Arch Virol (2014) 159:3005–3011
DOI 10.1007/s00705-014-2169-1

ORIGINAL ARTICLE

Molecular investigation and cultivation of camelpox virus in Iran

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Received: 18 November 2013 / Accepted: 28 June 2014 / Published online: 10 July 2014

Abstract Camelpox virus (genus *Orthopoxvirus*, family *Poxviridae*) is the etiologic agent of camel pox. The clinical manifestations of this virus range from inapparent infection to mild, moderate and, less commonly, severe systemic infection and death. Following an outbreak of camelpox, samples that were collected from camel flocks suspected to have camelpox in Qom Province in central Iran and Khash city, Sistan and Baluchestan Province and South Khorasan Province in eastern Iran were sent to Razi Vaccine and Serum Research Institute in Mashhad. DNA extraction was performed primarily by the phenol-chloroform method, and PCR was carried out using a Bioneer kit. 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) was amplified. The size of the PCR product, specific for camelpox virus, was 881 bp. The PCR product was purified, and to confirm its sequence, it was sent to the reference laboratory. The sequence was subjected to a BLAST search and then phylogenetically analyzed using CLC software. The results showed that all samples were nearly 100 % identical to each other and to strains CMS and M-96. These isolates also had 99 % and 95 % similarity to the CP-1 strain and isolate FIN/T2000, respectively. In Vero cell culture, inoculation with this virus caused a cytopathic effect (CPE), which appeared 2-5 days post-inoculation. Characteristic CPE showing foci of rounded cells, ballooning, giant-cell formation and syncytia with degenerative changes appeared.

Introduction

Camelpox occurs in almost every country in which camel husbandry is practised, apart from the introduced dromedary camel in Australia and tylopods (llama and related species) in South America. 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, Egypt, Ethiopia, Kenya, Mauritania, Morocco, Niger, Somalia and Sudan) [18] and in the southern parts of Russia and India [17]. The disease is endemic in these countries.

Camelpox virus (genus *Orthopoxvirus*, family *Poxviridae*) is the etiologic agent of camelpox. Camels have been successfully vaccinated against camelpox with vaccinia virus strains [11]. The nucleic acid is a double-stranded linear DNA. The virus replicates in the cytoplasm of the host cell, and at the histopathological and cytological level, these viral particles are found in inclusion bodies [11].

The disease is characterized by fever, enlarged lymph nodes and skin lesions. The skin lesions appear 1–3 days after the onset of fever, starting as erythematous macules, developing into papules and vesicles, and later turning into

Electronic supplementary material The online version of this article (doi:10.1007/s00705-014-2169-1) contains supplementary material, which is available to authorized users.

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pustules. Crusts develop on the ruptured pustules. Skin lesions may take up to 4–6 weeks to heal. In the systemic form of the disease, the pox lesions can be found in the mucous membranes of the mouth, respiratory and digestive tracts [19].

Transmission occurs either by direct contact between infected and susceptible animals or indirectly via a contaminated environment. The role of an arthropod vector in the transmission of the disease has been suspected [18], and potential vectors such as biting flies and mosquitoes may be involved [11].

Camelpox virus is very host specific and does not infect other animal species, including cattle, sheep and goats. However, it appears that this disease is of public-health importance because one suspected case of human camelpox was described in 1982 [7]. Recently, Bera et al. reported camelpox virus (CMLV) zoonosis on the basis of clinical and epidemiological evidence, combined with serological and molecular characterization of the responsible agent in three human cases. This was the first incidence of laboratory-confirmed cases of camelpox zoonosis in India as well as in the world [2]. Because of the importance of camelpox and the lack of a comprehensive study in this field, the following study was designed to clarify the molecular pattern of camelpox virus.

Materials and methods

Sample collection

Scabs from skin lesions were collected from infected camels from Qom in central Iran and South Khorasan Province and Khash city in Sistan and Baluchestan Province in eastern Iran. The specimens were sent to Razi Vaccine and Serum Research Institute in Mashhad in special tubes on ice. In the laboratory, the samples were kept at −20 °C before testing. If a longer period was required, the specimens were placed at −70 °C [11].

