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Research Paper

Identification and Characterization of Virulence-Associated Genes from Pathogenic *Aeromonas Hydrophila* Strains.

Alaa El-Dein Omar A, Moustafa Moustafa E and Mamdouh Zayed M.

World's Vet. J. 6(4): 185-192, 2016; pii: S232245681600025-6

ABSTRACT

Aeromonas hydrophila is a freshwater, Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacterium that exists frequently in aquatic environments producing disease, not only to fish but also to human causing gastroenteritis. The present study aims to isolate, identify and characterize *A. hydrophila* isolated from *Oreochromis niloticus* fish in Kafr El-Sheikh governorate, Egypt using selective differential cultural medium (Rimler Shotts agar), morphological and biochemical tests (oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatin hydrolysis, glucose, triple sugar iron, urease, starch hydrolysis, lactose and trehalose tests). Besides, to search for the presence of the virulence genes in the pathogenic *A. hydrophila* isolates. In the present study we screened the presence of five virulence-associated genes of *A. hydrophila* isolated from diseased cultured fish. The detection of virulence factors of *A. hydrophila* is a key component in determining potential pathogenicity because these factors act multifunctionally and multifactorially. Pathogenesis of *A. hydrophila* was checked by experimental infection to *Oreochromis niloticus* fish together with screening of the five virulence genes which are heat-stable enterotoxin (ast), cytotoxic enterotoxin, hemolysin and aerolysin and heat-labile enterotoxin (alt). The obtained results revealed that the five screened virulence genes were positively correlated with *A. hydrophila* pathogenicity and the presence of virulence genes in pathogenic *A. hydrophila* strains may help in disease diagnosis, prevention and control.

Key words: *Aeromonas hydrophila*, Identification, Characterization, *Oreochromis niloticus,* Virulence genes

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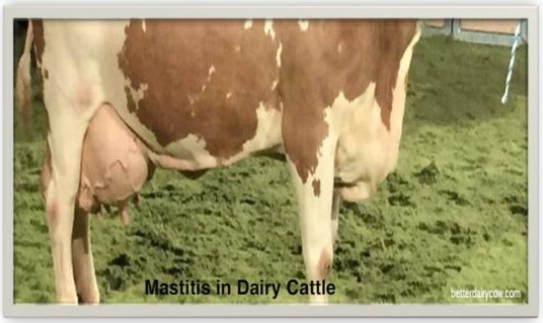
Research Paper

Molecular Detection of *Streptococcus* species Isolated from Cows with Mastitis.

Elsayed Eldesouky I, Allah Abd Elnaby Refae M, Saad Nada H and Ragab Hassb Elnaby G.

World's Vet. J. 6(4): 193-202, 2016; pii: S232245681600026-6



ABSTRACT

Streptococcal mastitis is considered as one of the most common infectious diseases in the dairy cattle, which threatens the dairy industry all over the world. The aim of this study was to determine the prevalence of *Streptococcus* species in mastitic cows with molecular investigation to detect the presence of some virulence genes of the recovered isolates by PCR. A total of 150 milk samples were collected from dairy cattle with clinical and subclinical mastitis from different areas in El- Gharbia governorate, Egypt. *Streptococcus* species were isolated with an incidence of 38%. *S. agalactiae, S. dysgalactiae, S. uberis*, *S. pyogenes*, *S. pneumoniae* and *S. faecalis* were isolated from the milk samples of the examined cows with the percentage of 14.7%, 6%, 9.3%, 4.7%, 1.3% and 2%, respectively. Molecular investigation of virulence associated genes revealed that sip, cfb and bca genes of *S. agalactiae* were found with the percentage of 77.7%, 88.8% and 33.3%, respectively. The mig gene of *S. dysgalactiae* was found with an incidence of 77.8%. Of the examined *S. uberis* isolates, 55.5%, 22.2% and 33.3% were carrying the cfu, oppF and has A genes, respectively. The present study revealed the prevalence of *Streptococci* and distribution of virulence associated genes among the isolates. The high frequency of virulence genes in the isolates suggests an important role of these virulence genes in the pathogenesis of *Streptococci* in cattle mastitis.

Key words: Mastitis, Cows, *Streptococci*, Virulence genes

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Research Paper

Constraints of Small-Scale Commercial Poultry Farms Analyzed by Garett's Ranking Technique in and around Debre Markos, Amhara Region, Ethiopia.

Bezabih Yitbarek M, Tamir Mersso B and Mengistu Wosen A.

World Vet. J. 6(4): 203-209, 2016; pii: S232245681600027-6

ABSTRACT

This study was carried out to assess constraints of small scale commercial poultry farms in and around Debre Markos, Amhara region, Ethiopia. Cross sectional study was involved on the assessment of socio demographic characteristics, flock size and constraints faced by employing structured questionnaire for personal interviews. The socio demographic and flock size data were analyzed by $\chi 2$ and one way analysis of variance, respectively. The constraints faced were ranked by the respondents and the factors were analyzed by Garrett's ranking technique. The result showed that sex, age, marital status, religion, occupation, family size, experience years, educational level had statistically significant effect ($p< 0.05$) on the operation of small scale poultry farms. The mean flock size was 844.3 chicks. Flock size was influenced ($P< 0.05$) by sex of birds rather than breed. The flock size of female chicks were significantly ($P< 0.05$) higher than male chicks. Among the constraints; high cost of feed, unavailability of feed and feed ingredients, unavailability of land , lack of market linkage and promotion, high cost of birds for starting business, lack of finance, lack of extension service and lack of training were listed as 1-10 ranks sequentially. Therefore, for successful poultry production and further expansion in the study area; there is a need to improve market linkage and promotion, provide training on poultry husbandry practice, provision of land and fulfilling the financial needs through facilitating credit services are among the imperatives for improving the current status of small scale commercial poultry production

Key words: Constraints, Flock size, Personal interview, Questionnaire

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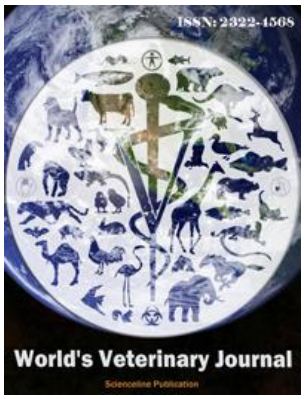

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Identification and Characterization of Virulence-Associated Genes from Pathogenic *Aeromonas Hydrophila* Strains

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ABSTRACT

Aeromonas hydrophila is a freshwater, Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacterium that exists frequently in aquatic environments producing disease, not only to fish but also to human causing gastroenteritis. The present study aims to isolate, identify and characterize *A. hydrophila* isolated from *Oreochromis niloticus* fish in Kafr El-Sheikh governorate, Egypt using selective differential cultural medium (Rimler Shotts agar), morphological and biochemical tests (oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatin hydrolysis, glucose, triple sugar iron, urease, starch hydrolysis, lactose and trehalose tests). Besides, to search for the presence of the virulence genes in the pathogenic *A. hydrophila* isolates. In the present study we screened the presence of five virulence-associated genes of *A. hydrophila* isolated from diseased cultured fish. The detection of virulence factors of *A. hydrophila* is a key component in determining potential pathogenicity because these factors act multifunctionally and multifactorially. Pathogenesis of *A. hydrophila* was checked by experimental infection to *Oreochromis niloticus* fish together with screening of the five virulence genes which are heat-stable enterotoxin (ast), cytotoxic enterotoxin, hemolysin and aerolysin and heat-labile enterotoxin (alt). The obtained results revealed that the five screened virulence genes were positively correlated with *A. hydrophila* pathogenicity and the presence of virulence genes in pathogenic *A. hydrophila* strains may help in disease diagnosis, prevention and control.

Key words: *Aeromonas hydrophila*, Identification, Characterization, *Oreochromis niloticus*, Virulence genes

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INTRODUCTION

Aeromonas hydrophila, belonging to the family *Aeromonadaceae*, is an environmental microorganism which is cosmopolitan in distribution. Mesophilic motile *aeromonads* are ubiquitous and autochthonous aquatic microorganisms occurring in fresh water, sewage and brackish water and in chlorinated and un-chlorinated drinking water. *Aeromonas hydrophila* is a freshwater, Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacterium (Garrity et al., 2006) that occurs frequently in aquatic environments such as surface waters, as well as food. Besides, it is an opportunistic pathogen implicated in human illnesses such as gastroenteritis, wound infections, septicaemia, pneumonia, necrotizing fasciitis and soft tissue conditions, has gained increasing interest (Janda and Abbott, 2010**;** Oleiwi et al., 2014; Praveen et al., 2016).

Some virulent strains of *A. hydrophila*, under stress conditions can invade most of freshwater fish species infecting them with hemorrhagic septicemia (MAS). In China, MAS has become the most important and dangerous bacterial fish disease to date leading to periodical high economic losses per year (Yang et al., 2016). The pathogenesis of *A. hydrophila* is multifactorial and the virulence factors work together to produce the virulence of this bacterium, these factors include extracellular products and S-layer that help the bacteria to resist the host immunity (Pang et al., 2015).

The pathogenicity of *Aeromonads* has been linked to exotoxins such as cytolytic enterotoxin, hemolysin⁄aerolysin, lipases and proteases (Yogananth et al., 2009). Hemolysins are exotoxins and the lytic activities on red blood cells are reported to be important for nutrient acquisition and⁄or for causing anemia (Sarkar et al., 2013). Haemolytic toxins as haemolysin and aerolysin released by *A. hydrophila* and their pathogenicity have been linked to hemagglutinins, adhesins and several hydrolytic enzymes. These toxins enter the tissue in culture and play a significant role in the pathogenesis of the bacteria. ExoPolySaccharides (EPS), importantly, play a very great role for the bacteria and their as they are organic molecules formed by polymerization of organic fractions, carbohydrates, proteins, and humic substances (Sarkar et al., 2013).

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The objectives of this study were to (i) isolate, identify and characterize *A. hydrophila* isolated from *Oreochromis niloticus* fish in Kafr El-Sheikh governorate, Egypt, using various culture media, morphological and biochemical tests, and (ii) search for the presence of the virulence genes in the pathogenic *A. hydrophila* isolates. In the present study we screened the presence of five virulence-associated genes of *A. hydrophila* isolated from diseased cultured fish.

MATERIALS AND METHODS

Ethical approval

Animal ethics committee, faculty of veterinary medicine, Kafr El-Sheikh University, Egypt, approved the protocol and conducting of the study.

Isolation and identification of *Aeromonas hydrophila*

A total number of 140 *Oreochromis niloticus* fish were collected from Kafr El-Sheikh governorate farms, Egypt, over the seasons of the year 2015. The collected fish showed petechial haemorrhages externally over the body, fins and particularly in the head region. The peritoneum was swollen with bloody ascitic fluid with gross visible hemorrhages on the internal organs of the affected fish. For bacteriological examination, sterile swabs were collected from the infected parts of the fishes. The collected swabs were firstly pre-enriched on Tryptic Soy Broth (TSB, Oxoid). After which (pre-enrichment on TSB) they were streaked on Rimler Shotts (RS) agar for presumptive identification and incubated at 28 °C for 24 hours. Well-differentiated single bacterial colonies were further streaked onto TSA (TSA, Oxoid) for obtaining pure culture.

Biochemical analysis

For phenotypic identification, pure cultures were subjected to Gram staining and viewed under light microscope (Leica). Further biochemical tests like motility, oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatin hydrolysis, glucose, triple sugar iron, urease, starch hydrolysis, lactose and trehalose tests, were performed for the identification as well as strain differentiation of bacteria and results were compared with the reference strain of *A. hydrophila* (obtained from the food analysis center, faculty of veterinary medicine, Banha university, Egypt).

