J. Basic. Appl. Sci. Res., 4(4)74-79, 2014
© 2014, TextRoad Publication
ISSN 2090-4304
Journal of Basic and Applied Scientific Research
www.textroad.com

Test System for Camelpox Virus Detection by Polymerase Chain Reaction Field Testing

Kulyaisan T. Sultankulova*, Vitaliy M. Strochkov, Yerbol D. Burashev, Olga V. Chervyakova, Kamshat A. Shorayeva, Nurlan T. Sandybayev, Abylay R. Sansyzbay, Mukhit B. Orynbayev, Murat A. Mambetaliyev, Sarsenbayeva GJ

Research Institute for Biological Safety Problems (RIBSP), Gvardeiskiy, Republic of Kazakhstan

Received: January 18 2014
Accepted: March 23 2014

ABSTRACT

High level of sensitivity ($1x10^2$ DNA copies) and specificity of the developed test system for detection of the camelpox virus by polymerase chain reaction is a result of using CPV-f and CPV-r primers flanking the fragment sized 266 bp at the DNA site 2230 bp in length that encodes the B22R-like protein from CMLV185 to CMLV187. The test system has been tested using the strains of the camelpox virus and organ tissue materials collected from camels in Manghistauskaya oblast, the Republic of Kazakhstan. The developed test system for detection of the camelpox virus DNA by PCR ensures high level of diagnostic assays and can be used in epidemiological monitoring of the camelpox foci in nature.

KEY WORDS: camelpox virus, DNA, polymerase chain reaction, primer, test system.

INTRODUCTION

Camel breeding is a traditional branch of animal husbandry in Kazakhstan and an important reserve of meat, milk and wool production. During recent years the camel population in the republic has grown considerably in the result of well-directed efforts.

It is well known that camels are amenable to different diseases. As it concerns infections, tuberculosis, tetanus, trichophytosis, camel plague *(Pestis camelorum)* and some other are most frequent in these animals [1]. Special attention is paid to spread of such infectious disease as camelpox. It covers the countries of Middle East, north and east Africa, India, and the United Arab Emirates [2, 3, 4, 5].

During epizootic camelpox outbreaks mortality is up to 10% of the adult animals, abortion among pregnant camels is up to 25-27%, decrease in camel yield is up to 85% and more. The infection is diagnosed on the basis of epizootological data, typical symptoms of the disease, positive results of microscopy, electron microscopy, as well as the results of laboratory tests: polymerase chain reaction, restriction enzyme digest analysis, enzyme-linked immunosorbent assay (ELISA). Camelpox was repeatedly registered in the adjacent countries, so the risk of carrying the infection into Kazakhstan is high. The last large-scale outbreak of camelpox was recorded here in 1996 (three districts of Manghistauskaya oblast). Over 10% of the camel population was infected [6]. Evaluation of statistical data on the camelpox morbidity among camels in Manghistauskaya oblast of Kazakhstan enables to ascertain cyclical recurrence of epizootics, which is about 10-20 years. Between epizootics the pathogen persists in camels, causing their sickness in the latent form.

Camelpox virus (CMLV) is a member of *Orthopoxvirus* genus, *Poxviridae* family and an etiological agent of the infectious disease of camels [7]. Viruses of this group affect humans as well as many animal and insect species [8]. Emergence of pox in a large camel farm leads to significant economic losses caused by productivity reduction, forced animal slaughter, as well as by use of preventive quarantine and veterinary-sanitary measures.

It should be noted that success in controlling viral diseases depends much on timely detection and identification of the causing agents that is why much attention is paid to improvement of diagnostic methods and tools. Timely and exact diagnosis of camelpox enables prevention or considerable reduction of economical losses in the result of urgent quarantine measures.

Development of a PCR test system for detection of the camelpox virus DNA and its practical application will contribute to solving many pressing tasks, such as rapid preliminary diagnosis, effective detection of agents in biological materials and environmental objects, thus enabling adequate appraisal of the current epizootic situation and timely performance of counter-epizootic measures.

Application of molecular and genetic methods of new generation in diagnosis and development of means for specific prophylaxis is very urgent for the Republic of Kazakhstan. Conventional methods of detecting viral disease agents sometimes appear to be laborious or ineffective. In many cases PCR can provide a rapid and accurate test [9].

The objective of the work was to develop and approve a test system for detection of the camelpox virus by polymerase chain reaction.

