



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

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ABSTRACT

Capri Pox Virus (Ca PV) is the causative agent of important diseases in sheep and goats 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 goats) 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 led to lower results than molecular methods, the findings indicated 11 and 13 positive samples out of 54 sheep as well as 1 and 2 positive samples out of 26 scab biopsy samples derived from goats, 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 GTPV 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

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molecular assays have been developed for the detection of CaPVs (Zeedan et al., 2014; Abdallah et al., 2018). Most of 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 -40°C 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 -40°C 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.

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 μ l PBS. Ten μ l 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 μ l of the clear DNA was taken from the walls or from lid of the tube according to Melendez (2016). Alternatively, for enduring storage, 30 μ l 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 μ l capacity PCR tube.

Table 1. Preparation of PCR reaction tube component

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

12.5 μ l = Green PCR Master (Gendirex, Inc, USA), 1 μ l of each primer (20 pmol/ μ l), 2 μ l of extracted DNA, and completed with nuclease free water up to 25 μ l. 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

Gene	PCR amplification conditions					Final extension
	Primary denaturation	Main Cycle for amplification			No. of cycles	
		Secondary denaturation	Annealing	Extension		
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 μ l 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 μ l 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 μ l) was added to the reaction mixture in a 25- μ l 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.

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.

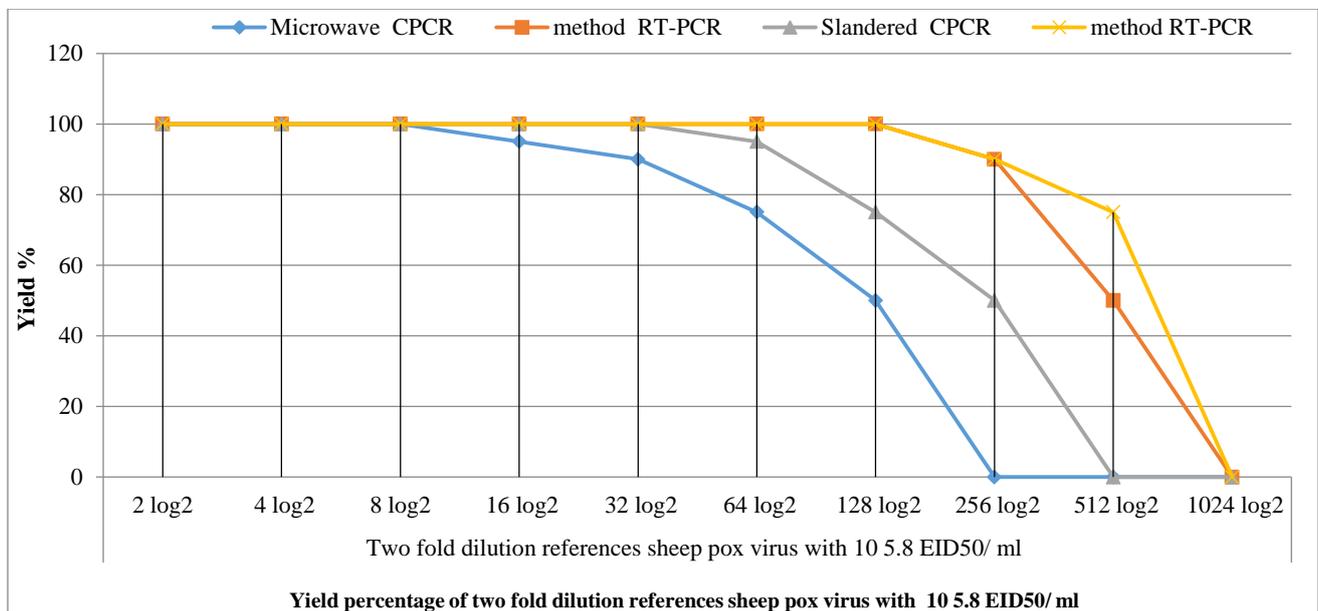


Figure 1. Different extraction methods used to SPPV DNA amplification by conventional PCR and Real-Time PCR