Molecular identification by PCR

DNA Extraction using QIA amp kit: DNA Extraction was performed using QIA amp kit (QIAamp: Qiagen inc., USA). Accurately, one ml of the young cell suspension was centrifuged at 10000 g for 10 min at 4^{o}C and the cell pellet was mixed with 600 µl of guanidine hydrochloride buffer (pH 8.0), incubated at room temperature for 30 min and again centrifuged at 10000 x g for 10 min at 4oC. From that, 500 µl of the supernatant was transferred to another tube and mixed with 100% ice cold ethanol and centrifuged at 13000 x g for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 95% and 90% ethanol, respectively followed by centrifugation at 10000 g for 10 min at 4°C. The pellet was then re-suspended in 50 µl of molecular grade water and then stored at -20°C until used as PCR template (Haldar et al., 2005).

Primer sequences used for PCR identification system of A. hydrophila: Application of PCR for identification of *A. hydrophila* by 16S rRNA and identification of virulence factors including, heat-stable enterotoxin (ast), cytotoxic enterotoxin, hemolysin and aerolysin (act/hlyA/aer) complex and heat-labile enterotoxin (alt) of the isolated *A. hydrophila* was performed essentially by using Primers (Pharmacia Biotech) as shown in table 1.

Table 1. The primers used for the amplification of different virulence associated genes of *A. hydrophila* isolated from *O. niloticus*

Primers	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
A16S1 (F)	16S rRNA	5′ CTACTTTTGCCGGCGAGCGG ′3	953	Pinto et al., (2012)
A16S1 (R)		5′ TGATTCCCGAAGGCACTCCC ′3		
AHS (F)	Ast	5′ GACTTCAATCGCTTCCTCAACG ′3	536	Bin Kingombe et al., (2010)
AHS (R)		5′ GCATCGAAGTCACTGGTGAAGC ′3		
act/hlyA/aer complex (F)	act/hlyA/aer comlex	5′ AGAAGGTGACYACCAAGAACA ′3	400	Balsalobre et al., (2009)
act/hlyA/aer complex (R)		5′ CCACTTCACTTCACCCGGGA ′3		
AHL (F)	Alt	5′ TGCTGGGCCTGCGTCTGGCGGT ′3	361	Bin Kingombe et al., (2010)
AHL (R)		5′ AGGAACTCGTTGACGAAGCAGG ′3		

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DNA amplification for the selected virulent genes

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 µl of PCR mixture. The reaction mix invariably consisted of 10X PCR buffer (100 mM Tris [pH 9.0], 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin), 10 mM $MgCl_2$, 100 mM of each dNTP, 25 pmol of each forward and reverse primers and 1.25 U of Taq DNA polymerase. The PCR cycling protocol was applied as following: An initial denaturation at 94°C for three minute, followed by denaturation at 94°C for 60 sec, extension at 72°C for 90 sec for 45 cycles, and final extension at 72°C for three min. Finally, 5 µl of each amplicon was electrophoresed in 1.5% agarose gel (Sigma –USA), stained with ethidium bromide and visualized as well as captured on UV trans-illuminator. A 100 bp DNA ladder was used as a marker for PCR products (Hussain et al., 2013).

Experimental infection

Total Bacterial count: The pour plate method for estimation of *A. hydrophila* strains per one ml, was used in demonstration of the inoculum dose for the experimental studies according to Cruickshank et al. (1975).

Lethal Dose fifty (LD_{50}): A total number of 210 apparently healthy *Oreochromis niloticus* fish weighting 40±0.5 gm, were divided into three main groups in which each group was subdivided into seven subgroups, 10 fish per each group and the seventh group was kept as a control group. All fishes were kept for 15 days under observation prior to injection for accommodation and to confirm that they are free from diseases.

24 hours colony cultures of *A. hydrophila* strains on TSA were used. The colonies were picked up and suspended in sterile saline in a tenfold serial dilution with subsequent incubation at 28 °C for 24 hours for plate counts on TSA. Only the dilutions (10^2-10^7CFU) were used. Each group was intra-peritoneally injected with 0.5 ml/fish of each bacterial dilution. The fishes in the control group were injected with 0.5 ml PBS/fish. All the injected fish were kept for one week post-inoculation for observation. The mortalities were recorded twice/day according to Ibrahim et al. (2011). The freshly dead fishes were moved for further post-mortem examination. The LD_{50} (the dose which kill 50% of the injected fish) was calculated according to Reed and Muench (1938).

Pathogenicity test: Experimental infection was carried out to determine the pathogenicity of *A. hydrophila* strains using intra-peritoneal route injection according to Li et al. (2011). A total number of 100 apparently healthy *O. niloticus* fish weighting 40±0.5 gm, were divided into five groups, 20 fishes per each. Each fish in the first group was intra-peritoneally injected with 0.2 ml/fish of LD_{50} dose of *A. hydrophila* strain (A1) which was determined previously (2.3 x 10^6cfu). Each fish in the second group was intra-peritoneally injected with 0.2 ml/fish of LD_{50} dose of *A. hydrophila* strain (A2) which was determined previously (2 x 10^6cfu). Each fish in the third group was intra-peritoneally injected with 0.2 ml/fish of LD_{50} dose of *A. hydrophila* strain (A3) which was determined previously (1.8 x 10^6cfu). Each fish in the fourth group (control negative group), was intra-peritoneally injected with 0.2 ml/fish of PBS. Each fish in the fifth group (Control positive group), was intra-peritoneally injected with 0.2 ml/fish of the obtained reference *A. hydrophila* strain. All injected fishes were observed for a period of 28 days post-inoculation. Mortalities were recorded daily and freshly dead fishes were moved for further pm examination and histopathological studies.

Histopathological examination

Specimens from liver, kidney and spleen of experimentally infected fishes were taken. Specimens were fixed immediately in 10% neutral buffered formalin, dehydrated and embedded in paraffin blocks. Paraffin blocks were sectioned at 4-5 µm thickness and stained with Hematoxylin and Eosin (H&E) and examined under light microscope (Leica) using ×200 and ×400 magnification power according to Bancroft and Gamble (2007).

RESULTS AND DISCUSSION

External examination of naturally infected *Oreochromis niloticus* revealed hemorrhages on the external surface and surrounding the anus (Figure 1 and Figure 2) and cutaneous ulcers (Figure 3). The gross lesions displayed in the current study are similar to those reported by several author**s** (Harikrishnan and Balasundaram, 2005; Sarkar and Rashid, 2012; Yardimci and Aydin, 2011). However, the postmortem findings exhibited the presence of abundant ascetic fluid in the abdominal cavity together with hemorrhagic gas bladder and congested liver (Figure 4). The postmortem findings displayed in the present study are similar to those reported by (Miyazaki and Kaige, 1985; Popovic et al., 2000; Harikrishnan and Balasundaram, 2005). The current postmortem findings may be due to the action of extracellular products of *A. hydrophila* which secretes haemolysin, aerolysin and cytotoxic toxins that possess a haemolytic, cytolytic and enterotoxic activities **(**Harikrishnan and Balasundaram, 2005).

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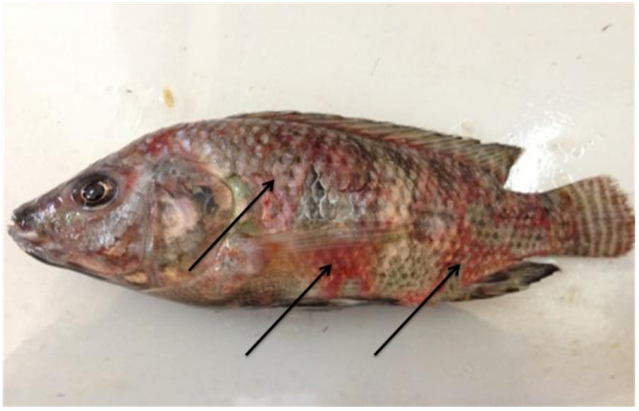

Figure 1. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *A. hydrophila* showing hemorrhagic patches (black arrows) on different parts of the body

Figure 2. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in autumn, naturally infected with *A. hydrophila* showing exophthalmia and blindness (black arrow)

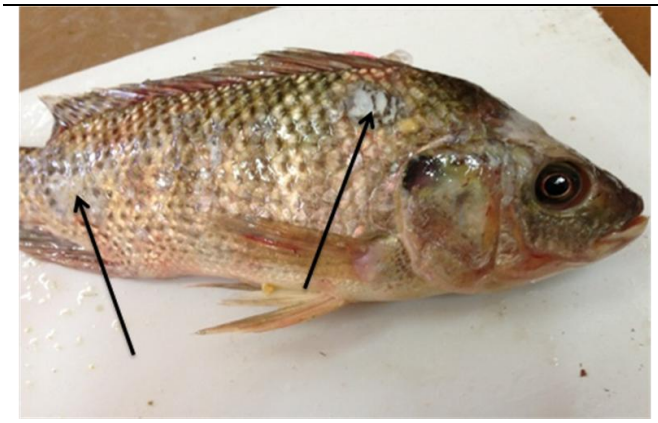

Figure 3. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *A. hydrophila* showing cutaneous ulcers on the body (black arrows)

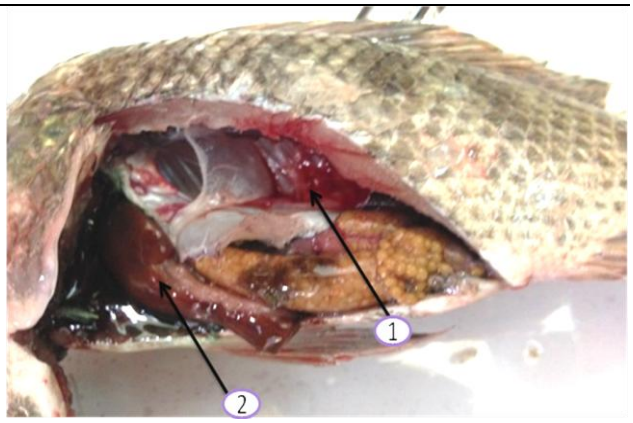

Figure 4. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in autumn, naturally infected with *A .hydrophila* showing hemorrhagic gas bladder (black arrow-1) and congested liver (black arrow-2)

Table 2. Comparison of the phenotypic and biochemical characteristics between the isolated *A. hydrophila* strains and the reference strain

Biochemical Tests	**RESULTS**			
	***Aeromonas hydrophila* strains**			
	A1	**A2**	**A3**	**Reference strain**
Motility	+	+	+	+
Gram staining	-	-	-	-
Indole	+	+	+	+
MR*	-	-	-	-
VP**	+	+	+	+
Citrate utilization	+	+	+	+
Catalase	+	+	+	+
Triple Sugar Iron	+	+	+	+
Urease	-	-	-	-
Oxidase	+	+	+	+
Lactose	+	+	+	+
Glucose	+	+	+	+
Trehalose	+	+	+	+
Starch Hydrolysis	+	+	+	+
Gelatin Hydrolysis	+	+	+	+

*MR: methyl red test; ** VP: Voges Proskauer

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The presumptive identification of the bacteria in the recent study was carried out from the colony morphology over (RS) medium which acts as a selective medium for *A. hydrophila*. They produced deep cream or light yellow colonies with entire margin over the RS-medium. This obtained result is similar to that obtained by Sarkar et al. (2013). The bacteria were observed as Gram-negative motile rods microscopically**;** the result which coincides with those reported by Cartwright et al. (1994). Biochemical characterizations have proven to be a valuable method for typing and differentiation of bacterial fish pathogens (Sarker et al., 2012). In the present study, based on 15 biochemical tests, a total number of 45 isolates were positively identified as *A. hydrophila*. All strains were positive for catalase, indole test, voges proskauer, oxidase test, citrate utilization, carbohydrate utilization, lactose, trehalose, glucose, starch, gelatine hydrolysis and for triple sugar iron agar medium. The strains are observed to be negative for methyl red and urease test (Table 2). Most of the phenotypic characteristics of the isolates were similar to those reported in Bergey's manual of determinative bacteriology (Holt 1994). Based on the biochemical tests results, all obtained isolates were similar to *A. hydrophila* reference strain. The obtained biochemical results were similar to findings of Jayavignesh et al. (2011). The PCR amplification with *A. hydrophila* specific primers (*A. hydrophila* specific-16S rRNA) identified 45 isolates into *A. hydrophila* species. Three of *A. hydrophila* positive strains were screened for the presence of five virulence genes (ast), cytotoxic enterotoxin, (act/hlyA/aer) complex and (alt) by PCR technique. The results revealed that two of the three screened *A. hydrophila* strains had the five virulence genes (ast, act, hlyA, aer and alt), while the third strain had only four virulence genes (ast, act, hlyA and aer) (Figure 5).

