INNOVATION OF INDOOR REAL-TIME POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF CAMEL POX VIRUS IN CLINICAL FIELD SAMPLES USING PRIMER SITE BELONGS TO CAPRIPOXVIRUS

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ABSTRACT

Real-time amplification techniques are currently used to determine the viral nucleic acid (NA) in clinical samples for diagnostic purposes. In disease management contexts, until now, a little have been described for the molecular detection of camel pox virus (CPV). This study reports the development of a Real-time polymerase chain reaction (RT-PCR) for detection of CPV using SYBR green I chemistry. A total of 15 specimens from camels suspected of being infected with CPV were collected from Riyadh Province during 2009 and submitted for virological investigation at the Central Veterinary Diagnostic Lab. (CVDL), Riyadh, Ministry of Agriculture; KSA. In solution; detection and identification of CPV was achieved in 10 samples by conventional polymerase chain reaction (PCR). During further studies performed, it was shown that CPV was isolated in Chorio-allantoic membranes (CAMs) and in Vero cell as well as demonstration of pock lesions and cytopathic effect (CPE) due to CPV were observed while CPV virus antigen was detected and identified by indirect immune-fluorescent assay (IFAT) in Vero cells. A trial for development of a simple and rapid qualitative Real-time polymerase chain reaction (RT-PCR) was applied using primer site belongs to *Capripoxvirus* to detect CPV load in prepared tissue samples comparing to inoculated

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CAMs and Vero cells [reveal pocks or CPE]; where NA of CPV in samples were (13/15), while; CAMs and Vero cells were (7/15) and (9/15) positive respectively. The obtained SYBR Green dye–based Real-time PCR results; comparing to ordinary PCR or CPV isolation in eggs or cell cultures; proved that this development RT- PCR assay was rapid, accurate and effective for the direct and qualitative detection of CPV (viral DNA) in both necropsy specimens and inoculated egg or tissue culture samples. To the authors knowledge, this is the first report to describe a primer set of *Capripoxvirus* gene–based Real-time PCR for specific diagnosis of CPV infection in clinical samples.

Keywords: Camel pox; Real-time PCR; Vero cell.

INTRODUCTION

Camel pox is a highly contagious viral skin disease and occurs in almost every country in which camel husbandry is practiced. Outbreaks have been reported in the Middle East (Bahrain, Iran, Iraq, Oman, Saudi Arabia, United Arab Emirates and Yemen), in Asia (Afghanistan and Pakistan), in Africa (Algeria, Egyptethiopia, Kenya, Mauritania, Morocco, Niger, Somalia and Sudan) **(Mayer and Czerny, 1990 and Wernery *et al.*, 1997)** and in the southern parts of Russia and India. The disease is endemic in these countries and a pattern of sporadic outbreaks occurs with a rise in the seasonal incidence usually during the rainy season **(OIE, 2008).**

Camel pox virus (CPV) is a member of the genus *Orthopoxvirus* (OPV), subfamily *Chordopoxvirinae* of the family *Poxviridae*, a group of large double-stranded DNA viruses that replicate in the cytoplasm **(Moss, 2001).** Camel pox is diagnosed based on clinical signs, epizootiological and pathological findings **(Buchnev, 1987),** isolation of virus in chicken eggs and cell lines, electron microscopic detection of virus particles in pox lesions, genus-specific antigen capture enzyme-linked immunosorbent assay **(Davies *et al.*, 1975 and Czerny *et al.*, 1989)** and immuno–histochemistry assay for detection of CPV-antigens **(Wernery and Kaaden, 2002).** Polymerase chain reaction (PCR) and restriction enzyme protocols

for detection and differentiation of species of the genus OPV were established **(Meyer *et al.*, 1994).** Further; DNA detection of OPV by Real-time PCR was done **(Nitsche *et al.*, 2004).** A specific diagnostic PCR for the detection of CPV has not yet been reported **(Balamurugan *et al.*, 2009).**

In this work, trials for molecular detection (PCR), and isolation of CPV in clinical samples obtained from affected camels were applied; in addition, development of a simple and rapid qualitative Real-time PCR using primer site belongs to *Capripoxviruse* was described to detect CPV load in prepared tissue samples comparing to inoculated CAMs and Vero cells.

