeria on June 2018 showing an intact intestinal glandular cell (arrow) and E × 200

DISCUSSION

Diarrhea is one of the symptoms of coccidiosis and may be bloody in some cases. Coccidian oocyst shed in the feces of carrier animals that did not reveal symptoms as well as affected animals expressing symptoms of the disease (Richards et al., 2016). The findings of the present study indicated that the kids started shedding the coccidian oocysts at day 14 post inoculation. This finding agrees with the earlier report of Rakhshandehroo et al. (2013) that indicated oocysts shedding could start at days 13 to 14 post inoculation. However, some researchers reported the shedding of *E. arloingi* oocysts by infected kids at days 16 to 18 post inoculation (Hashemnia et al., 2012).

Animals affected by coccidian parasite show specifically dehydration and weight loss during the period of diarrhea (Khodakaram-Tafti and Hashemnia, 2017). The current study demonstrated that clinical coccidiosis is associated with diarrhea and shedding of *Eimeria* oocysts in the feces of infected animals.

Damage to the epithelial cells with a subsequent reduction in the number of oocysts is reflected histologically by villous atrophy, crypt hyperplasia and cellular infiltration (Khodakaram-Tafti and Hashemnia, 2017). The present study revealed that treatment with oxytetracycline has ceased the development and multiplication of coccidian parasites and protected the glandular cells of the intestine from being damaged by the developmental stages of *E. arloingi* oocysts (schizonts and merozoites). However, severe damages to the intestinal glandular cells of the infected untreated negative control group were seen due to cystic degenerative changes that took place therein.

CONCLUSION

The data obtained from the present study revealed that both oxytetracycline and sulfadimidine are effective in reducing and/or clearing fecal oocysts load in the infected animals however oxytetracycline was more effective in stopping the developmental stages of the Eimeria parasites. In conclusion, the current finding suggests that oxytetracycline can be effectively used in treating coccidiosis in small ruminants and particularly in goats.

DECLARATIONS

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Competing interests

The authors declared no competing interest exist.

Consent to publish

All authors were aware and agreed to the fact that the manuscript be published in this journal. This study was not published elsewhere partially or totally.

Authors' contribution

Mikail HG, Saidu SNA and Mamman M designed the work. Mikail HG conducted the experiment and wrote the manuscript, while Saidu SNA and Mamman M reviewed the manuscript. All authors confirmed the final form of the article.

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Kids' Survivability as Affected by Their Body Weight, Blood Biochemical Indices and Maternal and Kids' Behavior in Baladi and Shami Goats under Semi-Arid Condition

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ABSTRACT

The present study was conducted to investigate the effect of body weight, blood biochemical parameters and post parturient behavioral activities of goats and their kids on kids' mortality rate in Baladi and Shami breeds during neonatal period. Twenty-five adults does of each breed (average age: 18 months old) were selected during breeding season. All female goats were estrus synchronized and naturally mated. After parturition, one hundred and one kids (39 Baladi and 62 Shami) were followed for up to 30 days of their age. The overall mean birth weights of female kids of Baladi and Shami goats were 2.47 and 2.81 Kg, respectively. For male kids, birth weights were 2.43 and 2.47 kg, respectively. There was no significant difference in average daily gain (g/day) between Baladi and Shami kids during the first 30 days of age. Male kids recorded higher mortality rate than female kids. The percent of death for male and female kids were 87.50% and 36.84% in Shami while, were 33.33% and 14.28% in Baladi respectively. However, death stopped in Baladi kids after 14 days, but continued in Shami kids to 28 days post-partum. Present data revealed that goat breed and neonatal period showed a significant effect on urea concentration and alkaline phosphatase (ALP) and insignificant effect on creatinine concentration, alanine transferase (ALT), gamma glutamyl transferase (GGT), total lipids, cholesterol and triglycerides in Baladi and Shami Kids. While aspartate transferase (AST), were not affected by goat breed and was significantly affected by both age of birth and interactions. Baladi breed showed significantly better maternal activity than Shami does as they spend lesser time to concern their newly born kids. Baladi kids had more strong behavior towards their dams when compared to Shami ones. It was concluded that body weight, blood biochemical parameters and Maternal and kid's behavior had notable effect on kid's survivability. Our results might declare superiority of Baladi kids than Shami ones which reflected on the significant reduction of mortality rate in Baladi kids as compared to Shami ones.

Key words: Body weight, Goat, Kids behavior, Maternal behavior, Offspring survival

INTRODUCTION

Goats (*Capra hircus*) have a variety of functions and display a high ability to adapt and maintain themselves in harsh environment (Girma et al., 2011). The population of Egyptian goats is estimated by 4.2 million heads (FAOSTAT, 2012). This population is distributed across the country, especially in the Nile valley and delta region followed by the north-western coastal region and at oases (Galal et al., 2005). However, in Sinai goat population is almost three folds of that of sheep CAPMAS (2016). Kids' mortality has a direct effect on genetic progress by its effect on selection pressure. Generally, greater kids' mortality happens at birth and from birth to weaning, while mortality is comparatively low from weaning to breeding age in several production systems (Donkin and Boyazoglu, 2004). Several factors had been reported in the literature that affected mortality rate in goat kids such as kind of birth, sex, weight of birth, parity, kidding season, age of the kids and kidding year (Hailu et al., 2006; Mtenga et al.,2008 and Khalil et al., 2018).

Awemu et al. (2002) and EL–Tonsy et al., (2018) reported that the kid's mortality was significantly affected by birth weight. Madibela et al. (2002) stated that persistence rates were similar (93.9 versus 91.9%) between singles and multiples in Tswana goats. Steve and Marco (2001) and Snyman (2010) stated that survival to weaning of goat kids appeared higher for male gender than for females. However, Ingo (2002) reported that sex is not significant factor in kid survival.

The haemato-biochemical profiles can be previously reported to assess the immunity in offspring (Al-Seaf and Al-Harbi, 2012). Gwaze et al. (2012) showed that there was a negative relationship ($P<0.05$) between age and ALP, while there was no relationship ($P>0.05$) between age and selected liver enzymes. Also, Herosimczyk et al. (2011) reported that the decrease in plasma creatinine concentrations after 14th day of age is may be associated with increased glomerular filtration rate in calves.

In mammals, maternal behavior (nursing of the infant and lactation) represent important behavioral and energy investments indispensable for the successful rearing of the offspring (Nowak et al., 2000). However, the degree to which maternal recognition contributes to preferential care and survival of the progeny may vary, depending on the type of mother-young relationships (Martínez et al., 2009). Vulnerable kids physically, especially kids resulting from mothers

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who did not receive good nutrition during pregnancy, have mortality rates higher, as they fail to show the behavior of survival such as searching for nipples and feeding the same most powerful child force of dams that feed well (Browning et al., 2011). Disturbance in maternal care, maternal selectivity, or mutual recognition may have adverse effects on the survival of the young during the first week of life (Poindron et al., 2007; Nowak et al., 2007). In goats, immediately after birth, the mother goats started licking their kids and allowing the kids to breastfeed are the first signs of a mother's behavior (Kaymakci, 2013). Dam behaviors help to clean and dry their offspring from fetal membranes, stimulate the newborn to seek teat and form an exclusive and selective attachment to her own offspring (Nowak et al., 2000; Dwyer, 2009).

The present study was undertaken to investigate the role of birth weight, biochemical parameters, sex, maternal behavior and kid's behavior in kids' mortality rate in both Baladi and Shami goat under semi-arid conditions.

MATERIALS AND METHODS

Experimental region

The present study was carried out at Ras-Sudr Research Station (located in190 km East of Cairo) that belongs to the Desert Research Center (DRC), South Sinai Governorate, Egypt. The prevailing climate is characterized by long hot summer, short winter, low rainfall and high evaporation intensities. However, winter nights might be so cold. The experiment was conducted during one month from February to March 2016.

Ethical approval

This experiment was performed according to all ethics and animal rights regulated by desert research centre, Egypt. As much as this work had considering all rules and regulations in conformity with the European union directive for the protection of experimental animals (2010/63/EU).

Animals

Twenty-five Baladi does (averaged 21.12 kg LBW) and 25 Shami does (averaged 35.78 kg LBW) all averaged 18 months old, were used in this study to evaluate kids' mortality rate and its reasons. All does were clinically examined. Baladi goats breed was well-adapted to the environmental conditions in North and South Sinai in Egypt, while Shami goat breed was originated in Syria and imported to North Sinai.

Feeding and management

All goats were housed in semi open shaded pens and were fed uniformly twice a day (08:00 and 14:00 h) on Berseem hay and concentrate feed mixture to cover their nutrient requirements during different physiological status according to Kearl (1982). The chemical compositions of feed stuffs were determined according to AOAC (1990) and showed in table 1. The two groups were fed these diets for an adaptation period of 21days before mating season and all over the pregnancy period and after parturition till one month after birth. During the trial, all animals drank 3 times a day (08:00, 14:00 and 20:00 h). Chemical composition of drinking water is showed in table 2. All animals were kept under natural photoperiod and ambient temperature. Vaccination for all goats Before the mating season against the main prevalent epidemic diseases, internal and external parasites were controlled in a timely manner in a good way.

Table 1. Chemical composition of experimental feed stuffs (as percentage of dry matter)

Ingredients	DM	OM	CP	EE	CF	NFE	Ash
Berseem hay (%)	85.1	86.85	12.09	1.36	27.57	45.83	13.15
Concentrate feed mixture (%)	91.42	88.61	15.61	3.01	16.33	53.66	11.39

DM: dry matter; OM: organic matter; CP: Crude protein; EE: ether extract; CF: crude fiber; NFE: Nitrogen free extract

Table 2. Chemical composition of tap water used in the experiment

Items	TDS	Na^+	K^+	Mg^+	CL^-
Tap water (ppm)	600	145.2	9.09	15.3	179.88

TDS: Total dissolved salts

Live body weight

All kids in both breeds were weighed immediately after birth and each week then after to the end of the experiment. Weighing was performed early in the morning before suckling. Electronic balance was used for measuring weights of kids to the nearest 0.01 kg.

Blood sampling

Five ml of blood were collected from the outer jugular vein of Shami and Baladi kids in vacuum tubes containing heparin once a week at the same time in the morning (8 am) during the first month after birth. The first sampling in the

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lambs was performed immediately after birth. Plasma was obtained for chemical analysis by centrifugation of blood at 3000 rpm then preserved at −20 ^{o}C. Biochemical analyses including plasma alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), urea, creatinine, total lipids, cholesterol and triglycerides assays were recorded in plasma using kits supplemented by Diamond Company.

Behavioral studies

For behavior observations, does and their kids were kept in open housing pen (observation pen) with dimensions of 6 m x 6 m and a floor made from cement and padded with rice straw. Does were entered in observation pens with their kids and were observed for 24 hours. All animals (dams and kids) were identified by painting their sides with spray by clear number for this study. Behavioral activities were recorded with eye observation.

Does behavior criteria

The following criteria were recorded for all does during post-partum till the first 24 h of birth:
DS: Does stood after delivery (sec).
FAK: First attention to kid (min)
DSL: Does started licking (min)
S/L: Sniffing/ licking during the first two hrs. after birth.

Kids' behavior criteria

All offspring were observed from parturition up to 24 hours of their age to assess the following patterns:

1. **Standing attempts including**
   - First standing attempt (min)
   - First success standing (min)
   - Time standing during the first two hours after birth (min)
2. **Suckling attempts including**
   - First attempted of suckling (min)
   - Kids starting suckling (min)
   - Duration of first suckling (min)
   - Total time of suckling during the first two hours after birth (min)
   - Suckling frequency during the first two hours after birth (bouts)

Statistical analysis

Results were presented as Mean ±SE. A two-way analysis of variance (ANOVA) considering repeated measurements; was used to determine statistical differences between mean values of the studied parameters during the first month postpartum. Data were analyzed by the least square of analysis variance using the general linear model procedure (SAS, 2004). Duncan's New Multiple Range test (Duncan, 1955) separated differences ($P<0.05$) among treatment means {Goat breed (Baladi and Shami); Neonatal period (Immediately, 7-day, 14-day, 21-day, 28-day after birth)}.

RESULTS AND DISCUSSION

Mortality rate of Baladi and Shami goat kids during neonatal period

As presented in table 3, one hundred and one kids (39 Baladi and 62 Shami) were obtained and followed for up to 30 days of their age. Forty-four (9 Baladi and 35 Shami) died before they achieved this age. The age of kids in weeks had a significant impact ($P<0.05$) on the death of kids. The 44 dead kid were distributed as follows; 21 (47.72%) occurred within the first week of their age and then death cases reduced significantly during the following 3 consequent weeks (29.54, 13.63 and 9.09%, respectively). The overall mortality rate in this study was also significantly affected by the breed that recorded 23.07% for Baladi and 56.45% for Shami kids during neonatal period. Sex had a significant effect on mortality rate. Male kids recorded higher mortality rate than females (33.33% versus 14.28%, respectively for Baladi kids and 87.50% versus 36.84% for Shami kids, respectively).

Present results were totally in agreement with Aganga et al. (2005) and Hailu et al. (2006) as they recorded higher mortality rates for male kids compared to females. On the other hand, Petros et al. (2014) found insignificant effect of sex on pre-weaning mortality, while Debele et al. (2011) recorded higher mortality rates in females than males' kids in Arsi-Bale kids maintained in a similar environment. This might reflect higher immunity of female as stated by Tambuwal et al. (2002) and Ashour et al. (2015) who found in goats a higher leucocyte counts in females than in males.

The results of present study indicated that 13 of Baladi dams gave one kid, 9 gave twins kids and 3 gave triple kids. For Shami dams, the corresponding numbers were 0, 13 and 12, respectively. Mortality tended to increase with a larger size litter where single kids had always a higher birth weight hence higher survival rate than twins and triplets (Awemu et al., 1999). Nevertheless, Madibela et al. (2002) found that survivability rates were similar (91.9 versus 93.9%)

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between manifold and singles in Tswana goats. They added that survival rates and growth rates can be improved by supplementation. In the present study, kids' mortality occurred in Baladi during the first two weeks, while continued in Shami kids all over the first month with different percentage per week.

Table 3. Means of twinning and mortality rates of Baladi and Shami kids during neonatal

Breed	Sex	Total live-born kids (%)	Sex ratio	Twinning rate (%l)	kids die during neonatal period as a percentage of total live-born kids								Total mortality rate (%)	
					1st week		2nd week		3rd week		4th week			
					N	%	N	%	N	%	N	%	N	%
Baladi	Female	21	53.85	156	2	11.11	1	5.56	0	0.0	0	0.0	3	14.28
	Male	18	46.15		4	19.05	2	9.52	0	0.0	0	0.0	6	33.33
	Total	39	100		6	15.38	3	7.69	0	0.0	0	0.0	9	23.07
Shami	Female	38	61.29	248	7	18.42	4	10.52	2	5.26	1	2.63	14	36.84
	Male	24	38.71		8	33.33	6	25.00	4	16.67	3	12.50	21	87.50
	Total	62	100.0		15	24.19	10	16.13	6	9.78	4	6.45	35	56.45

N: number of kids

Body weight changes and growth rates in kids during neonatal period

The body weight results of Baladi and Shami kids are illustrated in tables 4 and 5. The overall mean birth weights of female kids of Baladi and Shami goats were 2.47 and 2.81 Kg with significant differences (P<0.01). For male kids, birth weights were 2.43 and 2.47 kg, respectively without significant differences. Male kids' birth weight was significantly lower than female kids. These results were contrary to several reports recording higher birth weight of male than female kids (Gubartalla et al., 2002; ELimam et al.,2007; Bushara et al., 2013). On the other hand, Hanford et al. (2006) recoded that there was no significant change in birth weight due to kids' sex. These differences between author's results might be due to breed differences and management. Baladi male kids showed the lowest birth weight (2.43 kg) and the highest percentage mortality rate (66.66%). Kid's birth weight was found to have a significant effect on kids' mortality (Awemu et al., 2002 and Debele et al., 2011) and final economic benefit (Portolano et al., 2002 and Hanford et al., 2006). Mtenga et al. (1992) reported that the lowest pre-weaning mortality rate occurred in kids with a birth weight greater than 2.6 kg.

The rate of change in live body weight (LBW) for Baladi and Shami was respectively 29.99 versus 33.18% for females, while was 31.35 versus 26.73% for males, with the differences being significant (P<0.01). However, there was no significant change in average daily gain (ADG) between Baladi and Shami kids during neonatal period. In agreement, Khazaal (2009) recorded that ADG of kids was similar between Saanen and Shami during the first three months of age (suckling period). It was apparent that Shami female kids had the highest birth weight, growth rate and final LBW. Baladi male kids that could stay alive compensated the lower birth weight by high relative growth rate and attained the same final LBW as Shami males. The data concerning body weight changes of kids that died then after are presented in table 6. The defects in birth weight and growth rated were higher in Shami kids that might give reason to higher mortality rates in this breed.

