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Genetic characterization of poxviruses in *Camelus dromedarius* in Ethiopia, 2011–2014

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ABSTRACT

Camelpox and camel contagious ecthyma are infectious viral diseases of *camelids* caused by camelpox virus (CMLV) and camel contagious ecthyma virus (CCEV), respectively. Even though, in Ethiopia, pox disease has been creating significant economic losses in camel production, little is known on the responsible pathogens and their genetic diversity. Thus, the present study aimed at isolation, identification and genetic characterization of the causative viruses. Accordingly, clinical case observations, infectious virus isolation, and molecular and phylogenetic analysis of poxviruses infecting camels in three regions and six districts in the country, Afar (Chifra), Oromia (Arero, Miyu and Yabello) and Somali (Gursum and Jijiga) between 2011 and 2014 were undertaken. The full hemagglutinin (HA) and partial A-type inclusion protein (ATIP) genes of CMLV and full major envelope protein (B2L) gene of CCEV of Ethiopian isolates were sequenced, analyzed and compared among each other and to foreign isolates. The viral isolation confirmed the presence of infectious poxviruses. The preliminary screening by PCR showed 27 CMLVs and 20 CCEVs. The sequence analyses showed that the HA and ATIP gene sequences are highly conserved within the local isolates of CMLVs, and formed a single cluster together with isolates from Somalia and Syria. Unlike CMLVs, the B2L gene analysis of Ethiopian CCEV showed few genetic variations. The phylogenetic analysis revealed three clusters of CCEV in Ethiopia with the isolates clustering according to their geographical origins. To our knowledge, this is the first report indicating the existence of CCEV in Ethiopia where camel contagious ecthyma was misdiagnosed as camelpox. Additionally, this study has also disclosed the existence of co-infections with CMLV and CCEV.

A comprehensive characterization of poxviruses affecting camels in Ethiopia and the full genome sequencing of representative isolates are recommended to better understand the dynamics of pox diseases of camels and to assist in the implementation of more efficient control measures.

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1. Introduction

Camels are economically important animals that are well adapted to the arid and semi-arid climates. Two major poxviruses, camelpox virus (CMLV) and camel contagious ecthyma virus (CCEV), infect camels causing camelpox (Gubser et al., 2004; OIE, 2012) and camel contagious ecthyma (Abubakr et al., 2007; Khalafalla et al., 2015a, 2015b), respectively. The two diseases are characterized by the appearance of pox-like lesions on the skin of camels of all ages and sex.

CMLV belongs to the genus *Orthopoxvirus* (OPV) of the family *Poxviridae* (Afonso et al., 2002; Gubser et al., 2004; Duraffour et al., 2011; King, 2012; Bilbao-Sieyro et al., 2014). Genomic and phylogenetic studies have demonstrated that CMLV is closely related to

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variola virus, the cause of smallpox (Gubser and Smith, 2002). Camelpox is endemic in the Middle East (Bahrain, Iran, Iraq, Oman, Saudi Arabia, United Arab Emirates, and Yemen), in Asia (Afghanistan, India, and Pakistan), and in Africa (Algeria, Egypt, Ethiopia, Kenya, Mauritania, Morocco, Niger, Somalia, and Sudan) (Bhanuprakash et al., 2010; Duraffour et al., 2011; OIE, 2012). CMLV is transmitted through skin abrasions and by aerosols in a contaminated environment, although mechanical transmission might also be considered (Duraffour et al., 2011; OIE, 2012). Camel pox disease ranges from local and mild to severe systemic infections and is characterized by fever, local or generalized pock lesions on the skin preferentially in the head, neck, extremities, inguinal region and in the mucous membrane of the mouth, respiratory and digestive tracts (Duraffour et al., 2011; OIE, 2012; Balamurugan et al., 2013). In general, young calves and pregnant females are more susceptible to the virus (Al-Zi'abi et al., 2007). Camel pox has a strong impact on the economy as camels are valued for transportation, racing, and production of milk, wool and meat (Renner-Muller et al., 1995; Duraffour et al., 2011). The disease also incurs considerable losses in terms of morbidity, mortality, loss of weight and reduction in milk production (Bhanuprakash et al., 2010; Duraffour et al., 2011). Additionally, the zoonotic importance of CMLV was established during an outbreak that involved camel handlers and attendants in India (Bera et al., 2011; OIE, 2012).