DNA extraction

A small aliquot of the crusted scabs was suspend in 90 µl of lysis solution (50 mM Tris/HCl, pH 8.0, 100 mM Na_2EDTA, 100 mM NaCl, 1 % sodium dodecyl sulphate), and 10 µl of proteinase K (20 mg/ml, Invitrogen) was added. The sample was digested for 10 minutes at 37 °C prior to disruption of the scab or tissue with a microfuge tube pestle. Another 350 µl of lysis solution and 50 µl of proteinase K was added, and the sample was gently mixed and incubated for 3 hours at 37 °C. The lysed suspension was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and centrifuged at 8000g at 4 °C for 1 minute. The upper aqueous phase was collected and mixed again with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1). It was centrifuged at 8000g at 4 °C for 1 minute, and the upper aqueous phase was transferred to a new tube. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and two volumes of ice-cold absolute ethanol. The mixture was placed at −70 °C for 30 minutes or −20 °C overnight. It was then centrifuged at 15,000g for 5 minutes at 4 °C. The supernatant was discarded, and the pellet was washed with 0.5 ml of 70 % ethanol. It was then centrifuged at 15,000g for 5 minutes. The supernatant was discarded and the pellets were air-dried. The pellets were resuspended in 10 µl of nuclease-free water [11].

PCR

PCR was done by generic assay described by Meyer et al. [9]. In this assay, due to the different sizes of the PCR products, members of different species in the genus *Orthopoxvirus* can be differentiated. The size of the PCR product of camelpox virus, cowpox virus and vaccinia virus is 881 bp, 1672 bp and 1596 bp, respectively [9].

DNA amplification was carried out using a Bioneer PCR kit that contained a PCR premix microtube. The PCR reaction was carried out in a final volume of 25 µl containing 1 µl of each primer, 1 µl of DNA template and an appropriate volume of nuclease-free water. The samples were incubated in a thermal cycler using the following conditions: 5 minutes at 94 °C (initial denaturation step), followed by 29 cycles of 1 minute at 94 °C, 1 minute at 45 °C and 2.5 minutes at 72 °C, and a final elongation step of 10 minutes at 72 °C. The temperature was then held at 4 °C until analysis. Ten microliters of the sample was mixed with loading dye solution and loaded onto a 1 % agarose gel in TBE (Tris/Borate/EDTA) buffer containing ethidium bromide. A parallel lane was loaded with a 100-bp DNA-marker ladder. The products were separated at 100 V for 30–40 minutes and visualized using a UV transilluminator. The positive reaction was confirmed according to the size [11].

Bioinformatics

Gel extraction was carried out using a Bioneer kit. The extracted DNA was sent to the companies Pishgam and Sinaclon for sequencing. The sequences were subjected to a BLAST search using NCBI internet resources. Sequences that were aligned by CLC Main Workbench version 5.5. are seen in Table 1. Phylogeny studies were carried out, using the UPGMA program CLC Main Workbench version 5.5. The distance matrix method was also used (Table 2).

Table 1 Orthopoxvirus sequences used for alignments

Sequence no.	Virus isolate (strain)	Organism	Country	Reference	Accession no.
1		Cowpox virus		Meyer et al. [9]	S72087
2	Swedish isolates	Cowpox virus		Hansen et al. [6]	Y18386
3	Norwegian isolates	Cowpox virus	Finland	Hansen et al. [6]	Y18385
4	FIN/T2000	Cowpox virus	Sweden	Okeke et al. [14] DS[a]	HQ680378
5	Swe-H2	Cowpox virus	Sweden	Okeke et al. [14] DS	HQ680377
6	Swe-H1	Cowpox virus	Norway	Okeke et al. [14] DS	HQ680376
7	No-F2	Cowpox virus	Norway	Okeke et al. [14] DS	HQ680375
8	No-F1	Cowpox virus	Norway	Okeke et al. [14] DS	HQ680374
9	No-H2	Cowpox virus	Norway	Okeke et al. [13]	HQ680373
10	No-H1	Cowpox virus	Saudi Arabia	Okeke et al. [13]	HQ680372
11	Al-Ahsaa	Camelpoxvirus		Yousif et al. [20]	GU937751
12	CPXV/MVA-Rec 3b	Cowpox-Vaccinia virus		Okeke et al. [12]	EF591311
13	CPXV/MVA-Rec 3a	Cowpox-Vaccinia virus		Okeke et al. [12]	EF591310
14	CPXV/MVA-Rec 3	Cowpox-Vaccinia virus		Okeke et al. [12]	EF591309
15	CPXV/MVA-Rec 2	Cowpox-Vaccinia virus		Okeke et al. [12]	EF591308
16	CPXV/MVA-Rec 1	Cowpox-Vaccinia virus		Okeke et al. [12]	EF591307
17	H1	Cowpox virus	Norway	Okeke et al. [12]	EF591305
18	Al-Ahsaa	Camelpox virus	Saudi Arabia	Yousif et al. [21]	JQ901104
19	CP-1	Orthopoxvirus	Iran	Meyer et al. [8]	X69774
20	CMS	Camelpoxvirus	Iran	Gubser & Smith [4]	AY009089[b]
21	M-96	Camelpoxvirus	Kazakhstan	Afonso et al. [1]	AF438165[c]

