**Original Paper****Development of duplex real time PCR assay based on C18L and DNA pol genes for rapid diagnosis of camel pox in dromedary camels**Amir A. Shehata¹, Ehab M. El-Nahas², Eman M. Abo Hatab³, Hanaa A. Ahmed¹, Saad S.A. Sharawi²¹ Genome Research Unit, Animal Health Research Institute, Agricultural Research Center, Giza, Egypt² Department of Virology, Faculty of Veterinary Medicine, Benha University, Benha, Egypt³ Department of Virology, Animal Health Research Institute, Agricultural Research Center, Giza, Egypt**ARTICLE INFO****ABSTRACT****Keywords**

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Camel pox is a viral contagious notifiable zoonotic disease-causing variable rate of mortality, morbidity and cutaneous lesions in camels and humans. It caused by camel pox virus (CMLV), an ortho-poxvirus and can be fatal in young camels. Confirmative, rapid and sensitive diagnostic techniques are required for accurate diagnosis of the disease, so in the present study, a duplex real-time PCR assay was developed for rapid detection of camel pox virus (CMLV) based on targeting both C18L and DNA pol genes. The detection limit was 0.4 pg and 4 pg of viral DNA for C18L and DNA pol genes, respectively. The duplex assay detected CMLV DNA in different types of spiked samples and provides a rapid diagnostic tool for camel pox especially in borders where testing of large number of animals during importation and exportation is applied. Further evaluation of the assay on other clinical samples or samples from experimentally infected animal may be required.

1. INTRODUCTION

Camel pox is a highly contagious skin disease affecting camels (Dahiya et al., 2016). The disease characterized by papules and pustules in skin and mucous membrane (Bhanuprakash et al., 2010). The causative agent is camel pox virus (CMLV), a member of *ortho-poxvirus* genus, *chordo-poxvirinae* subfamily, *poxviridae* family (Haller et al., 2014). The other members of the genus include Monkey pox virus, Vaccinia virus, Buffalo pox virus, Cow pox virus, Ectromelia virus, Rabbitpox virus, Raccoon pox virus, Monkey pox virus, horse pox virus (Haller et al., 2014).

The average size of the virus is 224 × 389 nm. Ortho-pox viruses are brick-shaped and consist of an envelope, outer membrane, two lateral bodies and a core (King et al., 2012). The CMLV genome is a single molecule of lineard DNA around 205.719 kbp (Bhanuprakash et al., 2010). It contains more than 211 putative genes, which code for different proteins with host range, virulence, immunomodulation, and other functions (Aregawi and Feyissa, 2016). The central region of the genome containing genes that are highly conserved amongst ortho-pox viruses and the terminal regions contain genes that has variability between different pox viruses (King et al., 2012).

The disease transmitted by direct contact between infected and susceptible animals through skin abrasions and indirect contact with contaminated objects. Saliva, milk, nasal and ocular discharge can be source of infection (Narnaware et al.,

2021). The disease has two forms mild or localized form in adult camels and severe and generalized in young ones (Mosadeghesari et al., 2014). The disease may include fever, face edema, lachrymation, pendulous lips and characteristic pox lesions. Papules and vesicles appear on the lips and nostrils and then involve the head, neck, buttock, legs and groin (Narnaware et al., 2021).

Outbreaks have been reported in Asia (Saudi Arabia, Bahrain, Oman United Arab Emirates, Yemen, Iran, Iraq, Syria, Afghanistan, southern parts of Russia and India, and Pakistan) (Mohammadpour et al., 2020) and in Africa (Egypt, Ethiopia, Algeria, Kenya, Mauritania, Niger, Morocco, Somalia and Sudan) (Ayelet et al., 2013; Bassiouny et al., 2014). Camel pox has socio-economic significance and cause losses through morbidity and mortality (Bhanuprakash et al., 2010). The zoonotic importance of camel pox was reported in transmission of CMLV to camel handlers in India (Bera et al., 2011).

Camel pox is routinely diagnosed based on clinical signs, pathological findings beside cellular and molecular assays (Aregawi and Feyissa, 2016). The classical methods of laboratory diagnosis as virus isolation of embryonated chicken eggs and cell culture are time consuming and the use of transmission electron microscope (TEM) is difficult to be done in many laboratories (Bhanuprakash et al., 2010).