***Corresponding Author:** Sarsenbayeva GJ, Laboratory Control Technology and Biopreparations, Research Institute for Biological Safety Problems, Republic of Kazakhstan, Zhambyl region, Korday district, Guardeyskiy, 080409. e-mail: gukacool@mail.ru

MATERIALS AND METHODS

Viruses

The following poxvirus strains were used as objects of research:
- camelpox virus, strain "M-96" epizootic;
- camelpox virus, strain "KM-70" vaccinal;
- sheeppox virus, strain "A", epizootic;
- sheeppox virus, strain "NISKHI", vaccinal;
- goatpox virus, strain "LKB-G 20", vaccinal;
- goatpox virus, strain "Pellor", epizootic;
- cowpox virus, strain "CowpoxCam", epizootic;
- chickenpox virus, strain "NISKHI-9-95", epizootic;
- chickenpox virus, strain "Taraz-K", vaccinal;
- vaccinia virus, strain "BIEMG-51", vaccinal;
- horsepox virus, strain "MNR-76", epizootic;
- contagious pustular dermatitis virus, "NISKHI-MTM", vaccinal;
- ectromelia virus (an isolate);
- samples of organ and tissue materials from infected camels.

Extraction of camelpox virus DNA

DNA was extracted by phenol-free method.

Viral DNA is extracted according to the procedure with use of SiO_2 sorbent [10, 11]. Purity of the resulted DNA preparations is checked by spectrophotometry judging on optical densities at 260 nm and 280 nm wavelengths (E_{260}/E_{280}).

Oligonucleotide primers

Orthopoxvirus nucleotide sequences were analyzed with use of the BioEdit v.7.0 program package. Primers were selected with the help of Primer 3 program [12].

Primers CPV-f (AAAATGCTGAATGCTGAAGCTGTA) and CPV-r (GCTGATGCAAATCTCATTGATGTTA) [13] to DNA sequence of the camelpox virus were synthesized in Expedite 8900TM Nucleic Acid Synthesis System (Applied Biosystems).

PCR-amplification and electrophoresis

Reaction mixture and temperature-time conditions for PCR were used pursuant to the instruction enclosed with the enzyme and to the primers characteristics.

Product amplification was carried out in thermocyclers GeneAmp PCR 9700, Applied Biosystems.

Electrophoretic analysis of the DNA amplification products was performed on 1.0% agarose in tris-borate buffer (TBE) at 60V (3-5 V/cm) in DNA-100 ("Pharmacia", Switzerland)d unit. The DNA bands were visualized using ethidium bromide. The gels were photographed with an electronic gel documentation system "BioRad"[e].

Sequencing DNA fragments

DNA was sequenced by BigDye Terminator v.3.1 Sequencing kit (Applied Biosystems) in the automatic 16-channel Genetic Analyzer 3130 xl, Applied Biosystems[g]. POP-7 was used as a polymer for capillaries. DNA terminating products were produced by the method of cycle sequencing.

RESULTS

Extraction of nucleic acids is one of the most important steps in development of test systems. As the published data show various methods including treatment with sodium dodecyl sulfate (SDS), proteinase K or protease inactivation with guanidine isothiocyanate followed by DNA sorption on silicium dioxide particles are used to extract nucleic acids of DNA-containing viruses [10, 11]. The key criterion for the methods of DNA isolation is the highest possible level of the nucleic acid purity from cellular DNAs and proteins. The performed research resulted in selection of the phenol-free method with use of SiO_2 sorbent for the camelpox virus DNA extraction. Unlike the other methods it does not use toxic reagents. The DNA extraction set includes the lysing buffer, washing buffer, SiO_2 sorbent and eluting solution. It is stored at 2-8°C.

The subsequent experiments were aimed at construction of specific primers and optimization of the PCR procedure [12].

Primers CPV-f (5'-AAAATGCTGAATGCTGAAGCTGTA-3') and CPV-r (5'-GCTGATGCAAATCTCATTGATGTTA-3') were chosen with the help of the computer program Primer 3 and BLAST algorithm on the basis of nucleotide sequences of various camelpox virus strains from the database of GenBank (AF438165.1 and AY009089.1) in accordance with the requirements to oligonucleotide primers. These primers flank the fragment sized 266 bp that is localized on the 2230 bp long DNA encoding B22R-like protein

from CMLV185 to CMLV187 and is absent in other orthopoxviruses [14]. These primers served a basis for development of a test system for detection of the camelpox virus by the method of polymerase chain reaction.