a: Direct submission

b: Sequences 136224 to 137040

c: Sequences 138104 to 138920

Cell culture

Camelpox virus can be propagated in a large variety of cell cultures, including the following cell lines: Vero, MA-104, MS monkey kidney, baby hamster kidney (BHK), lamb testes, lamb kidney, camel embryonic kidney, calf kidney, and chicken embryo fibroblasts [17].

Samples were prepared for virus isolation as follows: The size of a sample was at least 30–50 mg. The scabs or tissue samples were minced with a disposable blade or sterile scissors and forceps. The samples were ground in a five-fold volume of phosphate-buffered saline (PBS) with antibiotics (105 international units [IU] of penicillin and 10 mg streptomycin per ml), using a mortar and pestle with sterile sand. The sample was transferred to a centrifuge tube and frozen and thawed two to three times to release the virus from the cells. The samples were vortexed while thawing. The tubes were placed on ice and sonicated once for 30 seconds at 80 Hz. They were then centrifuged at 1000g for 10 minutes to remove large particles and to collect the supernatant [15, 16].

Four hundred microliters of the supernatant was incubated for 1 hour at room temperature and overnight at 4 °C. The supernatant was filtered through a 0.45-μm filter and used to inoculate confluent cells in a 25-cm^2 flask. The filter was flushed with 0.5 ml of the maintenance medium used for cell culture, and the flask was incubated at 37 °C for 1 hour.

Six to seven ml of the fresh medium was added to the flask, and the incubation was continued for about 10 days. If there was any reason to suspect fungal contamination, the contaminated medium was discarded and fresh medium containing 5 μg of amphotericin B per ml was added. The flasks were monitored daily for 10–12 days.

Once CPE was observed, the flask was transferred to an inverted microscope for photography. The second passage was used from both the supernatant of the first culture and primary sample. The CPE resulting from inoculation with supernatant appeared to be more marked.

The growth of camelpox virus in cell culture can be confirmed by TEM, PCR or antigen capture enzyme-linked immunosorbent assay (ELISA) [17].

Results

Typical clinical signs of CMLV were observed in which the affected camels showed signs that varied from acute to

mild. The affected animals were off food with edematous face and ocular lacrimation. Papules, vesicles and thick scabs were observed on the lips and nostrils, and in some instances such lesions involved the whole head and neck. It is also necessary to mention that cattle, sheep, goats, rabbits, guinea pigs, rats, hamsters, and mice in the area where camelpox occurred were not infected with camelpox virus. In addition, no cases of human infection were observed in the infected areas.

Polymerase chain reaction

PCR is a fast and sensitive method for detection of orthopoxviral DNA that allows detection and differentiation of viruses 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) was amplified. The size of the PCR product that is specific for camelpox virus is 881 bp. Samples from the entire infected area were positive for camelpox virus and similarly yielded a 881-bp band (Fig. 1).