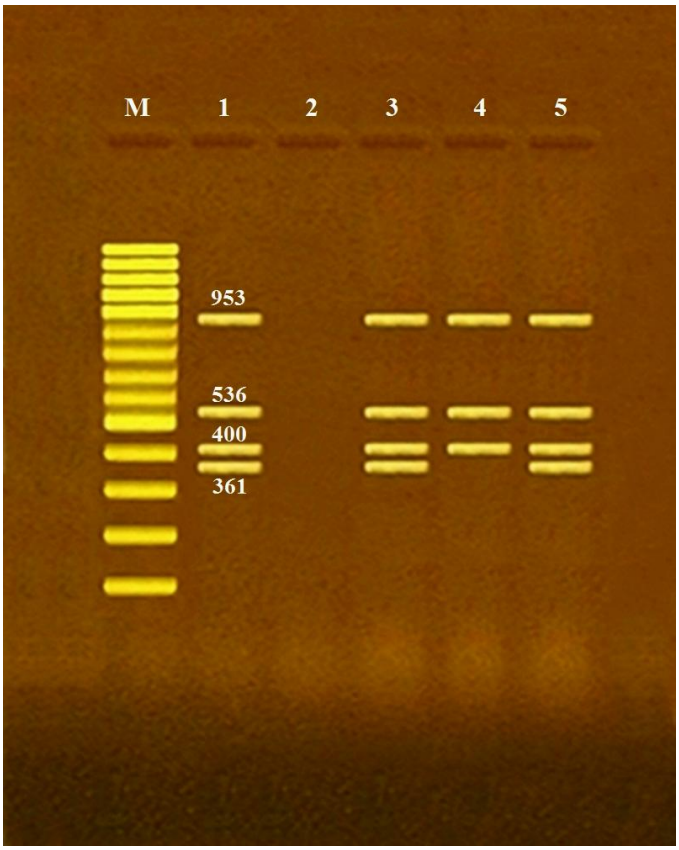

Figure 5. Agarose gel electrophoresis of multiplex PCR of 16S rRNA (953 bp), ast (536 bp), act/hlyA/aer complex (400 bp) and alt (361 bp) virulence genes for characterization of *A. hydrophila.*

Lane M: 100 bp ladder as molecular size DNA marker; Lane 1: Control positive *A.hydrophila* for 16S rRNA, ast, act/hlyA/aer complex and alt genes; Lane 2: Control negative; Lane 3 and Lane 5: Positive *A. hydrophila* strains for 16S rRNA, ast, act/hlyA/aer complex and alt genes; Lanes 4: Positive *A. hydrophila* strainsfor 16S rRNA, ast and act/hlyA/aer complex genes

In *Aeromonas* spp., as with all pathogens, disease is the result of complex molecular interactions between bacterium, environment, and host; however, the literature on *A. hydrophila* remains limited by the lack of experimental data on validated members of *A. hydrophila*. While there was numerous virulence factors shared between members of *A. hydrophila* (Rasmussen-Ivey et al., 2016). It has been reported that the five virulence genes (*ast, act, hlyA, aer* and *alt*), obtained in the current study were specific to pathogenic *A. hydrophila* (Cartwright et al., 1994; Paniagua et al., 1990). The virulence of *A. hydrophila* is complex and involves multiple virulence factors, which may work in concert enabling the bacteria to colonize, gain entry, establish, replicate and damage the host tissues and to evade the host defense system and spread, eventually killing the host. Haemolytic toxins; haemolysin and aerolysin released by *A. hydrophila* may be used as a marker of pathogenicity of *A. hydrophila* (Al-Maleky et al., 2011).

The LD_{50} experiments in the present study revealed that the concentration 10^6cfu was the most potent dilution causing 50% mortalities within (24-48 hr). This result is similar to Pachanawan et al. (2008) and Omar (2014) but, disagrees with Viji et al. (2011) and Li et al. (2011) who obtained LD_{50} at concentration of 10^3 -10^5cfu. The differences in LD_{50} between different authors may be due to the number of the virulent genes in *A. hydrophila* strains, pathogen properties as well as size and weight of fish species. The LD_{50} experiments with *A. hydrophila* strains in *O. niloticus* revealed a higher mortality rate for A1, A2 and A3strains which may be due to the higher number of virulence genes, five virulence genes in A1 and A3 and 4 virulence genes in A2 which may be responsible for the production of extracellular toxins as well as, the synergistic effects conferred by combination of several virulence genes (Viji et al., 2011).

The clinical signs of experimentally infected *O. niloticus* with *A. hydrophila* strains revealed that the fish became lethargic and anorexic, with increased respiratory frequency and mucus production. Abnormal movement and loss of

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balance were observed. The injected fish were characterized by swollen abdomen, red mouth, hemorrhages on the external surface and surrounding the anus. Hemorrhages on the external body surface may be attributed to the hemolytic effect of the exotoxins, haemolysin (α- hemolysins) and aerolysin (β-hemolysins). Both hemolysins induce pore-formation in the cell membrane of the RBCs, leading to haemolysis Zhang et al. (2000); Singh et al. (2008); Singh et al. (2010) and Hidalgo and Figueras (2013).

The Pathogenesis of motile aeromonas septicemia is complex and associated with multiple virulence factors. The recorded mortalities of the experimentally infected fishes may be attributed to the presence of more than one virulent gene in the same strain as well as the synergistic effects conferred by combination of several virulence genes including (ast) and (alt), which increase the vascular permeability of the gut causing detachment of the intestinal mucosa and cytotoxic enterotoxin which possess a hemolytic, cytolytic, and enterotoxic activities Harikrishnan and Balasundaram (2005).

The histopathological findings of the experimentally infected *O. niloticus* in the present study were represented by diffuse degenerative lesions especially in parenchymatous organs such as liver and kidneys. The liver showed diffuse hepatocytes vacuolation, degeneration of the pancreatic portion and multifocal necrotic foci (Figure 6). Similarly, the kidney showed diffuse degenerative lesions extended to coagulative necrosis of the renal tubules (Figure 7). These findings were nearly similar to those reported by Harikrishnan and Balasundaram, (2005); Oliveira et al. (2012). The findings of histopathological changes might be attributed to different virulence genes (hemolysin, aerolysin, cytotoxic enterotoxin and cytotonic heat stable enterotoxin). These virulence factors exhibit hemolytic, cytotoxic and enterotoxic activities that play a role in the establishment of *A. hydrophila* infection (Li et al., 2011).

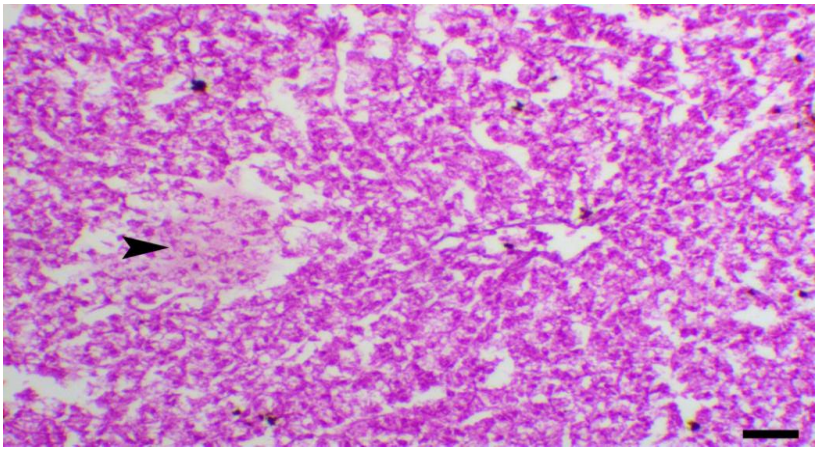

Figure 6. Liver of *Oreochromis niloticus* infected with *A. hydrophila* showing necrotic foci (arrowhead), H&E, bar=100µm, ×200

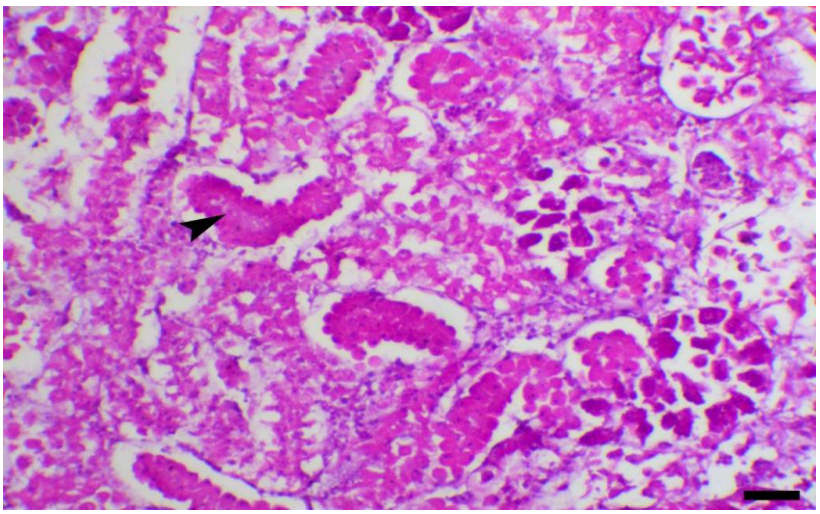

Figure 7. Kidney of *Oreochromis niloticus* infected with *A. hydrophila* showing coagulative necrosis of the renal tubules (arrowhead), H&E, bar=100µm, ×200

CONCLUSION

A. hydrophila is a Gram-negative, rod-shaped, facultatively anaerobic bacterium; a pathogen, not only to fish but also to human causing gastroenteritis. *A. hydrophila* strains possess a wide range of virulence factors enabling the pathogen to evade the host defense mechanisms, spread and eventually kill the host. Virulence genes act as a key component in determining the potential pathogenicity of the micro-organism, acting multifunctionally and multifactorially and can be used for virulence typing of *A. hydrophila* isolates. In the present work, the five screened

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virulence genes were positively correlated with *A. hydrophila* pathogenicity and the presence of virulence genes in pathogenic *A. hydrophila* strains may help in diagnosis, prevention and control of the disease.

Competing interests

Authors have declared that there is no competing interest.

