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4 **Another case of mistaken identity? Vaccinia virus in another live Camelpox vaccine.**
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26 **Abstract**
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28 Camelpox virus is the causative agent of Camelpox, a highly contagious disease of camels. A high
29 passage Camelpox virus strain has previously been reported to contain several genes which more closely
30 resemble Vaccinia, a virus species with no known natural host, encompassing various strains that show
31 high inter-strain genomic variation. In this study, we demonstrate that yet another high passage, live
32 attenuated vaccine, comprising a different strain of Camelpox virus, contains genomic sequences that
33 match a differing strain of Vaccinia virus. These results are discussed in the context of hypotheses put
34 forward to explain the unknown origins of Vaccinia virus, suggesting further studies to elucidate
35 evolutionary trajectories of Orthopoxviruses through passaging.
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47 **Keywords**
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49 Orthopox, Vaccinia, Camelpox, Sequencing, Passaging, Attenuation
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52 **1. Introduction**
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55 Camelpox virus (CMLV) is the causative agent of the highly contagious Camelpox disease [1,2], endemic
56 in all regions where camel husbandry is practiced except Australia [1,3]. Several CMLV vaccines are
57 produced worldwide, either in a live attenuated or inactivated adjuvant form [3]. Live attenuated CMLV
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4 vaccines contain highly passaged live viral particles, produced using methods not much different from
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6 those used by Pasteur to develop the first live attenuated vaccine over a century ago [4].
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10 In an earlier study, sequencing of 4 genomic regions of a tissue culture attenuated CMLV isolate (Jouf-
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12 78), used in a live attenuated vaccine in Saudi Arabia [5], showed that all sequences closely resembled the
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14 Vaccinia (VACV) strain Lister [6]. In this study, we show that gene regions from another live attenuated
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16 CMLV vaccine, Ducapox, show 100% identity to the corresponding gene regions of VACV strain
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18 Modified Virus Ankara (VACV-MVA).
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21 **2. Methods**