Phylogenetic analysis

The samples analyzed in this study included those from Qom and MSPKH and MSPKHJ, which were obtained in the city of Khash and South Khorasan Province, respectively. ATIP gene sequences from this study were aligned with previously published sequences (Table 1), and a phylogenetic tree was constructed using the program CLC version 5.5 (Fig. 2). CPXV/MVA-Rec 2 formed a distinct branching pattern, implying high divergence from other members of the genus *Orthopoxvirus*. The sequences from the present study formed a cluster together with Norwegian and Swedish isolates of cowpoxvirus that had been isolated

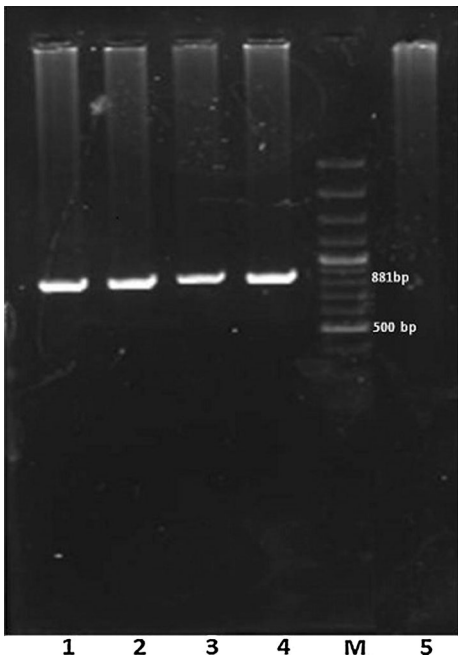

Fig. 1 Visualization of camelpox virus PCR products by agarose gel electrophoresis. Lane 1, positive control from Razi Institute, Tehran, Iran; lane 2, Qom sample; lane 3, Khash sample; lane 4, South Khorasan sample; lane 5, negative control, distilled water; lane M, 100-bp ladder. The image was made using a gel documentation system

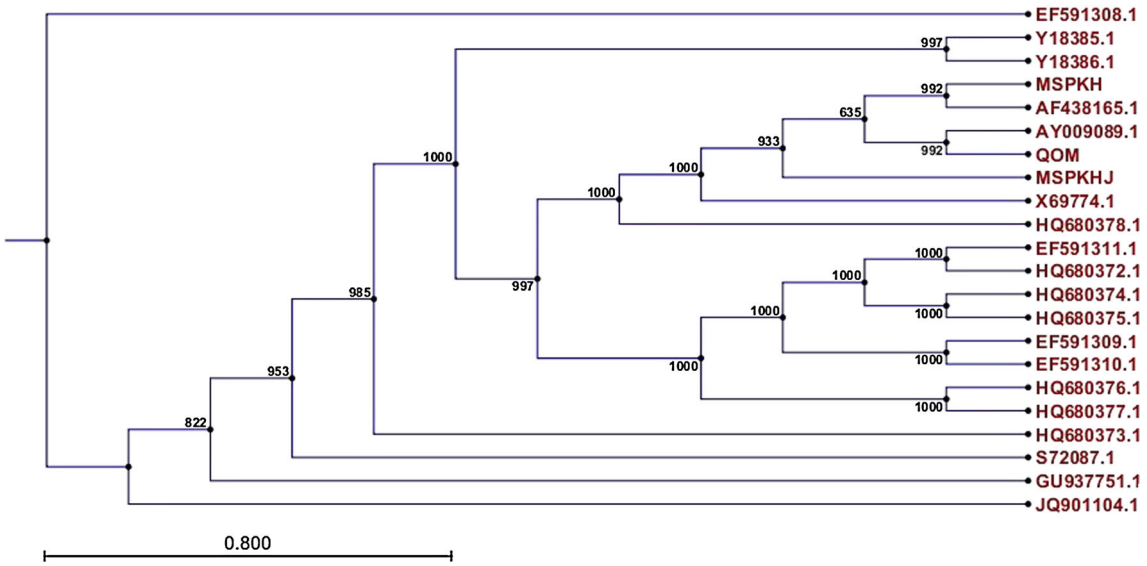

Fig. 2 Phylogenetic tree of the orthopoxviruses constructed based on the gene encoding the A-type inclusion protein (ATIP) by the UPGMA method in CLC Main Workbench 5.5, with 1000 bootstrap replicates. The numbers at each branch represent bootstrap values. The bar represents the genetic distance