MATERIAL & METHODS

MATERIALS

Clinical examinations and sample collection:

During summer 2009, specimens including nodular and proliferative skin lesions, and mouth crusted scabs were collected from 15 camels suspected of infection with CPV from different camel herds near Riyadh Provence. According to the history, the animals had not been vaccinated against CPV infection. The samples were sent in ice boxes to the Central Veterinary Diagnostic Laboratory (CVDL), Riyadh Ministry of Agriculture, KSA.

Camel pox virus isolate strain:

Infected Vero cell with a virulent field camel poxvirus isolate 433/28/07 (obtained from CVDL) was used as positive control.

Antiserum:

Both standard anti-Jouf-78 strain camel pox virus rabbit antiserum (Abdel Baky et al., 2006); obtained from CVDL; and fluorescent conjugated swine anti-rabbit IgG (Dokocytomation, Lot No. 4918, Denmark), were used for detection of CPV through immunofluorescent assay.

Primers:

A generic PCR assay, described by Meyer et al., (1994), allows the detection and differentiation of species of the genus *Orthopoxvirus* because of the size differences of the amplicons. Using the primer pair: 5′-AAT-ACA-AGG-AGG-ATC-T-3′ and 5′-CTT-AAC-TTT-TTC-TTT-CTC-3′; the gene sequence encoding the A-type inclusion protein (ATIP) will be amplified. The size of the PCR

product specific for the camel pox virus is 881 bp. Another primer set belongs to *Capripoxviruse*, was used in amplification of Real-time PCR assay. Primer sequences of the first and second strands of Capripox (Ks.1.5/Ks1.6), were F 5/ GTG-TGA-CTT-TCC-TGC-CGA-AT 3/ and P_2 5/ TCTATTTTATTTCGTATATC 3/ (Gershon et al., 1989 and Mangana-Vougiouka et al., 1999) respectively. All primers were obtained from, TIB-MOL Biol syntheslabor Gmb H Berlin, Germany.

METHODS

Samples preparation:

The specimens were ground and supplemented with 200 U of penicillin, 200 mg of streptomycin and, 250 mg of gentamycin per ml, making up a 50% (volume/volume) solution. Post-incubation at 37°C for 1 hour, the suspension was frozen at -20C° and thawed triple times. After centrifugation at 3000 rpm for 15 min. under refrigeration the supernatant was aspirated and stored at -65C°, until it was used.

Virus isolation and titration:

According to **Burleson *et al.* (1997)**, 0.1 ml of prepared sample was inoculated onto chorio-allantoic membrane (CAM), eggs were incubated at 38.5C° **(Tantawi *et al.*, 1974)**, Twelve-day chiken embryos were used. The harvested CAMs were examined for any changes till the 3rd passage. For tissue culture inoculation, monolayers of Vero (African green monkey kidney) cells adapted to growth in Minimum Essential Medium Eagle (MEM - Cultilab®) with 10% of bovine fetal serum (Cultilab®) were used. One ml of clarified sample preparation supernatant was inoculated on to a 25 cm^2 tissue culture flask (Nunc®) of 90% confluent monolayers of Vero cells, and allowed to absorb for 1 hour under stirring at 37 °C. The culture is then washed with warm PBS and covered with 10 ml of MEM containing antibiotics (100 U penicillin sodium and 100 ug streptomycin sulphate /1ml) and 2% fetal calf serum. The flasks were examined daily for up to 10 days for evidence of cytopathic effect (CPE), and the medium is replaced if it appears to be cloudy, If no CPE is apparent, the culture should be freeze-thawed three times, and clarified supernatant inoculated onto fresh cell culture. Isolation was considered negative if the CPE did not observe till the next 3rd passage. Both harvested

CAMs and tissue cultures isolated virus were titrated following the procedure described by Villegas and Purchase (1983) and 50% infective dose end point was estimated in accordance with the method of **Reed and Muench (1938),** and expressed as EID_{50} / 0.1 ml and TID_{50} /1 ml respectively. The harvested inoculated CAMs and cell cultures were stored at -65 °C for further investigation.

Indirect fluorescent antibody test (IFAT):

The indirect fluorescent antibody assay was applied according to **Abdel Baky *et al.* (2006)** in guidance of **Woldehiwet and Hussein (1994).** At the first sign of CPE, the flasks were PBS-washed and air-dried then fixed in cold acetone for 15 minutes, where camel pox virus rabbit antiserum was added and all examined flasks were incubated at 37 °C for 1 hour followed by triple washing with PBS, the fluorescent conjugated swine anti-rabbit IgG were added and incubated at 37 °C for 1 hour followed by triple washing with PBS containing 0.1% bovine albumin (BD-BBL, Ireland) to avoid non specific illumination, then examined under fluorescent microscope. Uninfected tissue culture was included as a negative control.