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Molecular Detection of *Streptococcus* Species Isolated from Cows with Mastitis

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ABSTRACT

Streptococcal mastitis is considered as one of the most common infectious diseases in the dairy cattle, which threatens the dairy industry all over the world. The aim of this study was to determine the prevalence of *Streptococcus* species in mastitic cows with molecular investigation to detect the presence of some virulence genes of the recovered isolates by PCR. A total of 150 milk samples were collected from dairy cattle with clinical and subclinical mastitis from different areas in El- Gharbia governorate, Egypt. *Streptococcus* species were isolated with an incidence of 38%. *S. agalactiae, S. dysgalactiae, S. uberis, S. pyogenes, S. pneumoniae* and *S. faecalis* were isolated from the milk samples of the examined cows with the percentage of 14.7%, 6%, 9.3%, 4.7%, 1.3% and 2%, respectively. Molecular investigation of virulence associated genes revealed that sip, cfb and bca genes of *S. agalactiae* were found with the percentage of 77.7%, 88.8% and 33.3%, respectively. The mig gene of *S. dysgalactiae* was found with an incidence of 77.8%. Of the examined *S. uberis* isolates, 55.5%, 22.2% and 33.3% were carrying the cfu, oppF and has A genes, respectively. The present study revealed the prevalence of *Streptococci* and distribution of virulence associated genes among the isolates. The high frequency of virulence genes in the isolates suggests an important role of these virulence genes in the pathogenesis of *Streptococci* in cattle mastitis.

Key words: Mastitis, Cows, *Streptococci*, Virulence genes

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INTRODUCTION

Bovine mastitis is one of the most frequent diseases in dairy cattle which causes major economic losses due to the reduced milk quantity and increased costs of treatment (Koskinen et al., 2009). Mastitis is an inflammation of the mammary gland that occurs as a response to injury. This disease is mainly caused by microorganisms usually bacteria, including Gram-negative and Gram-positive bacteria, mycoplasma, yeasts and algae (Zadoks et al., 2011).

Streptococci are Gram-positive bacteria widespread in the environment and are also commensal organisms of the cow's udder, mucosa and skin. Some of the commensal *Streptococcus* are opportunistic bacteria causing infection if the balance between bacteria and host is broken. Others are considered primary pathogens of mastitis (Cleary and Cheng, 2006). Among *Streptococcus* species, *S. agalactiae* (contagious agent), *S. uberis* (environmental agent) and *S. dysgalactiae* are the predominant group of organisms isolated from mastitis (Kuang et al., 2009).

Streptococcus agalactiae, the lone member of the Lancefield group B, is an important cause of chronic and contagious bovine mastitis. Its presence is frequently associated with high somatic cell counts in milk and decreased milk yield (Jain et al., 2012). However, *S. agalactiae* possesses several virulence factors including structural components, toxins and enzymes that play an important role in the intra-mammary infections (Krishnaveni et al., 2014). *Streptococcus dysgalactiae* is considered as a contagious pathogen, which adapts to the udder environment and can be spread from cow to cow during milking (Yanliang et al., 2016). However, this bacterial pathogen may contain several putative virulence associated genes which are not fully understood (Calvinho et al., 1998). One of these genes, a surface- expressed M-like protein, named mig, has been found to bind to the Immunoglobulin G (IgG), K2-macroglobulin (K2-M) (Jonsson and

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Muller, 1994) and Bovine Immunoglobulin A (B-IgA) and plays a role in anti-phagocytosis by bovine neutrophils in the presence of bovine serum (Song et al., 2001).

Streptococcus uberis is one of the prime causative agents among the environmental pathogens and is predominantly associated with the clinical and subclinical mastitis in both lactating and non lactating cows (Reinoso et al., 2011). Despite the great economical loss due to high prevalence of *S. uberis*, several virulence factors associated with the pathogenesis are not well understood. Among these factors, resistance to phagocytosis conferred by hyaluronic acid capsule (Ward et al., 2001), Christie, Atkins and Munch Peterson (CAMP) factor (Jiang et al., 1996) and Opp proteins (Smith et al., 2002) have been found. The identification and characterization of *Streptococci* virulence associated genes causing bovine mastitis will open the way for the development the new strategies to prevent and control this bacterial pathogen in dairy herds (El-Behiry et al., 2015). Few reports exist on the prevalence of *Streptococci* as well as the occurrence of virulence-associated genes among *Streptococcus* isolates recovered from cattle with mastitis in Egypt. Therefore, the aim of the present study was to determine the prevalence of *Streptococci* involved in mastitis in cattle with molecular investigation the pattern distribution of some virulence associated genes of the recovered isolates.

MATERIALS AND METHODS

Ethical approval

Handling of animals were according to the guidelines of animal ethics committee, faculty of veterinary medicine, Kafrelsheikh University, Egypt.

Sampling

A total of 150 milk samples (115 from clinical mastitic cows and 35 from apparently healthy cows) were collected from lactating cows (Friesian and Jersey breeds) at different stages of lactation during the period from January 2015 to November 2015 from different areas in El- Gharbia governorate, Egypt. The udder of each animal was examined before sampling for detection of clinical signs of mastitis such as inflammation, asymmetry, hotness, swelling or any physical changes. Each udder was washed and carefully dried with clean towel then the teats were swabbed with 70% alcohol. Before sampling, the first jets of milk were rejected, then each quarter milk sample (nearly 3ml) was collected into sterile screw capped MacCarteny bottle as recommended by Blood and Handerson (1986) and submitted to the laboratory in an ice container as soon as possible for further bacteriological and molecular examination.

Isolation and identification of *Streptococcus* species

Isolation of suspected *Streptococci* was done according to Quinn et al. (2002). Briefly, the collected milk samples were incubated aerobically at 37°C for 24 h, then centrifuged at 3000 rpm for 20 min. The cream and supernatant fluid were discarded. Methylene blue stain was used routinely to detect the suggestive bacterial causes. The sediment was streaked on the surface of 5% sheep blood agar and Edward's media (Oxoid). The inoculated plates were incubated at 37°C for 24-48 h, and examined for bacterial growth. Suspected streptococcal colonies were sub-cultured, purified and preserved in semisolid agar for further identification which was done according to Cowan (1979); Carter and Cole (1990). Also, CAMP test was carried out according to Koneman et al. (1988). The subclinical samples were subjected to total somatic cell count using California Mastitis Test (CMT) in order to confirm the subclinical status of the collected samples.

Molecular detection of *Streptococcus* virulence associated genes

Bacterial DNA extraction was done by using QIA amp DNA mini and Blood mini kit according to the manufacturer's instructions. A molecular identification of *Streptococcus* species was performed by using species-specific oligonucleotide primers for the genes encoding 16S rRNA for the identification of *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. For detection of some virulence associated genes, *S. agalactiae* isolates were screened for Sip, cfb and bca. *S. dysgalactiae* isolates were screened for mig while *S. uberis* isolates were screened for cfu, oppE and hasA genes. The primer pairs used in PCR protocols were selected from published papers based on specificity, compatibility and ability to target the potential virulence genes of interest.

The nucleotide sequences and anticipated molecular sizes of PCR amplified fragments for these gene-specific oligonucleotide primer sets are outlined in table 1. PCR reaction mixtures and conditions are given in table 2. The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The PCR products were analyzed by electrophoresis using a 1.5 % agarose gel in Tris-Borate EDTA (TBE) buffer. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

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Table 1. Nucleotide sequence and product length of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* virulence gene specific primers

Streptococcus spp.	Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
S. agalactiae	16S rRNA(F)	5′ ATTGATAACGACGGTGTTACTGT ′3	487	Raemy et al. (2013)
	16S rRNA(R)	5′ CATAGTAGCGTTCTGTAATGATGTC ′3	487	Raemy et al. (2013)
	sip (F)	5′ ACTATTGACATCGACAATGGCAGC ′3	266	Nithinprabhu et al. (2010)
	sip (R)	5′ GTTACTGTCAGTGTTGTCTCA′3	266	Nithinprabhu et al. (2010)
	Cfb (F)	5′CAAAGATAATGTTCAGGGAACAGATTATG′3	320	Krishnaveni et al. (2014)
	Cfb (R)	5′ CTTTTGTTCTAATGCCTTTACGTT ′3	320	Krishnaveni et al. (2014)
	bca (F)	5′ TAACAGTTATGATACTTCACAGAC ′3	535	Manning et al. (2006)
	bca (R)	5′ ACGACTTTCTTCCGTCCACTTAG ′3	535	Manning et al. (2006)
S. dysgalactiae	16S rRNA(F)	5′ GTGCAACTGCATCACTATGAG ′3	279	Raemy et al. (2013)
	16S rRNA(R)	5′ CGTCACATGGTGGAT TTTC ′3	279	Raemy et al. (2013)
	mig (F)	5′ CGTTTTTAGTTTCGGGAGCA ′3	188	Nithinprabhu et al. (2010)
	mig (R)	5′ TGCCTTCAATTGAGTCTGCTG ′3	188	Nithinprabhu et al. (2010)
S. uberis	16S rRNA(F)	5′ TGATTCCGACTACTACGCTAGAT ′3	723	Raemy et al. (2013)
	16S rRNA(R)	5′ ATACTTTGAGTTTCACCGAGTTC ′3	723	Raemy et al. (2013)
	cfu (F)	5′ TATCCCGATTTGCAGCCTAC ′3	205	Reinoso et al. (2011)
	cfu (R)	5′ CCTGGTCAACTTGTGCAACTG ′3	205	Reinoso et al. (2011)
	oppE (F)	5′ GGCCTAACCAAAACGAAACA ′3	419	Smith et al. (2002)
	oppE (R)	5′ GGCTCTGGAATTGCTGAAAG′3	419	Smith et al. (2002)
	hasA (F)	5′ GAAAGGTCTGATGCTGAT ′3	600	Ward et al. (2001)
	hasA (R)	5′ TCATCCCCTATGCTTACAG ′3	600	Ward et al. (2001)

Table 2. PCR assay conditions and reaction mixture for molecular identification of *Streptococcus* species and detection of the virulence associated genes

Reaction	Assay (1) (Raemy et al.,2013)			Assay (2) (El-Behiry et al., 2015)			Assay (3) (Krishnaveni et al., 2014)			Assay (4) (Reinoso et al., 2011)		
	Temp.(°C)	Duration	Cycle	Temp.(°C)	Duration	Cycle	Temp.(°C)	Duration	Cycle	Temp.(°C)	Duration	Cycle
Denaturation	94	60 sec	1	94	30 sec	1	94	30 sec	1	94	30 sec	1
Annealing	58	60 sec	35	45	60 sec	25	55	30 sec	30	48-58	30 sec	25
Extension	72	10 min		72	90 sec		72	30 sec		72	10 min	
Elongation	-	-	-	-	-	-	72	10 min		-	-	
Cooling	4	Infinite	-	4	Infinite	-	4	Infinite	-	4	Infinite	-
Reaction mixture	(total volume = 25 µl): 1x HotStarTaq Master Mix + 2.5 µl of diluted lysate, and 300 µM of each primer (16S rRNA of *S. agalactiae*, *S. dysgalactiae* and *S. uberis*)			25 µl containing PCR master mix with 3 mm of $MgCl_2$ + 3 µl of template DNA and 0.5 µM of each primer (*sip*, *cfb*, *bca* genes of *S. agalactiae*)			25 µl of 10X PCR Taq Buffer A+ 1µl (20 pmol) of *mig* primers of *S. dysgalactiae* + 1 µl (100 µM) of each dNTPs and 3 µl (150 ng) of template DNA			50 µl containing 1.50 U Taq polymerase with 1.5 mM $MgCl_2$, 1 µM of each primer(*cfu*, *opp*F, *has*A genes of *S. uberis*) , 0.4 µM of each of the dNTPs and 20 ng template DNA		

Assay 1 is designed for molecular identification of *S. agalactiae, S. dysgalactiae* and *S. uberis*. Assays 2, 3 and 4 are for molecular detection of virulence associated genes of *S. agalactiae, S. dysgalactiae* and *S. uberis*, respectively

RESULTS

Incidence of *Streptococcus* species in mastitic cows

Among the 150 examined milk samples, 115 from clinical cases and 35 from apparently healthy cows were subjected to CMT for detection of subclinical mastitis, 25 were positive for CMT and 10 were negative. A total of 57 isolates of *Streptococcus* species were isolated and identified [41 from clinical mastitis (35.7%) and 16 from subclinical

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cases (64%)]. In clinical mastitis samples, *S. agalactiae* was the predominant species (13.9%) followed by *S. uberis* (9.6%), *S. dysgalactiae* (6.1%), *S. pyogenes* (3.5%), *S. pneumoniae* (1.7%) and *S. fecalis* (0.9%). While in subclinical mastitis, *S. agalactiae* was also the most frequent isolated species (17.1%), followed by *S. uberis*, *S. pyogenes* (8.6% each) *S. dysgalactiae* and *S. fecalis* (5.7% each) (Table 3).