CCEV is the causative agent of camel contagious ecthyma, also known as Auzdyk disease, classified under the genus *Parapoxvirus* (PPV) of the *Poxviridae* family (Abubakr et al., 2007; King, 2012). Camel contagious ecthyma is widely present in camel rearing regions of the world and reported in Somalia (Moallin and Zessin, 1988), Kenya (Gitao, 1994), Libya (Azwai et al., 1995), Saudi Arabia (Housawi et al., 2004), Bahrain (Abubakr et al., 2007), India (Nagarajan et al., 2010), Israel (van et al., 2001) and Sudan (Khalafalla et al., 1994, 2015a, 2015b). Camel contagious ecthyma is characterized by the appearance of papules which develop into pustules on the lips, muzzles, and eyelids of infected camels (Abubakr et al., 2007; Khalafalla et al., 2015b). The morbidity rate was reported as 100% while mortality reached up to 9% in young camels in Arabian Peninsula (Abubakr et al., 2007). It can also lead to losses in milk and meat production, labor, and skin quality (Mombeni et al., 2013). No report is available on the zoonotic potential of CCEV. Camelpox is a notifiable animal disease by the World Organization of Animal Health (OIE, 2012), unlike camel contagious ecthyma.

Ethiopia has an estimated population of 1.1 million camels, reared in the sedentary areas of the country (CSA, 2014). Despite the increased occurrence of pox-like diseases in camels in Ethiopia, there is only one report on the identification of CMLV (Ayelet et al., 2013), with no data available on the existence of camel contagious ecthyma or the identification of the causative agent.

On the basis of the hemagglutinin (HA, CML176, similar to vaccinia virus strain Copenhagen A56R) and the A-type inclusion protein (ATIP, CMLV143/4, similar to vaccinia virus strain Copenhagen TA29L/TA31L) gene sequences it is possible to differentiate CMLVs from other orthopoxviruses (Meyer et al., 1994; Pfeffer et al., 1996; King, 2012). Similarly, the major envelope glycoprotein (B2L, ORF11, EEV envelope phospholipase) gene can separate CCEV into another cluster independent from other members of parapoxviruses (Abubakr et al., 2007). Since the full genome has not yet been sequenced, phylogenetic analysis was based on the parapoxvirus homolog of B2L gene, which indicated a subclade of pseudocowpox viruses that has adapted to camels (Abubakr et al., 2007; Khalafalla et al., 2015b).

The present study was designed for the isolation and molecular characterization of the causative viruses and the phylogenetic comparison of the Ethiopian isolates with foreign isolates using the full length gene sequence of the HA and B2L of CMLV and CCEV, respectively.

2. Materials and methods

2.1. Study areas, observations and samples collection

The study was conducted using pox-like disease outbreak samples collected between 2011 and 2014 in the regions of Afar, Oromia and Somali, which are the three major camel-rearing areas of Ethiopia. The following six districts were covered: Chifra (11°45′ N 40°20′ E) in the Afar region; Gursum (9°20′ N 42°35′ E) and Jijiga (9°20′ N 42°50′ E) in the Somali region; and Yabello (5°00′ N 38°15′ E), Arero (4°40′ N 39°00′ E), and Miyu (4°15′ N 38°15′ E) in the Oromia region (Fig. 1).

These areas are arid and semi-arid environments where the livelihoods of animal owners mainly depend on livestock breeding.