Viral DNA extraction:

Viral DNA was extracted from prepared samples or harvested inoculated cells and CAM homogenates centrifuged at 4000 rpm for 15 min., using automated MagNA pure Compact extraction machine (Roch) and MagNA pure Compact Nucleic Acid isolation Kit I (Roch, Cat. No. 03730964001). The sample volume was 400 ul, and elution volume was 100 ul. The extraction procedures were carried out according to the manufacture's instruction. The suspected extract DNA was storied at -20°C.

Polymerase chain reaction (PCR):

The PCR condition was applied as described by Meyer et. al (1994); DNA amplification is carried out in a final volume of 50 µl containing 2 µl of each dNTP (10 mM), 5 µl of 10 × PCR buffer, 1.5 µl of MgCl2 (50 mM), 1 µl of each primer, 2.5 U Taq DNA polymerase, 1 µl DNA template and an appropriate volume of nuclease-free water. Incubate the samples in a thermal cycler: first cycle: 5 minutes at 94°C (initial denaturation step), second cycle: 1

minute at 94°C, 1 minute at 45°C, 2.5 minutes at 72°C. Repeat the second cycle 29 times. Last cycle: 10 minutes at 72°C (final elongation step to ensure complete extension of amplification products) and hold at 4°C until analysis. Mix 10 µl of a sample with loading dye solution and load in 1% agarose gel in TBE (Tris/Borate/EDTA) buffer containing ethidium bromide (Promega, Madison, USA, 10 mg /ml). A parallel lane was loaded with a 100 bp DNA-marker ladder (Roch). Separate the products at 100 V for 30-40 minutes and visualise using an UV transilluminator. Confirm the positive reactions according to the sizes of amplicon obtained.

SYBR Green Real-time PCR:

Real-time PCR in the presence of SYBR Green I was performed on extracted DNA using Light Cycler 2.0 (Roch) using, primer set for *Capripoxvirus*, and Light Cycler Fast Start DNA master plus SYBR Green Kit (Roch, Cat. No. 03515885001) . PCR assay was performed in final volume of 20 ul reaction mix containing 9 ul water (PCR grade), 4 ul master mix 5x conc., 1ul of each 10 uM forward and reverse primer (final conc. of 0.5 uM each primer), and 5 ul of DNA (diluted 1:10 in PCR grade water). Cycling parameters were the following: 10 min at 95°C for pre-incubation following by 40 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 5 s and extension at 72°C for 15 s, then one cycle of melting and as described in the back-insert of the Kit, during the melting cycle, the temperature was increased by increments of 0.1°C/s between 65°C and 95°C.

RESULTES

The clinical signs of camel pox reveal face edema, lachrymation, papules formation and pendulous lips, excessive salivation and nasal discharge. Papules, vesicles and thick scabs present on the lips and nostrils and may involve the whole head, neck, buttock, abdomen, legs and groin **(Figure 1).** The amplicons of 881bp specific for CPV were observed in PCR assay **(Figure 2).** Ten positive samples of 15 for CPV could be detected migrated similarly in the gel at respective locations by PCR. Six out of 15 examined suspected CPV were successfully isolated on eggs, where CAMs reveal dense, grayish white pock lesions **(Figure 2),** while all inoculated samples don't

cause any deaths for the inoculated chicken egg embryos.

Characteristic, plaque-type cytopathic effect (CPE) showing foci of rounded cells, cell detachment, giant cell formation and syncytia appear. Syncytia may contain up to 10 nuclei **(Figure 3)**. At first only small areas of CPE can be seen, sometimes as soon as 4 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. Nine samples out of 15 were confidently isolated in Vero cell. The titer of the obtained virus at the 3rd passage became $10^{3.25}$ EID_{50}\0.1ml and $10^{5.2}$ $TCID_{50}$ \1 ml. The inoculated Vero cells exposed positive intracytoplasmic immunofluorescent for CPV **(Figure 4).**