Table 4. Means of live body weight total gain daily gain and percentage of body weight change of Baladi and Shami female kids during neonatal period

Parameter	Breed	Neonatal period					Overall	± Standard error		
		Immed. After birth	7 d after birth	14 d after birth	21 d after birth	28 d after birth		B	N	B × N
BW (kg)	Baladi	1.83^{e}	2.23^{de}	2.37^{cde}	2.77^{bcd}	2.90^{bc}	2.42^{F}	0.09^{**}	0.12^{**}	0.13^{**}
	Shami	2.09^{de}	2.32^{cde}	2.59^{cd}	3.21^{ab}	3.71^{a}	2.78^{E}			
	Overall	1.96^{D}	2.28^{C}	2.48^{C}	2.99^{B}	3.30^{A}				
TG (kg)	Baladi	0.00^{c}	0.40^{abc}	0.13^{bc}	0.40^{abc}	0.12^{bc}	0.21	0.09^{NS}	0.11^{**}	0.17^{*}
	Shami	0.00^{c}	0.23^{abc}	0.23^{abc}	0.60^{a}	0.48^{ab}	0.30			
	Overall	0.00^{C}	0.31^{AB}	0.18^{BC}	0.50^{A}	0.30^{AB}				
DG (g)	Baladi	0.00^{c}	57.94^{abc}	18.71^{bc}	58.17^{abc}	18.05^{bc}	30.57	9.10^{NS}	15.89^{**}	18.20^{*}
	Shami	0.00^{c}	32.85^{abc}	32.85^{abc}	85.95^{a}	68.69^{ab}	44.07			
	Overall	0.00^{C}	45.39^{AB}	25.78^{BC}	72.06^{A}	43.37^{AB}				
BWC (%)	Baladi	0.00^{c}	19.01^{de}	7.30^{bc}	17.16^{abc}	4.89^{bc}	9.67	3.89^{NS}	4.71^{*}	7.25^{*}
	Shami	0.00^{c}	11.07^{abc}	10.13^{abc}	24.50^{a}	13.37^{a}	11.81			
	Overall	0.00^{B}	15.04^{A}	8.71^{AB}	20.83^{A}	9.13^{AB}				

B: Breed; N: Neonatal period; LBW: live body weight; TG: total gain; DG: daily gain; BWC%: percentage of body weight change; A, B, C, D: values with different letters on the same row are significantly different (P< 0.05); E, F: values with different letters on the same column are significantly different (P< 0.05); a, b, c, d, e; values in the experimental sub-groups within certain trait with different superscripts are significantly different (P< 0.05); NS: non-significant, *: P<0.05; **: P<0.01.

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Table 5. Means of live body weight total gain daily gain and percentage of body weight change of Baladi and Shami male kids during neonatal period.

Parameter	Breed	Neonatal period					Overall	± Standard error		
		Immed. After birth	7 d after birth	14 d after birth	21 d after birth	28 d after birth		B	N	B × N
BW (kg)	Baladi	1.90^{d}	1.99^{d}	2.55^{b}	2.57^{b}	2.95^{b}	2.39	0.03^{NS}	0.06^{*}	0.18^{**}
	Shami	1.93^{d}	2.14^{cd}	2.55^{b}	2.62^{b}	3.18^{a}	2.48			
	Overall	1.91^{D}	2.06^{C}	2.55^{B}	2.59^{B}	3.06^{A}				
TG (kg)	Baladi	0.00^{c}	0.08^{c}	0.56^{a}	0.36^{ab}	0.49^{a}	0.30	0.05^{NS}	0.07^{*}	0.11^{*}
	Shami	0.00^{c}	0.20^{bc}	0.39^{ab}	0.06^{c}	0.55^{a}	0.24			
	Overall	0.00^{C}	0.14^{C}	0.47^{A}	0.21^{B}	0.52^{A}				
DG (g)	Baladi	0.00^{c}	12.21^{c}	81.00^{a}	51.73^{bc}	70.31^{a}	43.05	0.06^{NS}	7.69^{*}	10.87^{*}
	Shami	0.00^{c}	29.57^{c}	55.86^{ab}	9.34^{c}	79.20^{a}	34.79			
	Overall	0.00^{C}	20.89^{B}	68.43^{A}	30.54^{B}	74.75^{A}				
BWC (%)	Baladi	0.00^{d}	4.60^{cd}	27.73^{a}	16.99^{b}	21.26^{ab}	14.12	1.83^{NS}	3.33^{*}	3.17^{*}
	Shami	0.00^{d}	10.53^{cd}	16.56^{b}	4.57^{cd}	21.17^{ab}	10.57			
	Overall	0.00^{C}	7.56^{BC}	22.15^{A}	10.78^{B}	21.22^{A}				

B: Breed; N: Neonatal period; LBW: live body weight; TG: total gain; DG: daily gain; BWC%: percentage of body weight change; A, B, C, D: values with different letters on the same row are significantly different (P< 0.05); a, b, c, d; values in the experimental sub-groups within certain trait with different superscripts are significantly different (P< 0.05); NS: non-significant; *: P<0.05; **: P<0.01.

Table 6. Live body weight (kg) and percentage change of alive and dead Baladi and Shami kids during neonatal period

Breed	Type of birth	No of does	Neonatal period									
				Females					Male				
			Case	0 Day	7 day	14 Day	21 day	28 day	0 Day	7 day	14 day	21 day	28 Day
Baladi	Single	13	Alive	1.83	2.23	2.37	2.77	2.90	1.90	1.99	2.55	2.57	2.95
			Dead	1.55	1.61	1.68	.	.	1.67	1.70	1.97	.	.
			% change	-15.3	- 27.8	- 29.1	.	.	-12.1	-14.6	-22.7	.	.
	Twins	9	Alive	1.74	1.89	1.95	2.10	2.90	1.79	1.90	2.23	2.30	2.41
			Dead	1.35	1.39	1.44	.	.	1.41	1.43	1.49	.	.
			% change	-22.4	-26.5	-26.2	.	.	-21.8	-24.7	-33.2	.	.
	Triplets	3	Alive	1.61	1.68	1.75	1.89	2.01	1.68	1.72	1.80	1.92	2.04
			Dead	1.30	1.32	1.37	.	.	1.35	1.38	1.40	.	.
			% change	-19%	-21%	-21%	.	.	-19%	-19%	-22%	.	.
Shami	Single	0	Alive	.	.	.	.	.	.	.	.	.	.
			Dead	.	.	.	.	.	.	.	.	.	.
			% change	.	.	.	.	.	.	.	.	.	.
	Twins	13	Alive	1.77	1.82	1.95	2.13	2.23	1.87	1.90	2.02	2.18	2.30
			Dead	1.40	1.44	1.49	1.55	1.61	1.47	1.50	1.52	1.58	1.67
			% change	-20.9	-20.9	-23.6	-27.2	-27.8	-21.4	-21.1	-24.8	-27.5	-27.4
	Triplets	12	Alive	1.70	1.78	1.86	1.96	2.14	1.75	1.81	1.99	2.11	2.33
			Dead	1.35	1.38	1.43	1.48	1.55	1.40	1.44	1.49	1.55	1.63
			% change	-20.6	-22.5	-23.1	-24.5	-27.6	-20.0	-20.4	-25.1	-26.5	-30.0

Physiological changes in hepatic enzyme of Baladi and Shami kids during neonatal period

Enzymes concentrations of alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) are those conventionally manipulated for diagnosing hepatic damage. The data presented in table 7 illustrated that goat breed, neonatal period and their interactions (B × N) showed insignificant effect on plasma ALT and GGT activities. In disagreement to Njidda et al. (2014) who indicated that there is an influence of age, sex and breed on plasma GGT and ALT activities. On the other hand, present findings revealed that goat breed, neonatal period and their interactions (B × N) showed a significant effect on ALP activities in Baladi and Shami Kids. While AST was not affected by goat breed and was significantly affected by both age of birth and interactions (B × N). These results are in agreement with Antunovic et al. (2004) who reported a decrease in ALP level with an increase in age of goats. Similar results recorded by Tibbo et al. (2008) and Piccione et al. (2010) who had similar conclusions in Arsi-Bale goats and Girgentana goats, respectively. Elitok (2012) reported that higher values of ALP were found in the first 40 days of life due to the more intense bone remodeling. The differences were also detected due to both sex and age in accordance with the study of Red Sokoto goats (Tambuwal et al., 2002, Daramola et al., 2005). Smith and Sherman (2009) reported that quality, quantity, and timing of colostrum feeding have an influence on the activity of GGT during the first few days of life. In addition to colostrum intake, the greater activity of ALP in growing goats may be correlated

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to Bone-ALP iso-enzyme. As shown in table 8 generally all liver enzymes (ALT, AST, ALP and GGT) for dead kids in both breeds were decreased as compared to alive ones. The percentages of ALT decreased in Baladi dead kids as compared with a live kids within the 14 days post-partum were between (-73.49, -73.61 and -73.20 %) and (-72.05, -73.54 and -75.71%) for Shami kids through the same period , then increased within the following two weeks to reach (-75.91 and -73.89 %) in Shami kids, while in AST, ALP and GGT were lower in Baladi dead kids as compared with live kids within the first two weeks post-partum it were between (-56.69, -65.82 and -65.06%), (-76.89, -76.19 and -74.08%), (-54.67, -53.85 and -46.58%) for Baladi dead kids and the percentage in Shami dead kids were (-64.46, - 67.29 and -68.18%), (-76.30, -75.56 and -74.75%), (-55.70, -52.55 and -52.24%) then, AST increased (-72.58 and -76.56%), while ALP and GGT decreased in (-74.47 and -71.37%), (-47.58 and -39.91%) respectively in Shami kids within the following two weeks. Decreasing in ALT, AST and ALP activities were higher in Shami kids that might give reason to higher mortality rates in this breed.

Table 7. Hepatic enzyme activity of Baladi and Shami kids during the first month of post-partum

Parameter	Breed (B)	Neonatal periods (S)					Overall mean	±Standard error		
		0 day	7 day	14 day	21 day	28 day		B	N	B x N
ALT (IU/L)	Baladi	64.22	68.38	68.66	67.01	69.96	67.65	2.84 NS	4.10 NS	6.36 NS
	Shami	53.72	56.89	66.00	68.92	66.66	62.44			
	Overall mean	58.97	61.05	67.33	67.96	68.31				
AST (IU/L)	Baladi	35.10^{b}	44.83^{b}	46.00^{b}	43.83^{b}	46.50^{b}	43.10	1.56 NS	2.26 **	3.50 **
	Shami	34.33^{c}	37.92^{b}	39.60^{b}	46.55^{b}	56.32^{a}	43.02			
	Overall mean	34.72^{B}	41.38^{B}	42.80^{B}	45.19^{B}	51.41^{A}				
ALP (IU/L)	Baladi	82.66^{ab}	80.66^{bc}	74.35^{de}	77.50^{e}	71.50^{cd}	77.40 C	0.50 **	0.73 **	1.13 **
	Shami	84.44^{a}	82.66^{a}	82.88^{ab}	82.31^{ab}	74.77^{de}	81.41 D			
	Overall mean	82.66^{A}	82.55^{A}	78.61^{B}	77.30^{B}	76.13^{B}				
GGT (IU/L)	Baladi	13.26	13.48	13.33	13.40	12.33	13.16	0.62 NS	0.90 NS	1.39 NS
	Shami	13.75	12.92	12.61	13.23	11.90	13.13			
	Overall mean	13.50	13.20	13.12	13.32	12.12				

B: Breed; N: Neonatal period; ALT: alanine transferase; AST: aspartate transferase; ALP: alkaline phosphatase; GGT: gamma glutamyl transferase; A, B: values with different letters on the same row are significantly different (P< 0.05); C, D: values with different letters on the same column are significantly different (P< 0.05); a, b, c, d, e; values in the experimental sub-groups within certain trait with different superscripts are significantly different (P< 0.05); NS: non-significant, **: P<0.01.

Table 8. Means of liver enzymes and percentage change of alive and dead Baladi and Shami kids during the postnatal period

Breed	Parameter	The neonatal period (Days)					
		Case	0 day	7 day	14 day	21 day	28 day
Baladi	ALT (IU/L)	Alive	64.22	68.38	68.66	67.01	69.96
		Dead	17.02	18.04	18.40	.	.
		% change	-73.49	-73.61	-73.20		
	AST (IU/L)	Alive	35.10	44.83	46.00	43.83	46.50
		Dead	15.20	15.32	16.07	.	.
		% change	-56.69	-65.82	-65.06	.	.
	ALP (IU/L)	Alive	82.66	80.66	74.35	77.50	71.50
		Dead	19.10	19.20	19.27	.	.
		% change	-76.89	-76.19	-74.08		
	GGT (IU/L)	Alive	13.26	13.48	13.33	13.40	12.33
		Dead	6.01	6.22	7.12	.	.
		% change	-54.67	-53.85	-46.58	.	.
Shami	ALT (IU/L)	Alive	53.72	56.89	66.00	68.92	66.66
		Dead	15.01	15.05	16.03	16.60	17.40
		% change	-72.05	-73.54	-75.71	-75.91	-73.89
	AST (IU/L)	Alive	34.33	37.92	39.60	46.55	56.32
		Dead	12.20	12.40	12.60	12.76	13.20
		% change	-64.46	-67.29	-68.18	-72.58	-76.56
	ALP (IU/L)	Alive	84.44	82.66	82.88	82.31	74.77
		Dead	20.01	20.20	20.35	21.01	21.40
		% change	-76.30	-75.56	-74.75	-74.47	-71.37
	GGT (IU/L)	Alive	13.75	12.92	12.61	13.23	11.90
		Dead	6.09	6.13	6.17	7.03	7.15
		% change	-55.70	-52.55	-52.24	-47.58	-39.91

ALT: alanine transferase; AST: aspartate transferase; ALP: alkaline phosphatase; GGT: gamma glutamyl transferase

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Urea and Creatinine concentrations of Baladi and Shami kids during neonatal period

The data presented in table 9 revealed that goat breed, neonatal period affected urea concentration significantly increases in Baladi kids as compared to Shami kids, while, they exhibited insignificant effect on creatinine level. Concerning to the experimental period in present study that plasma urea had significantly decreased at day 0 and 7th day of life while it showed significant increase at 14th, 21th and 28th day, while creatinine significantly increased at day 0 and 7th as compared to the 14th and 12th day and continued to decline until the 28th day of life (Abdolvahabi, 2016) concluded that plasma concentrations of urea and creatinine were below the adult reference intervals (ARI), the decrease in plasma urea concentrations during the first 10 days of life in neonates was in accordance with previous studies by Herosimczyk et al. (2011) and Antunović et al. (2017). The decrease in plasma creatinine concentrations after 14th day of birth is may be associated with increased glomerular filtration rate (Herosimczyk et al., 2011). As shown in table 10 generally all kidney functions parameters (urea and creatinine) for dead kids in both breeds were insignificantly decreased as compared to alive ones, the percentages of urea within the 14 days post-partum were between (-47.06:-54.06%) in and (-46.93: -52.97%) in urea for Baladi and Shami kids respectively through the same period, then increased within the following two weeks to reach (-56.36: -57.48%) in Shami kids, while percentages of creatinine within the 14 days post-partum were between (-10.31: -35.50%), for Baladi dead kids and the percentage in Shami dead kids were (-27.14: -34.59%), then decreased (-13.33: -28.93%). The reductions of kidney functions (urea and creatinine) were higher in Shami kids that might give reason to higher mortality rates in this breed.

Table 9. Evaluating kidney function of Baladi and Shami kids during the first month of post-partum

Parameter	Breed (B)	Post-partum period (S)					Overall	±Standard error		
		0 day	7 day	14 day	21 day	28 day		B	N	B x N
Urea (mg/dl)	Baladi	43.82	40.50	43.38	44.39	43.68	43.75^{D}	0.88 **	1.28 **	1.98 NS
	Shami	35.84	36.05	40.83	44.28	45.63	39.93^{E}			
	Overall	39.83^{BC}	38.28^{C}	42.10^{AB}	44.34^{A}	44.65^{A}				
Creatinine (mg/dl)	Baladi	1.69	1.45	1.26	1.35	1.33	1.42	0.05 NS	0.07 NS	0.11 NS
	Shami	1.40	1.59	1.56	1.59	1.35	1.50			
	Overall	1.54	1.52	1.41	1.47	1.34				

B: Breed; N: Neonatal period; A, B, C: values with different letters on the same row are significantly different (P< 0.05); D, E: values with different letters on the same column are significantly different (P< 0.05); NS: non-significant; **: P<0.01.