Molecular assays as PCR and real time PCR offer a better solution for the rapid diagnosis of camel pox especially at countries borders during importation of large number animals. The present study aimed to develop and evaluate a

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duplex real time PCR assay for rapid diagnosis of camel pox in dromedary camels based on C18L gene (ankyrin repeat protein) and DNA pol gene (viral DNA polymerase).

2. MATERIAL AND METHODS

Reference camel pox virus:

Camel pox virus (Jouf-78) was used for validation of the assay, this strain used for vaccination of camels in Egypt against camel pox (Bassiouny et al., 2014).

Samples:

Thirty samples (ocular and nasal swabs, skin tissue scrapings from healthy camels) in Aswan and Behera Governorates were collected for spiking and evaluation of the test in different matrices and as negative controls as well.

Primer and probes design:

Primers and probe targeting the C18L gene were developed by Venkatesan et al.(2012)(Table 1)but the probe dye changed to VIC. For DNA pol gene, primers of Balamurugan et al. (2009) and new TaqMan probe labeled by FAM was designed for targeting this gene (table 1). Nucleotide sequences of camel pox virus were retrieved from GenBank and aligned together using BioEdit software version 7.2.6.1(Hall, 1999).The probe designed in the region between the forward and reverse primers of DNA pol gene then checked using BLAST tool on Gene Bank.

Table 1 Primers and probes sequences used in this study

Name	Sequence 5'-3'	Reference
CMLV C18L F	GCGTTAACGGACGTCGTG	(Balamurugan et al., 2009a)
CMLV C18L R	GATCGGAGATATCATACTTACTTTAG	(Balamurugan et al., 2009a)
CMLV C18L Probe	CATCAATACAGCCATCCAGAAAAGTAG-BHQ-1	(Venkatesan et al., 2012)
DNA pol FP1	ATTACCTGTGGTTAAACCTTTGT	(Balamurugan et al., 2009a)
DNA pol RP1	AGAGAAATGACGTTTATCGGTGAC	(Balamurugan et al., 2009a)
DNA pol probe	FAM-GTTTGCCCTTAGCGTCCGTAGTAT-BHQ1	This study

DNA Extraction and concentration:

DNA Extraction of the reference strain and the collected samples was done by QiampDNA Mini Kit (Qiagen, Germany) according to manufacturer instructions. The DNA was measured by Nanodrop (ThermoFisher Scientific, USA). A stock DNA from the reference strain (400 ng/ul) was diluted 10-fold serial dilution (400 ng to 0.4 pg) to determine the limit of detection of the assay.

Duplex real time PCR:

It was performed by Maxima Probe/ROX qPCR Master Mix (ThermoFisher Scientific, USA). The reaction was 25 μ L containing 12.5 μ L 2x Maxima probe master mix, 1 μ L from each primer (10 uM) and 0.5 μ L from each probe (5 uM) and extracted DNA 4 μ L. the cycling conditions was as described previously (Venkatesan et al., 2012) using StepOne real time thermocycler (AppliedBiosystems, USA).

Detection of CMLV in spiked samples:

Due to unavailability of clinical camel pox samples, Negative oral, ocular and nasal swabs and skin tissue samples (n=30) were collected from healthy dromedary camels from Aswan and Behera. The samples were tested for CMLV as described by Venkatesan et al.(2012) then each sample was divided into two parts; the first part was spiked with the reference CMLV strain and the second part was used as negative control. The viral DNA from spiked and non-spiked samples was extracted simultaneously in the same conditions. The duplex real time PCR assay was used

to detect the viral C18L and DNA pol genes in the spiked and non-spiked samples.

3. RESULTS

The assay design:

The primers and probe of C18L gene were already tested before and the new probe of DNA pol gene was designed in this study to combine the two genes together in a TaqMan duplex real time assay format. DNA pol probe was 100 % matched with members of ortho-poxviruses including camel pox virus as this gene is conserved among ortho-poxvirus genus members.