As SYBR Green I dye binds to any double stranded DNA product; the specificity and the absence of non-specific amplification or dimer were determined by melting curve analysis post amplification. Real-time PCR assay revealed positive amplification **(Figure 5)** and melting curve analysis **(Figure 6)** for CPV viral DNA in both skin specimens and inoculated egg or tissue culture harvests like that detect for positive control CPV, corresponding, none of the CPV negative controls tested was positive by Real-time PCR assay. Reference CPV, Saudia field isolate 433/28/07 was detected by the 27th cycle of amplification with temperature of melting (Tm) score of 81.53°C, whereas the samples were detected between the 19th and 26th cycle of amplification with one peak of Tm (81.04°C ~ 81.28°C). Detection of CPV loaded in prepared samples comparing to inoculated CAMs and Vero cells [reveal pocks or CPE] could be confirmed. The findings are summarized in **(Table 1);** where NA of CPV in samples were (13/15), while CAMs and Vero cells were (7/15) and (9/15) positive respectively.

Figure 1. Examined camel shows typical pox lesions with nasal and ocular discharge.

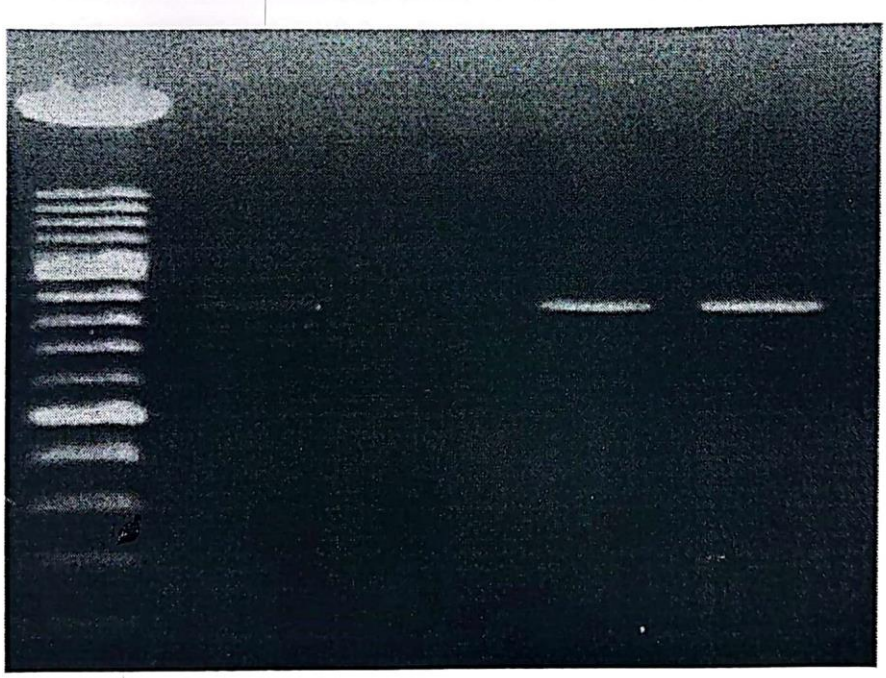

Figure 2. Polymerase chain reaction (PCR) to detect pox viruse. Lanes: M, 100-bp molecular weight marker; 1, camelpox positive control (881bp): 2, negative control; 3&4 camel pox positive examined samples.