Table 10. Evaluating of kidney function and percentage change of alive and dead Baladi and Shami kids during the postnatal period

Breed	Parameter	The neonatal period (Days)					
		Case	0	7	14	21	28
Baladi	Urea (mg/dl)	Alive	43.82	40.50	43.38	44.39	43.68
		Dead	20.13	21.44	22.20	.	.
		% change	-54.06	-47.06	-48.82	.	.
	Creatinine (mg/dl)	Alive	1.69	1.45	1.26	1.35	1.33
		Dead	1.09	1.11	1.13	.	.
		% change	-35.50	-23.44	-10.31	.	.
Shami	Urea (mg/dl)	Alive	35.84	36.05	40.83	44.28	45.63
		Dead	19.11	19.13	19.20	19.32	19.40
		% change	-47.15	-46.93	-52.97	-56.36	-57.48
	Creatinine (mg/dl)	Alive	1.40	1.59	1.56	1.59	1.35
		Dead	1.02	1.04	1.12	1.13	1.17
		% change	-27.14	-34.59	-28.20	-28.93	-13.33

Physiological changes in total lipids, cholesterol and triglycerides concentration of kids during neonatal period

The data presented in table 11 demonstrated that, Baladi kids showed insignificant increase in plasma total lipids, cholesterol and triglycerides levels when compared to Shami kids, while plasma HDL Baladi kids showed insignificant decrease level when compared to Shami kids. Concerning to post-partum days in this study showed that plasma total lipids were insignificantly higher at the first day after birth and showed reduction at the rest of post-partum period, in agreement with Öztabak and Özpinar (2006) findings that indicated that plasma total lipid concentrations of the Ewes Rearing (ER) lambs at 24 h ($P < 0.05$) and first week ($P < 0.01$) were significantly higher than Artificial Rearing (AR) lambs. From the point of view, the high levels on total lipids after birth could be explained by the fact that fat concentration continuously increased in colostrum, Earlier, Mersmann and McNeilm (1985) reported that fatty acids in blood were the source of triglyceride synthesis in adipose tissue.

In this study plasma cholesterol and triglycerides insignificantly increase at the 7th day till 14th day after birth, however it showed insignificant decrease from 21th day and reached to its highest decline at day 29th after birth. The

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results reported herein were in according to Chniter et al. (2013) who indicated that plasma cholesterol and triglycerides showed significant increasing levels in newborn lambs, this increase was attributed to that the main source was through the fatty acids in colostrum (Blum et al., 1997 and Bittrich et al., 2002). The present results showed that plasma HDL concentrations insignificant increased gradually from birth onwards. There is no previous information about the plasma lipoproteins of goats. Results in table 12 illustrated that all parameters of lipids profile analysis showed lowering in activity of these parameters in dead Baladi and Shami kids comparing with alive kids. The percentage of this reduction in Baladi dead kids within the 14 days post-partum were between (-31.79: -40.97%) and (-29.86: -38.60%) for Shami kids through the same period, then decreased within the following two weeks to reach (-28.96: -32.32%) in Shami kids, while in cholesterol, triglycerides were lower in Baladi dead kids as compared with living kids within the first two weeks post-partum it were between (-41.97: -49.15%), (-20.24: -21.36%) for Baladi dead kids and the percentage in Shami dead kids were (-40.02: -51.55%) , (-19.83: -23.09%) then decreased (-7.95: -33.48), (-16.59: -19.71%), respectively in Shami kids within the following two weeks. On the other hand, in HDL were lower in Baladi dead kids as compared with living kids within the first two weeks post-partum, it were between (-33.54: -40.47%) for Baladi dead kids and the percentage in Shami dead kids were (-36.99: -50.40%) then increased (-50.09: -52.82%) respectively in Shami kids within the following two weeks. The decreasing of lipids profile measure were higher in Shami kids could be explain by higher mortality rates in this breed.

Table 11. Means of lipid parameters of Baladi and Shami kids during the first month of post-partum

Parameter	Breed (B)	Experimental periods (S)					Overall	±Standard error		
		0 day	7 day	14 day	21 day	28 day		B	N	B x N
Total Lipids (g/l)	Baladi	545.74	483.86	508.51	458.51	473.93	494.11	14.39 NS	20.77 NS	32.18 NS
	Shami	506.61	509.69	456.50	474.58	453.31	480.14			
	Overall	526.18	496.77	482.50	466.54	463.62				
Cholesterol (g/l)	Baladi	189.79^{bc}	216.64^{ab}	194.89^{bcd}	185.20^{abc}	189.50^{d}	195.20	9.79 NS	14.13 NS	21.89 **
	Shami	178.58^{bcd}	187.94^{bc}	221.90^{a}	162.69^{cd}	117.77^{bc}	173.78			
	Overall	184.18	202.29	208.39	173.94	153.63				
Triglycerides (g/l)	Baladi	136.33	137.46	136.80	136.73	128.20	135.10	3.49 NS	5.04 NS	7.80 NS
	Shami	127.37	128.22	135.37	124.88	129.82	129.13			
	Overall	131.85	132.84	136.08	130.81	129.01				
HDL (g/l)	Baladi	180.78	195.22	202.16	225.81	245.29	209.85	14.70 NS	21.22 NS	32.87 NS
	Shami	206.51	208.93	262.58	261.37	278.79	243.63			
	Overall	193.64	202.08	232.37	243.59	262.04				

B: Breed; N: Neonatal period; a, b, c, d; values in the experimental sub-groups within certain trait with different superscripts are significantly different (P< 0.05); NS: non-significant; **: P<0.01.

Table 12. Means of lipid parameters and percentage change of alive and dead Baladi and Shami kids during the postnatal period

Breed	Parameter	The neonatal period (Days)					
		Case	0	7	14	21	28
Baladi	Total Lipids (g/l)	Alive	545.74	483.86	508.51	458.51	473.93
		Dead	322.12	330.01	332.11	.	.
		% change	-40.97	-31.79	-34.68	.	.
	Cholesterol (g/l)	Alive	189.79	216.64	194.89	185.20	189.50
		Dead	110.12	110.14	110.15	.	.
		% change	-41.97	-49.15	-43.48	.	.
	Triglycerides (g/l)	Alive	136.33	137.46	136.80	136.73	128.20
		Dead	108.05	108.09	109.11	.	.
		% change	-20.74	-21.36	-20.24	.	.
	HDL (g/l)	Alive	180.78	195.22	202.16	225.81	245.29
		Dead	120.14	120.24	120.33	.	.
		% change	-33.54	-38.40	-40.47	.	.
Shami	Total Lipids (g/l)	Alive	506.61	509.69	456.50	474.58	453.31
		Dead	311.02	314.12	320.16	321.15	322.02
		% change	-38.60	-38.37	-29.86	-32.32	-28.96
	Cholesterol (g/l)	Alive	178.58	187.94	221.90	162.69	117.77
		Dead	107.11	107.41	107.50	108.21	108.40
		% change	-40.02	-42.84	-51.55	-33.48	-7.95
	Triglycerides (g/l)	Alive	127.37	128.22	135.37	124.88	129.82
		Dead	102.10	102.12	104.11	104.15	104.22
		% change	-19.83	-20.35	-23.09	-16.59	-19.71
	HDL (g/l)	Alive	206.51	208.93	262.58	261.37	278.79
		Dead	130.11	130.13	130.22	130.44	131.51
		% change	-36.99	-37.71	-50.40	-50.09	-52.82

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Maternal behavior of Baladi and Shami does after kidding

Dam behavior around kidding just after parturition has a major impact on kids' survival. Specific behaviors of the does (licking and grooming, helping kids with suckling) promote doe–kid recognition and a close relationship between them. The data presented in figures 1, 2 and 3 showed that overall means of does stood (sec), does started licking (sec) and first attention to kid (min) was highly significant in Shami does in comparison with Baladi does (34.70 versus 25.10) and (19.3±2.61 versus 9.8± 2.61) and (12.60±1.05 versus 5.70± 1.05), respectively. Moreover, sniffing/licking during the first two hours after birth (frequency) showed higher (P<0.01) frequency by Baladi does than Shami (193.60 versus 99.50). Within few minutes of parturition, the mother starts to lick her offspring, especially starting with its head and neck. This behavior of licking the newborn after birth optimizes the offspring's respiratory system and encourages them to stand up for suckling. The licking action of female goats is an important first stage in preparing the neonatal lamb for its new environment and life. The licking also makes the lamb clean and dry. Gul et al. (2017) reported that the average time for first licking after parturition in Awassi sheep was 138.3 sec. in single births and 154.2 sec. in twin births, while the first sniffing time intervals were 118.31 sec. and 123.6 sec., respectively.

Maternal behavior parameters showed that Baladi does stand significantly earlier than Shami does to offer care to their offspring. In addition, Baladi does significantly licked and sniffed their kids more frequently than Shami does did. These results of maternal behavior in the Baladi breed might directly relate to the reduced kid's mortality compared to Shami kids. Nowak and Poindron (2006) has made it clear that the chances of the newborn's survival and protection against external conditions will be greatly affected by the successful association between the mother and her lamb. Poindron et al. (2010) stated that the first hour after birth is critical to establishing a maternal bond towards her newborn, as pregnancy nutritional supplements can be obtained through this association regularly. for the establishment of the maternal bond towards her newborn, as nutritional supplements for a newborn can be obtained through this association regularly. The recognition by the mother of the offspring of the ewes through licking and continuous inhalation during the first postpartum hours has been described in many local hooves (Lévy et al., 2004; Lévy and Keller, 2009).

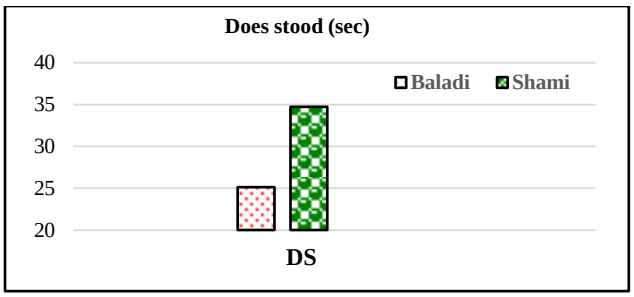

Figure 1. Overall means for Does stood (sec) of Baladi and Shami does started from kidding time. DS: Does stood (sec)

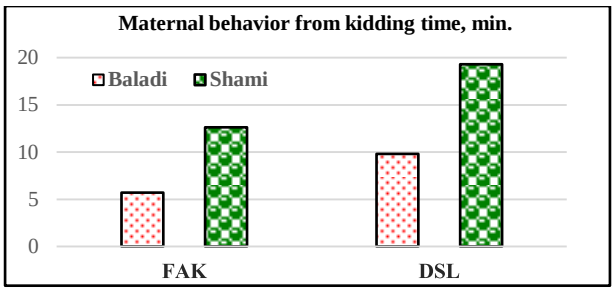

Figure 2. Overall means for first attention and does started licking kids of Baladi and Shami does. FAK: First attention to kid (min), DSL: Does started licking (min)

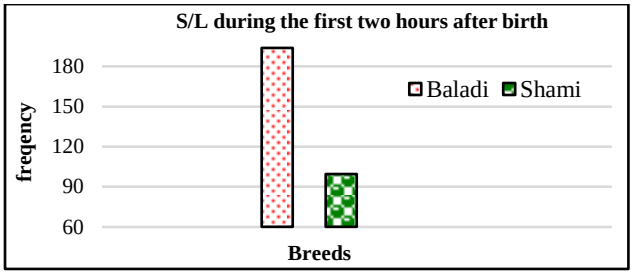

Figure 3. Overall means for maternal sniffing and licking kids during the first two hours after birth of Baladi and Shami does. S/L: Sniffing/ licking

Standing and suckling behavior of Baladi and Shami kids after delivery

The data presented in table13 show the mean values of standing and suckling behavior parameters of newly born kids, either still alive kids or those died then after. The parameters included first standing attempt (min) and the first standing success (min), first attempted to suckling (min), kids starting suckling (min), suckling frequency during the first two hours after birth (bouts). For alive kids, all parameters were affected significantly (P<0.01) by breed differences except duration of the first suckling (min). Baladi kids showed earlier activity toward their dams than Shami did. The duration of these parameters for Baladi and Shami kids respectively were 20.40 versus 30.30 min for the first standing attempt, 35.10 versus 46.40 min for the first standing success, 43.2 versus 64.0 min for the first attempted to suckling and 50.0 versus 70.0 min for kids starting suckling. However, suckling frequency during the first two hours after birth was greater (P<0.01) in Shami kids (16.6 bouts) than in Baladi kids (12.25 bouts). This might due to higher number of kids seeking and competing one mother.

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Everett et al. (2005) explained that viability rate of offspring depends on suckling the first milk, colostrum. For this purpose, offspring must stand up as soon as possible. The maternal behavior of the dam plays an important role in the behavioral interactions that occur between ewe and their offspring during the first few hours following birth and ultimately ends in the suckling behavior. Ceyhan et al. (2012) reported that mothers must help their offspring to stand up and reach the udder. Most recently, Gul et al. (2017) studied the behavioral characteristics of Awassi sheep and their offspring during the first hour after parturition and found that all lambs tried to stand up for suckling and succeeded during the first hour after they were born. Earlier, Dwyer (2003) reported the first attempt for standing up time as 5.7 min, and successful standing-up time was 19.04 min. Abdul-Rahman and Yaro (2010) reported that the standing-up times ranged from 4 to 79 min, averaging 24.01 min, in Djallonke lambs. Madani et al. (2013) found the successful standing-up time after birth to be about 23.45 min in sheep.

Dwyer (2003) reported an average successful suckling time of 19.04 min, while Abdul-Rahman and Yaro (2010) found a mean time for first successful suckling of 35.35 min (a range from 5 to 105 min) in Djallonke lambs. Madani et al. (2013) Noticed that standing after delivery and reaching the udder occurred within 32.7 ± 19.02 minutes and suckling started within 41.9 min in Djellal lambs. Mahboub et al. (2013) indicated that lambs and kids born in well-fed dams with a high level of concentration (135%) exhibited more suckling behavior during the first week after birth and spent significantly more time licking their offspring and preparing them.

Total time of suckling during the first two hours after birth (min) was higher (P<0.01) in Baladi kids (536.0 min) than in Shami ones (38.5 min). Suckling and high milk production plays an important role in establishing a good relationship between dams and their offsprings (Nowak et al., 1997; Mahboub et al., 2013).

Kids died then after in both Baladi and Shami breeds were born very weak (physically); due to their lower birth weight; took very long time seeking their mothers and starting suckling when compared to those still alive to the end of study (Table 7). The dead Shami kids took a highly significant (P<0.01) longer time to stand and suckle than the dead Baladi kids. First standing attempt, the first standing success, first attempted to suckle and kids starting suckling in Baladi and Shami kids that died then after were 35.15 versus 44.14 min, 43.11 versus 51.09 min, 61.11 versus 73.14 and 69.10 versus 80.14, respectively. During the first two hours, those Shami kids took less time standing (26.63 min) and duration of the first suckling (1.19 min) when compared with Baladi kids (33.13 and 1.40 min, respectively). Again, this might explain partially the higher kids' mortality in Shami goats.

The differences in does and their kid's behavioral activities after birth could explain partially the higher kids' mortality in Shami goats.

Table 13. Means for standing and suckling behavior of Baladi and Shami kids after birth

Indices	Still alive kids				Later dead kids			
	Baladi kids	Shami kids	Overall Mean	Breed ±SE	Baladi kids	Shami kids	Overall Mean	Breed ±SE
First standing attempt (min)	20.40^{b}	30.30^{a}	25.35	1.15^{**}	35.15^{B}	44.12^{A}	39.63	1.35^{**}
First standing success (min)	35.10^{b}	46.40^{a}	40.75	2.34^{**}	43.11^{B}	51.09^{A}	47.50	2.38^{**}
standing time during the first two hours after birth (min)	71.60^{a}	51.80^{b}	61.70	2.79^{**}	33.13^{A}	20.14^{B}	26.63	1.22^{**}
First attempted to suckling (min)	43.20^{b}	64.0^{a}	53.60	3.02^{**}	61.11^{B}	73.14^{A}	67.12	3.16^{**}
Kids starting suckling(min)	50.00^{b}	70.00^{a}	60.00	4.81^{**}	69.10^{B}	80.14^{A}	74.62	4.88^{**}
Duration of first suckling (min)	5.70	4.60	5.15	0.71^{NS}	1.40	1.19	1.29	0.26^{NS}
Total time of suckling during the first two hours after birth (min)	52.00^{a}	38.50^{b}	45.25	2.02^{**}	10.13^{A}	8.21^{B}	9.17	0.77^{**}
Suckling frequency during the first two hours after birth (bouts)	12.20^{b}	16.60^{a}	14.40	0.88^{**}	5.20^{B}	7.11^{A}	6.15	0.70^{**}

SE: standard Error; a, b: values with different letters on the same row differ at (P<0.05); A, B: values with different letters on the same row differ at (P<0.05); NS: non-significant; ** P<0.01

CONCLUSION

In the present study, kids' mortality occurred in Baladi during the first two weeks, while continued in Shami kids all over the first month with different percentage per week. Also, it was apparent that Shami female kids had the highest birth weight, growth rate and final LBW. Moreover, Baladi male kids that could stay alive compensated the lower birth weight by high relative growth rate and attained the same final LBW as Shami males. Also, that decreasing in liver enzymes (AST, ALT, ALP) activities, kidney functions (urea and creatinine) and lipids profile analysis were higher in Shami kids that might give reason to higher mortality rates in this breed.