earlier, except isolate No-H2. The Qom sample formed a clade with the CMS strain (sequences 136224-137040). MSPKH and M-96 (sequences 138104 to 138920) also formed a clade. Qom/CMS formed a group with the clade MSPKH /M-96. This group is sister to MSPKHJ, CP-1 and FIN/T2000. The branch length between our samples, isolate FIN/T2000 and the CP-1 strain illustrates the very close distance between our samples' clade and FIN/T2000 and CP-1. Thus, a clade containing our samples and the camelpox virus CP-1 strain (X69774.1) is sister to isolate FIN/T2000 (HQ680378). Therefore, using the CLC program, based on the distance method, we concluded that the studied samples showed most similarity to the CMS and M-96 strains. They also showed high similarity to isolate FIN/T2000 and the CP-1 strain. As shown in Table 2, the samples in the present study showed 100 % similarity to each other and to CMS and M-96. As shown in Table 2, they showed 95 % and 99 % similarity to FIN/T2000 and CP-1, respectively. As expected, the shortest distance was found between our samples and the CMS and M-96 strains. The next closest distances were 0.01 and 0.05, to CP-1 and FIN/T2000, respectively. These results correspond to the phylogenetic tree. We can say that the similarity of our samples to strains from Iran (CMS, CP-1) is understandable. Also, the relationship to a strain from Kazakhstan (M-96), which is a neighbor of Iran, is logical. But we do not have an explanation for the relationship to the Swedish isolate (FIN/T2000), and this question requires more research. The reliability of the phylogenetic relationship was statistically evaluated from 1000 bootstrap replicates.

Virus isolation in cell culture

In this study, camelpox virus was cultured in Vero cells. Fig. 3a shows normal Vero cells. All other images in Fig. 3 were taken on different days postinfection, and the monolayers shown in Fig. 3 are from two consecutive experiments. Fig. 3b-e and g were taken on day 5 postinfection