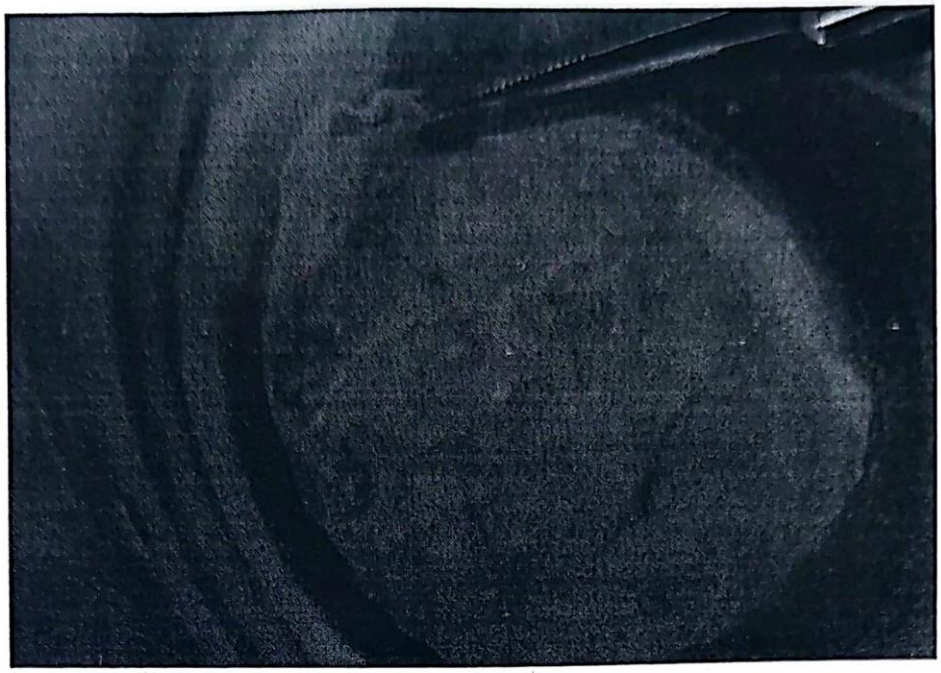

Figure 3. Numerous pock lesions post CAM inoculation.

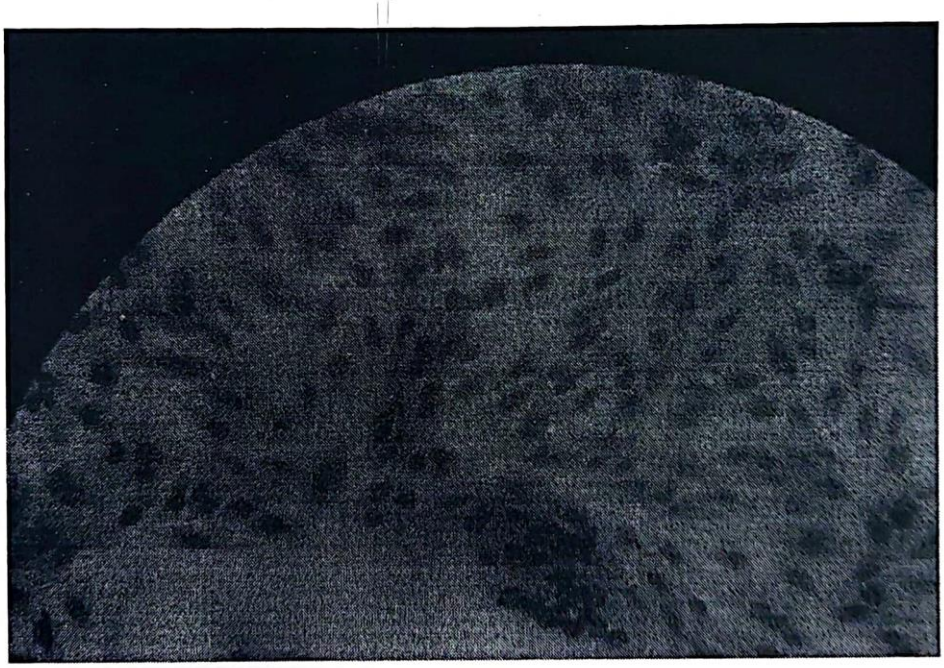

Figure 4. Characteristic CPE of CPV in Vero cells 4 days post-inoculated.