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So that, Baladi does and their kids overpowered their Shami counterparts in some biochemical parameters and some behavioral characteristics, which might enhance kids' viability and reduce mortality rates. Moreover, the number of weak kids with lower body weight was more pronounced in Shami goats that led to higher mortality rate.

DECLARATIONS

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Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article. The authors declare that they have no competing interests.

Author's contribution

Dr. N. H. Ibrahim designed the experiment, helped in field study, tabulation of experimental data, statistical analysis, article writing and revision. Dr. M. T. Badawy facilitates the field study, manuscript writing and revision. Dr. I. A. Zakzouk helped in field study, collected data, tabulation of experimental data and manuscript writing. Dr. F. E. Younis helped in field study. All authors have read and approved the final manuscript.

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Determination of the Appropriate Inoculum Dose and Incubation Period of Cassava Leaf Meal and Tofu Dreg Mixture Fermented with *Rhizopus Oligosporus*

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ABSTRACT

The present study was conducted to determine the appropriate inoculum dose and incubation period for the mixture of Cassava Leaf Meal (CLM) and Tofu Dreg (TD) fermented with *Rhizopus oligosporus*. This experiment was carried out in a completely randomized design in a 3 x 4 factorial treatments arrangement with 4 replications. The first factor was the inoculum dose (6, 8 and 10%), and the second factor was the incubation period of the fermentation (2, 3, 4, and 5 days). Measured variables were the changes in Dry Matter (DM), Organic Matter (OM), crude fat, Crude Fiber (CF), and Crude Protein (CP). The experimental results showed that there was no interaction between the inoculum dose and an incubation period of the fermentation in the reduction of DM, organic matter, and crude fat as well as the increase in the CP of fermented CLM and TD with *Rhizopus oligosporus*. However, the interaction was occurred between inoculum dose and incubation period in the reduction in CF. The inoculum dose significantly decreased the DM, OM, crude fat and CF and also increased the CP. The best inoculum dose effect was at 10%. The incubation period had a significant reduction in the DM, OM, crude fat, and CF and also increased the CP. The best incubation period of fermentation was at 3 days. The results indicated that the appropriate inoculum dose to ferment CLM and TD mixture with *Rhizopus oligosporus* was 10% at each incubation period. In the meanwhile, the appropriate incubation period was 3 days for each inoculum dose.

Keywords: Fermentation, Inoculum dose, Incubation time, *Rhizopus oligosporus*

INTRODUCTION

Cassava Leaf Meal (CLM) has been widely studied as a feed ingredient in a mixture of chicken rations because it contains quite high crude protein. According to Iheukwumere et al. (2008), CLM contains dry matter 25.30%, crude protein 25.1%, crude fiber 11.4%, crude fat 12.7%, nitrogen retention 46.1%, and ash 9.1%. Although the protein content of CLM is quite high, it cannot be utilized in large quantities in poultry rations because it contains high crude fiber and HCN. The CLM could only be used up to 5% in broiler chicken rations because the utilization of up to 10 and 15% reduced the efficiency of feed conversion (Melesse et al., 2018; Wyllie and Chamanga, 1979). According to Ravindran et al. (1986) and Eggum (1970), the utilization of cassava leaves is still limited due to high CF, tannins, HCN, alkalis, low digestibility and deficiency of sulfur-containing amino acids, especially methionine. To improve the quality of CLM, it needs to be processed. One of the processing methods that can be done is by fermentation method. Fermentation is a process of microorganism or microbial activity that can produce a product whose texture, taste, aroma, and nutritional quality changes better than the raw material and result in an increase in the quality and nutritional content of feed ingredients (Aisjah and Abun, 2012; Mirnawati et al., 2019a). Fermentation of cassava waste has been carried out by many researchers, including Sugiharto (2019), who performed a solid-fermentation with fungi which was a simple method to improve the nutrient qualities of cassava pulp and thus increased the production of such a cheap agro-industrial by-product in chicken rations. Therefore, microbes were needed to overcome all of the problems of CLM as a feed ingredient. Yuniza et al. (2017) found that the fermentation of the combination of CLM and palm kernel cake by using *Bacillus amyloliquefaciens* improved the nutritional values of this fermented mixture of CLM and palm kernel cake. In the present study, therefore, microbes were needed that could overcome all problems of cassava leaves as feed component. According to Han et al. (2003), Dewi (2015) and Sumiati et al. (2011), *Rhizopus oligosporus* produces enzymes such as protease, lipase, alfa-amylase, glutaminase, and alfa-galactosidase and cellulase. The presence of the enzymes produced by *Rhizopus oligosporus,* is expected to reduce anti-nutrients and toxins content in CLM while increasing its nutritional values.

The success of solid fermentation is dependent heavily on the optimal conditions that exist. Some factors that influence solid fermentation are substrate composition, inoculum dosage and fermentation time/incubation period. The

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composition of the substrate must be considered as it affects the enzymes produced by microbes. Microbes produce enzymes based on the availability of inducers in the substrate. Therefore, in the present study the addition of tofu dregs (TD) as a combination of CLM substrate aims to provide the adequacy of nitrogen sources.

The inoculum dose and incubation period of the fermentation also affect fermentation. The higher the dose of the inoculum, the more microbe grow, and the longer the incubation period of fermentation, the more chances for microbes to break down nutrients in the substrate (Mirnawati et al., 2012; Mirnawati et al., 2013; Mirzah and Muis, 2015; Mirnawati et al., 2019b). Thus, the combination of inoculum dose and incubation period of fermentation might improve the quality of CLM and TD. However, with the increasing in incubation period, the availability of nutrients in the fermentation media was exhausted, so that the bacteria would eventually die and thereby the decline in organic matter was reduced (Agustina et al., 2015; Iwansyah et al., 2019). In order to obtain the optimal conditions, it was necessary to determine the best inoculum dose and incubation period for the fermentation of CLM and TD mixture with *Rhizopus oligosporus*.

MATERIALS AND METHODS

The present study aimed to investigate the effects of inoculum dose and incubation period of CLM and TD mixture that were fermented with *Rhizopus oligosporus* on the alteration in DM, OM, crude fat, CF and CP, and to determine the appropriate inoculum dose and incubation period for the fermentation process.

Materials

The materials used in this study consisted of CLM, TD, Rice Bran (RB), *Rhizopus oligosporus*, Potato Dextrose Agar and brook minerals (Brook et al., 1969). This CLM was taken from the cassava waste when the cassava farmers harvested the roots. The cassava leaves were obtained from the two-thirds of the shoots or from the top part (where the leaves were still green). The equipment used in this study consisted of 5 kg capacity scale, 1 kg glass plastic size, autoclave, test tube, laminar flow, incase, oven, grinding machine and a set of proximate apparatus.

Methods

This experiment was carried out by the experimental method using a completely randomized design in a 3 x 4 factorial treatment arrangements with 4 replications. The first factor was the fresh inoculum dose (D refers to inoculum dose, D6 = 6%, D8 = 8%, and D10 = 10%). The second factor was the incubation period (L refers to long term fermentation, L2 = 2 days, L3 = 3 days, L4 = 4 days, and L5 = 5 days). Measured variables were alteration in DM, OM, crude fat, CF, and CP after fermentation.

Data collection

The CLM was prepared by drying CLM in the sun and then grinding it in a finely milled two mm filter size. The *Rhizopus oligosporus* inoculum was the RB and CLM mixture at the ratio of 90% to 10%. Then, the substrate composition consists of 80% CLM and 20% TD. Finally, the fermentation process was carried out. The fermentation of CLM and TD by using *Rhizopus oligosporus* was performed with the inoculums dose of 6, 8 10% and incubation period of 2, 3, 4 and 5 days according to the treatment. This fermentation process was as follows: each of the CLM-TD samples was inserted into a heat-resistant and homogenized glass plastic, afterwards the samples, distilled water, and fermentation equipment were sterilized with autoclave, at a pressure of 15 lbs, and temperature 121°C for 15 minutes, and then cooled in laminar flow and UV for 15 minutes. In the next step the inoculum was dissolved in accordance with the treatment in 27 ml water that was sterilized (as an addition of water to 45-65%), then it had put into plastic substrate, then homogenized, fixed surface, and covered with plastic clips, and pierced with sterile sticks flat surface. Subsequently, the samples were incubated in open incase of airflow for 2, 3, 4 and 5 days according to the treatment (facultatively anaerobic). After completing the fermentation process according to the treatment, the DM, OM, crude fat, CF and CP of samples were analyzed according AOAC (1990) procedures.

Data Analysis

The data obtained were analyzed statistically using analysis of variance of completely randomized design in a 3 x 4 factorial treatment arrangements. If there was a real effect, then the Duncan's Multiple Range Test (DMRT) according to Steel and Torrie (1980) should be used to test the significant differences among treatment means.

RESULTS

The effects of inoculum dose and incubation period of fermentation of CLM and TD mixture with *Rhizopus oligosporus* on the alterations in DM, OM, crude fat, CF and CP are shown in table 1.

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Table 1. Effect of inoculum dose and the incubation period of cassava leaves and tofu dregs fermented with *Rhizopus oligosporus* on the reductions of dry matter, organic matter, crude fat, crude fiber and the increase in crude protein in the Non-Ruminant Nutrition Laboratory, at the Faculty of Animal Husbandry, Andalas University, Padang, Indonesia in January 2019.

Observed variables	Inoculum dose (%)	Incubation period (days)				Average
		L2	L3	L4	L5	
Reduction in dry matter (%)	D6	0.67	0.83	0.82	0.81	0.78 [b]
	D8	0.68	0.84	0.82	0.82	0.79 [b]
	D10	0.72	0.86	0.84	0.84	0.82 [a]
	Average	0.69 [c]	0.84 [a]	0.83 [b]	0.82 [b]	
Reduction in organic matter (%)	D6	2.96	3.82	3.36	3.44	3.40 [b]
	D8	3.04	3.86	3.62	3.45	3.49 [b]
	D10	3.10	3.86	3.91	3.70	3.64 [a]
	Average	3.04 [c]	3.85 [a]	3.63 [b]	3.53 [b]	
Reduction in crude fat (%)	D6	14.04	24.30	24.19	23.93	21.62 [b]
	D8	14.24	24.22	24.20	24.32	21.74 [b]
	D10	14.69	25.47	24.82	24.71	22.42 [a]
	Average	14.32 [c]	24.66 [a]	24.40 [b]	24.32 [b]	
Reduction in crude fiber (%)	D6	18.27 [c]	25.36 [b]	24.66 [b]	24.66 [b]	23.24 [b]
	D8	18.39 [c]	26.05 [b]	25.27 [b]	24.90 [b]	23.65 [b]
	D10	24.73 [b]	27.40 [a]	26.40 [b]	25.86 [b]	26.10 [a]
	Average	20.46 [c]	26.27 [a]	25.44 [b]	25.14 [b]	
Increase in crude protein (%)	D6	22.40	25.44	23.51	23.84	23.80 [b]
	D8	22.44	26.92	24.97	24.39	24.68 [b]
	D10	24.07	28.47	27.20	25.79	26.38 [a]
	Average	22.97 [c]	26.94 [a]	25.23 [b]	24.67 [b]	

[a, b, c,] : Different in superscripts at the same rows and columns of each variable indicate significantly different effects ($p < 0.05$).

Reduction in dry matter

The result of experiment showed that there was no interaction between the inoculum dose and the incubation period of CLM and TD mixture fermented with *Rhizopus oligosporus* in the reduction in DM. The inoculum dose showed a significant effect ($p < 0.05$) on decreasing in DM. Further DMRT test indicated that inoculum dose had showed that D6 treatment had no difference with D8 treatment. However, D6 and D8 treatments were significantly different from D10 treatment. The incubation period showed a significant effect ($p < 0.05$) on the reduction in DM from the fermentation of CLM and TD mixture with *Rhizopus oligosporus.* The results of the DMRT indicated that the incubation period of L2 treatment was significantly different from L3, L4, and L5 treatments. L3 treatment was significantly different from L4 and L5. Then the L4 treatment was not different from the L5 treatment. The highest reduction in DM was found in the inoculum dose of 10%. The more reduction in DM was found at the incubation period of 3 days. The reduction in DM increased with the incubation time of 3 days, compared to the incubation time of 2 days, then decreased again after 4 days and 5 days. Reduction in DM indicated that the optimal incubation period for CLM and TD mixture fermented with *Rhizopus oligosporus* was 3 days.

Reduction in organic matter

Based on the results of an analysis of variance, it was found that there was no interaction between the inoculum dose and incubation period of CLM and TD mixture fermented with *Rhizopus oligosporus* to reduce OM. The inoculum dose showed a significant effect ($p < 0.05$) on the reduction in OM of CLM and TD mixture fermented with *Rhizopus oligosporus*. Based on the result of further tests using DMRT showed that D6 treatment was not different from D8 treatment. Furthermore, D6 and D8 treatments were significantly different from D10 treatment. The incubation period showed a significant effect ($p < 0.05$) on the reduction in OM of CLM and TD mixture fermented with *Rhizopus oligosporus*. The result of DMRT of incubation period showed that L2 treatment was significantly different from L3, L4, and L5 treatments. L3 treatment was significantly different from L4 and L5 treatments. Meanwhile, L4 treatment was not different from L5 treatment. In the inoculum dose factor, the highest reduction in OM was found at the inoculum dose of 10%. Increasing the inoculum dose had a positive effect on the reduction in OM. During the incubation period, the greatest reduction in OM was found in the incubation period of 3 days. The reduction in OM increased during incubation period of 3 days compared to the incubation period of 2, 4 and 5 days. The result of experiment showed that the incubation period of 3 days was the optimal one for fermenting of CLM and TD mixture with *Rhizopus oligosporus*.

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Reduction in crude fat

From the result of the analysis of variance, it was obtained that there was no interaction ($p > 0.05$) between the inoculum dose and incubation period of CLM and TD mixture fermented with *Rhizopus oligosporus* in the reduction in crude fat. The inoculum dose significantly affected ($p < 0.05$) the reduction in crude fat. The results of the DMRT analysis showed that D6 treatment was not significantly different from D8 treatment. Furthermore, D6 treatment was significantly different from D10 treatment.

The results of the analysis variance of incubation period showed a significant effect ($p < 0.05$) on the reduction in the crude fat of cassava leaves and tofu dregs were fermented with *Rhizopus oligosporus*. The DMRT test results showed that the L2 treatments differed significantly from L3, L4, and L5 treatments. In addition, L3 treatment was significantly different from L4 and L5 treatments. Then L4 treatment did not differ significantly from L5 treatment.

In the inoculum dose factor, the highest decrease in crude fat was found at the treatment of the 10% inoculum dose The increasing inoculum dose had a positive effect on the reduction in crude fat. In the incubation period, the highest decrease in crude fat was found in the incubation period of 3 days. The reduction in crude fat increased at an incubation period of 3 days when compared to a 2 days incubation period, then decreased again at 4 days and 5 days. The incubation period of 3 days was the optimal for fermentation of cassava leaves and tofu dregs with *Rhizopus oligosporus*.