The sensitivity and limit of detection of the assay:

The CMLV DNA was detected successfully in the different serially diluted samples ranging from 400ng to 0.4 pg for C18L gene (Fig1. A), while for DNA pol gene, it was detected till 4 pg only in 6 dilutions from 7 as shown in Fig.1 B. The efficiency of the duplex real time PCR assay for C18L gene was 100.6 % and 96.9% for DNA pol.

Detection of CMLV in the spiked samples:

The CMLV isolate (Jouf-78) showed positive amplification signals in both targets C18L and DNA pol while no signal or fluorescence in the negative controls (Fig. 2).

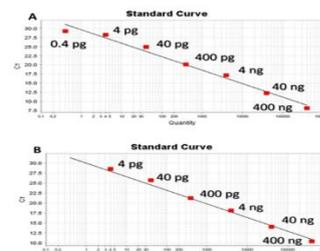


Figure 1 Standard curve of the Duplex real time PCR assay. A) C18L gene. B) DNA pol gene. (pg= picogram), (ng=nanogram).

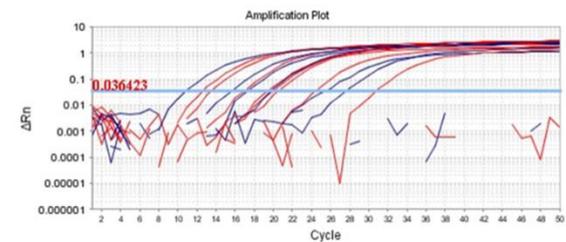


Figure 2 Detection of CMLV in the spiked samples.C18L (Blue), DNA pol (Red)

4. DISCUSSION

Camel pox is a notifiable disease from OIE affecting camel population in many countries, causes losses in milk and wool production(OIE, 2018)and has a zoonotic impact as stated before (Bera et al., 2011; Mohammadpour et al., 2020). This importance of camel pox needs control measures at the same level. Several diagnostic methods were developed before but some of them are traditional and time consuming, so this makes the molecular methods more reliable and sensitive. In the current study, a duplex probe based real time PCR was developed by targeting 2 genes in CMLV genome. The first is C18L gene (ankyrin repeat protein) which can be used in differentiation of members of ortho-pox viruses (Singh et al., 2008) the other one, is DNA

pol gene which is conserved among most of genus members (Bhanuprakash et al., 2010).

The limit of detection CMLV was 0.4 pg for C18L gene when applied for serially diluted CMLV DNA. This is better than the conventional PCR and the SYBR green Based real time PCR developed by Balamurugan et al. (2009) which can detect till 4 ng and 4 pg, respectively. The detection limit of the assay developed by Venkatesan et al. (2012) was very close to the developed assay, 0.35 pg of viral DNA. For DNA pol gene, the detection limit was 4 pg but for same gene in Balamurugan et al. (2009) it was 4 ng. In general, probe based real time PCR assays are more specific than SYBR green based assays. Targeting 2 different genes will provide confirmative diagnosis for this virus which has a critical health impact if reach to humans as reported previously (Bera et al., 2011). For trade also Egypt import very large number of camels every year from Sudan and Ethiopia (Napp et al., 2018) and this requires rapid, specific diagnostic assays for detection of camel diseases .

5. CONCLUSION

The developed duplex real time PCR assay for detection of CMLV will provide a sensitive, rapid and confirmative molecular diagnosis for camel pox and reducing the chance for false positive or negative diagnosis for this critical zoonotic disease.

