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Letter

Analysis of TK and C18L Genes of Wild-type and Cell Culture Passaged Camelpox Virus

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Dear Editor,

Camelpox is an infectious skin disease of camels caused by camelpox virus (CMLV). It is confined to camel rearing belts of developing countries (Wernery U, *et al*., 2002). CMLV is classified in the *Orthopoxvirus* (OPV) genus of the subfamily *Chordopoxvirinae* in the *Poxviridae* family. Camelpox is considered a possible zoonosis and under certain circumstances it can be mildly pathogenic in humans (Duraffour S, *et al*., 2011). The disease, restricted to camels, is enzootic in almost every region where camel breeding is practiced with the exception of Australia (Duraffour S, *et al*., 2011). Camelpox occurs in epizootics that lasts for 2-5 months with higher prevalence in winter and it mostly affects young animals of less than four years old (Khalafalla A I, *et al*., 1996). As a result of major economic losses from disease outbreaks, research has been oriented toward the development of live-attenuated and killed vaccines (Hafez S M, *et al*., 1992; Nguyen Ba-Vy, *et al*., 1996; Pfeffer M, *et al*., 1996; Wernery U, *et al*., 1999). CMLV can be adapted to grow in a variety of primary and transformed cells derived from camel, lamb, calf, pig, monkey, chicken, hamster and mouse (Duraffour S, et al., 2011). However, the molecular mechanisms of its adaptation are still not completely understood. As new approaches may be required for the development of better attenuated vaccine candidates, we have been interested in the molecular mechanisms underlying the phenotypic changes which followed the adaptation and passage of CMLV in cell culture. Furthermore, the sequencing of highly passaged strains with an attenuated phenotype, could be of great value in the search for virulence and host-range genes (Duraffour S, *et al*., 2011). It has been assumed that mutations in genes interfering with the virulence and or host range genes are responsible for the loss of pathogenicity (Massung R F, *et al*., 1993; Monath T P, 2005; Perkus M E, *et al*., 1991). Therefore, the purpose of the present study was to determine whether mutations in the *thymidine kinase gene* (TK) a potential determinant of virulence and Orthopoxvirus C18L gene (*OPVC18L,* encoding an ankyrin repeat protein) a determinant of the host range (Perkus M E, *et al*., 1991) play a role in cell culture adaptation and attenuation of CMLV.

The CMLV used in this study is a pathogenic field strain (CMLV/DB/93) isolated from sick camels during a field outbreak that occurred in Butana area, eastern Sudan (Khalafalla A I, et al., 1998). The virus was serially propagated in confluent monolayers of African green monkey kidney cells (Vero). Vero cells were grown in 25 cm^2 cell culture flasks using Eagle's minimal essential medium (MEM) with Earle's salts, supplemented with glutamine, penicillin, streptomycin and 5% fetal bovine serum and inoculated with the virus at a multiplicity of infection (MOI) of 1. The virus was serially passaged 100 times. During serial passaging the virus was clone purified at passages 30 and 90 by the limiting dilution technique according to the standard method. The purpose of cell culture attenuation was the development of candidate vaccine. And to ensure homogeneity, purification was done twice at passages 30 and 90. Then we selected the following

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passages 50 and 100 for genetic analysis and also for testing in animals. The viruses at passages 50 and 100 were described as CMLV/50 and CMLV/100, the wild-type virus was described as CMLV/WT. The tissue culture infective dose 50% ($TCID_{50}$/mL) was determined for the three above virus preparations following standard techniques. Results showed that the wild-type virus (CMLV/WT) displayed similar characteristics in cell culture to other CMLVs described already in the literature (Duraffour S, *et al*., 2011). The result of virus propagation in Vero cells showed that the cytopathic effects (CPE) appeared within five days post inoculation (pi) of Vero cells with CMLV/WT and complete destruction of the cell sheet took nine days to complete. On the other hand, it took three days to complete the cycle in CMLV/50 compared to two days in CMLV/100. The infectious titers in cell culture increased from $10^{3.66}$ for CMLV/WT to $10^{4.75}$ for CMLV/50 and $10^{5.5}$ $TCID_{50}$/mL for CMLV/100.