Reduction in crude fiber

Based on the results of the analysis variance, showed that there was an interaction ($p < 0.05$) between the dose of inoculum and the incubation period of cassava leaves and tofu dregs was fermented with *Rhizopus oligosporus* on the decrease in CF. Based on the results of the DMRT test the highest the decrease in CF was found in the treatment of D10L3, then D10L4, D10L5, D10L2, D8L3, D8L4, D8L5, D6L3, D5L4, D6L5, D8L2, and D6L2. This is caused by the fact that the higher the dose of inoculum the more the decrease in CF, the better the effect on increasing the nutritional value of fermented feed ingredients with *Rhizopus oligosporus.* Increasing the dose had a positive effect on the decrease in CF later. The incubation period in the treatment of 3 days was the optimal time to ferment cassava leaves and tofu dregs with *Rhizopus oligosporus*. The longer the fermentation time used, the ingredients were overhauled by enzymes. However as the fermentation time increased, the availability of nutrients in the fermentation media decreased, so that the microbes eventually died and the CF was reduced.

Increase in crude protein

Based on the results of the analysis of variance, it was found that there was no interaction ($p > 0.05$) between inoculum dose and fermentation period of a cassava leaf and fermented tofu dregs with *Rhizopus oligosporus* to increase CP. The results of the analysis variance of inoculum dose factors indicated a significant ($p < 0.05$) increase in CP fermented from cassava leaves and tofu dregs with *Rhizopus oligosporus*. Based on the DMRT test, it was found out that although D6 treatment was not significantly different from D8 treatment, but it was significantly different from D10 treatment. Increasing the inoculum dose had a positive effect on the increase in CP.

The incubation period factor also influenced ($p < 0.05$) the increase in CP from cassava leaves and TD mixture fermented with *Rhizopus oligosporus*. The DMRT test results indicated that L2 treatment was significantly different from L3, L4, and L5. L3 treatment was significantly different from L4 and L5. Then, L4 treatment was not significantly different from L5 treatment. The highest increase in CP was found at an incubation period of 3 days. Thus, the optimal incubation period was 3 days for fermented of cassava leaves and tofu dregs with *Rhizopus oligosporus*.

DISCUSSION

Reduction in dry matter

The reduction in DM could be an indicator of the success of fermentation, since the fermentation process could decrease the amount of DM and increase the use of nutrients from the substrate by microbes as a source of carbon, nitrogen, and minerals, and release of CO2 and energy in the form of heat that evaporates with air particles created by the catabolic process that transform complexes into simpler materials (Astuti et al., 2017). The more mold grew, the more substance was in the substrate material, which was renewed as an energy source. The increase in water molecules resulting from the mold growth also increased (Kasmiran, 2011).

The reduction in DM also depended on the incubation period factor. The reduction in DM began to tie up for 3 days and began to decrease for 4 days, and this reduction happened because the longer the incubation period was used, the more nutrients were overtaken by enzymes. However, as the incubation period increased, the availability of nutrients in the fermentation media became increasingly limited, which led to decreased microbial growth and ultimately to death (Agustina et al., 2015; Iwansyah et al., 2019). According to Wang et al. (1979), the growth of microbes has reached a stationary phase, the growth rate would decrease due to increased use of food and the accumulation of metabolic substances which inhibit the growth and the growth rate. That condition was increased until the value became zero (the

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number of growing cells corresponds to the number of dying cells), then the total cell mass was constant and the number of living cells continued to decrease due to lysis.

Reduction in organic matter

The reduction in OM indicated a better fermentation process by increasing the provision of tofu dregs compared to the without fermentation. The reduction in DM showed success indicators of fermentation as the fermentation process reduced the amount of OM, due to the use of nutrients from the substrate by microbes as a carbon source, nitrogen and minerals, and release of CO2 and energy in the form of heat that evaporates with air particles whereby water molecules were formed from catabolic processes that convert complex compounds into simpeler materials (Astuti et al., 2017). The reduction in OM caused to decrease in the nutrients supply of the overhauled material that has been utilized by mold (Kasmiran, 2011).

The reduction in OM at the incubation duration factor was limited to 3 days and began to decrease after 4 days. The OM reduction happened because the longer the incubation period, the more ingredients were overhauled by enzymes produced by microbes. With increasing incubation period, however, the availability of nutrients in the fermentation media would exhausted, so that the bacteria will eventually die off, thereby reducing the reduction in OM (Agustina et al., 2015; Iwansyah et al., 2019). Moreover, Wang et al. (1979) explained that the growth rate of microbes decreased due to the declining in nutrients supply when they reached stationary phase, and the microbes died overtime.

Reduction in crude fat

The reduction in crude fat caused by lipase enzymes produced by *Rhizopus oligosporus* converted fat content into lighter fatty acids (Yuliani, 2008). The higher reduction in crude fat in the substrate was eventually caused by increasing the inoculum dose.

The reduction in crude fat with the incubation period factor increased with the incubation period of 3 days and began to decrease with the incubation period of 4 days. The reduction in crude fat happened because the longer the fermentation incubation had been used, the more ingredients were degraded by enzymes produced by microbes. However, with the increasing in incubation period, the availability of nutrients in the fermentation media decreased, so that the microbes eventually died, and the reduction in crude fat also declined (Agustina et al., 2015; Iwansyah et al., 2019).

Reduction in crude fiber

The reduction in CF was reduced due to heat due to the fermentation process, whereby the binding of fibers could be broken down into simpler bonds, thereby increasing the dissolved fiber and decreasing the CF. *Rhizopus oligosporus* with a substrate made from a mixture of cassava leaves and tofu pulp as an inducer could use the carbohydrates and hemiselolosa contained in the substrate as an energy source for the metabolism, so that the rough fiber decreased (Wattiheluw, 2012). In addition, the reduction in CF was also caused by cellulase enzymes produced by the fungus *Rhizopus oligosporus*. Mold secreted enzymes according to the inductors available in the substrate (Mirza, 2019). Cassava leaves and tofu dregs as inducers in the substrate contain CF which could induce *Rhizopus oligosporus* to secrete cellulase enzymes which are needed to degrade the available fiber in the substrate. *Rhizopus oligosporus* cellulase enzymes could degrade carbohydrates and hemicelluloses such as galactose and pentose into simpel sugars and then used for the growth of these molds to about 50 percent (Wattiheluw, 2012).

The reduction in CF increased with increasing in inoculum doses, since the reduction in CF declined.. The success of solid medium fermentation was highly dependent on the optimal conditions. Some factors that influence solid media fermentation were substrate composition, substrate thickness, inoculum dosage, fermentation period, temperature and pH. The longer the incubation period used, the more ingredients were broken down by enzymes. However, as incubation period increased, the availability of nutrients in the fermentation medium decreased, so that the microbes eventually had died and then the reduction in CF decreased (Agustina et al., 2015; Iwansyah et al., 2019).

Increase in crude protein

The increase in CP occurred because of the protein was donated by microbial cells. Increased in growth resulted in a Single Cell Protein (SCP) product or cell biomass containing approximately 40-65% protein (Krishna et al., 2005). During the fermentation process, the microbes released enzymes, which was a protein, and the microbe itself was also a source of single-cell protein. According to Han et al. (2003), *R. Oligosporus* produces the enzymes including protease, lipase, a-amylase, glutaminase, and a-galactosidase. According to Dewi (2015), *R. Oligosporus* also produces cellulase, B-glucosidase, tananase, phytase enzymes. The presence of these enzymes was expected to overcome all limiting factors, reduce anti-nutrients and toxins contained in cassava leaves, while increasing their nutritional value (Sumiati et al., 2011). The presence of body cells and enzymes produced by *R. oligosporus* during the fermentation of cassava leaves and tofu dregs could increase the CP of the substrate because the body cells and these enzymes are proteins.

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According to Mirzah and Muis (2015) and Mirnawati et al. (2019b) the more CP would be produced when the inoculum dose was increased because the higher inoculum dose resulted in more microbes to degrade substrates. The increase in CP in 3 days and began to decrease in 4-5 days. This happened because the longer the fermentation time used, the more ingredients were degraded by enzymes. However, with the increase in fermentation time, the availability of nutrients in the fermentation medium decreased and the bacteria eventually had died, so that CP decreased (Agustina et al., 2015; Iwansyah et al., 2019).

CONCLUSION

The inoculum dose as well as incubation period affected the chemical composition of CLM and TD mixture fermented with *Rhizopus oligosporus.* The fermentation process altered DM, OM, crude fat, CF, and CP. There was a reduction in DM, OM, crude fat and CF, but an increase in crude protein of fermented CLM and TD mixture. The appropriate inoculum dose for fermenting the CLM and TD mixture with *Rhizopus oligosporus* was 10% for each incubation period, while the appropriate incubation period for this fermentation was 3 days for each inoculum dose.

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Author's contribution

Annisa and Yose Rizal participated in writing and revising the final edition of the manuscript. Mirnawati, Irfan Suliansyah, and Amri Bakhtiar participated in design of the experimental procedure of the manuscript. All authors confirm the final accepted article.

Competing interests

The authors have declared that no competing interest exists.

Consent to publish

The authors guarantee that this work has not been published elsewhere and any person named as a co-author of this study is aware of the facts and has agreed to the designation.

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Gene Expression Profile and Enzymatic Activities of Frozen Buck Sperm Supplemented with Melatonin in Cold and Hot Temperatures

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ABSTRACT

This study was carried out to improve the freezability of buck semen using two different types of cryoprotectants supplemented with melatonin as antioxidant in cold and hot temperature of breeding season. Ejaculates from four mature Egyptian baladi bucks were pooled after collection. Semen was extended with Tris-fructose-citric containing egg yolk using glycerol and dimethyl sulfoxide supplemented with two doses of melatonin (10^{-6}M and 10^{-3}M) in addition to control group. Types of motility as well as velocity, enzymatic activity and expression profile of selected genes were measured. The results revealed that the progressive motility percentage was significantly higher in samples supplemented with low dose of melatonin (10^{-6} M) compared to high dose (10^{-3}M) in glycerol (74.4 versus 64.4) and Dimethyl Sulfoxide (DMSO) based extender (35.5 versus 32.9) in cold temperature. The same trend was found in samples cryopreserved with glycerol (75.1 versus 53.5) and DMSO (32.1 versus 22) in hot temperature. The results also demonstrated that CASA parameters (VAP and VCL) were significantly increased in low compared to high melatonin dose in glycerol based extender during cold and hot temperature. The activity of total antioxidant capacity (TAC) was significantly higher in samples supplemented with low (0.49 mM/L) than high melatonin dose (0.16 mM/L) in DMSO extender. CPT2, ATP5F1A and SOD2 genes were up regulated in glycerol based extender groups in cold temperature compared to other groups of this study. On the other hand, NFE2L2 gene was up-regulated in groups cryopreserved with DMSO in hot temperature compared with all other experimental groups. Therefore, it could be concluded that the glycerol based extender in cold season supplemented with low dose of melatonin improved semen quality, antioxidant defense capacity and transcriptional profile, which may maintain the post-thaw fertilizing ability of buck semen.

Keywords: Antioxidant enzymes, Bucks, Melatonin, Motility, Transcript abundance

INTRODUCTION

Goat population contributes 3.37% of local meat production and approximately 0.3% of local milk production in Egypt (Khalifa et al., 2009). This contribution is considered low that is due to low genetic merit and performance of Egyptian goat (Khalifa et al., 2009). Therefore, genetic improvement of goats under Egyptian conditions is a necessary demand for improving the productivity of this species (Khalifa et al., 2009). Indeed, cryopreservation of semen is an important technique for breeding schemes focus on increasing livestock production, However, technical and biological factors limit the commercial application of this technique (Silva et al., 2000). The freezing technique, extender, as well as the type and concentration of cryoprotectant agent determine the quality of frozen semen. Glycerol and Dimethyl Sulfoxide (DMSO) have been extensively used as effective agents for preserving sperms (Fleming and Hubel, 2006).

Variation in annual environmental temperature and photoperiod represent key factors determining reproductive seasonality and defining the reproductive seasons of some farm animal species (Aguirre et al., 2007 and Abecia et al., 2012). Several breeds of sheep and goats are short-day seasonal breeders (Carolan et al., 1994; Hafez, 2000 and Abecia et al., 2012). In addition breed, season and type of ration are main factors affect sexual maturity of buck (Barkawi et al., 2006; Zarazaga et al., 2009; Elhammali and Elsheikh, 2014). Moreover, semen quality was improved during the breeding season in the late summer and early autumn, which coincides with decreasing photoperiod (Farshad et al., 2012). It is reported that semen quality and freezability were higher in breeding season than in non-breeding season (Wang et al., 2014). On the other hand, the decline in semen quality of bucks during hot months was due to increased alkalinity of semen that changed pH and subsequently compromised spermatozoa physical characteristics (Elsheikh et al., 2013).

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Oxidative stress is referred to an imbalance between the oxidant and antioxidant systems that is caused by the accumulation of free radicals such as Reactive Oxygen Species (ROS) and reactive nitrogen species (Deng et al., 2016). Free radicals have critical roles in sperm hyperactivation, capacitation, and the acrosome reaction (Zhang et al., 2016). Nevertheless, excessive level of ROS is produced during cryopreservation process that may cause lipid peroxidation and destroy the lipid bilayer structure of the spermatozoon membrane (Zhang et al., 2016).

Melatonin is a neuroendocrine hormone with a natural antioxidant activity. Melatonin stimulates the activity of antioxidant enzymes such as Superoxide Dismutase (SOD) and glutathione peroxidase (GSH-Px) (Jang et al., 2010). Melatonin improves semen characteristics in goat (Ramadan et al., 2009), rat (Sonmez et al., 2007), boar (Jang et al., 2010), ram (Ashrafi et al., 2011), and in human spermatozoa (Ortiz et al., 2011). Related enzymes includes catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase represent the most important enzymatic system involved in maintaining balance between ROS production and scavenging (Gadea et al., 2011 and Saraswat et al., 2014).

Transcriptional profile of specific candidate genes was linked with conventional semen physical and biochemical characteristics predicted male fertility in different farm animal species (Kadivar et al., 2016; Kim et al., 2019). The implication of sperm related genes in fertility was suggested by the involvement of sperm transcripts in different crucial reproductive processes like spermatogenesis, sperm motility (Bissonnette et al., 2009), fertilizing ability and subsequent embryonic development (Boerke et al., 2007). Therefore, identification of the possible role of genes regulating sperm quality, the transcripts related to mitochondrial activity (CPT2, ATP5F1A and SOD2) as well as oxidative stress (NFE2L2) for profiling all experimental groups was selected.

The objective of this study was to improve the freezability of buck semen through testing the effect of glycerol and DMSO as two different cryoprotectants in combination with melatonin supplementation of (10^{-6}M and 10^{-3}M) in cold and hot temperature on motility characteristics of buck sperm post thawing. Moreover, to correlate between the activity of biomarkers and transcriptional profile of the selected genes and the antioxidant competence of melatonin in buck sperm.

MATERIALS AND METHODS

Experimental region

This study was carried out at the experimental farm station located on the Faculty of Agriculture and Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. Semen samples were collected during breeding season in cold temperature (April and May, 2018) with average maximum and minimum of ambient temperatures 31.4˚C and 17.4˚C that corresponds to temperature-humidity index (THI) values of 79.31±0.68 and 55.73±0.38, respectively. In addition, environmental data recorded during hot temperature (July and August 2018) indicated average maximum and minimum of ambient temperature 36.3˚C and 22.5˚C that correspond to THI values of 98.32±0.37 and 73.97±0.22, respectively. The formula used to calculate THI was;

THI = T – 0.55 (1 – RH)(T – 58),

T = air temperature in ˚F; RH = relative humidity percentage.

After cryopreservation, the analysis of semen samples was done in semen laboratory belongs to Animal Production Department, Faculty of Agriculture Cairo University.

Experimental animals, feeding and management

Four healthy Egyptian native Baladi bucks were used in this experiment. The animals are sexually mature and their age ranged from 2.5 to 3 years. The bucks were nearly at the same body condition and with an average body weight of 45 kg. The bucks were kept under shaded yard and drinking water was provided *ad libitum*. Animals were fed concentrated feed (16 % total protein and 65 % total digestible nutrients), and roughage (rice straw).