Molecular detection of *Streptococcus* species virulence associated genes by PCR

As a result of the molecular screening of 27 *Streptococcus* species using species specific PCR, nine isolates for each *S. agalactiae*, *S. dysgalactiae* and *S. uberis* were identified (Figure1). PCR based screening of virulence genes revealed the presence of cfb, sip and bca in *S. agalactiae* isolates with the percentage of 88.8%, 77.7% and 33.3%, respectively (Figure 2),whereas 77.8% of *S. dysgalactiae* were carrying mig gene (Figure 3). Similarly, cfu, oppF and hasA genes were detected in *S. uberis* isolates with the percentage of 55.5%, 22.2% and 33.3%, respectively (Figure 4) (Table 5).

Table 3. The incidence of *Streptococcus* species among the examined Cow's milk samples in El- Gharbia governorate, Egypt during the period from January 2015 to November 2015

***Streptococcus* Species**	Clinical mastitis (n=115)		Subclinical mastitis (n=35)		Total (n=150)	
	No.	**%**	**No.**	**%**	**No.**	**%**
S. agalactiae	16	13.9	6	17.1	22	14.7
S. dysgalactiae	7	6.1	2	5.7	9	6
S. uberis	11	9.6	3	8.6	14	9.3
S. pyogenes	4	3.5	3	8.6	7	4.7
S. pneumonia	2	1.7	0	0	2	1.3
S. fecalis	1	0.9	2	5.7	3	2
Total	41	35.7	16	45.7	57	38

No = positive number; % was calculated according to the total number of examined animals

Table 4. The incidence of some virulence associated genes in *Streptococcus* species isolated from Friesian and Jersey breeds in El- Gharbia governorate, Egypt during the period from January 2015 to November 2015

***S. agalactiae* (n=9)**						***S. dysgalctiae* (n=9)**		***S. uberis* (n=9)**					
Sip		Cfb		bca		Mig		cfu		oppF		hasA	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
7	77.8	8	88.8	3	33.3	7	77.8	5	55.5	2	22.2	3	33.3

No = positive number; % was calculated according to the positive examined samples for each species

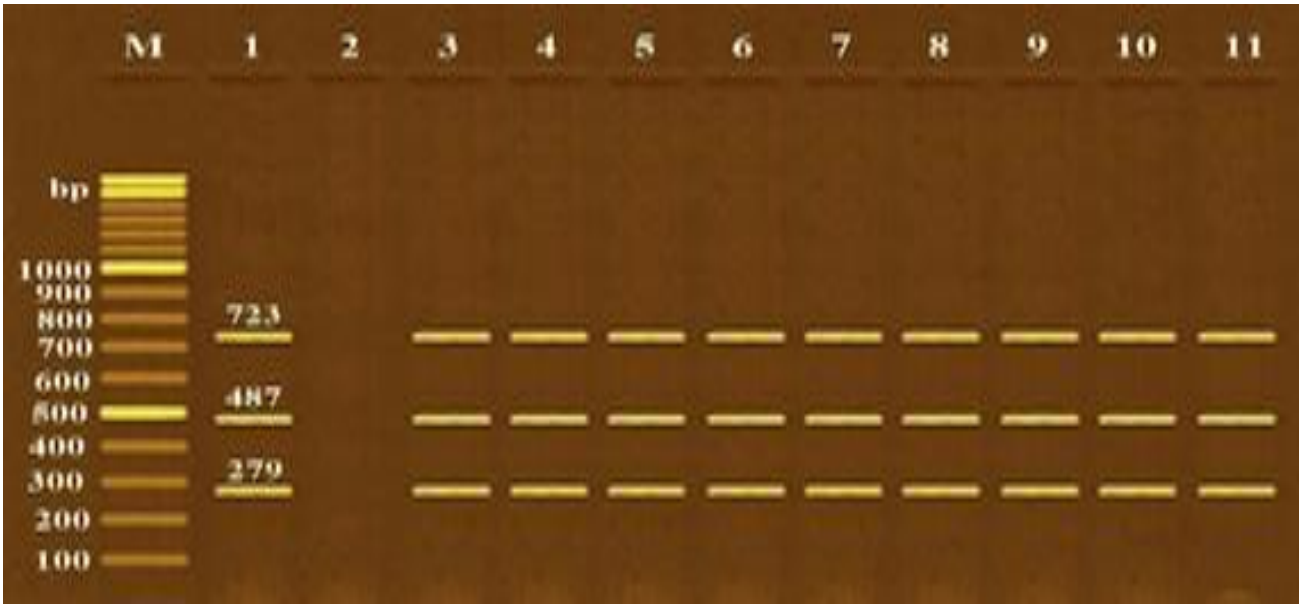

Figure 1. Agarose gel electrophoresis of multiplex PCR of GSag (487 bp) for *Streptococcus agalactiae*, GSdys (279 bp) for *Streptococcus dysgalactiae* and GSub (723 bp) for *Streptococcus uberis* as species specific genes for their identification (M=100 bp ladder, Lane 1: Control positive *Streptococcus* species for GSag, GSdys and GSub genes, Lane 2: Control negative, Lanes 3-9: positive for GSag, GSdys and GSub for *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, respectively)

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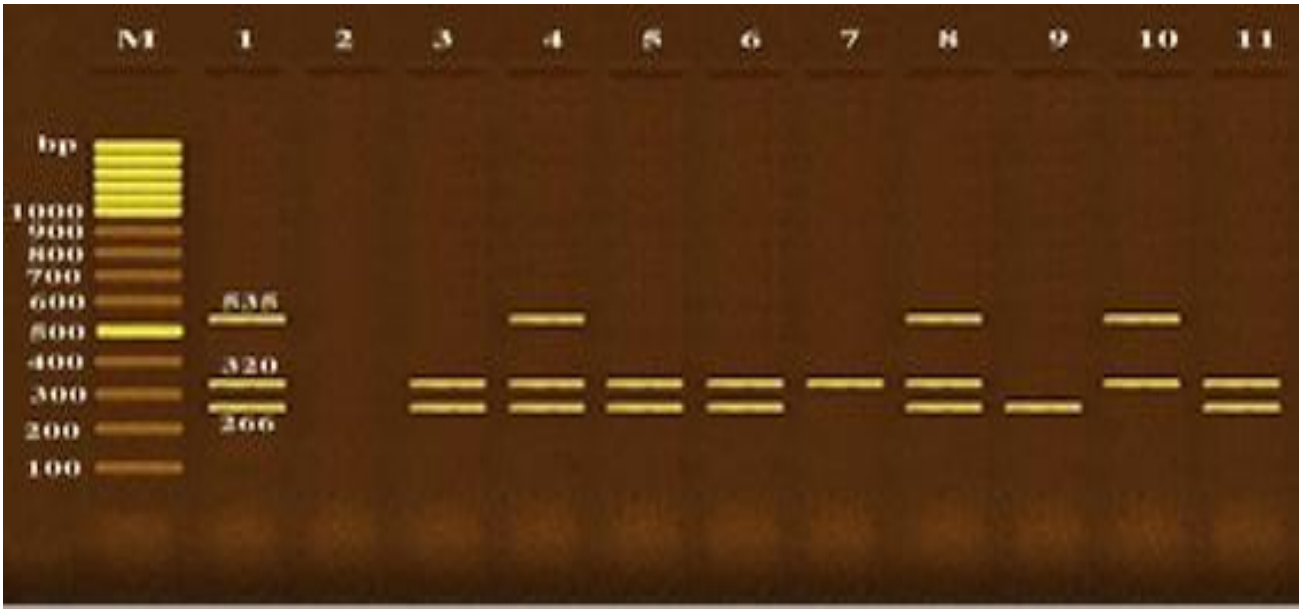

Figure 2. Agarose gel electrophoresis of multiplex PCR of *sip* (266 bp), cfb (320 bp) and bca (535 bp) as virulence genes for characterization of *Streptococcus agalactiae* strains (M= 100 bp ladder, Lane 1: Control positive *Streptococcus agalactiae* for sip, cfb and bca genes, Lane 2: Control negative, Lane 3, 5-6 and 11: Positive *Streptococcus agalactiae* for sip and cfb genes, Lanes 4 and 8: Positive *Streptococcus agalactiae* for sip, cfb and bca genes, Lane 7: Positive *Streptococcus agalactiae* for cfb gene, Lane 9: Positive *Streptococcus agalactiae* strain for sip gene and Lane 10: Positive *Streptococcus agalactiae* strain for cfb and bca genes)

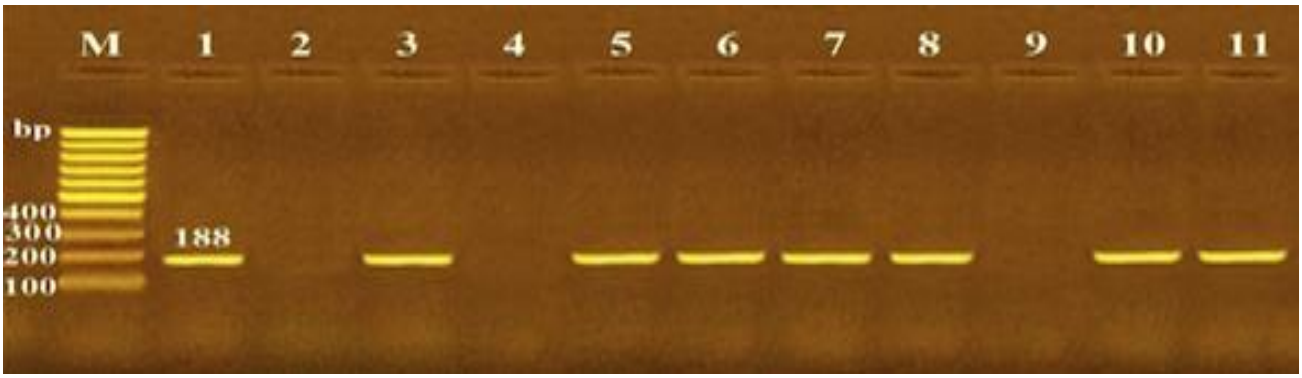

Figure 3. Agarose gel electrophoresis of PCR of mig protein gene (188 bp) virulence gene for characterization of *Streptococcus dysgalactiae* strains (M=100 bp ladder, Lane 1: Control positive *Streptococcus dysgalactiae* for mig gene, Lane 2: Control negative, Lane 3, 5-8, 10 and 11: Positive *Streptococcus dysgalactiae* strains for mig gene and Lanes 4 and 9: Negative *Streptococcus dysgalactiae* strains for mig gene)