Numerous experiments towards determining the best composition of the PCR reaction mixture as well as optimizing temperature and time parameters of amplification enabled choosing optimal conditions of the PCR process.

For amplification of the camelpox virus DNA the following components of the reaction mixture (50 µl in total) were used:

x10 (100 mM Tris HCl, pH 8,3, 15 mM $MgCl_2$, 250 mM KCl) PCR buffer	- 5 µl
dNTP mix10 mM[i]	- 1 µl
Primer 1 CPV-f (10 pM)	- 1 µl
Primer 2 CPV-r (10 pM)	- 1 µl
Camelpox virus DNA	- 2 µl
Taq DNA polymerase (5 U)[j]	- 0.4 µl
Deionized water	- 39.6 µl

Also the research aimed at optimization of the Taq DNA polymerase, primers, and dNTF was carried out. The resulted experimental data show that 2 units of the enzyme Taq DNA polymerase[j] activity, 10 pM of each primer and 10 mM of dNTF in reaction mixture are enough for accumulation of the camelpox virus specific product. It was found out that the magnesium concentration does not influence markedly the amplification process.

In the course of the experiments towards optimization of the time and temperature conditions for PCR procedure in camelpox diagnosis the following parameters were chosen:

pre-denaturation	94°C - 5 min	
denaturation	94°C - 20 sec	35 cycles
annealing	55°C - 20 sec	
replication	72°C - 20 sec	
post-replication	72°C- 10 min	

On the basis of this PCR method a test system for laboratory diagnosis of camelpox was composed. In case of a positive reaction after separation of the PCR product in agarose gel supplemented with ethydium bromide a band 266 bp long appears. Absence of this band or presence of the band of different size means negative result.

The PCR kit includes the following components: specific direct and reverse primers, deoxynucleotide mixture, Taq DNA polymerase, PCR-buffer, deionized water, negative and positive control samples of the camelpox virus DNA.

The kit is stored at minus 20°C.

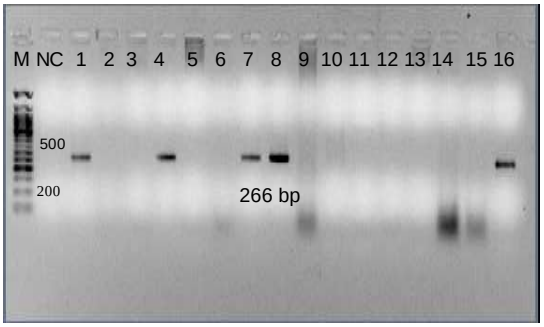
So, the PCR test system for camelpox diagnosis includes two kits:

- a kit for DNA extraction;
- a PCR kit.

Diagnostical characteristics of the test system for detection of the camelpox virus by PCR

One of the key steps in the process of a test system construction is a step of its testing. The RIBSP has a collection of poxviruses, so there is a good possibility to test the developed system with a large number of viruses that vary in origin, epidemiological and biological characteristics.

Analytical specificity of the test system has been proved by testing camelpox virus-containing materials ("M-96" and "KM-70" strains), as well as of organ and tissue materials from infected camels. DNAs of sheeppox, goatpox, cowpox, chickenpox, horsepox, contagious pustular dermatitis, ectromelia viruses were used as DNAs of heterologous poxviruses. The obtained results are shown on Figure 1.



M – 50 bp marker, *Bio*Labs; NC – negative control; 1 – virus DNA extracted from scabs of a pox infected camel; 2 – DNA of the sheeppox virus, strain "A", epizootic; 3 – DNA of the goatpox virus, strain "LKB-G-20", vaccinal; 4 – DNA extracted from serous fluid of a pox infected camel; 5 – DNA of the sheeppox virus, strain "NISKhI", vaccinal; 6 – DNA of the goatpox virus, strain "Pellor", epizootic; 7 – DNA extracted from scabs of a pox infected camel; 8 – DNA of the camelpox virus, strain "M-96"; 9 – DNA of the vaccinia virus, strain "BIEMG-51", vaccinal; 10 – DNA of the horsepox virus, strain "MNR-76", epizootic; 11 – DNA of the contagious pustular dermatitis virus, "NISKhI-MTM", vaccinal; 12 – ectromelia virus (an isolate) DNA; 13 – DNA of the cowpox virus, strain "CowpoxCam", epizootic; 14 – DNA of the chickenpox virus, strain "NISKhI-9-95", epizootic; 15 – DNA of the chickenpox virus, strain "Taraz-K", vaccinal; 16 – DNA of the camelpox virus, strain "KM-70".