Epidemiological information on the clinical signs, age and sex of animal, location of lesions, morbidity and mortality were recorded through interview and physical examination. Representative skin scrapings of scab lesions were collected from fifty-five camels showing suspected clinical signs of poxvirus infection. Representative skin scrapings of lesions were collected from clinically diseased camels of different age and sex group originating from all six districts. About 2 g of tissue samples were collected from each animal and transferred into a labeled sterile universal tube containing phosphate buffered saline (PBS), pH 7.2 supplemented with antibiotics and antifungal. Samples were immediately stored in a cold box and transported to the National Veterinary Institute (NVI) of Ethiopia under the cold-chain system. The samples were then kept at −20 °C until laboratory analysis.

2.2. Virus isolation

Skin scraping samples were washed three times with sterile PBS containing antibiotics and antifungal and ground using a sterile pestle and mortar. Tissue homogenates (10% w/v) were prepared using sterile PBS and centrifuged at high speed in a refrigerated centrifuge. The supernatant (0.5 mL) was inoculated onto a confluent monolayer of Vero cells grown in a 25 cm^2 tissue culture flask containing 10 mL Glasgow Minimum Essential Medium (Sigma-Aldrich) supplemented with 2% fetal calf serum (Gibco). The inoculated cultures were incubated at 37 °C, 5% CO_2 and observed daily for the appearance of virus-induced cytopathic effects (CPEs). Samples were considered negative when no CPE was observed following three blind passages.

2.3. Virus DNA extraction

The pathological tissue homogenates (10% w/v in PBS) were centrifuged at 10,000 g for 10 min at +4 °C. DNA was extracted directly from the supernatant of clinical specimens using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instruction. The eluted DNA was labeled and stored at −20 °C until further testing.

2.4. Primer design

The full genome nucleotide sequences of two CMLVs (NC003391/AY009089) and two fragments containing the full B2L gene of CCEVs (GQ390365/GU460370) were retrieved from the GenBank and the sequences were aligned separately using MAFFT. The primers (Table 1) were designed on the conserved regions flanking the full nucleotide sequence of the HA gene of CMLV (NC003391:163139-164431) and the B2L gene of CCEV