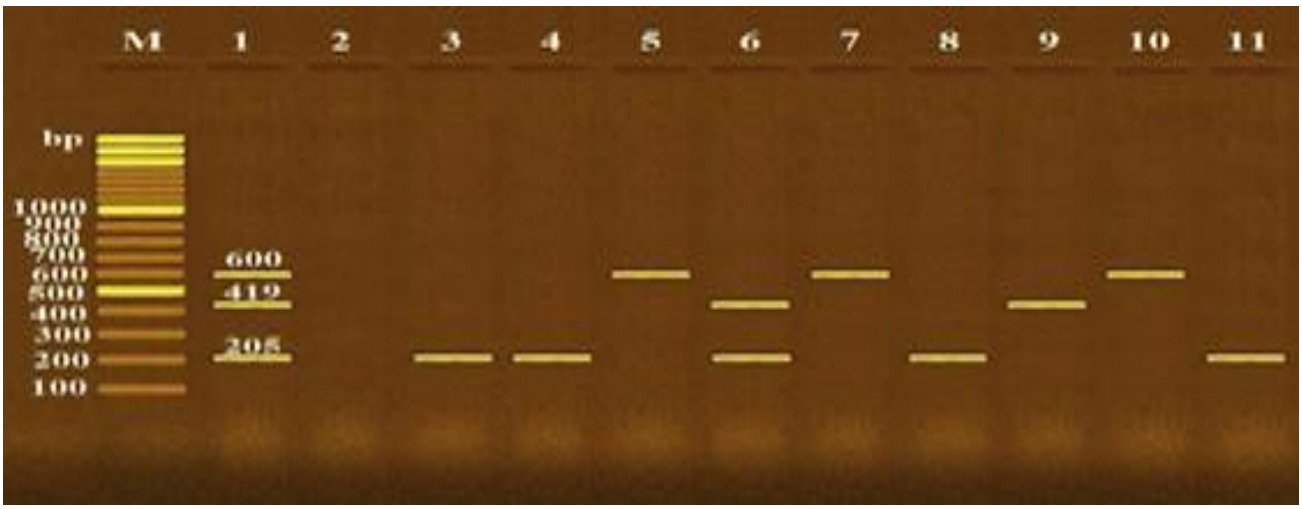

Figure 4. Agarose gel electrophoresis of multiplex PCR of cfu (205 bp), oppF (419 bp) and hasA (600 bp) as virulence genes for characterization of *Streptococcus uberis* strains (M=100 bp ladder, Lane 1: Control positive *Streptococcus uberis* for cfu, oppF and hasA genes, Lane 2: Control negative, Lane 3-4, 6, 8 and 11: Positive *Streptococcus uberis* strains for cfu gene, Lanes 6 and 9: Positive *Streptococcus uberis* strains for oppF gene and Lanes 5, 7 and 10: Positive *Streptococcus uberis* strains for *has A gene*)

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DISCUSSION

Streptococcus species belong to a large group of organisms which are associated with bovine udder infections (Wyder et al., 2011). In the current study, a total of 150 milk samples were collected from cattle with mastitis (115 from clinical cases and 35 from apparently healthy cows, 25 were California mastitis test positive and represent subclinical mastitis and 10 samples were CMT negative and were discarded) and examined for presence of *Streptococci*. Fifty-seven *Streptococci* were recovered with a prevalence rate of 38%. This finding is nearly consistent with the report described by Mohanty et al. (2013). However, the present study shows much lower isolation rate than El Jakee et al. (2013) who isolated *Streptococci* with an incidence of 55%. In the present investigation, 16 *Streptococci* isolates were recovered from subclinical cases of mastitis with an incidence of 64%. Present result was in agreement with those obtained by Wahba et al. (2005). While a higher incidence of *Streptococci* in subclinical mastitis was obtained by Kia et al. (2014) with an incidence of 75%. However, other studies have reported a lower incidence of *Streptococci* from subclinical mastitis (Esron et al., 2005; Ranjan et al., 2011; chen et al., 2012; Jeykumar et al., 2013) who recovered *Streptococci* with the percentage of 9.8%, 5.7%, 15.5% and 16.1%, respectively.

With regard to clinical cases of mastitis, 41 isolates of *Streptococci* were recovered with a percentage of 35.7%. Contrary to our results, a lower incidence was recently reported by Demme and Abegaz (2015) who isolated *Streptococcus* from clinical cases at a rate of 16.7%.In our study, six different species of *Streptococci* (*S. agalactiae, S. uberis, S. dysgalactiae, S. pyogenes, S. pneumoniae* and *S. fecalis)* were isolated and identified.

Our results showed a clear overall predominance of *S. agalactiae* among *Streptococcus* species (14.7%). This finding confirms the results reported by Klimiene et al. (2005) and Kivaria and Noordhuizen (2007) who isolated *S. agalactiae* with an incidence of 15.1% and 15.4%, respectively. The high prevalence of *S. agalactiae* may indicate the poor management for the investigated cows. While higher incidences of *S. agalactiae* isolated from mastitic cows were recovered by Kuzma and Malinowski (2001), Khan and Mohammad (2005), Borkowoska et al. (2006), Momtaz et al. (2012) and El-Jakee et al. (2013) with isolation rate of 41.2%, 30%, 84.8%, 16%, 19.3%, respectively.

In our report, *S. uberis* was the second predominant *Streptococcus* species isolated from mastitic cattle with a percentage of 9.3%. A similar prevalence rate was also recently reported by El-Bagory and Zayda (2015) (9.4%). Other studies have been reported a higher incidence rate of *S. uberis* by Zadoks et al. (2003) (26%), Hussain et al. (2006) (15%), Bradley et al. (2007) (23%), Ericsson Unnerstad et al. (2009) (11%) and El Jakee et al. (2013) (15%).

A comparatively lower prevalence rate of *S. uberis* isolated from cattle with mastitis was also reported by other studies (Tenhagen et al., 2006 and Momtaz et al., 2012) with a percentage of 0.1% and 7.3 %, respectively. *S. dysgalactiae* was isolated in our study with an incidence of 6%. Other previous studied reported higher incidences such as Ericsson Unnerstad et al. (2002); Moges et al. (2011) and El Jakee et al. (2013) that isolated *S. dysgalactiae* with a prevalence rate of 15.6%, 14% and 17% respectively. Other *Streptococcus* species were isolated from the examined milk samples as *S. pyogenes, Enterococcus* species and *S. pneumoniae.* Yet these differences may be attributed to other factors rather than geographical location, such as the differences in the samples taken or type of mastitis.

S. pyogenes, a beta-hemolytic bacterium that belongs to Lancefield serogroup A, causes a wide variety of diseases in humans (Khan, 2012). The main reservoir of *S. pyogenes* includes, man, rarely cattle. *S. pyogenes* is almost exclusively associated with man, and contact with infected individuals or asymptomatic carriers is the most common source of infection (McDougall, 2005). However, previous study carried out by Khalil et al. (2014) reported that, the *S. agalactiae* and *S. pyogenes* represent the most important bacterial isolates responsible for severe losses to milk industry, in addition to the zoonotic importance of *S. pyogenes*.

Streptococcus pneumoniae colonizes the nasopharynx in mainly human at any time, and causes serious infectious diseases, such as pneumonia, septicemia, meningitis, and otitis media (Musher et al., 2005). *S. pyogenes* and *S. pneumoniae* were isolated with an incidence of 4.7% and 1.3% respectively. This is in contrast to El Jakee et al. (2013) who isolated *S. pyogen* and *S. pneumoniae* from mastitic cows in a lower percentage (2.7% and 0.7%, respectively). Their presence in the examined milk samples may be due to the bad manipulation during milking from the hand milkers. In cattle, enterococci have been associated with diarrhea in calves and bovine mastitis in dairy cattle (Rogers et al., 1992). *Enterococcus* species were recovered in our study with an incidence of 2%.

In the present study, a total of nine isolates of *S. agalactiae* were screened for some associated virulence genes, cfb, bca and sip by using multiplex PCR. Out of nine isolates, seven (77.8%) contained *sip* gene. Similarly, high percentages of *sip* gene in *S. agalactiae* have been reported by Krishnaveni et al. 2014 and El-Behiry et al. 2015, who reported *sip* gene with an incidence of 100% and 90.69%, respectively.

The cfb gene was detected in eight isolates (88.8%) of the examined *S. agalactiae*. Our results confirm the finding reported by El-Behiry et al. (2015) (93 %). However; other previous studies (Shome et al., 2012; El-Gedawy et al., 2014; Krishnaveni et al., 2014) have been reported that cfb was detected in the all obtained isolates. On the other hand, lower incidence of cfb gene was reported by Ding et al. (2016) who found cfb gene in only 50% of isolates. The cfb gene is a

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cell surface protein that produces a traditional CAMP phenomenon with the typical half-moon forming hemolytic zones on blood agar plates (El-Behiry et al., 2015).

The results in the present study revealed that three isolates of *S. agalactiae* (33.3%) carried the *bca* gene. The *bca* gene codes for Alpha-C protein, a surface protein that helps the bacteria to enter the host cells (Bolduc et al., 2002). Higher incidences of bca gene was reported by Duarte et al. (2004) (64.7%) and Duarte et al. (2005) (78.9%). On the other hand, lower incidences of bca gene was recorded by El-Behiry et al. (2015) (20.93%) and Ding et al. (2016) (3.7%). While Jain et al. (2012) found that none of *S. agalactiae* isolates was carrying the bca gene.

The hyaluronic acid capsule production of *S. uberis* is dependent on the has operon (hasA, hasB and hasC). In this study, hasA gene was detected in three isolates out of nine *S. uberis* (33.3%). This result was nearly agreeing with Matthews et al. (1994) who found that 44% of *S. uberis* strains were carried hasA. However, higher incidence of hasA among *S. uberis* isolates have been reported by other studies (Reinoso et al., 2011; Mirta-Lasagno et al., 2011; Almeida et al., 2013) with the percentage of 74.3, 59.4 and 100%, respectively.

Another potential virulence factor analyzed in the present study was the CAMP factor (*cfu*) which was observed in five isolates out of nine *S. uberis* strains (55.5 %). This finding is consistent with the finding described by Shome et al. (2012) (46.15%). However, other previous studies have been reported high frequency of cfu gene in *S. uberis* isolates (Shalka and Smola 1981; Hassan et al., 2000; Reinoso et al., 2011). While lower incidence was reported by Lammler et al. (1991) and Mirta-Lasagno et al. (2011) who detected cfu in a percentage of 28% and 25%, respectively among *S. uberis* isolates.

With regard to oppF gene, the data presented here showed that only two isolates of *S. uberis* were carring oppF gene with an incidence of 22.2%. OppF gene plays an important role during growth in milk (Smith et al., 2002). Higher incidences of oppF have been described by previous study (Reinoso et al. 2011) (64.1%). Moreover, Almeida et al. (2013) detected oppF in all *S. uberis* isolates.

The mig protein of *S. dysgalactiae* is involved in resisting phagocytosis by bovine neutrophils in the presence of bovine serum (Song et al., 2001). Thus, the mig protein, an M-like protein, is considered as a potential virulence factor of *S. dysgalactiae* (Krishnaveni et al., 2014). This protein could act as the sensory component of a multiple component system, whereby, binding of IgG and or IgA to mig could trigger a conformational change on this protein, resulting in the activation of secondary proteins with histidine-kinase activities that result in the modulation of gene expression of factors involved in virulence (Krishnaveni et al. 2014). The DNA sequence encoding the alpha 2-M receptor portion of the mig gene was different from other *Streptococci* and was highly specific to *S. dysgalactia*e (Jonsson et al., 1994). In the current study, Mig gene was detected in seven out of nine *S. dysgalactiae* isolates with an incidence of 77.8 %. Song et al. (2001) reported that out of 16 *S. dysgalactiae* isolates, only five strains (31%) were found to carry the *mig* α_2-M-homologous sequences linked to the IgG-binding domains.