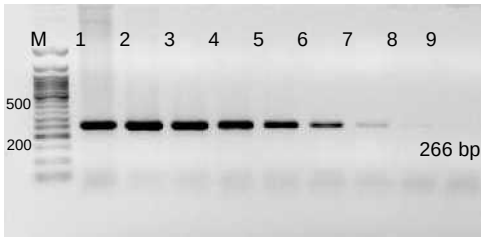
Fig. 1 – Electrophoregram of the camelpox virus DNA amplification products

In all cases of using heterologous poxviruses negative results were obtained. Usage of deionized water also resulted in the absence of any amplification products.

Nucleotide sequences of the PCR products were analyzed to check the reliability of the results concerning specificity of the PCR test system. Direct sequencing of PCR-products was performed in 16-channel Genetic Analyzer 3130 xl, Applied Biosystems. The resulted nucleotide sequences were analyzed with the help of the BLAST program. The analysis data show 100% homology of the nucleotide sequences of the camelpox virus PCR products with sequences of the camelpox virus strains "M-96" ID (AF438165.1) and "CMS" ID (AY009089.1) in Genbank.

So, high homology of the compared nucleotide sequences confirms specificity of the developed test system.

The chosen time and temperature reaction conditions and 10-fold dilutions (from $1x10^8$ to 1.0) of the DNA of the camelpox virus, strain "M-96", were used to assess sensitivity of the PCR test system. The results are shown on Figure 2.



M – 50 bp marker, *Bio*Labs;
1 – $1x10^8$; 2 –$1x10^7$; 3 – $1x10^6$; 4 –$1x10^5$; 5 – $1x10^4$; 6 – $1x10^3$; 7 – $1x10^2$; 8 – $1x10^1$; 9 – 1.0 DNA copies

Fig. 2 – Appraisal of sensitivity of the PCR test system for camelpox diagnosis

Testing of 10-fold dilutions of the camelpox virus DNA (from $1x10^8$ to 1.0 DNA copies) for appraisal of the test system sensitivity has shown the sensitivity threshold to be $1x10^2$ DNA copies.

DISCUSSION

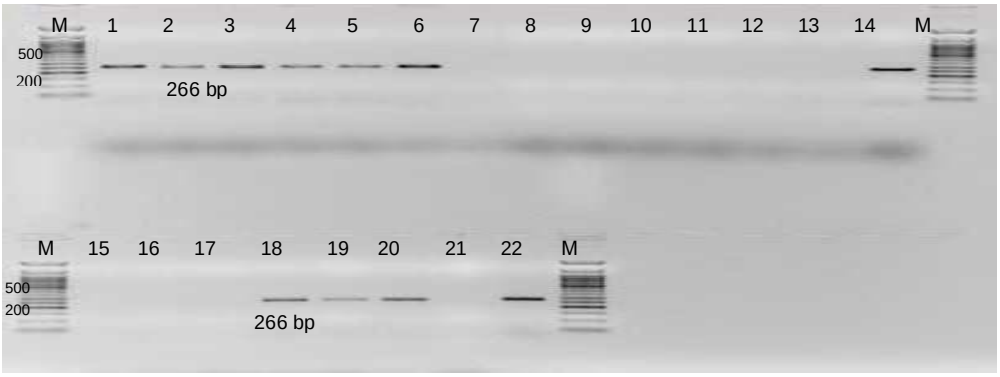
To take timely counterepizootic measures in case of camelpox emergence it is necessary to have at one's disposal the methods of laboratory assay that enable to encode the etiology of the virus as soon as possible. The infection is diagnosed by traditional methods that are very time-consuming.

The PCR test systems are the most perfect diagnostic means in molecular biology, molecular genetics and clinical laboratory diagnosis that allow detecting single cells of many infectious agents in tissues and biological fluids of a body. Currently the requirements to diagnostic preparations are much higher. Apart of rapid response the employed PCR test systems should demonstrate high sensitivity, ensure virus detection in materials with its low content and to provide differentiation of closely-related orthopoxviruses.