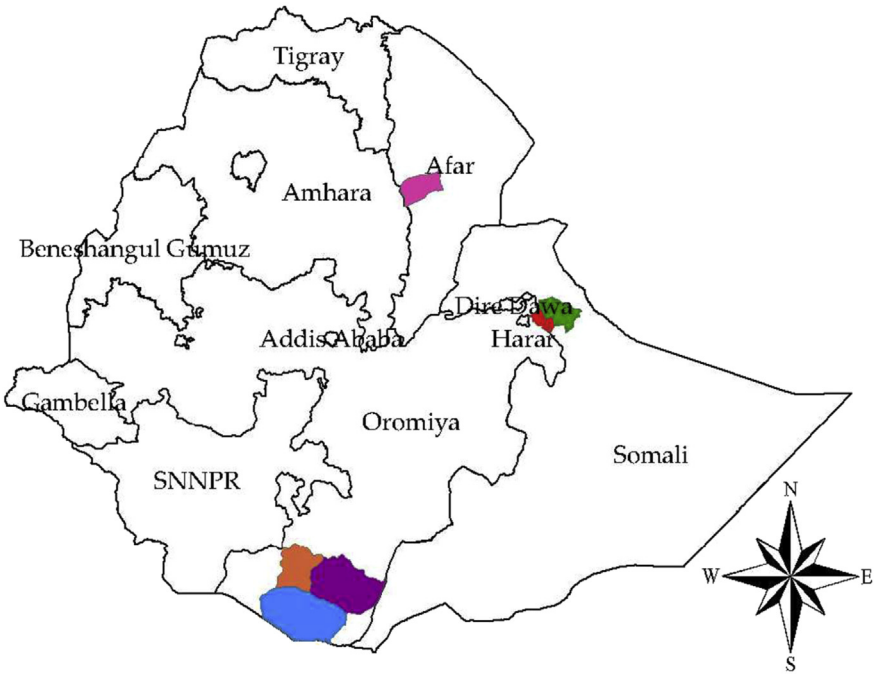


Fig. 1. Map of Ethiopia showing the samples collection sites. The different geographical districts (n = 6) where representative nodular skin lesion samples have been collected during pox suspected outbreaks from clinically diseased camels are presented using different colors. Rose – Chifra, Red – Gursum, Green – Jijiga, Orange – Yabello, Purple – Arero, and Blue – Miyu. The map was sketched using ArcGIS 9 software (ArcMap™ version 9.3, California, USA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(GU460370:31-1190) using Allele ID version 6 software package (Premier Biosoft International, Palo Alto, CA, USA). The primers for the amplification of the ATIP gene were adopted from OIE (2012). For each gene, additional walking primer pairs were also designed (Table 1). The specificity of each primer sequence was checked using blastn: http://blast.ncbi.nlm.nih.gov./Blast.cgi. All primers were synthesized by Eurofins Genomics, Austria.

2.5. Amplification of virus genes

For each of the two genes, the PCR reaction mix consisted of 500 nM for each of the forward and reverse primers, 2 mM dNTPs, 1x PCR Buffer (Qiagen), 2.5U Taq Polymerase (Qiagen) and 6 μL template DNA in a total volume of 50 μL. The cycling conditions for the amplification of the full HA gene were: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 50 °C for 40 s and 72 °C for 60 s, and final extension at 72 °C for 7 min. The cycling conditions for the full B2L gene were similar to HA gene amplification except that the annealing was performed at 50 °C for 30 s. The ATIP gene was amplified as described in OIE (2012). An individual aliquot (5 μL) of each PCR product was checked using electrophoresis on a 1.5% agarose gel stained with GelRed for 1 h at 100 V.

2.6. Sequencing and phylogenetic analysis

The amplified PCR products of the full HA and partial ATIP genes of CMLV and full B2L gene of CCEV were purified using Wizard SV Gel and PCR clean-up system kit (Promega). The purified products were quantified spectrophotometrically using the NanoDrop 2000 spectrophotometer (Thermo Scientific) and sequenced by the Sanger method at LGC Genomics (Germany) using the four primers indicated in Table 1. The sequences were assembled and edited using Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). The sequences of the local CMLV and CCEV isolates together with the homologous genes of orthopoxviruses and parapoxviruses respectively, retrieved from GenBank, were aligned using BioEdit version 7.1.3.0 (Hall, 1999). Multiple sequence alignment of the nucleotides sequences were performed separately for each gene using the ClustalW. Phylogenetic trees were reconstructed using

Table 1
List of oligonucleotide sequences used in this study. The primers for the amplification and sequencing of the HA (948bp) and ATIP (881bp) genes of CMLV and the B2L (1137bp) gene of CCEV are presented with the estimated PCR product size.

SI No	Primer's name	Sequences (5′–3′)	Application	PCR product size (bp)
1	OPV-HAf-For	GAAAAAGATTGTTGGACATTGGA	PCR/Sequencing	1293
2	OPV-HAf-Rev	GTGGTATGGGACACCACAAA		
3	OPV-HAi-For	TCTCCATACGATGATCTAGTTACAA	Sequencing	–
4	OPV-HAi-Rev	GGACGAGGAAATCTAGTGGTATG		
5	OPV-ATIP-For	AATACAAGGAGGATCT	PCR/Sequencing	881
6	OPV-ATIP-Rev	CTTAACTTTTCTTTCTC		
7	CCEV-B2Lf-For	AATAAATGTGGCCGTTCTCC	PCR/Sequencing	1160
8	CCEV-B2Lf-Rev	ACCTTCCGCGCTTTAATTTT		
9	CCEV-B2Li-For	GCGGGCGTGAACTACTACA	Sequencing	–
10	CCEV-B2Li-Rev	GTCCGCGTTCTTCCACTC		

the nucleotide sequences for each of the HA and the B2L genes, including representative sequences retrieved from GenBank. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was computed using MEGA version 6 (Tamura et al., 2013). The robustness was tested by performing 1000 bootstrap replicates.