Semen collection and processing

The procedure of semen collection and physical analysis was done according to Hafez and Hafez (2000). Semen samples were collected using artificial vagina twice a week from four mature healthy bucks during cold (April and May) and hot temperature (July and August). The collection was done for eight successive weeks in each period. The volume, color and viscosity of all ejaculates (n=128) were recorded throughout the experiment. Progressive motility percentage was evaluated subjectively and only ejaculates of at least ≥ 70% motility with 2 x 10^9 sperm/mL concentrations were pooled and cryopreserved according to experimental design (Evans and Maxwell 1987 and Leboeuf et al., 2000). Pooled samples were diluted at 1: 8 (v:v) with each extender. The concentration of sperm was adjusted to be 50 million/straw. The composition of 100 ml extender contained 3.605 g of Tris base (buffer), 1.490 g of fructose, 20 ml of egg yolk, 100,000 IU penicillin, 50,000 IU streptomycin, and 2.024 g of citric acid that all were mixed well and dissolved in distilled water. Two different cryoprotective agents, glycerol and DMSO were used for the composition of the two different types of extenders at 5% each. The melatonin (M5250, Sigma – Aldrich, 3050 Spruce Saint Louis, MO 63103,

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USA) was diluted in each extender before extension at (10^{-6}M and 10^{-3}M). Supplementation of melatonin was done by weighing 0.0023 g of melatonin powder, which was then dissolved in 10 ml of each extender to get 10^{-3} M melatonin concentration, and further diluted to reach 10^{-6} M as the second concentration. Fresh extender was prepared on every collection day and left in a fridge for maximum of 1 to 1.5 hours until semen was collected. The extender was pre-warm at 37˚C in water bath prior to extension. Extended semen was packed in 0.25 ml French straws (IMV). The straws were cooled at 5ºC for four hours as an equilibrium period. Thereafter, straws were collected and spread on a rack above liquid nitrogen for 15 minutes. Finally, the straws were submerged slowly into liquid nitrogen and kept at -196 ºC.

Post-thawing evaluation motion characteristics

According to Ashrafi et al. (2013), three straws from each batch of semen were removed from liquid nitrogen tank and immersed immediately into a water bath at 37˚C for 30 seconds. Thawed semen was evaluated immediately and the motion characteristics of spermatozoa were determined by using Computer Assisted Sperm Analysis (CASA; instrument SpermVision™ software Minitube Hauptstraße 41. 84184 Tiefenbach, Germany). The motion characteristics included the following measurements; distance curved line (DCL, µm), distance average path (DAP, µm), distance straight line (DSL, µm), velocity curved line (VCL, µm/sec), velocity average path (VAP, µm/sec), velocity straight line (VSL, µm/sec), linearity (LIN=VSL/VCL), straightness (STR=VSL/VAP), wobble (WOB=VAP/VCL), beat cross frequency (BCF, H_z) and amplitude of lateral head displacement (ALH, µm).

Antioxidant biomarkers assays

Two straws from each group were thawed for 30 seconds at 37ºC and packed into 1.5 ml eppendorf tube. Then 1 ml of Dulbecco's phosphate buffer saline was added to each eppendorf tube and they were centrifuged for 10 minutes at 1030 rcf at 4ºC. This procedure was done three times and every time the supernatant was discarded. Afterward, 1 ml of distilled water was added to each eppendorf and frozen in liquid nitrogen. Later on the contents were centrifuged at 1030 rcf at 4ºC for 15 minutes. The pellets were discarded and supernatants were collected for enzymatic activity analyses. Three different biological replicates of each experimental group were used to profile all antioxidant enzymes; catalase, Total Antioxidant Capacity (TAC), Glutathione Peroxidase (GPX), and Malondialdehyde (MDA). The analyses of all enzymes were achieved by colorimetric assay using spectrophotometer (Sunostk SBA 733 plus, Bio diagnostic - Egypt) at 505 nm wavelength

Catalase activity

The activity of catalase was measured using standard procedure described by Fossati et al. (1980) and based on the company information (Cat No. CA 25 17, Bio Diagnostic, Giza, Egypt). The reaction was initiated by mixing the phosphate buffer, pH 7.0 (R_1) with known quantity of H_2O_2 (R_2). Catalase inhibitor (R_3) was added for one minute to inactivate the reaction. In the presence of peroxidase (HRP) (R_4), remaining H_2O_2 reacted with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample. After incubation for 10 minutes at 37ºC, samples were read at 510 nm (500 – 520 nm).

Total antioxidant capacity (TAC)

The TAC was determined using the same method according to Koracevic et al. (2001) by mixing antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (R_1) and incubated for 10 minutes at 37˚C. The antioxidants in the sample eliminated a certain amount of the provided hydrogen peroxide. In the presence of chromogen (R_2), the residual H_2O_2 was measured by an enzymatic reaction which resulted in the conversion of 3,5,dichloro–2–hydroxy benzensulphonate (R_3) to a colored product. The absorbance of blank (distilled water) and sample were read immediately at 505 (500 – 510 nm). All the chemicals were manufactured and the method was described in the manual of the company (Cat. No. TA 25 13, Bio Diagnostic, Giza, Egypt)

Glutathione peroxidase activity

The measurement of the GPx activity was performed as described by Paglia and Valentine (1967) and followed the company instruction (Cat. No. GP 25 24, Bio Diagnostic, Giza, Egypt). The chemical conversion of NADPH (R_2) to $NADP^+$ was followed by a reduction in measured absorbance by spectrophotometer at wavelength 340 nm (A_{340}) which was a tool to measure the activity of GPx enzyme. Phosphate Buffer (R_1) pH 7.0 was added to a solution containing glutathione, glutathione reductase, and NADPH (R_2). The enzyme reaction was initiated by adding the substrate hydrogen peroxide (R_3) and the decrease of absorbance at 340 nm/ min was recorded over a period of 3 minutes against deionized water.

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Malondialdehyde concentration

To measure the concentration of MDA, thiobarbituric acid (TBA) (R_2) was reacted with MDA in the presence of chromogen in acidic medium at 95°C for 30 minute and formed thiobarbituric acid reactive product (Ohkawa et al., 1979). The absorbance of the resultant pink product was measured for sample against blank and standard (R_1) against distilled water according to the procedure mentioned by (Cat No. MD 25 29, Bio Diagnostic, Giza, Egypt) at 534 nm.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Three straws from each treatment were removed from the liquid nitrogen tank and thawed at room temperature. The contents were transferred to 1.5 ml eppendorf tube and centrifuged for 12 minutes on 680 rpm at 4 ˚C. The pellets of all samples were kept at -80˚C till the extraction of RNA. The isolation of RNA was performed using GeneJET kit (Thermofisher Sci., Vilnius, Lithuania), following the instructions of manufacturer. The digestion of genomic DNA was done by adding DNase I recommended by RNase-free kit (Thermo Scientific, California, USA). The Nano-drop 2000C instrument (Thermo Scientific, Wilmington, DE, USA) was used to measure the concentration of total RNA and purity was determined by the ratio reading at A260/280 nm. Reverse transcription Kit (Life Technologies Corporation, California, USA) was used to synthesize cDNA which includes MultiScribe™ Reverse Transcriptase and random primers. Gene-specific primers were designed from sequences available in GenBank database (www.ncbi.nlm.nih.gov) using Primer3 software (http://primer3.wi.mit.edu//) as shown in Table1. The real-time PCR reaction was done using using a StepOnePlus™ Real-Time PCR instrument (Applied Biosystems, California, USA). The reaction contained 0.2 µl reverse and forward primers, 10ul of SYBR Green Master Mix (Thermofisher Scientific, California, USA), 7.6µl nuclease-free water and 2.0µl cDNA. The settings of PCR reactions were as following: 10 minutes at 95°C, 40 cycles of 15 seconds at 95 °C, 20 seconds at 60 °C, and 30 seconds at 72 °C and finally 1 min at 60°C. Delta Ct method was used for gene expression data analysis after normalization of the target transcript (CPT2, ATP5F1A, NFE2L2 and SOD2) to the housekeeping gene (GAPDH).

Table1. The primers used for quantitative real-time PCR analysis

Gene Name	Gene bank accession number	Primer sequence	Fragment size (bp)	Reference
ATP5F1A	NM_174684.2	F: 5′-CTCTTGAGTCGTGGTGTGCG-3′ R: 5′-CCTGATGTTGGCTGATAACGTG-3′	184	(Ghanem et al., 2014)
CPT2	NM_001045889	F: 5'-CCGAGTATAATGACCAGCTC-3' R: 5'-GCGTATGAATCTCTTGAAGG-3'	152	(Ghanem et al., 2014)
NFE2L2	NM_001011678	F: 5'-TAAAACAGCAGTGGCTACCT-3' R :5'-GAGACATTCCCGTTTGTAGA-3'	159	(Ghanem et al., 2014)
SOD2	NM_201527	F: 5'-GTGATCAACTGGGAGAATGT-3' R: 5'-AAGCCACACTCAGAAACACT-3'	163	(Ghanem et al., 2014)
GAPDH	NM_001034034.2	F: 5'- AGGTCGGAGTGAACGGATTC -3' R: 5'- GGAAGATGGTGATGGCCTTT -3'	219	(Ghanem et al., 2014)

PCR: polymerase chain reaction; bp: base pair.

Experimental design

The impact of the two different levels of melatonin supplementation (10^{-6}M and 10^{-3}M) in combination with the two different types of buck extenders (glycerol and DMSO based extenders) during cold and hot temperature were evaluated after cryopreservation in the present study. Motion characteristics and antioxidant enzymes activity were analyzed in all experimental groups. In addition, expression profile of SOD2, CPT2, NFE2L2, and ATP5F1A genes was quantified using Real-time PCR.

Data analysis

The data of the current study was analyzed using GLM procedure (SAS, 2004) by applying the following statistical model: $Y_{ijklm}= \mu + E_i + M_j + S_k + (E^{\times}M^{\times}S)_l + e_{ijklm}$. The measured traits were as following: μ = overall means; E_i = Effect of extender type; M_j = Effect of melatonin level; S_k = Effect of season; $(E^{\times}M^{\times}S)_l$ = The interaction between extender type, melatonin level and season and e_{ijklm} = Experimental error. Duncan's multiple range tests was used to detect differences among means. The significance level was set at ($P<0.05$).

RESULTS

Motion characteristics

The percentage of total motility (Tables 2 and 3) was higher significantly ($p \leq 0.05$) in samples extended with glycerol (control, low and high melatonin level) in cold and hot temperature than those supplemented with DMSO. The

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progressive motility (Tables 2 and 3) was significantly higher in all groups of glycerol-based extender than in DMSO based extender in both cold and hot temperature. The percentages of total and progressive motility in samples supplemented with (10^{-6}M) melatonin was insignificantly higher than in samples supplemented with (10^{-3} M) melatonin in both two types of extenders in cold temperature however, these differences were significant in hot temperature. In addition, samples supplemented with (10^{-6}M) melatonin in glycerol extender recorded the highest percentages of total and progressive motility compared with other samples in hot temperature. All CASA parameters tended to be higher in semen samples extended with glycerol than those extended with DMSO during cold temperature (Table 2). However, there were no clear variations in CASA parameters between samples extended with either glycerol or DMSO during hot temperature (Table 3).

Table 2. Spermatic parameters of buck semen as affected by types of extender and levels of melatonin supplementation during cold temperature

Traits	Glycerol 5%			DMSO 5%		
	Control	Low melatonin	High melatonin	Control	Low melatonin	High melatonin
Total Motility (%)	83.5^{a}±2.2	84.1^{a}±2.3	80.8^{a}±2.4	48.9^{bc}±2.5	50.2^{b}±2.3	43.5^{c}±2.6
Progressive motility (%)	71.6^{a}±2.3	74.4^{a}±2.4	64.4^{b}±2.5	32.9^{cd}±2.5	35.5^{c}±2.4	29.8^{d}±2.6
DAP (um)	24.9^{a}±0.5	24.6^{a}±0.5	21.8^{b}±0.5	18.9^{c}±0.6	18.7^{c}±0.5	18.8^{c}±0.6
DCL (um)	45.5^{a}±1.2	44.5^{a}±1.3	39.2^{b}±1.4	33.5^{c}±1.4	33.4^{c}±1.3	33.8^{c}±1.4
DSL (um)	18.3^{a}±0.3	18.2^{a}±0.4	15.9^{b}±0.4	14.5^{b}±0.4	14.3^{b}±0.4	14.2^{b}±0.4
VAP (um/s)	58.6^{a}±1.1	57.4^{a}±1.1	51.3^{b}±1.2	45.0^{c}±1.2	44.0^{c}±1.1	44.5^{c}±1.2
VCL (um/s)	106.6^{a}±2.8	103.5^{a}±2.9	91.8^{b}±3.0	78.8^{c}±3.1	77.4^{c}±2.9	78.7^{c}±3.2
VSL (um/s)	43.0^{a}±0.7	42.5^{a}±0.8	37.7^{b}±0.8	34.8^{bc}±0.8	34.0^{bc}±0.8	33.8^{c}±0.9
STR (%)	72.9 ^{b}±0.6	73.6^{b}±0.6	73.2 ^{b}±0.7	77.6^{a}±0.7	76.8^{a}±0.6	75.8^{a}±0.7
LIN (%)	39.9 ^{c}±0.8	40.7^{bc}±0.8	40.9 ^{c}±0.9	45.8^{a}±0.9	44.7^{a}±0.8	43.4^{ab}±0.9
WOB (VAP/VCL)	0.54^{d}±0.01	0.55^{bcd}±0.01	0.55^{cd}±0.01	0.58^{a}±0.01	0.57^{ab}±0.01	0.56^{abc}±0.01
ALH (um)	5.0^{a}±0.0	4.7^{bc}±0.1	4.8^{b}±0.1	4.4^{cd}±0.1	4.2^{d}±0.1	4.2^{d}±0.1
BCF (H2)	23.8^{a}±0.5	24.4^{a}±0.5	23.5^{a}±0.5	19.1^{b}±0.5	19.1^{b}±0.5	18.9^{b}±0.6

Means having different superscripts within the same row differ significantly (P<0.05). DAP: Distance Average Path (microns); DCL: Distance Curved Line (microns); DSL: Distance Straight Line (microns); VAP: Velocity Average Path (microns/sec); VCL: Velocity Curved Line (microns/sec); VSL: Velocity Straight Line (microns/sec); STR: Straightness (VSL/VAP); LIN: Linearity (VSL/VCL); WOB: Wobble (VAP/VCL); ALH Amplitude of Lateral Head Displacement (microns); BCF: Beat Cross Frequency (H_2); DMSO: Dimethyl Sulfoxide.

Table 3. Spermatic parameters of buck semen as affected by types of extender and levels of melatonin supplementation during hot temperature

Traits	Glycerol 5%			DMSO 5%		
	Control	Low melatonin	High melatonin	Control	Low melatonin	High melatonin
Total Motility (%)	67.4^{b}±2.1	82.0^{a}±2.3	59.0^{c}±2.3	24.1^{f}±2.3	41.2^{d}±2.0	31.1^{e}±1.8
Progressive motility (%)	57.9^{b}±2.0	75.1^{a}±2.2	53.5^{b}±2.2	19.7^{d}±2.2	32.1^{c}±1.9	22.0^{d}±1.8
DAP (um)	20.9^{d}±0.5	24.7^{a}±0.5	23.1^{bc}±0.5	23.3^{ab}±0.5	21.6^{cd}±0.4	20.5^{d}±0.4
DCL (um)	35.6^{bc}±0.8	42.3^{a}±0.9	35.6^{bc}±0.9	38.0^{b}±0.9	35.6^{bc}±0.7	34.3^{c}±0.7
DSL (um)	16.4^{c}±0.5	19.4^{a}±0.5	19.5^{a}±0.5	19.3^{a}±0.5	18.2^{ab}±0.5	16.7^{bc}±0.4
VAP (um/s)	47.5^{c}±1.0	56.2^{a}±1.1	51.2^{b}±1.1	51.5^{b}±1.1	48.6^{bc}±0.9	45.5^{c}±0.8
VCL (um/s)	81.0^{bc}±1.8	95.8^{a}±2.0	79.0b^{c}±2.0	83.3^{b}±2.0	79.5^{bc}±1.7	76.0^{c}±1.6
VSL (um/s)	37.4^{b}±1.0	44.1^{a}±1.1	43.2^{a}±1.1	42.6^{a}±1.1	40.8^{a}±0.9	36.9^{b}±0.9
STR %	78.5^{b}±0.0	78.0^{b}±0.0	83.8^{a}±0.0	82.2^{a}±0.0	83.4^{a}±0.0	80.5^{ab}±0.0
LIN %	45.8^{c}±0.0	45.4^{c}±0.0	54.2^{a}±0.0	50.6^{ab}±0.0	50.8^{ab}±0.0	48.2^{bc}±0.0
WOB (VAP/VCL)	0.58^{c}±0.0	0.58^{c}±0.0	0.64^{a}±0.0	0.61^{b}±0.0	0.60^{bc}±0.0	0.59^{bc}±0.0
ALH (um)	3.4^{ab}±0.1	3.9^{a}±0.1	2.8^{c}±0.1	3.1^{bc}±0.1	2.9^{c}±0.1	3.0^{bc}±0.1
BCF (H2)	26.4^{b}±0.9	28.3^{ab}±1.0	29.6^{a}±1.0	25.1^{b}±1.0	26.5^{b}±0.9	26.3^{b}±0.8

Means having different superscripts within the same row differ significantly (P<0.05). DAP: Distance Average Path (microns); DCL: Distance Curved Line (microns); DSL: Distance Straight Line (microns); VAP: Velocity Average Path (microns/sec); VCL: Velocity Curved Line (microns/sec); VSL: Velocity Straight Line (microns/sec); STR: Straightness (VSL/VAP); LIN: Linearity (VSL/VCL); WOB: Wobble (VAP/VCL); ALH Amplitude of Lateral Head Displacement (microns); BCF: Beat Cross Frequency (Hz); DMSO: Dimethyl Sulfoxide.