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24 Commercial live attenuated ‘Ducapox’ vaccines were sourced from Al Bashayer Veterinary Supplies
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26 (Dubai, United Arab Emirates), manufactured by Design Biologix (Pretoria, South Africa) and
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28 commercialized by Highveld Biological Ltd (Johannesburg, South Africa). The CMLV strain CaPV298-
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30 2, the parent strain of this vaccine, was originally isolated in the United Arab Emirates and attenuated
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32 through serial passage in Vero cell culture [7]. Manufacture and expiry dates were 08-2016 and 08-2018
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34 for batch number: DPV0816 (batch 1), and 07-2018 and 06/2019 for batch number DPV0618 (batch 2).
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36 Viral titer was stated as $1 \times 10^{6.5}$ TCID₅₀/ml.
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40 DNA was extracted using the QIAamp DNA Mini kit (Catalog # 51304, Qiagen, Hilden, Germany),
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42 following the DNA purification from tissues protocol, adding 180 uL of Buffer ATL to 25 mg of
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44 lyophilized vaccine and following the manufacturer’s guidelines with the addition of adding 5 ug of
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46 Carrier RNA Poly A (Catalog # 1017647, Qiagen, Hilden, Germany) to the 200 uL of Buffer AL solution.
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50 Primers designed to amplify the H3L gene region of CMLV comprised; H3L forward primer-
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52 ATGGCGGCGGTGAAACTC and H3L reverse primer- TTAGATAAATGCGGTAACAAATGTTC.
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54 Primers targeting a sub-region of the CMLV DNA polymerase gene (3021 bp full size) were developed
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56 using Primer-BLAST [8]: CMLV DNA POL forward primer- AGTCACAAGGGGGTAGGTGG and
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58 CMLV DNA POL reverse primer- GCACATATGTTGACGCACCC. This primer set would also amplify
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4 homologous polymerase gene regions in several Orthopoxvirus species. Another forward primer was
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6 designed to better match the gene sequence of VACV-MVA; VACV DNA POL forward-
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8 AGTCATAAGGGAGTAGGCGG, differing by 3 nucleotides in comparison to the original CMLV DNA
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10 POL forward primer. The reverse primer binding site was identical between both species, hence the
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12 original CMLV DNA POL reverse primer was used in conjunction with both forward primers targeting
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14 each respective species. The latter primer set showed 100% complementarity in-silico to several strains of
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16 VACV, Cowpox, Horsepox, and Rabbitpox. In order to confirm the absence of CMLV DNA polymerase
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18 gene region DNA, individual primer sets specific to the DNA polymerase regions of both CMLV and
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20 VACV-MVA were designed using DECIPHER, by inputting the DNA polymerase genes of both species,
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22 and keeping the default parameters [9]. The following primer sets were designed for VACV-MVA:
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24 VACV DNA POL SP forward- CGGAAAGGATGACGTTGATTTAGCT and VACV DNA POL SP
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26 reverse- AATCAGTCGTTCTAACTCTTTCGC. For CMLV, the following primer sets were designed:
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28 CMLV DNA POL SP forward- CCATTTAACGCGAGGCCGA and CMLV DNA POL SP reverse-
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30 TCTCTCTCATAATCCATTTCCGTCAG.
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36 PCR mixtures were prepared in 25 ul volumes using AmpliTaq Gold® 360 Master Mix (Catalog #
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38 4398876, ThermoScientific, Rochester, USA), following manufacturer guidelines and adding 2ul of
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40 template Ducapox DNA, with initial activation of polymerase at 95°C for 10 minutes followed by 40
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42 cycles of DNA denaturation at 95°C for 30 seconds, annealing of primers at 50°C (and 55°C for all
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44 reactions targeting the DNA polymerase gene) for 30 seconds and elongation at 72°C for 1 minute, with a
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46 subsequent elongation hold at 72°C for 7 minutes after these cycles.
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50 PCR reaction products were purified using a QIAquick PCR Purification Kit (Catalog # 28104, Qiagen,
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52 Hilden, Germany), following the manufacturer's guidelines. Sanger sequencing of purified amplicons was
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54 performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Catalog # 4337454,
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56 ThermoScientific, Rochester, USA) following the manufacturer's guidelines for 10 ul reactions, adding
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58 15 ng of template DNA, with an initial denaturation step at 96°C for 1 minute, followed by 25 cycles of
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4 denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes.
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6 Purification of reaction mixtures were performed using BigDye XTerminator Purification Kit (Catalog #
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8 4376484, ThermoScientific, Rochester, USA), following manufacturer’s guidelines. Sequencing was done
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10 on a SeqStudio Genetic Analyzer (ThermoScientific, Rochester, USA), equipped with a cartridge
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12 containing a 28 cm column (Catalog # A33671, ThermoScientific, Rochester, USA), using the
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14 ‘LongSeq_BDX’ run module. Sequence analyses was performed using Unipro UGENE software [10] and
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16 Genbank accession numbers for all sequences used in this research can be found in Appendix 1.
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20 21 **3. Results**

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23 The PCR amplification of the vaccine yielded a product of approximately 900 bp for the H3L gene
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25 region. For the polymerase gene region, reactions incorporating the CMLV DNA POL primers produced
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27 products with lighter bands in comparison to reactions incorporating VACV DNA POL primers, with
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29 both products measuring at around 500 bp in size (Fig. 1). Reactions incorporating the CMLV specific
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31 primer set CMLV DNA POL SP produced no visible amplicon, whereas reactions incorporating the
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33 VACV specific primer set VACV DNA POL SP produced a product band measuring around 900 bp (Fig.
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35 1). There was no observable difference between using a 50°C or 55°C annealing temperature (Fig. 1).
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40 Length of consensus sequences produced for each sample and percentage identity of consensus sequences
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42 to the corresponding genomic position of VACV-MVA are shown in table 1. Consensus sequences for
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44 each sample can be found in Appendix 2. Genomic mapping graphs for each sample, including the H3L
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46 gene region mapped to CMLV, can be found in Appendix 3. All sequenced amplicons showed 100%
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48 identity to corresponding gene regions in VACV-MVA.
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51 52 53 54 **4. Discussion**

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57 We confirmed the absence, to the sensitivity of the PCR method, of CMLV polymerase gene region DNA
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59 in the Ducapox vaccine, a gene involved in viral replication and known to be relatively conserved within
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4 the Orthopoxvirus genus [11]. Our findings corroborate the findings of Yousif et al. 2012 [6], who
5 showed that four gene regions of a tissue culture attenuated CMLV (isolate Jouf-78), more closely
6 resembled VACV, albeit strain Lister, whereas the Ducapox strain in this study more closely resembles
7 VACV-MVA. Given the nucleotide differences between the sequenced gene regions of these VACV
8 strains, it is unlikely that both seed viruses came from the same misidentified stock, or that the
9 observations are the result of a circulating VACV strain which was isolated, misidentified, and used as
10 seed stocks for both vaccines.