3. Results

3.1. Clinical observations

Pox-like skin lesions were observed on one-humped camels (*Camelus dromedarius*) reared in various geographical locations of Ethiopia where camels are the dominant animal species handled by the local pastoral communities. Skin lesions were observed equally in all age and sex groups. However, lesions were more severe in young camels of less than two years old in camel contagious ecthyma suspicions. In both camelpox and camel contagious ecthyma, the major clinical signs recorded were high fever (39–40 °C), and nodular skin lesions. In camel contagious ecthyma suspicion cases, skin lesions were observed mainly on the face and neck regions of young camels, unlike for camelpox where the lesions extended to other body parts like limbs and inguinal regions and seen mostly in adult camels (Fig. 2). In general, the morbidity rate was recorded between 10 and 45% depending on the visited outbreak areas.

3.2. Virus isolation and preliminary screening by PCR

Vero cell cultures that were infected with nodular lesion supernatants developed characteristic poxvirus-induced cytopathic effects (CPE) within 8–10 days post-inoculation. CPE included formation of multinucleated syncytia, rounding, ballooning and syncytia with degenerative changes. Further during infection, cells detached from the cell culture flask forming plaques. Around 70% of the cultured samples developed characteristic cytopathic effect.

Twenty-seven out of 32 viral DNAs suspected of CMLV infections were confirmed positive through amplification of the HA and ATIP genes. Geographically, 18 positive samples were collected from Oromia (Yabello and Miyu districts) and 9 positive samples from Somali (Jigjiga and Gursum districts). Similarly, 20 out of 23 viral DNAs suspected of CCEV infection were positive by amplification of the B2L gene. Eleven samples originated from Oromia (Arero district), six samples from Afar (Chifra district), and three samples from Somali (Jijiga and Gursum districts). Two DNA samples from Somali (Hordha/01/2011 and Golajo/02/2012) were positive for both CMLV and CCEV.

3.3. Sequence analysis and phylogenetic tree construction

The nucleotide sequences of the complete HA and partial ATIP genes of CMLV and the full B2L gene of CCEV were successfully acquired and deposited at Genbank with the accession numbers shown in Table 2.

Multiple alignments of the nucleotide sequences of the twenty-seven full HA and 27 partial ATIP genes revealed no nucleotide variation among the Ethiopian isolates, showing that they are highly identical. Similarly, no variability was observed in the full HA gene sequences between the Ethiopian isolates and CMLVs from Somalia (AF375081) and Syria (DQ853384). The Ethiopian CMLV presented a single nucleotide variation (T:C) at position 875 when compared to CMLV isolates from Iran (AY009089 and AF375079), and Saudi Arabia (AY902251); and two nucleotide mismatches T:C and G:T respectively at nucleotide 875 and 888 positions with and Indian isolate (GQ453436) and isolate from Kazakhstan (AF438165). Consequently, in the phylogenetic tree reconstructions