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Antioxidant biomarkers assays

There were no significant differences between all samples cryopreserved with glycerol and DMSO on activity of MDA during cold temperature (Figure 1). However, the control group extended with glycerol and DMSO increased the level of MDA compared with samples supplemented with melatonin in hot temperature. The semen samples supplemented with melatonin in either glycerol or DMSO based extender had higher levels of MDA in cold than hot temperature. The activity of GPX (Figure 2) revealed insignificant difference between samples extended with glycerol and DMSO in cold season although samples cryopreserved with glycerol tended to show higher level of GPX than those cryopreserved with DMSO. While, there were no differences between all semen samples cryopreserved in hot temperature. Regarding the level of catalase (Figure 3), the activity of this enzyme was reduced in control samples with glycerol in comparison to all other experimental samples in cold temperature. However, the level of CAT was reduced in semen samples supplemented with low melatonin dose compared to all semen samples cryopreserved during hot temperature. The total antioxidant capacity values (Figure 4) increased in control groups cryopreserved with glycerol and DMSO compared to other experimental groups in hot temperature. Moreover, control and low level of melatonin-supplemented groups extended with either glycerol or DMSO revealed higher level of TAC than the groups supplemented with high melatonin level during cold temperature.

Gene expression profile

Transcript abundance of carnitine palmitoyl transferase 2 (CPT2) was up-regulated (P< 0.0.5) in sperm samples cryopreserved in glycerol based extender compared with that cryopreserved with DMSO in cold temperature (Figure 5). Moreover, supplemented samples with low melatonin dose in DMSO based extender recorded higher (P< 0.05) transcript abundance than all other sperm samples cryopreserved during hot temperature. The samples supplemented with low level of melatonin in both DMSO and glycerol based extender recorded the highest expression profile of this gene during cold and hot temperature. Expression profile of the ATP synthase F1 subunit Alpha (ATP5F1A) in sperm of glycerol based extender was increased significantly in comparison to those cryopreserved by DMSO during cold temperature (Figure 6). However, this gene had been down-regulated significantly in control group with DMSO compared to all semen samples cryopreserved during hot temperature. The expression of Nuclear Factor erythroid-derived 2-like 2 (NFE2L2, NRF2) was higher in samples cryopreserved in hot than cold temperature (Figure 7). In addition, the expression profile of this transcript was up-regulated in semen samples extended with DMSO than those extended with glycerol in hot temperature. There was no significant differences between all samples in the expression pattern of NFE2L2 during cold temperature although sperm samples cryopreserved with DMSO tended to show higher expression than those cryopreserved with glycerol. The transcript abundance of superoxide dismutase 2 (SOD2) gene was higher (P<0.05) in samples cryopreserved with glycerol than DMSO based extender during cold temperature (Figure 8). The expression of SOD2 was increased in control group extended with glycerol and low level of melatonin-supplemented group as well as with DMSO compared to all semen samples in hot temperature.

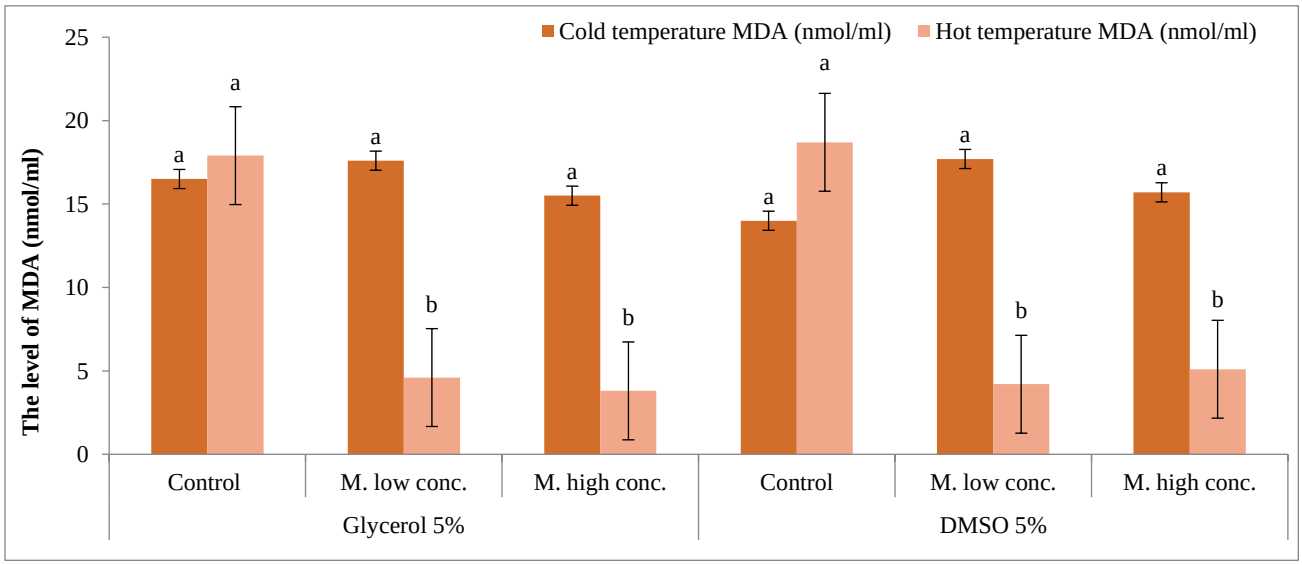

Figure 1. Enzymatic activity of Malondialdehyde of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. MDA: Malondialdehyde; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration

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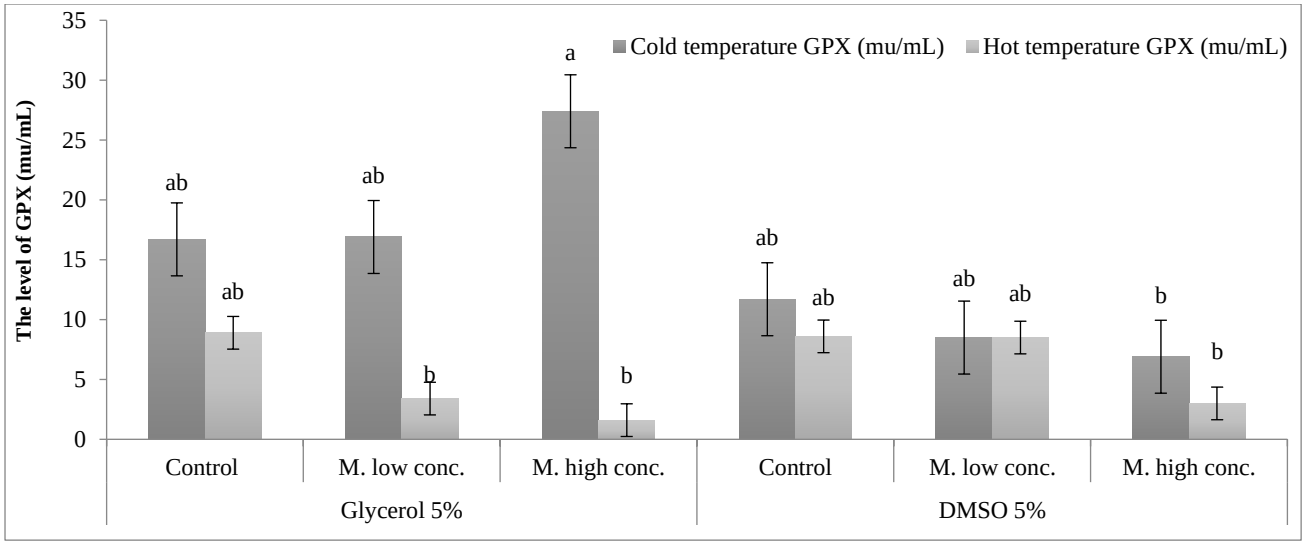

Figure 2. Enzymatic activity of glutathione peroxidase of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. GPx: glutathione peroxidase; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

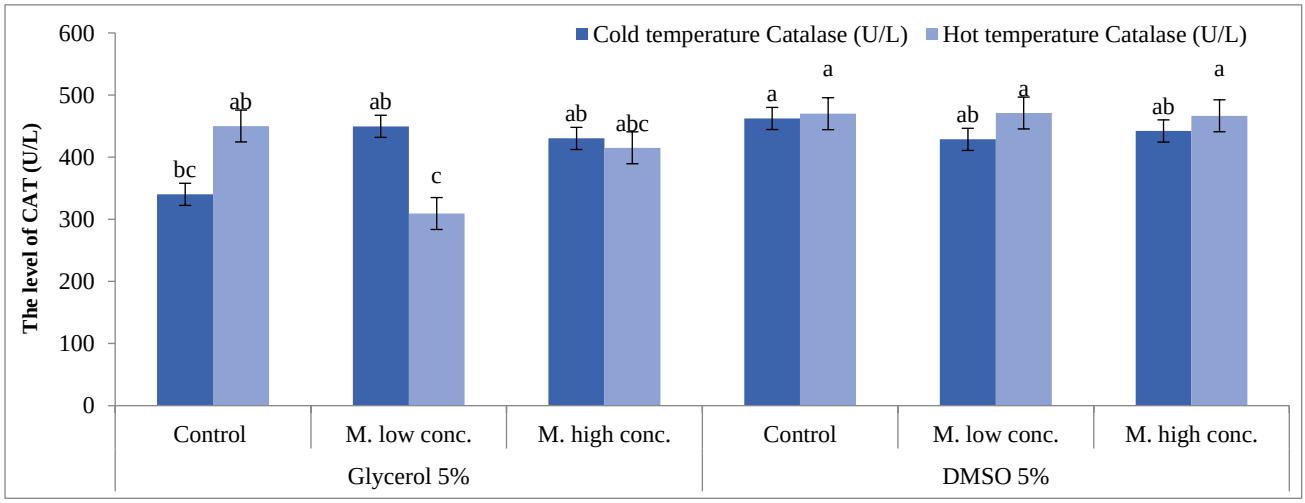

Figure 3. Enzymatic activity of Catalase of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. CAT: Catalase; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

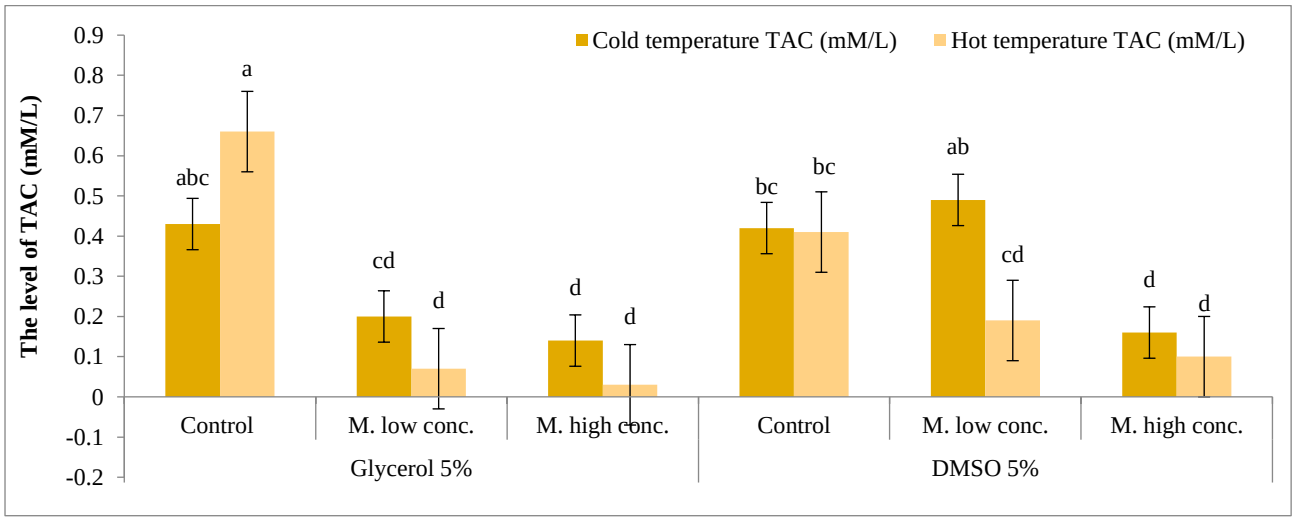

Figure 4. Total antioxidant capacity of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. TAC: Total Antioxidant Capacity; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

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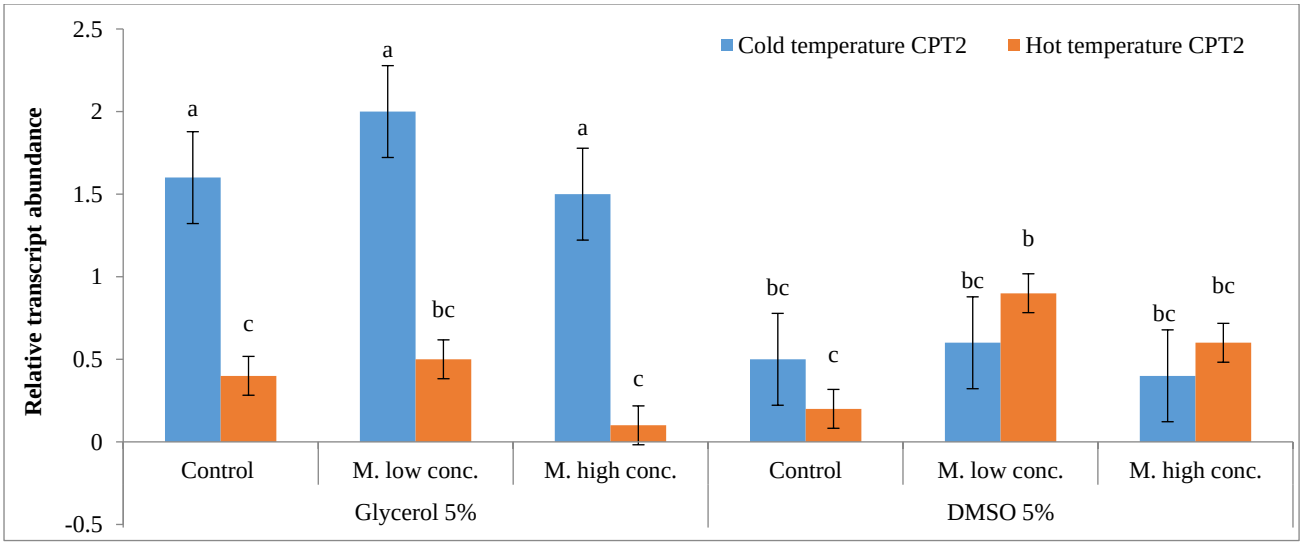

Figure 5. Relative transcript abundance of CPT2 gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

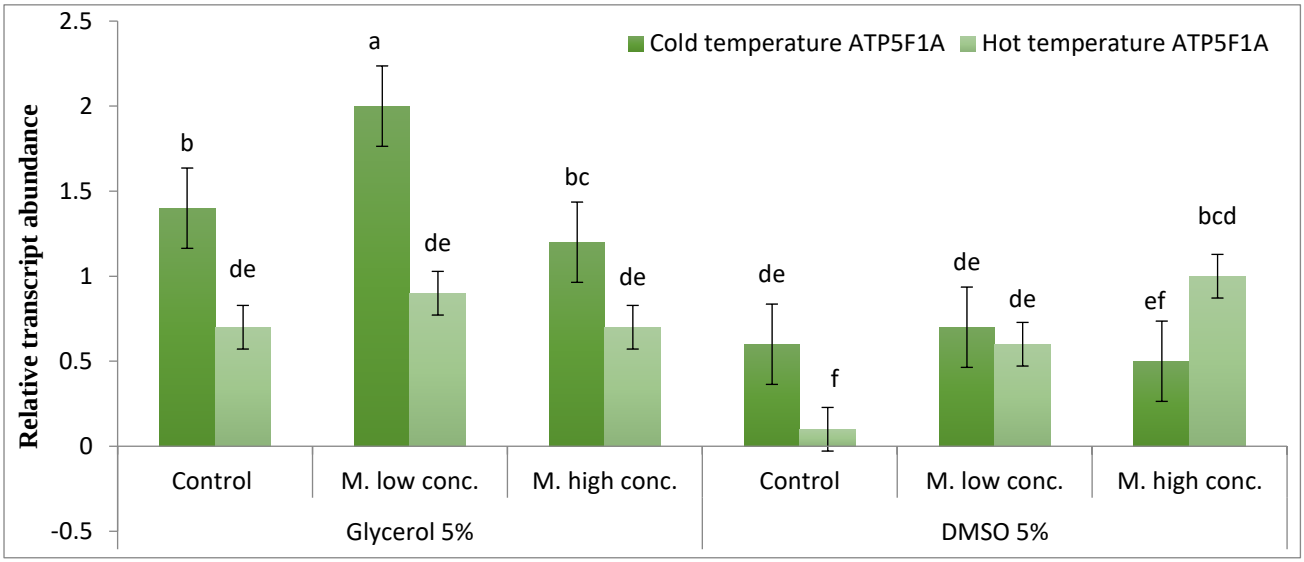

Figure 6. Relative transcript abundance of ATP5F1A gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