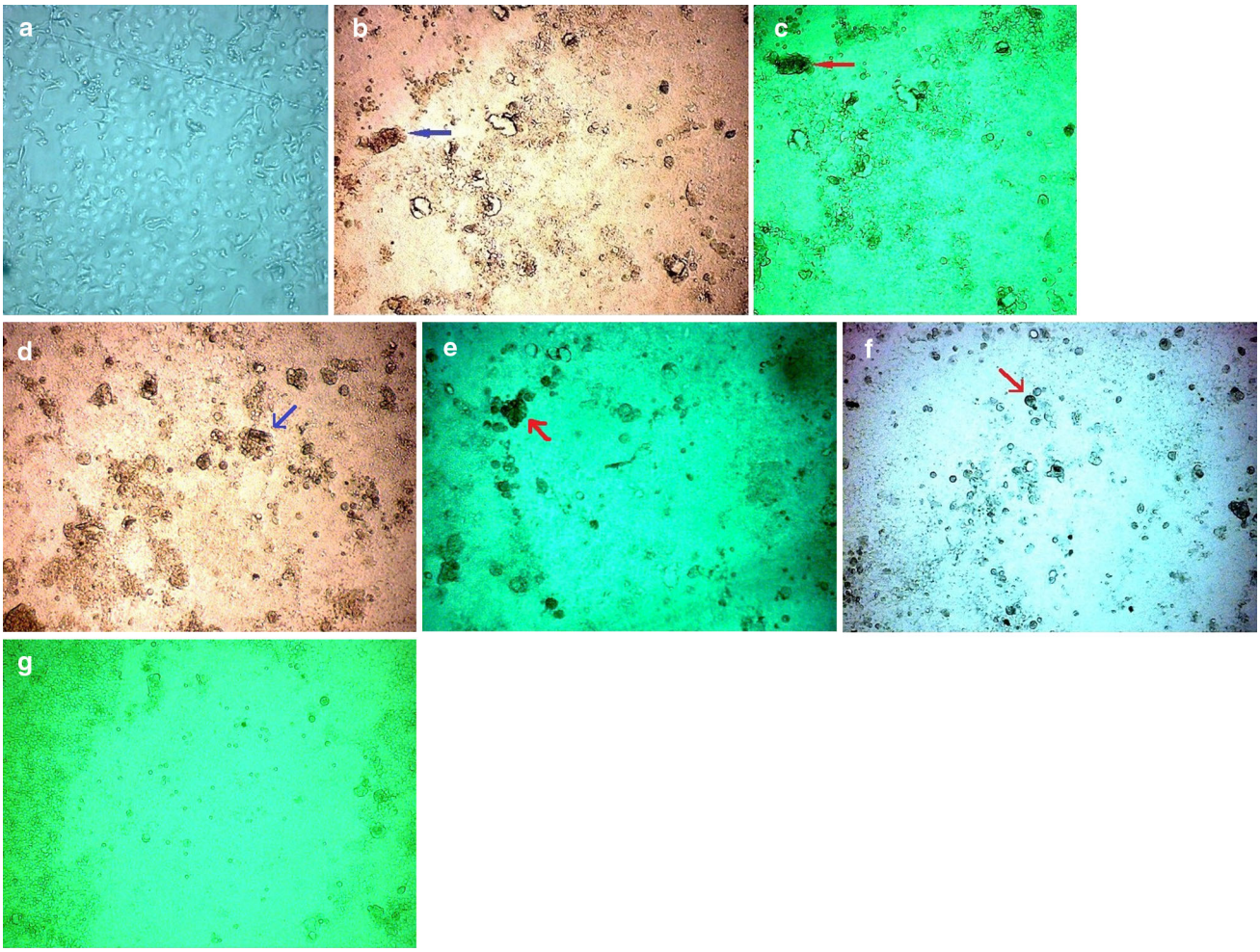

Fig. 3 Cytopathic effects of camelpox virus on Vero cells. a, uninfected Vero cells, ×100 magnification; b, c, d, e, syncitia resulting from the fusion of a few cells infected with camelpox virus, viewed at ×400 magnification; f, rounded cells, ×400; g, cell detachment, ×100 magnification. The image was made using an inverted microscope with the program Dino Capture

from the first culture. Fig. 3f was taken on day 6 postinfection from the first culture.

Characteristic plaque-type cytopathic effect (CPE) showing foci of rounded cells (Fig. 3f), cell detachment (Fig. 3g), and syncytia (Fig. 3b, 3c, 3d, 3e) were observed. The syncytia contained up to 10 nuclei.

As shown in Fig. 3, in the first passage, only small areas of CPE could be seen 3 days after inoculation, but over the subsequent 4-6 days, these areas expanded to involve the entire monolayer. The virus produced small holes in the cell sheet, and this resulted in detachment of the cells. This was observed on day 2 in the second culture. Cultures showing distinct cytopathogenic effect (CPE) on the 8 to 10th day were frozen and used for further *in vitro* passages.

Discussion

Camelpox virus causes a smallpox-like illness in camels. The disease is enzootic in almost all regions where camel husbandry is practiced and is responsible for severe economic losses. Although it is genetically the closest known virus to variola virus, the etiologic agent of smallpox, CMLV remains poorly studied [3]. This is true especially in the case of Iranian camelpox virus strains, which we tried to focus on in the current study. Therefore, the main objective of the present study was to propagate the virus and find a substitute for time-consuming serological techniques. In the present study, we detected camelpox virus using a PCR assay described by Meyer et al. [9], in which an 881-bp amplification product for CMLV was detected. Our results are in agreement with those of Hanan et al. [5] and Sharawi et al. [17], in which this method was used for detection and differentiation of viruses of the genus *Orthopoxvirus* based on size differences of the amplicons.

The PCR assay described in this study is a valuable addition to the current methods for diagnosis and differentiation of parapoxvirus (PPV) and orthopoxvirus (OPV) infections in camels. Camelpox virus infects camels and causes orf disease. Well-equipped laboratories with cell culture and electron microscopy facilities are not needed, and the results can be obtained in a small laboratory within 24 hours of receiving the sample. This is expected to help in the implementation of prompt measures to control these important diseases, resulting in improved health and productivity of national camel herds.

In the present study, we found that there is 100 % similarity between our samples and CMS and M-96 strains, and therefore there is a close relationship between our samples and isolate FIN/T2000 and the CP-1 strain. We

Table 2 Part of pairwise comparison of aligned sequences (upper right, percent identity; lower left, distance) conducted using CLC Main Workbench 5.5