CONCLUSION

This study revealed that *Streptococcus* species contribute to the occurrence of bovine mastitis in El- Gharbia governorate, Egypt. So, preventive measures must be taken to reduce the spread of infection. The high frequency of virulence genes in the isolates obtained in this work revealed the important role of these virulence genes in the pathogenesis of bovine mastitis. So, from our previous results we conclude that this multiplex PCR assay could be used as an alternative method in routine diagnosis for rapid, sensitive and specific simultaneous identification for *Streptococcus* spp. which play a role in the transmission and pathogenesis of bovine mastitis which is important for diseases control and epidemiological studies.

Competing interests

The authors have no competing interests to declare.

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Constraints of Small-Scale Commercial Poultry Farms Analyzed by Garett's Ranking Technique in and around Debre Markos, Amhara Region, Ethiopia

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ABSTRACT

This study was carried out to assess constraints of small scale commercial poultry farms in and around Debre Markos, Amhara region, Ethiopia. Cross sectional study was involved on the assessment of socio demographic characteristics, flock size and constraints faced by employing structured questionnaire for personal interviews. The socio demographic and flock size data were analyzed by $\chi 2$ and one way analysis of variance, respectively. The constraints faced were ranked by the respondents and the factors were analyzed by Garrett's ranking technique. The result showed that sex, age, marital status, religion, occupation, family size, experience years, educational level had statistically significant effect ($p<0.05$) on the operation of small scale poultry farms. The mean flock size was 844.3 chicks. Flock size was influenced ($P<0.05$) by sex of birds rather than breed. The flock size of female chicks were significantly ($P<0.05$) higher than male chicks. Among the constraints; high cost of feed, unavailability of feed and feed ingredients, unavailability of land , lack of market linkage and promotion, high cost of birds for starting business, lack of finance, lack of extension service and lack of training were listed as 1-10 ranks sequentially. Therefore, for successful poultry production and further expansion in the study area; there is a need to improve market linkage and promotion, provide training on poultry husbandry practice, provision of land and fulfilling the financial needs through facilitating credit services are among the imperatives for improving the current status of small scale commercial poultry production

Key words: Constraints, Flock size, Personal interview, Questionnaire

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INTRODUCTION

Food security and poverty reduction are priority policy issues in the Ethiopian government development plan due to the fact that about 29% of the population lives below the national poverty line (IFAD, 2012). Poor human nutrition has continued due to lack of sufficient energy and protein in the food or due to insufficient availability of food (Abedullah et al., 2007). Shortages of protein availability are a well-known problem in Africa (Haftu, 2016). To fulfil the protein requirements' of the population, animal sources play a significant role. Among the animal protein sources, poultry meat and eggs are important sources of edible animal protein (FAO, 2010). Thus, if food self-sufficiency is to be achieved and to combat malnutrition in developing countries particularly in Ethiopia, there is a need to give due attention to poultry production (Melkamu, 2013). Because poultry is needed not only to fulfill the protein requirements but it also plays a pivotal role in poverty reduction due to its enormous potential to bring about rapid economic growth, particularly benefiting the weaker sections of the society. Further, it requires low capital investment and assures quick returns (Rajendran and Samarendu, 2003).

The total poultry population in Ethiopia is estimated to be about 50.38 million and the estimated poultry populations in the Amhara Region and East Gojjam Zone is 14.6 million and 1.15 million, respectively (CSA, 2013). The prevailing poultry production systems in the mentioned areas include backyard, small-scale, and large-scale commercial production systems.

Commercial poultry production in Ethiopia is characterized by a large number of small scale farms, and a few medium to large scale poultry farms (Nebiyu et al., 2016). There are several emerging small-scale commercial poultry farms in the country in general and in Debre Markos in particular. These emerging farms have vital contribution to

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improve the livelihood, food security and poverty reduction as well as providing a handsome return in semi-urban and urban areas in the tropics (Pica-Ciamarra and Otte, 2010; Emebet, 2015). Also in Ethiopia, it plays a great role as a prime supplier of eggs and meat both in rural and urban area (Haftu, 2016). However, the contribution of poultry in both systems to the Ethiopian economy is only 2-3% due to different constraints (Dana, 1999). The information pertaining to constraints of small-scale poultry farms in and around Debre-Markos was generally scanty. Therefore, this study was carried out to assess the major constraints which hinder the sustainability of small scale poultry farms in the areas studied.

MATERIAL AND METHODS

The Study Area

The study was conducted in and around Debre-Markos, Ethiopia. Debre-Markos is located at 300 km from Addis Ababa in Northwest of the country and 265 km Southeast of Bahir Dar, capital of Amhara Region. The altitude ranges from 500-4154 meter above sea level. The annual rainfall ranges from 900-1800 mm and a minimum and maximum temperature of the area is 7.5^{O}C and 25^{O}C, respectively.

Ethical Approval

Before conducting this survey type research, Debre Markos University ethical approval committee critically assessed and approved the document to undertake this research on small scale commercial farms

Study population

All small-scale poultry farm owners who personally funded and were organized by small and micro enterprise offices in and around Debre Markos were considered as the study population.

Research design

A cross-sectional study was carried out to assess the constraints of small-scale commercial poultry farms. A checklist of constraints was developed to guide the ranking process and farm heads or leaders were asked to rank them according to their priority.

Data collection and analysis

Data were collected by the use of pretested structured questionnaire through personal interview method from heads (owner of the farm) and leaders of the farm (organized in small and micro enterprise offices) to generate in formation on constraints faced in small-scale commercial poultry farms. The farm heads or leaders were asked to rank the factors that were hindering poultry production. These factors were analysed by Garrett's ranking technique within sex steps to interpret the result

Step one: the ranking given by producers for each factor was analysed

Step two: Thus assigned ranks by the individual producers was counted into percent position value by using the formula.

$$Percentage\ position\ = \frac{100(Rij-0.50)}{Nj}$$

Where

Rij - Rank given for the ith factor by the jth individual

Nj - Number of factor ranked by the jth individual.

Step three: For each percent position scores were obtained with reference to Garrett's Ranking Conversion Table and each percent position value were converted into scores by reference to Garrett's Table (Garrett and Woodworth, 1969)

Step four: The summation of these scores for each factor were worked out for the number of respondents who gave ranking for each factor

Step five: Mean scores were calculated by dividing the total score by the number of respondents

Step six: These mean scores for all the factors were arranged in descending order and the most influencing factors were identified through the ranks assigned.

RESULTS

Socio demographic characteristics

The socio demographic characteristics of small scale poultry farmers are presented in table 1. Sex had a significant effect (P< 0.05) on the operation of small scale poultry farms. About 83.7% of the farms were run by males and 16.7%

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were by females. Age had a significant difference (P<0.05) in running of small scale poultry farms. Among the poultry farmers, 85.7% of the age profile ranged 15-30 years. There was no any significant (P>0.05) difference between married and unmarried in small scale poultry production. The unmarried poultry producers were 55.1% and the married were 44.9%. Religion had a significant (P<0.05) effect in poultry operation. Almost 98% of the farms were run by Orthodox Christianity believers and the rest was Muslim. The educational level were highly significant effect (P<0.05) in running poultry farming. Almost one third (36.7%) of small scale farming was run by first degree poultry producers. Nearly more than half (57.1%) of the producers had not any experience and the rest 42.9% of the producers run their farms with experiences. About 57.1% of the producers were new and 40.8% had 1-3 years of experience. Family size had a significant effect (P<0.05) in small scale poultry production. Almost 79.6% of the producers had 1-3 family sizes. Just about 79.6% of the poultry producers were engaged fully in poultry production and the rest 20.4% of the producers were a secondary occupation in and around Debre Markos small scale commercial poultry farms.

Table 1. Socio demographic characteristics of small scale poultry farms in and around Debre Markos, Amhara Region, Ethiopia from September 2015 to May 2016

Variables		N=49	%	χ^2	P-Value
Sex	M	41	83.7	10.694	< 0.001*
	F	8	16.3		
Age	<15	1	2.0		
	15-30	42	85.7	97.204	< 0.001*
	31-45	5	10.2		
	46-60	1	2.0		
Marital Status	Married	22	44.9	0.510	0.475
	Unmarried	27	55.1		
Religion	Orthodox	48	98	45.082	< 0.001*
	Muslim	1	2		
	Others	0	0		
Ethnic Group	Amhara	49	100	1.000	< 0.001*
	others	0	0		
Occupation	Poultry prod	39	79.6	17.163	<0.001*
	others	10	20.4		
Family size	1-3	39	79.6	49.143	<0.001*
	4-6	9	18.4		
	7-9	1	2.0		
Presence of experiences	Yes	21	42.9	1.000	0.317
	No	28	57.1		
Experience years	New	28	57.1	23.551	<0.001*
	1-3	20	40.8		
	4-6	1	2.0		
Educational level	5-8 grade	4	8.2		
	9-10 grade	8	16.3		
	11-12 grade	9	18.4		
	Diploma	10	20.4		
	Degree	18	36.7	10.694	0.030*

Note: *Shows a significant effect at P<0.05.

Flock size of chicks in small scale poultry farms in and around Debre Markos

The flock size and breeds of chicks in small scale poultry farms in and around Debre Markos is presented in table 2. The mean flock size per farm was 844.3, however the flock size was significantly (P<0.05) influenced by the sex of birds. Female chicks were higher (P<0.05) than male chicks. The flock was composed of four breeds of chicks like Bovans brown (egg type), Bovans white (egg type), Koekoek (dual), Sasso T44 (dual). The flock size did not become statistically (P>0.05) affected by breed. However, 71.4% of the producers had kept Bovans brown.

Constraints

The constraints faced to hinder the sustainability of small scale poultry farms are presented by their rank in table 3.