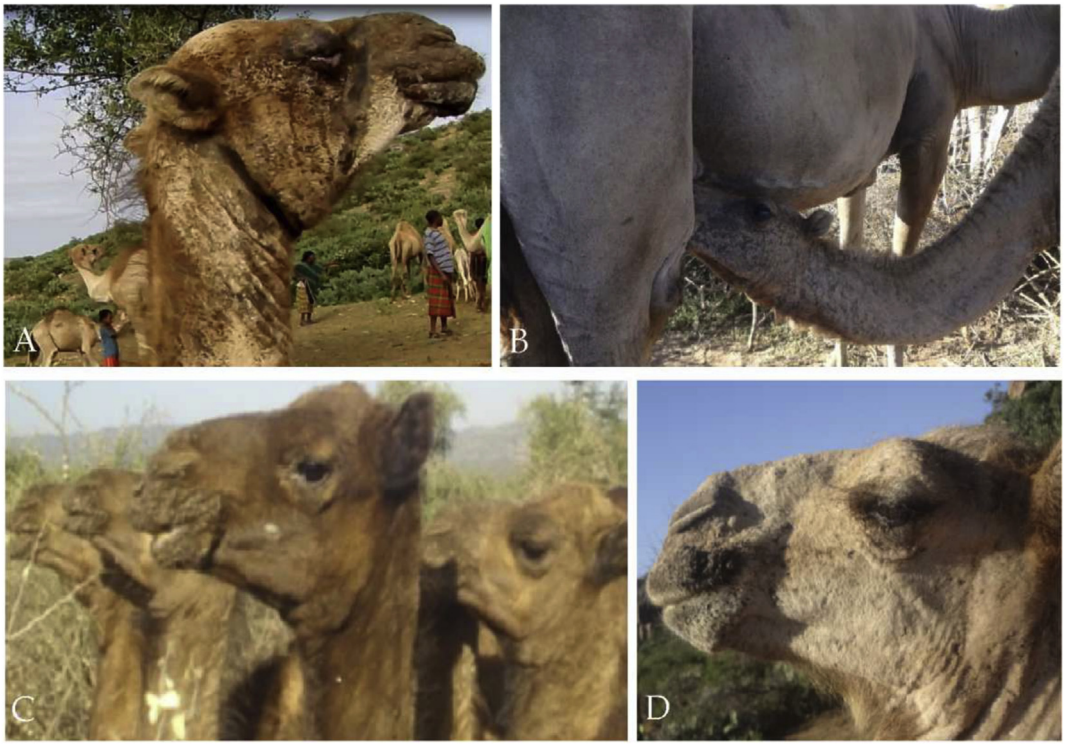


Fig. 2. Clinical manifestation of pox-like diseases observed on Ethiopian camels. (A). Adult camel showing signs of camelpox where skin lesions on the mouth, face and neck regions where both eyes were damaged. (B). Camel calf with skin lesions on the neck and face areas. (C). Young camels showing signs of camel contagious ecthyma where severe nodular lesions on the upper and lower lips and nostril. (D). Adult camel developed nodular lesions on the upper lip and nose area.

Table 2
List of the isolates characterized in this study. The Ethiopian CMLV (n = 27) and CCEV (n = 20) with isolate name, area and date of collection, genotype, and the GenBank accession numbers for the nucleotide sequences of the HA and ATIP genes of CMLV and the B2L gene of CCEV generated in this study are presented.