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20 Yousif et al. 2012 [6] attribute a possible contamination event to their findings. Although
21 misidentification of initial seed stock or contamination would also be a plausible explanation for our
22 findings, given that this is the second strain of CMLV found to resemble VACV after passage, other
23 possibilities should be considered for the following reasons. Firstly, the Ducapox CMLV strain was
24 claimed to have been cultured in Vero cells, a continuous cell lineage, originally isolated from kidney
25 epithelial cells extracted from an African green monkey [7]. VACV-MVA cannot be cultured in most
26 mammalian cells, or produces minimal viral titers in the few known permissive mammalian cell types in
27 which it can be cultured [12]. Secondly, the question of whether an Orthopox virus can be passaged to
28 create a more VACV-like strain is not a novel one, VACV has no known natural host, and the definitive
29 origins of the virus are still highly contested, with many hypotheses having been put forward [13]. Better
30 conclusions could be made from conducting whole genome sequencing of high passage strains and doing
31 whole genome comparisons. Also, it would be interesting to see the evolutionary trajectory with passage,
32 at the whole-genome level, of the same seed stock of Orthopoxvirus grown in different mammalian and/or
33 avian cell lines. Whole genome sequence analyses of CMLV passages at differing passage intervals
34 would determine the evolution of the virus through the passage stages. Deep-sequencing would also show
35 the abundance of viral quasi-species within the sample. However unlikely it may be, it cannot be
36 discounted that divergent Orthopoxviruses may gradually evolve to become more VACV like with greater
37 passage, especially given the fact that genomes of most VACV strains tend to be smaller than those of
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4 pathogenic Orthopoxviruses, with high inter-strain genomic variation [14]. This theory could be tested
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6 experimentally, as one could examine whether high passaging of a number of the most diverged
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8 Orthopoxvirus species causes them to separately evolve to become more VACV-like. This may certainly
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10 hold true for CMLV, as some strains of VACV are thought to have been derived from attenuating VARV,
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12 and, amongst the Orthopoxviruses, CMLV shows the closest genetic homology to VARV [15].
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16 To conclude, this paper has shown that another highly passaged live attenuated CMLV vaccine contains
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18 genomic regions that match VACV, highlighting the need for more robust and stringent testing of
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20 biologicals intended for veterinary applications.
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23 24 25 26 **Conflicts of Interest**

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29 None
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32 33 34 35 **Acknowledgement**

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Table 1: Lengths of consensus sequences produced for each primer set and percentage identity of the consensus sequences to the corresponding genomic position of VACV-MVA. No sequencing was attempted with primer sets CMLV DNA POL SP due to the lack of visible amplicon formation following PCR.

Primer set	Batch	Length of consensus sequence	Genomic position of VACV-MVA	% identity to VACV-MVA
H3L	1	879	83211 – 84080	100
	2	870	83211 – 84080	100
CMLV DNA Pol	1	507	50102 – 50608	100
	2	464	50102 – 50565	100
VACV DNA Pol	1	507	50102 – 50608	100
	2	507	50102 – 50608	100
VACV DNA Pol SP	1	829	49308 – 50136	100
	2	818	49319 – 50136	100
CMLV DNA Pol SP		No amplicon produced		

Figure 1: DNA polymerase gene region amplicons of Ducapox vaccine DNA. Reactions incorporating the CMLV DNA POL primers produced products with lighter bands in comparison to reactions incorporating VACV DNA POL primers, with both products measuring at around 500 bp in size. Reactions incorporating the CMLV specific primer set CMLV DNA POL SP produced no visible amplicon. Reactions incorporating the VACV specific primer set VACV DNA POL SP produced a product band measuring around 900 bp. There was no observable difference between using a 50°C or 55°C annealing temperature.

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