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

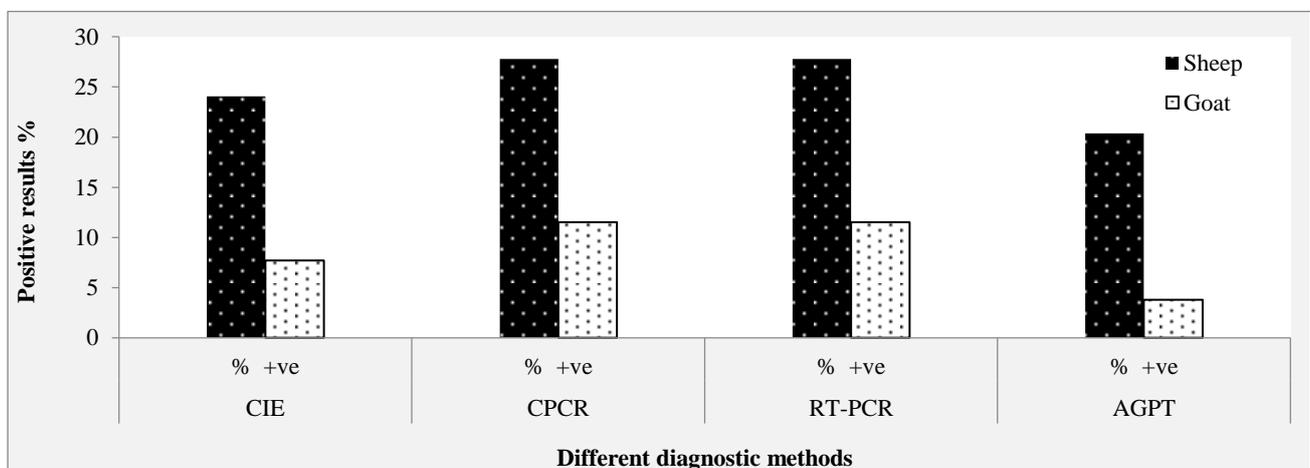


Figure 2. Positive percentage results for scab biopsy samples of suspected sheep and goat infected with Pox virus, which were examined using different diagnostic methods. % +ve CIE= Positive percent of counter immune electrophoresis, % +ve c-PCR = Positive percent of conventional PCR, % +ve RT-PCR= Positive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

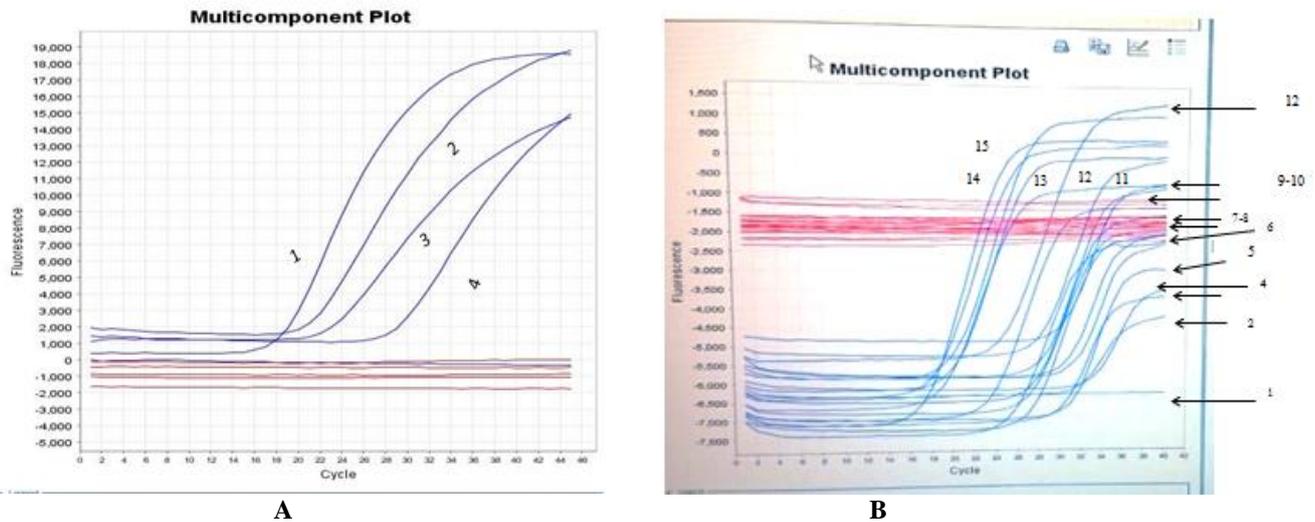


Figure 3. A) Amplification plot showed a positive control, and Ct2, Ct1 and Ct3 are positive tested samples for DNA extracted from a biopsy skin lesion. Ct 4 showed positive DNA extracted from infected CAM with pathogenic pock lesion for SPPV. Ct = DNA-free negative control (graphic generated by ES Equant). **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

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

Competing interests

The authors declared that they have no competing interests.

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