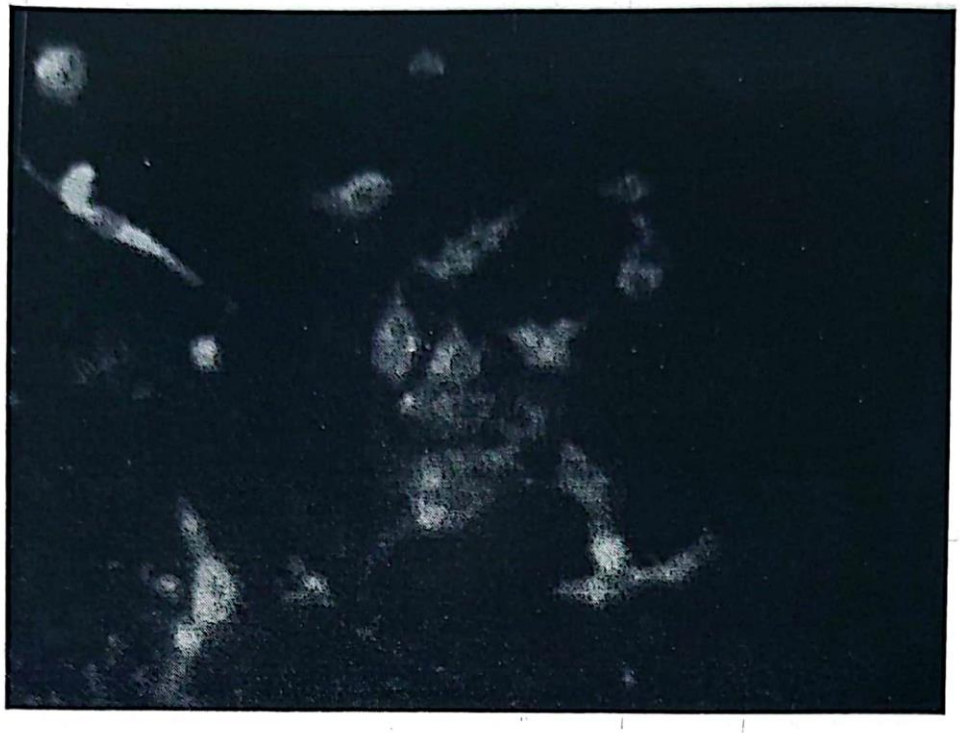

Figure 5. Inoculated Vero cells exposed positive intracytoplasmic immunofluorescence.

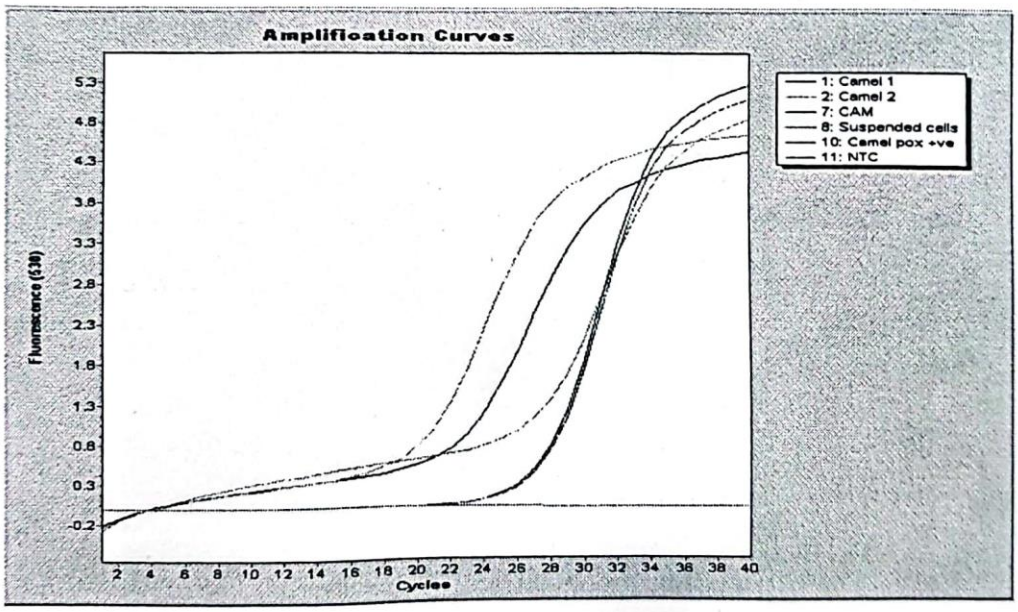

Figure 6. Real-time PCR amplification curves of examined CPV illustrate positive amplification.