6. REFERENCES

1. Aregawi, W. and Feyissa, P., 2016. Diagnostic Approaches towards Camel pox Disease. *Journal of Veterinary Science and Animal Husbandry*, 4 :1- 6.
2. Ayelet, G., Jenberie, S., Belay, A., Mohammed, A., Mola, B., Gizaw, Y., Muhie, Y., Gelaye, E., Asmare, K. and Skjerve, E., 2013. The first isolation and molecular characterization of camel pox virus in Ethiopia. *Antiviral Research*, 98 :417- 422.
3. Balamurugan, V., Bhanuprakash, V., Hosamani, M., Jayappa, K.D., Venkatesan, G., Chauhan, B. and Singh, R.K., 2009. A Polymerase Chain Reaction Strategy for the Diagnosis of Camel pox. *Journal of Veterinary Diagnostic Investigation*, 21: 231–237 (SAGE Publications Sage CA: Los Angeles, CA)
4. Bassiouny, A.I., Soliman, S.M., Hussein, H.A., Rohaim, M.A. and El-Sanousi, A.A., 2014. Sequence Analysis of Camel pox virus Isolated in Egypt. *International Journal of Virology*, 2: 121–128.
5. Bera, B.C., Shanmugasundaram, K., Barua, S., Venkatesan, G., Virmani, N., Riyesh, T., Gulati, B.R., Bhanuprakash, V., Vaid, R.K., Kakker, N.K., Malik, P., Bansal, M., Gadvi, S., Singh, R. V., Yadav, V., Sardarilal, Nagarajan, G., Balamurugan, V., Hosamani, M., Pathak, K.M.L. and Singh, R.K., 2011. Zoonotic cases of camel pox infection in India. *Veterinary Microbiology*, 152: 29–38.
6. Bhanuprakash, V., Prabhu, M., Venkatesan, G., Balamurugan, V., Hosamani, M., Pathak, K.M. and Singh, R.K., 2010. Camel pox: Epidemiology, diagnosis and control measures. *Expert Review of Anti-infective Therapy*, 8: 1187-1201.
7. Dahiya, S.S., Kumar, S., Mehta, S.C., Narnaware, S.D., Singh, R. and Tuteja, F.C., 2016. Camel pox: A brief review on its epidemiology, current status and challenges. *Acta Tropica*, 158: 32-38 .
8. Hall, T.A., 1999. BioEdit: a User-friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT (Nucluc Acids Symposium Series)
9. Haller, S.L., Peng, C., McFadden, G. and Rothenburg, S., 2014. Pox viruses and the evolution of host range and virulence. *Infection. Genetics and Evolution*, 21: 15–40.
10. King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J.B.T.-V.T. (eds), 2012. Family-Poxviridae In: *Virus Taxonomy*, Eleventh Edn. Elsevier: San Diego, USA, PP. 291–309
11. Mohammadpour, R., Champour, M., Tuteja, F. and Mostafavi, E., 2020. Zoonotic implications of camel diseases in Iran. *Veterinary Medicine and Science*, 6: 359–381.
12. Mosadeghesari, M., Oryan, A., Zibae, S. and Varshovi, H.R., 2014. Molecular investigation and cultivation of camel pox virus in Iran. *Archives of Virology*, 159: 3005–3011
13. Napp, S., Chevalier, V., Busquets, N., Calistri, P., Casal, J., Attia, M., Elbassal, R., Hosni, H., Farrag, H., Hassan, N., Tawfik, R., Abd Elkader, S. and Bayomy, S., 2018. Understanding the legal trade of cattle and camels and the derived risk of Rift Valley Fever introduction into and transmission within Egypt. *PLoS Neglected Tropical Diseases*, 12: 1–25
14. Narnaware, S.D., Ranjan, R., Dahiya, S.S., Panchbuddhe, A., Bajpai, D., Tuteja, F.C. and Sawal, R.K., 2021. Pathological and molecular investigations of systemic form of camelpox in naturally infected adult male dromedary camels in India. *Heliyon*, 7: 1-8
15. Office Internationale des Epizooties (OIE), 2018. Manual of diagnostic tests and vaccines for terrestrial animals. Camel pox Chapter 3.9.2.
16. Singh, R.K., Balamurugan, V., Hosamani, M., Kallesh, D.J. and Bhanuprakash, V., 2008. Sequence analysis of C18L gene of buffalopox virus: PCR strategy for specific detection and differentiation of buffalopox from orthopox viruses. *Journal of Virological Methods*, 154: 146–153
17. Venkatesan, G., Bhanuprakash, V., Balamurugan, V., Prabhu, M. and Pandey, A.B., 2012. TaqMan hydrolysis probe based real time PCR for detection and quantitation of camel pox virus in skin scabs. *Journal of Virological Methods*, 181: 192–196.