According to Balamurugan V. et al. 2009 in PCR-diagnosis of camelpox with use of primers to ORF CMLV004 (encoding ankyrin repeat protein) sensitivity of the duplex PCR is 0.4 ng of viral DNA while our test system with primers CPV-f and CPV-r localized at the 2230 bp long DNA site that encodes ORF from CMLV185 to CMLV187 demonstrates sensitivity as high as $1x10^2$ DNA copies.

The developed system was tested for detection of the camelpox virus in field materials.

An advantage of this test system is a possibility to assay organ and tissue materials from camels. Samples of blood, serous fluids from pustules and scabs collected from the infected camels in Manghistauskaya oblast of Kazakhstan in 1996 and 2011 were assayed for detection of the camelpox virus DNA with the help of the developed test system. Figure 3 shows electrophoretic profiles of the PCR products separation in the course of appraisal of the test system for detection of the camelpox virus DNA in pathology materials by PCR.



M – 50 bp marker, *Bio*Labs;1 – DNA isolated from scabs of pox-infected camels (1996); 2 – DNA isolated from saliva sampled from mouths of pox-infected camel colts (1996); 3 – DNA isolated from pocks of sick camels on 12^{th}-13^{th} days post vaccination (1996); 4 – DNA isolated from sera of camel colts on the 2^{nd}-3^{rd} days of sickness (1996); 5 – DNA isolated from sick camel colts on the 2^{nd}-4^{th} day of sickness (1996); 6 – DNA isolated from serous fluids collected from pustules of camelpox-infected animals (1996); 7 – DNA isolated from blood sample No. 2 (2011); 8 – DNA isolated from blood sample No. 3 (2011); 9 – DNA isolated from blood sample No. 8 (2011); 10 – DNA isolated from blood sample No. 10 (2011); 11 – DNA isolated from blood sample No. 16 (2011); 12 – DNA isolated from blood sample No. 17 (2011); 13 – negative control (water); 14 – positive control (camelpox virus, strain "M-96", DNA); 15 – DNA isolated from blood sample No. 19 (2011); 16 – DNA isolated from blood sample No. 34 (2011); 17 – DNA isolated from blood sample No. 35 (2011); 18 – DNA isolated from scabs collected from camelpox-infected animals (1996); 19 – DNA isolated from serous fluids collected from pustules of camelpox-infected animals (1996); 20 – DNA isolated from blood of camelpox-infected animals (1996); 21 – negative control (water); 22 – positive control (camelpox virus, strain "M-96", DNA).

Fig. 3 – Electrophoretic profiles of the PCR products separation in appraisal of the test system for detection of the camelpox virus DNA in pathology materials from the infected camels

As one can see from Figure 3 samples 1-6, 18-20 from Manghistauskaya oblast, Kazakhstan (1996) gave positive reactions. Negative were samples 7-12, 15-17 collected in the same region in 2011. The above data indicate that in 2011 the camelpox virus have not circulated among camels in Manghistauskaya oblast, Republic of Kazakhstan.

Hemagglutination inhibition test that reveals the presence of specific antibodies to the camelpox virus is also used. Basic value of serologic methods consists in retrospective diagnosis of viral diseases that provides indirect determination of the spectrum of the poxvirus circulating among camels.

The laboratory findings show that in 2011 there were no cases of camelpox in Manghistausskaya oblast, but the number of positive results was 40% higher in sera tested for antibodies to the camelpox virus. It is an evidence of the symptomless circulation of the agent among camels. The fact is that the specific antibodies to the camelpox virus appear in blood of sick animals late, as a rule, when they are getting better already and laboratory verification of the infectious agent is slightly important for them.

Between epizooties the disease agent persists among camels causing animal sickness in latent form, therefore it is supposed that the disease outbreaks can occur on the territory of Manghistausskaya oblast, Republic of Kazakhstan, in the next years [6]. Thereby it is important to conduct constant laboratory monitoring in camel herds with use of the test-system developed for detection of the camelpox virus by the method of polymerase chain reaction.

Effectiveness of the test system for detection of the camelpox virus by the method of polymerase chain reaction is 95% as compared to the hemagglutination inhibition test.

Usage of the PCR test system enables differentiated diagnosis of camelpox on early stages of the disease and therefore timely infection-oriented treatment.

The findings of quite a number of research works [15, 16] are an evidence of using PCR in the camelpox diagnosis. For instance, the PCR-based method with primers localized on sites of the virus DNA genes B5R and C18L was used for the agent identification in the course of the camelpox outbreak in India in 2010. Recent studies [17] have shown the camelpox to be confirmed also by sequencing region C18L of the virus DNA.