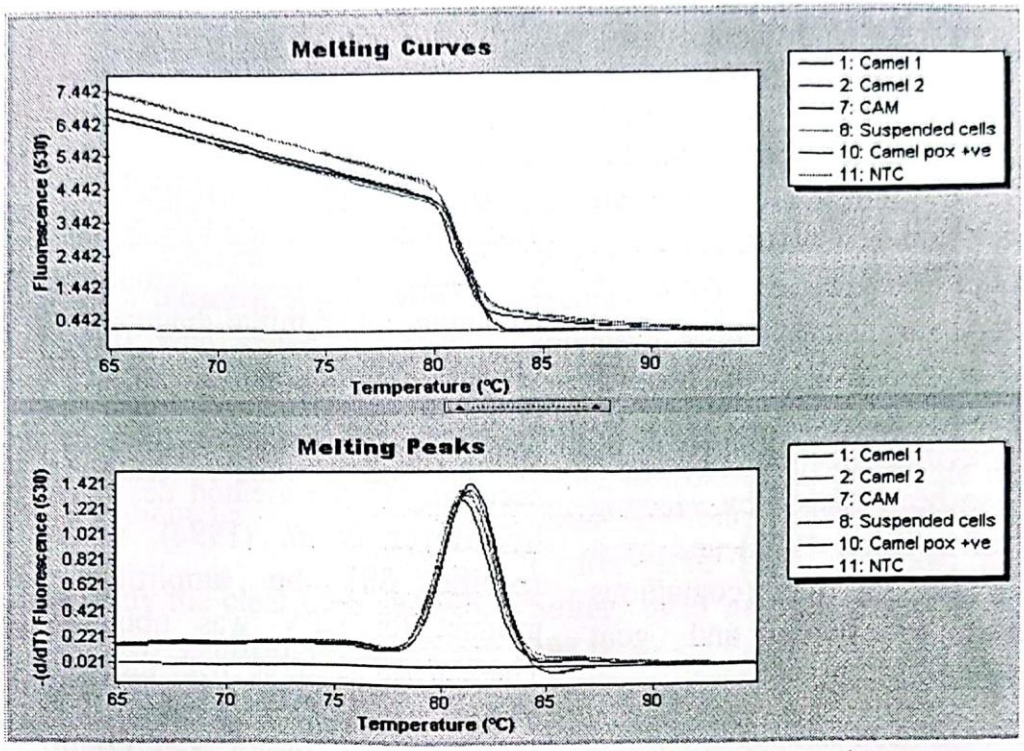

Figure 7. Melting curve profiles of SYBR Green I Real-time PCR in detecting CPV, T_m of CPV was (81.04oC ~ 81.28°C), no primer dimer or non-specific product was detected after amplification.

Table 1. Comparison of CPV detection by different assays.

No. of samples	PCR[1] of prepared samples		RT-PCR[2] of prepared samples		Pock lesions[3] on inoculated CAMs & RT-PCR		CPE[4] of cell culture & RT-PCR	
	+VE %	-VE %	+VE %	-VE %	+VE %	-VE%	+VE %	-VE %
15	10(66.7%)	5(33.3%)	13(87%)	2(13%)	7(47%)	8(53%)	9(60%)	6(40%)

1. conventional PCR. 2.RT-PCR 3.CAMs showed pock lesions and NA detection of CPV by RT-PCR. 4. Vero cells showed CPE and NA detection of CPV by RT-PCR.

DISCUSSION

Information is still required about the epidemiology and natural history of poxvirus infections among camels world widely, particularly with a view to assessing the distribution and relative importance of the various poxviruses involved. However; early poxvirus infections of camels have also been caused by vaccinia virus **(Krupenko, 1972)** and by a virus resembling Orf (contagious ecthyma) of sheep and goat **(Roslyakov, 1972).**

In the current study, typical clinical signs of CPV were observed, where affected camels showed signs varied from acute to mild infection, affected animals were off food with edematous face and ocular lacrimation. Papules, vesicles and thick scabs were observed on the lips, nostrils and may involved the whole head and neck; occasionally all ages were affected; This coincides with several reports from other investigators who regarded a typical CPV infections is showing the four usual stages of pox lesions: papules, vesicles, pustules and **crusts (Kritz, 1982, Munz 1992 and Murphy *et al.*, 1999)**, possibly reflecting differences between the strains of camel pox viruses **(Wernery and Kaaden, 2002).**

The main objective of the present study is overcoming difficulties in virus propagation, and to substitute the time consuming serological techniques. The initial diagnosis of the suspected CPV in direct prepared samples was made by conventional PCR performed according to the method described by **Meyer *et al.* (1994),** where specific 881 bp amplification product for CPV was obtained. These findings agree with those of **Meyer *et al.* (1997), Khalafalla *et al.* (2003) and Hanan *et al.* (2009)** in using PCR in diagnosis of CPV.

Camel pox virus can be propagated in chick embryos and large variety of cell cultures including Vero cells **(Tantawi *et al.*, 1974 and Davies *et al.*, 1975).** Our results interestingly demonstrated that a primary attempts for inoculation of material from the suspected camel pox lesions in CAMs and Vero cells were done successfully where distinct pock lesions and characteristic cytopathic effects of CPV were observed on CAMs and Vero cells respectively. The results obtained in this study confirm the observations of **Marennikova *et***

al. **(1974)** who reported that when CPV was propagated on CAM at 37 C°, monomorphic punctated, rather dense white pock 0.2-0.3 mm in size developed. The present study also yielded results which agree with those of **Khalafalla *et al.* (1998)** who stated that CPV grew on CAM and produced distinct pock lesions which were opaque-white in color, round and 0.5-1.5 in diameter.