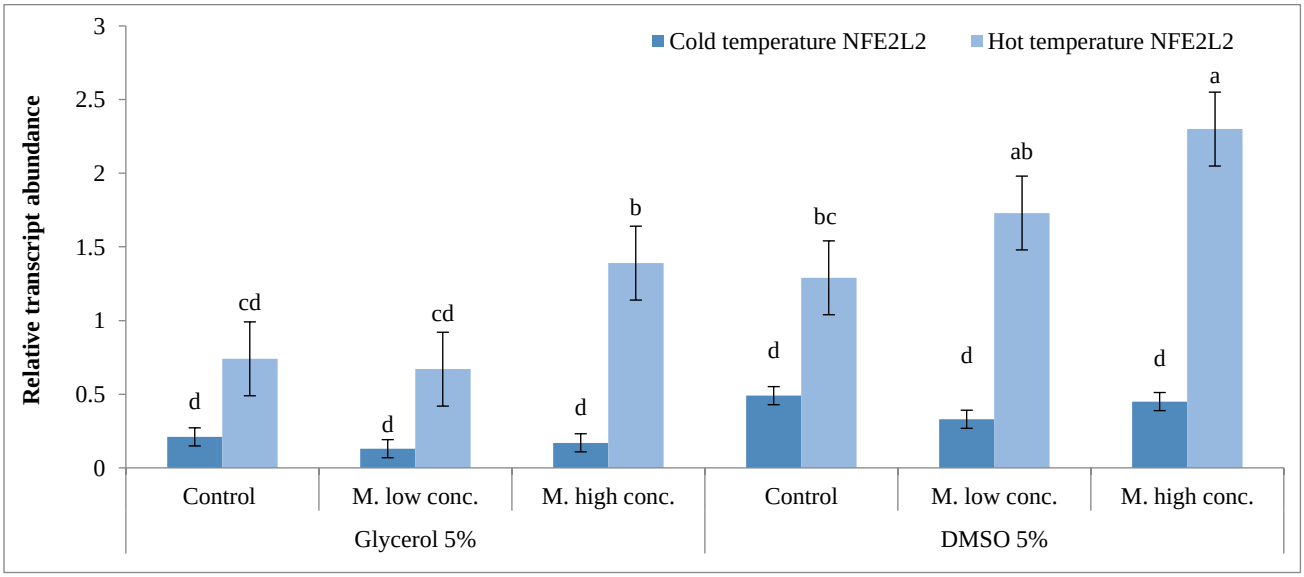

Figure 7. Relative transcript abundance of NFE2L2 gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

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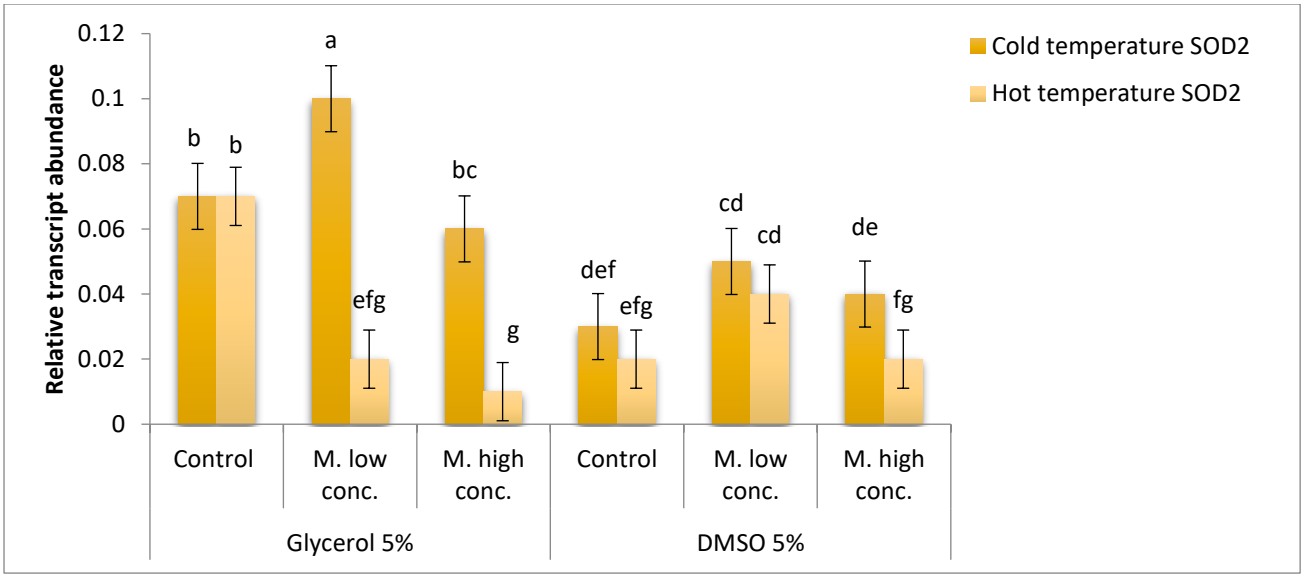

Figure 8. Relative transcript abundance of SOD2 gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

DISCUSSION

Cryopreservation is an important method used in biotechnology of reproduction to preserve male semen and store it in a bank for future use (Sikarwar et al., 2015). It is an important method used in biotechnology of reproduction to preserve male semen and store it in a bank for future use. All metabolic activities are minimized during this process due to the freezing at low temperature (Sikarwar et al., 2015). However, freeze–thawing process could cause damage to sperm, mitochondria, acrosome and DNA that reduced its fertilizing ability (Watson, 2000 and Baumber et al., 2003). Moreover, cryopreservation could alter transcriptional profile of genes that considered biomarkers of spermatozoa quality (Valcarce et al., 2013; Hezavehei et al 2018).

The results of current study revealed that the percentages of total and progressive motility were improved in samples supplemented by low dose of melatonin (10^{-6} M) in comparison to control and high dose (10^{-3}M) in glycerol and in DMSO based extenders in hot season. This result confirmed the positive action of melatonin on sperm motility and viability (Ramadan et al., 2009; Du Plessis et al., 2010; Ashrafi et al., 2011 and 2013). Indeed, melatonin is a potent free radical scavenger through activation of antioxidant enzymes, which protect cells from oxidative stress (Ashrafi et al., 2013; Kilic et al., 2017). In addition, researchers have reported beneficial effects of melatonin on reducing the rate of lipid peroxidation in a dose dependent manner (Du Plessis et al., 2010; Succu et al., 2011and Kilic et al., 2017). Succu, et al. (2011) have indicated that supplementing melatonin in a dose of 1.0 mM to ram extender maintained progressive motility, intracellular ATP concentrations, DNA integrity and fertilization rate in comparison to other doses (0.001, 0.01, 0.1, and 10 mM). In contrast, Souza et al. (2016) have stated that low concentration of melatonin (100 pM) increased the sperm motility, mitochondrial activity, integrity of plasma membrane and acrosome in ram semen in comparison to high concentrations (100 nM, and 100 µM). Moreover, supplementation of melatonin at low dose (0.1 mM) to semen extender of buffalo bulls maintained the ultrastructure integrity of the sperm and increased pregnancy rate in compared to control and high doses groups (0.250, 0.500, 0.750 and 1 mM) (EL-Raey et al., 2015). The variation in melatonin concentrations supplemented to extender and their wide response on semen quality parameters could be due to animal species and breed used in these different experiments.

Present results (figures 1, 2 and 4) demonstrated that, melatonin supplementation has negatively correlated with some biochemical parameters of semen like MDA, GPX and TCA in glycerol and DMSO-based extenders during hot temperature. Indeed, the interaction among melatonin, season of collection and type of extender may be another factor as the quality and biochemical parameters changed with season of semen collection (Marti et al., 2007; Elsheikh et al., 2013 and Gabr et al., 2019). However, there are no available data describing the interaction among these three factors and downstream effect on semen cryopreservation. In general term, glycerol-based extender was better than DMSO as indicated from results presented in the current work which is in agreement with (Rasul et al., 2007). In accordance with our result, glycerol-based extender has enhanced post-thaw sperm motility, velocities (straight-line and average path), compared to other extenders that included DMSO in semen of buffalo bulls (Rasul et al., 2007). The post thaw sperm progressive motility rate was almost double in buck semen cryopreserved with 6% glycerol compared to that cryopreserved in 6% DMSO (Sikarwar et al., 2015). This could be due to rapid flow rate of DMSO due to its lower

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molecular weight than glycerol that may be toxic to spermatozoa (Rasul et al., 2007). Interestingly, there is an interaction between breed of bucks and glycerol concentration (Kulaksiz et al., 2013).

Results of the current study demonstrated up-regulation of three mitochondrial transcripts (CPT2, ATP5F1A and SOD2) in glycerol based extender groups and this was more apparent in the group of low melatonin level compared with all other glycerol based extender groups. Moreover, the expression of these transcripts was not affected by season of semen collection. Developing spermatids and sperms rely on stored mRNAs to produce required proteins for development (Steger, 2001) and for energy production in form of ATP (Rodriguez-Martinez, 2001). Interestingly, sperm mitochondrial RNA is transcribed during testicular development (Alcivar et al., 1989). Chen et al. (2014) reported up-regulation of mitochondrial proteins (ATP5A1 and ATP5B) in frozen boar sperms. Present findings revealed up-regulation of these genes was coupled with increased post thaw sperm motility percentage in glycerol-based extender. The expression of mitochondrial transcripts seems to be crucial for sperm motility. In this regard, the transcript abundance of mitochondrial NADH dehydrogenase 2 (MTND2) genes were significantly lower in asthenospermia samples than in normal ones (Jodar et al., 2012).

Present study show that the melatonin supplementation (in low-level) has positive effect with glycerol-based extender on sperm motility in goat, under hot conditions. The protective effect of melatonin coupled with increased expression of NFE2L2 in samples cryopreserved in hot than in cold temperature. In addition, the expression profile of this transcript was higher in semen samples extended with high melatonin concentration and those extended with DMSO than with glycerol in hot season. The higher expression of this gene during summer could be due to its action as a transcription factor activating different antioxidant genes that protect cells from any cellular stress. In support to this idea, Nakamura et al. (2010) have reported reduction of mice fertility (seminiferous tubule damage) as a result of NRF2 and increased oxidative stress highlighting the importance of this gene in defense against oxidative stress during sperm formation. Moreover, Chen, et al. (2012) reported lower expression profile of NRF2 in human experiencing low sperm motility.

Marti et al. (2007) revealed the effect of season on GPx, glutathione reductase (GR) and superoxide dismutase (SOD) activities in seminal plasma of ram. Results of previous study indicated clearly an increase in protein content of seminal plasma during the breeding season of rams (Marti et al., 2007). While, there was a higher activity of GR, GPx, SOD and CAT enzymes during the non-breeding than in breeding season (Marti et al., 2007) indicating enhancement of antioxidant defense system during the non-breeding season when lower sperm motility and viability values were found (Marti et al., 2007). Our results revealed that the profile of CAT was almost the same in all experimental groups. On the other hand, the profile of MDA, GPX and TAC was reduced in both high and low-melatonin supplemented groups compared with control cryopreserved either with glycerol or DMSO-based extender during hot temperature. This is similar to the pattern of SOD2 expression reflecting partial reduction in antioxidant capacity. In support to this idea, the transcript abundance of NFE2L2 was up-regulated in response to reduced antioxidant capacity as compensatory mechanism. Interestingly, melatonin supplementation into mithun bull's extender has improved semen quality compared with control group at different seasons of the year (Perumal et al., 2015). Moreover, an increase in the activities of (SOD, CAT, GSH and TAC) in semen during spring season followed by a reduction in these values during autumn and winter seasons, while the most decline in the values were obtained during summer season (Perumal et al., 2015). Ashrafi et al. (2013) recorded an increase in the activity of CAT and SOD with melatonin supplementation in different levels in the semen extender, however the activity of GPX had no significant differences. Thus, it seems that melatonin supplementation to semen extender during cold period was more effective in improving enzymatic defense system of spermatozoa. The increased antioxidant system might help to ensure an adequate fertilizing potential of spermatozoa (Rasul et al., 2007).

CONCLUSION

Present results have revealed that the cryoprotectants (glycerol versus DMSO) that were used for semen cryopreservation and the season of collection (cold versus hot) had significant effects on the kinetics, biochemical and molecular characteristics after thawing the goat spermatozoa. Supplementation of melatonin at low dose to semen extender during cold period has positive effects on the antioxidant defense system of buck sperm.

DECLARATIONS

Author`s contribution

Sherif Mohamed Dessouki designed the experiment and worked on statistical analysis, semen analysis, antioxidant biomarker analysis and wrote the manuscript. Gamal Ashour designed the experiment and revised the manuscript. Moataz El-Gayar designed the study and revised the manuscript. Fakhri El-Hadi El-Azzazi prepared the extender and antioxidant, analyzed the semen samples and revised the manuscript. Elias Kodi designed the experiment, prepared the

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extender, collected and analyzed the semen samples, wrote the manuscript and revised the edited manuscript. Nasser Ghanem designed the experiment and worked on gene expression, analysis of data revisions of manuscript.

Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article.

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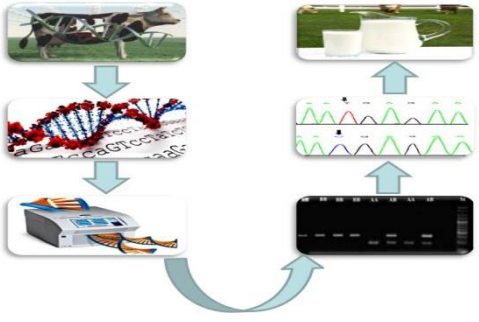

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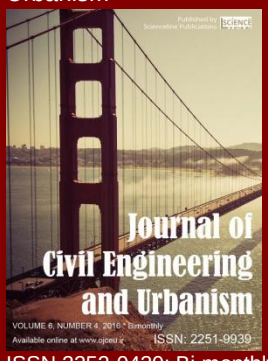

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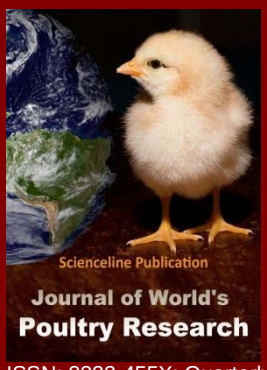

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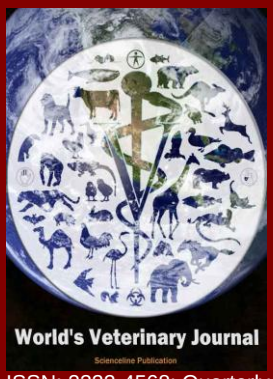

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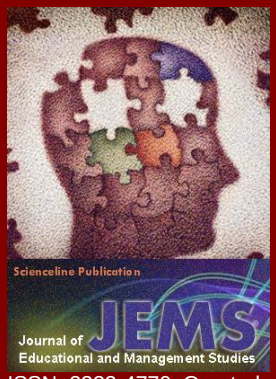

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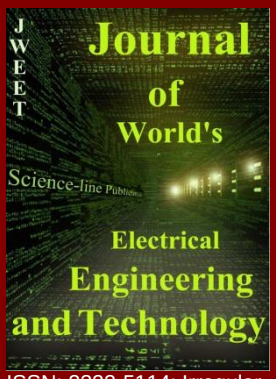

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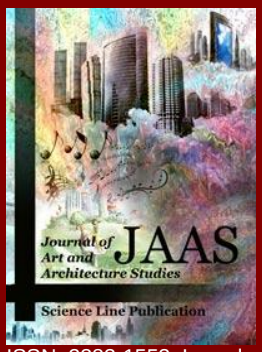

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