Viral DNA of both the wild type and passaged viruses from tissue culture supernatant was extracted, and PCR amplifications of *TK* gene and *OPVC18L* gene were performed following previous methods described by Binns M *et al,* (1992) and Balamurugan V *et al,* (2009), respectively. Using the Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystem, Japan) and ABI Prism 3730 Genetic analyzer PCR products were then sequenced. Amplicons with the expected size for the TK gene (Fig. 1A) and *OPVC18L* gene (Fig. 1B) were obtained. Sequence chromatograms were edited and analyzed using Edit Seq implemented in the software package DNASTAR v5.01. Multiple sequence alignments were performed using MeqAlign 5.01.

Sequence analysis of the *TK gene* revealed no changes between different viral passages.However, analysis of the OPVC18L gene revealed single nucleotide (nt) substitutions at position 41 (T41A) and 91 (T91A). Sequences were deposited to GenBank with accessory numbers: 1-TK gene; JX177681 for CMLV, 2- *OPVC18L* gene; JX409666 for CMLV/WT, JX409667 for CMLV/50 and JX409668 for CMLV/100. Changes in the amino acid sequence revealed that phenylalanine in CMLV/WT was replaced by tyrosine at position 14 (F14Y) in CMLV/50 and 100, and tyrosine in CMLV/WT was replaced by asparagine at position 31 (Y31N) in CMLV/50, but the latter reverted again to tyrosine (N31Y) in CMLV\100 as a reversion mutation compared with CMLV/WT (Y31Y). This switch back could indicate that the two alleles Y and N are present from the beginning in the parental WT strain as multiple alignments of *C18L gene* showed that several CMLV and OPXV strains possess tyrosine at positions 14 and 41 (Singh R K *et al*., 2004). Comparison of the *OPVC18L* gene revealed 99.6 - 99.8% sequence identity among CMLV strains at the nt level. However, CMLV/100 contained one amino acid mutation (F14Y) in an ankyrin repeat protein, encoded by *OPVC18L* gene, compared to the wild-type virus. It is postulated that this mutation alone or collectively with other mutations in other parts of the virus genome may be involved in the enhanced growth ability of the virus in cell culture and attenuation. In another communication CMLV/100 was found to be attenuated and experimentally inoculated dromedary camels conferred full protection against the virulent field virus (Abdellatif M M *et al*., Submitted).

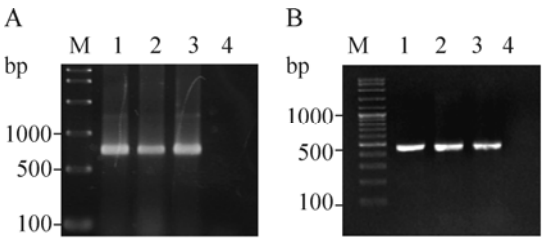

Fig. 1. Agarose gel electrophoresis of three CMLV DNA preparations, using ethidium bromide stained agarose gel (1.5%). A: Amplicons targeting *TK gene*. B: Amplicons targeting *OPVC18L* gene Lane M: DNA size marker (100 bp ladder); Lane 1: DNA from CMLV/WT; Lane 2: DNA from CMLV/50; Lane 3: DNA from CMLV/100; Lane 4: Double Distilled Water (negative control).

Ankyrin repeat proteins, though absent in most viruses, are common among poxviruses. Our result reinforces the previous indication, obtained from analysis of sheeppox virus and goatpox virus vaccines (Tulman E R, *et al*., 2002) and cell culture attenuated fowlpox virus (Laidlaw S M, Skinner M A, 2004), that attenuation of poxviruses by tissue culture passage could be driven by the loss of members of multigene families, including those encoding ankyrin repeat proteins.

Amino acid substitutions have been used for the attenuation of various viruses for vaccine production. In the present study we detected only one amino acid mutation. This is not uncommon as there are examples of a single amino acid mutation leading to the attenuation of a virus (Dietzschold B, *et al*., 1983; Westrop G D, *et al.*, 1989).

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Author contributions

MMA propagated the viruses in cell culture, participated in the design of the study. BS carried out the DNA sequencing and the analyses. AAI participated in the lab work and in the design of the study. EA participated in the analyses and helped to draft the manuscript. AIK participated in the design of the study and drafted the manuscript.

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