		6	7	8	9	10	11	12	13	14	15	16	17	18
HQ680375.1	1	83.81	100.00	100.00	100.00	82.80	44.38	82.29	82.29	82.29	82.29	82.12	65.41	77.93
HQ680374.1	2	83.81	100.00	100.00	100.00	82.80	44.38	82.29	82.29	82.29	82.29	82.12	65.41	77.93
HQ680372.1	3	83.81	100.00	100.00	100.00	82.80	44.38	82.29	82.29	82.29	82.29	82.12	65.41	77.93
HQ680377.1	4	84.82	97.12	97.12	97.12	83.64	44.94	83.14	83.14	83.14	83.14	82.97	67.03	75.62
HQ680376.1	5	84.82	97.12	97.12	97.12	83.64	44.94	83.14	83.14	83.14	83.14	82.97	67.03	75.62
HQ680378.1	6		83.81	83.81	83.81	95.62	41.99	95.11	95.11	95.11	95.11	94.94	65.60	64.76
EF591311.1	7	0.18		100.00	100.00	82.80	44.38	82.29	82.29	82.29	82.29	82.12	65.41	77.93
EF591310.1	8	0.18	0.00		100.00	82.80	44.38	82.29	82.29	82.29	82.29	82.12	65.41	77.93
EF591309.1	9	0.18	0.00	0.00		82.80	44.38	82.29	82.29	82.29	82.29	82.12	65.41	77.93
X69774.1	10	0.05	0.20	0.20	0.20		41.46	99.15	99.15	99.15	99.15	98.98	65.09	64.08
HQ660373.1	11	1.11	1.01	1.01	1.01	1.14		41.26	41.26	41.26	41.43	42.61	52.78	48.31
QOM	12	0.05	0.20	0.20	0.20	0.01	1.15		100.00	100.00	100.00	99.83	65.31	64.30
AY009089.1	13	0.05	0.20	0.20	0.20	0.01	1.15	0.00		100.00	100.00	99.83	65.31	64.30
AF438165.1	14	0.05	0.20	0.20	0.20	0.01	1.15	0.00	0.00		100.00	99.83	65.31	64.30
MSPKH	15	0.05	0.20	0.20	0.20	0.01	1.14	0.00	0.00	0.00		99.83	65.31	64.30
MSPKHJ	16	0.05	0.20	0.20	0.20	0.01	1.09	0.00	0.00	0.00	0.00		65.14	64.13
Y18386.1	17	046	046	046	046	0.47	0.74	0.47	0.47	0.47	0.47	0.47		82.50
Y18385.1	18	0.48	0.26	0.26	0.26	0.49	0.88	0.46	0.46	0.46	0.46	0.49	0.20	
EF591308.1	19	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
GU937751.1	20	1.48	1.23	1.23	1.23	1.39	∞	1.37	1.37	1.37	1.37	1.37	∞	∞
S72087.1	21	1.47	1.23	1.23	1.23	1.57	∞	1.59	1.59	1.59	1.59	1.59	1.16	0.80
JQ901104.1	22	1.98	1.58	1.58	1.58	1.93	∞	1.92	1.92	1.92	1.88	1.68	2.74	1.88

believe that this study is the first report that provides considerable information about phylogenetic analysis of Iranian camelpox viruses. Nagarajan et al. [10], based on the nucleotide and amino acid sequence identities and phylogenetic analysis of these genes, found that CMLV-India forms a cluster with Kazakhstan and Iranian CMLV isolates. Several camelpox virus strains such as CMLV-Tehran, CP-1, CML-1, CM-G1, G2 and CM-S have previously been identified [3].

The results of the present study interestingly demonstrate that our primary attempt to inoculate tissue specimens from the suspected camel pox lesions in Vero cells was successfully performed, resulting in a characteristic cytopathic effect. The clear CPE formation in Vero cells in the present study confirmed the report by Sharawi et al. [17], who reported that virus from scab samples collected from camels grows well in Vero cells. They showed that the CPE produced by CMLV in all cell culture types resulted in rounding of the cells, plaque formation, cytoplasmic elongation, and multinucleated giant cell formation.

In conclusion, we confirmed the incidence of CMLV in Iran using a number of methods recommended for its diagnosis [11]. We also analyzed CMLV phylogenetically and compared it with other orthopoxviruses.

Acknowledgments The authors are thankful to Ms. Majidi from the Razi Vaccine and Serum Research Institute for her valuable contribution to this study. They are also indebted to Dr. Barani for his assistance in preparing the samples. They also would like to thank the authorities of Shiraz University for partial financial support of the project.

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