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Table 2. Flock size and breeds of chicks in small scale poultry farms in and around Debre Markos, Amhara Region, Ethiopia from September 2015 to May 2016

Variables		N (%)	Mean (SEM)
Flock size		49(100)	844.3(98.257)
Sex	Female chicks	49(100)	774.7(98.257)[a]
	Male chick	13(26.5)	261.7(104.890)[b]
Breed	Bovans brown (egg type)	35(71.4)	982.0(121.952)[a]
	Bovans white (egg type)	2(4.1)	1105.0(605.000)[a]
	Koekoek (dual)	9(18.4)	354.4(82.481)[a]
	Sasso T44 (dual)	3(6.1)	503.3(115.518)[a]

Note: N (%) describes number or percent of producers; SEM-standard error of mean, means with the different letter of superscript in the same column did differ significantly (P<0.05)

Table 3. Rank of constraints faced in small scale poultry farms in and around Debre Markos, Amhara Region, Ethiopia, from September 2015 to May 2016

Constraints	Total score	Mean Score	Garrett's rank
High cost of feed	3080	62.86	1
Unavailability of feed and feed ingredients	2867	58.51	2
Unavailability of land/space	2579	52.63	3
Lack of market linkage and promotion	2326	47.47	4
High cost of birds	2177	44.43	5
Lack of finance	1974	40.29	6
Lack of extension service	1746	35.63	7
Lack of training	1734	35.39	8
Poor credit facilities	1725	35.20	9
High cost of medicaments	1477	30.14	10
Unavailability of improved birds near the farm	1339	27.33	11
Electric disturbance	1301	26.55	12
Lack of technical know-how in handling poultry	1291	26.35	13
Mortality of day-old/young chicks	1282	26.16	14
Losses due to environmental change	1277	26.06	15
High initial investment	1227	25.04	16
Cannibalism	1084	22.12	17
Lack of veterinary care	1063	21.69	18
Inability to diagnose sick birds	1005	20.51	19
High rate of interest on loans	956	19.51	20
Unavailability of desired breed	897	18.31	21
Lack of equipment	798	16.29	22
unavailability of waste disposal	779	15.90	23
Difficulty for water	634	12.94	24
Labour problem	552	11.27	25
Disease outbreak	542	11.06	26
Loss of birds due to predators	538	10.98	27
High rate of morbidity of birds	496	10.12	28
Inability to pay constant attention	395	8.06	29

DISCUSSION

In spite of the contribution of the poultry industry to the economy, the subsector is faced by challenges to hinder further growth. Among the constraints faced in and around Debre Markos small scale commercial poultry farms, high cost of feed stood at the forefront and unavailability of feed and feed ingredients near to the town was the second rank. This was in line with the report of Tadelle et al. (2003) who noted that poultry feed and nutrition is one of the most critical constraints to poultry production under both the rural small holder and large-scale systems in Ethiopia. The rank confirmed the report of Demeke (1996) who reported that the availability, quality and cost of feed are the major constraints to poultry production in Ethiopia which is not self-sufficient in cereal grains that form the bulk of concentrate feeds for poultry. The report coincides with the report of Nebiyu et al. (2016) who noted that the price of feed was the

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most noticeable constraint in Addis Ababa small scale intensive poultry farming. The result also agreed with the report of Aromolaran at al. (2013) who found that in small scale commercial poultry farms, 55.8% of the respondents lack of quality ingredient for feed formulation to be a major constraint while 51.7% believed that the high cost of feed for their layering birds was a major constraint encountered which prevented them from increasing their layers production. The result coincides with the report of Rajendran and Samarendu (2003) who noted that high cost of feed was the leading factor to hinder production in India.

Unavailability of land/space/ was the third factor among the constraints in small scale poultry farms in and around Debre Markos. Almost more than 71.4% of poultry producers keep their chicken within their own dwelling houses and 4.1% in hired houses. This is due to lack of capital to request land from the government and unable to establish their own farms/poultry house/. However, Nebiyu et al. (2016) explained that the unavailability of land was the second constraint in Addis Ababa small scale poultry production. The result also coincides with the report of Mengistu (2008) who noted that shortage of space was one of the major constraints among the others in smallholder broiler producers in and around Debre zeit, Ethiopia. Aromolaran et al. (2013) reported that 25% of the respondents replied that unavailability of land/space was the major constraint, and the other 57.5% and 17.5% of the respondents thought that unavailability of land was a minor and not as constraint, respectively in Nigeria.

Lack of market linkage and promotion was the fourth rank among the constraints in the study area. Most of the poultry producers replied that the involvement of the government to link the market and give promotion service is still limited. Due to this reason, they are unable to sell birds on their selling age and lead to expose for extra outlay. The result is in line with Jaafar and Gabdo (2010) who confirmed that inadequate market/market linkage is the major constraint in small scale poultry enterprise in Nigeria. Small-scale poultry farmers also encountered problems in marketing produce (Okantah et al., 2003). Lack of market for birds was the leading constraint compared with lack of market for eggs in India (Nath et al., 2012). A different result was reported by Nebiyu et al. (2016) who noted that market difficulties during selling was the eighth rank in Addis Ababa small scale poultry farming.

The purchasing price of day old chicks was very high and it had the fifth rank. The egg type day old chick was purchased around 25.50 Ethiopian Birr. According to the producers thought due to their expensiveness, unable to maximize the number of birds during production time. The result coincides with the report of Aromolaran at al. (2013), 54.2% poultry producers noted that purchasing a healthy day old chicks was the major constraint. The result also confirmed by Nath et al. (2012) who reported that high cost of day old chicks were the second constraint among the economic constraints.

The lack of finance was the sixth rank constraint in small scale poultry farms in and around Debre Markos, because almost half of the poultry producers were beginners in poultry production and they are not economically efficient. A similar result was reported by Micheal (2008) who noted that shortage of capital was one of the leading constraints among others in and around Debrezeit stallholder broiler producers, Ethiopia. The result was in agreement with the report of Okoli et al. (2005) who noted that to run small scale commercial poultry production, high cost of production inputs and lack of adequate finance were major constraint to the business in Nigeria. Another similar result was reported by Ghasura et al. (2013) who noted that lack of finance was the seventh leading constraint in Gujarat, India. Also Bishop et al. (2009) reported that in intensive small scale poultry production, lack of finance was the major constraint in Delta State Nigeria.

The agricultural extension service is one of the institutional support services that have a central role to play in the transformation process from backyard poultry production system to small scale intensive poultry production system. The service contributes to the development of the skill and knowledge of farmers to adopt new and improved technologies. Lack of extension service was the seventh leading constraint in the study area. Jaafar and Gabdo (2010) reported that among the six major constraints in small scale poultry production enterprises, lack of extension service was the leading constraint (100%). Lack of training was the eighth constraint in the study area; however inadequate training was the 11^{th} constraint in Addis Ababa small scale intensive poultry farming (Nebiyu et al., 2016). Poor credit facilities were the 9^{th} constraint in and around Debre Markos small scale commercial poultry farms. The result was confirmed by the report of Ghasura et al. (2013) who noted that the poor credit facilities were ranked as the 8^{th} factor in India small scale poultry farm entrepreneurs. The result coincides with the report of Nebiyu et al. (2016) who reported that the lack of access to credit was the 10^{th} factor in Addis Ababa small scale intensive poultry farming. High cost of medicaments was the 10^{th} constraint in and around Debre Markos small scale poultry farm producers. Vaccines were given by the town agriculture office came from the national veterinary institute at Bishofitu, Ethiopia and other medicaments were purchased from the town veterinary pharmacy and these medicaments are costy. The result was in agreement with the report of Nath et al. (2012) who noted that high cost of medicine was also the major constraint in India small scale poultry production. The result was also confirmed by Ghasura et al. (2013) who stated that the high price of medicine was the fifth major constraint in Gujarat poultry farm entrepreneurs, India.

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The other constraints ranked by Garret's ranking technique in small scale commercial poultry farm in and around Debre Markos were ranked from rank 11-29. According to their sequence, these were unavailability of improved birds near the farm, electric disturbance, lack of technical know-how in handling poultry, mortality of day-old/young chicks, losses due to environmental change, high initial investment, cannibalism, lack of veterinary care, inability to diagnose sick birds, high rate of interest on loans, unavailability of desired breed, lack of equipment, unavailability of waste disposal, difficulties accessing water, labor problem, disease outbreak, loss of birds due to predators, high rate of morbidity of birds, inability to pay constant attention. Nearly similar result was reported by Ghasura et al. (2013) who noted that the constraints in Gujarat poultry farm entrepreneurs beyond rank 10 were non-availability of improved birds in time, inability to pay constant attention, lack of supports from family members, high charge of electricity and non-availability of laborers for poultry enterprise.

CONCLUSION

There were 49 small scale commercial poultry farms in and around Debre Markos. Among them 75.5 % of the farms were established by their own initiation privately and 24.5% of the farms were established by youths organized by small and micro enterprise office. However the poultry production didn't achieve its full potential due to a number of constraints. Among the constraints high cost of feed, unavailability of feed and feed ingredients, unavailability of land/space, lack of market linkage and promotion, high cost of birds, lack of finance, lack of extension service, lack of training, poor credit facilities and high cost of medicaments were the leading major constraints that hindered to run successful poultry production through their production time. Therefore, the intervention of the government is very crucial to alleviate the constraints faced for efficient and successful poultry production, and for further expansion.

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Competing interests

The authors declare that they have no competing interests.

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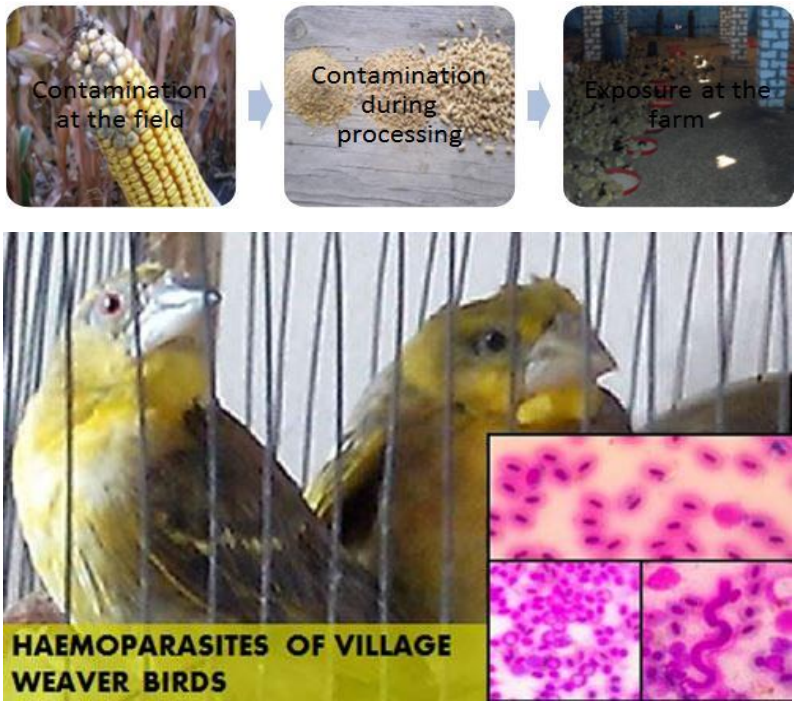

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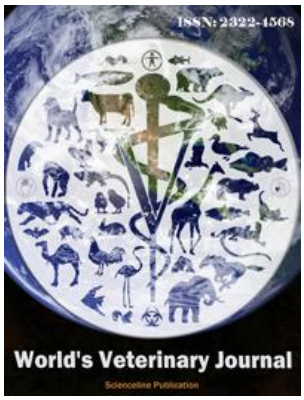

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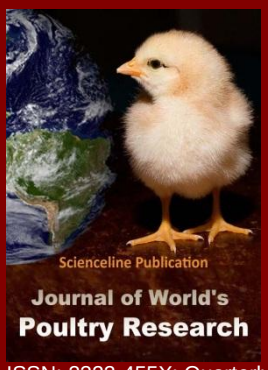

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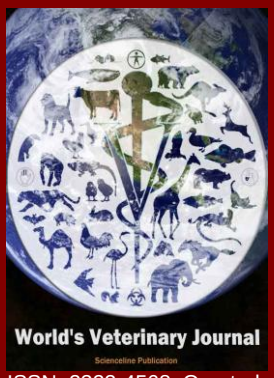

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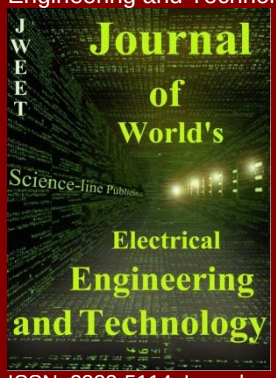

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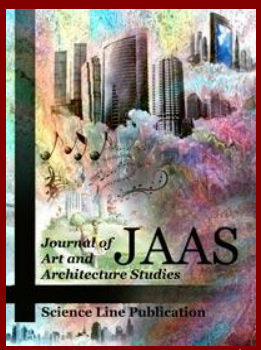

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