So, a sensitive and specific test system for detection of the camelpox virus DNA by the method of polymerase chain reaction in cultures and in specimens taken from animals has been developed.

Diagnosis of the camelpox with the help of the highly sensitive and specific test system on the basis of polymerase chain reaction allows accurate and very rapid detection of the viral DNA in organ and tissue materials and simultaneous assaying of many specimens is possible.

Conclusion

Detection of the unique fragment that is characteristic only for the camelpox virus DNA provides high specificity of the developed PCR test system. This specificity is defined by nucleotide sequences of primers that exclude false results in contrast to immunological methods that are prone to errors because of cross-reacting antigens.

The sensitivity threshold of the test system for the PCR-based camelpox diagnosis is $1x10^2$ virus DNA copies per specimen.

The developed test system for laboratory diagnosis of camelpox on the basis of PCR is specific and highly sensitive, ensures high level of diagnostic studies and can be used in epizootological monitoring of the natural camelpox foci.

Acknowledgement

The authors are grateful to Yevgeniya Chebotar for translation of the manuscript from Russian into English.

REFERENCES

1. Akimushkin I.I., 1974. Animals World. M.: "Mysl", pp: 268-269 (in Russian)

2. Al-zi'abi O., Nishikawa H, Meyer H., 2007. The first outbreak of camelpox in Syria. J Vet Med., 69 (5): 541-3

3. Wernery U., Meyer H., Pfeffer M., 1997 Camelpox in the United Arab Emirates and its prevention. J. Camel Pract Res., 4: 135–139

4. McGrane J.J., Higgines H.J., 1985. Infectious diseases of the camel: viruses, bacteria and fungi. Br. Vet., J 141: 529–547

5. OIE, 2009. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Camelpox. (Chapter 2.9.2) 1177–1184

6. Bulatov YE.A., Mamadaliyev S.M., Mambetaliyev M.A., Bitov N.T., 2010. About circulation of the camelpox virus in Manghistauskaya oblast, Republic of Kazakhstan, in latent form. J Aktualnye voprosy veterinarnoy biologii (Pressing issues of veterinary biology), 3: 10-13 (in Russian)

7. Caroline Gubser and Geoffrey L. Smith, 2002. The sequence of camelpox virus shows it is most closely related to variola virus, the cause of smallpox. J Gen Virol., 83:855-872

8. Jezek Z., Kriz B., 1983. Camelpox and its risk to the human population. J of Hygiene, Epidemiol, Microbiol and Immunol., (1): 29–42

9. Belak S., Ballagi-Pordany A.., 1993. Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. J Vet Res Commun., V17 (1): 55-72

10. Esposito J.J. et al., 1981. The preparation of orthopoxvirus DNA. J Virol Methods., 2: 175-179

11. Esposito, J.J., Knight J., 1985. Orthopoxvirus DNA: a comparison of restriction profiles and maps. J Virol., 143: 230-251

12. Li-Yeh Chuang, Yu-Huei Cheng, Cheng-Hong Yang, 2013. Specific primer design for the polymerase chain reaction. Biotechnol Lett., DOI: 10.1007/s10529-013-1249-8

13. Azhibayeva D.T., Strochkov V.M., Sultankulova K.T., Sandybayev N.T. et al., 2011. Primer construction and selection for detecting DNA of the camelpox virus by PCR, The International IMBG Conference for young scientists Molecular Biology Advances and Perspectives, September, 14-17, 2011 Kyiv, Ukraine, 80

14. Alfonso C.L., Tulman E.R., Lu Z., Zsak L., Sandybaev N.T., et al., 2002. The genome of Camelpox Virus. J Virol., 295: 1-9

15. Balamurugan V, Bhanuprakash V, Hosamani M, et al., 2009. A polymerase chain reaction strategy for the diagnosis of camelpox. J Vet Diagn Invest., 21 (2): 231-7

16. Bhanuprakash V, Prabhu M, Venkatesan G., et al., 2010a. Camelpox: epidemiology, diagnosis and control measures. Expert Rev. Anti Infect Ther., 8(10): 187-201

17. Bhanuprakash V., Balamurugan V., Hosamani M, et al., 2010b. Isolation and characterization of Indian isolates of camelpox virus. Trop Anim Health Prod., 42: 1271-1275