In this study the clear CPE showed in Vero cell confirm the reported evidences on the growth of CPV by **Wernery *et al.* (1997)** who reported that, the virus in scab samples collected from camels grow well in Vero cells and that of **Ramyar and Hessami (1972) Nguyen *et al.* (1989), Renner-Muller et al., (1995) and Khalafalla *et al.* (1998)** who observed that the CPE produced by CPV in all cell cultures types consisted of rounding of cells, plaque formation, cytoplasmic elongation and multinucleated giant cell formation. The harvested positive CAMs (pock lesions) and Vero cells (CPE) were collected and prepared individually, then inoculated separately on CAM and Vero cells for further viral propagation and titeration where the titer became $10^{3.25}$ EID_{50}\0.1ml and $10^{5.2}$ $TCID_{50}$ \1 ml at the 3rd passage, the low titer of CPV in CAM may be attributed to the fact that; pox viruses have not been shown to have more receptor mediated binding to cells as supposed by **Payne and Norrby (1973), Fearon and Wang (1983) and Doms *et al.* (1990),** so it is better to isolate and propagate pox viruses in tissue culture than that in CAMs **(Lal, 1975, Tantawi and Soker, 1975 and Chandra *et al.*, 1984).**

In our study IFAT has been used to stain infected Vero cells, where positive intra-cytoplasmic CPV antigens were detected, although, the staining results (positive) tend to lack detail on intensity/distribution of staining. Similar immunofluorescent reaction was applied for detection of CPV antigen either in tissue specimens or infected Vero cells **(Abdel Baky *et al.*, 2006),** so; cross-fluorescence between CPV and antisera of members of the *Orthopoxvirus* groups, includes camelpox virus, vaccinia and cowpox was recorded **(Davies *et al.*, 1975).**

Clinical diagnostic applications and the use of Real-time PCRs are

growing exponentially. The combined properties of high sensitivity and specificity, low contamination risk, and speed has made Real-time PCR technology a highly attractive alternative to tissue culture or immunoassay-based methods for diagnosing many infectious diseases **(Espy *et al.*, 2006).** An important outcome of this current study was that; for rapid detection, as well as qualitative of CPV; SYBR Green I Real-time PCR with *Capripox* KS.1 and KS.6 primer set of *Capripoxvirus* was applied either in skin lesions or inoculated CAMs and Vero cells comparing with reference CPV. The results obtained were astonishing where sigmoid curve obtained in the standard amplification curve confirmed the presence of amplification of a specific CPV amplicon without non-specific reactions or inter-assay cross-amplification , while the dissociation melting temperature of the product with peak-measured fluorescence [d(F)] was noticed.

In light of the antigenic relationship between Capripox- and Orthopoxviruses **(Ramyar and Hessami, 1970);** the reality of our results could be attributed to the fact that most of essential and conservative genes are placed in the central part of the poxviruses genomes while the most of nonessential and variable genes are located in the terminal ends **(Mercer *et al.*, 2002).** In addition the genomes of Capripox and Orthopox viruses appear to be more divergent in both sequence and size towards their termini than in a centrally placed collinear region **(Gershon *et al.*, 1989 and Kotwal *et al.*, 2000).** The nucleotide sequence analysis of the tow centrally located segments of *Capripoxvirus* DNA have shown a very similar gene orientation to that of corresponding segments of vaccinia virus DNA **(Gershon *et al.*, 1989)** so; a Capripoxvirus detection PCR and antibody ELISA based on the major antigen P32, the homolog of the vaccinia virus H3L gene were applied **(Heine *et al.*, 1999),** moreover; CPV were found to be related to vaccinia and variola viruses; that classified as a members of genus Orthpoxvirus.

In Conclusion we confirmed the incidence of CPV in KSA with the a viable methods recommended for it's diagnosis according to **OIE, (2008).** We can also say that our developed SYBR Green Real-time

PCR assay was accurate and effective in the direct detection and qualitative of CPV, however; the assay requires further testing on biological materials from animals suspected of camel pox and other viral and nonviral diseases of camels that would pose problems in the differential diagnosis of camel pox.

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