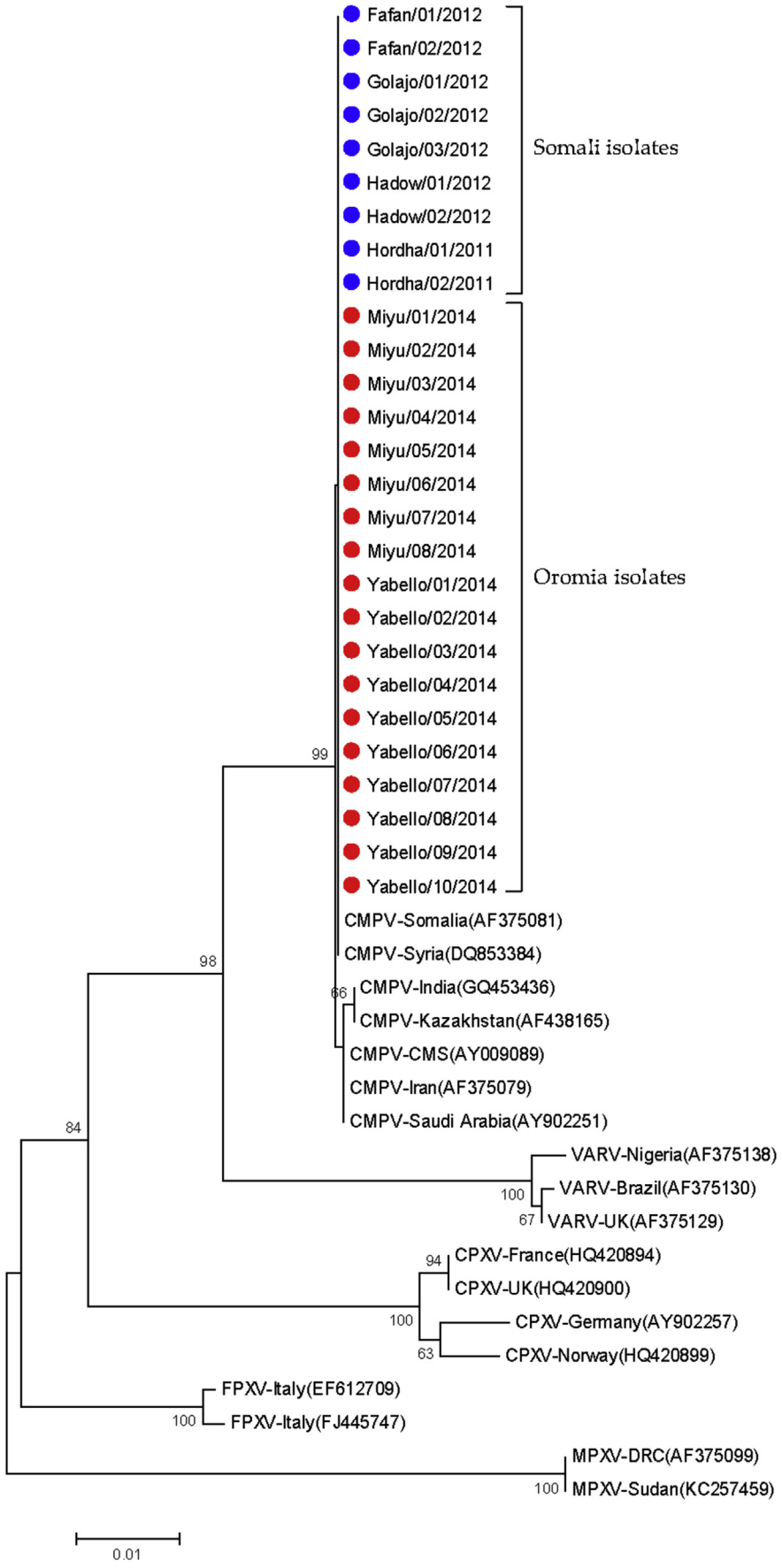
Sl No	Strain name	Area of collection	Date of collection	Genotype	GenBank accession number		
					HA	ATIP	B2L
1	Arero/01/2013	Oromia-Arero	14/11/2013	CCEV			KU645544
2	Arero/02/2013	Oromia-Arero	14/11/2013	CCEV			KU645545
3	Arero/02/2014	Oromia-Arero	21/03/2014	CCEV			KU64554650
4	Arero/03/2013	Oromia-Arero	14/11/2013	CCEV			KU645547
5	Arero/04/2013	Oromia-Arero	14/11/2013	CCEV			KU645548
6	Arero/05/2013	Oromia-Arero	14/11/2013	CCEV			KU645549
7	Arero/06/2013	Oromia-Arero	23/12/2013	CCEV			KU645550
8	Arero/07/2013	Oromia-Arero	23/12/2013	CCEV			KU645551
9	Arero/08/2013	Oromia-Arero	23/12/2013	CCEV			KU645552
10	Arero/09/2013	Oromia-Arero	23/12/2013	CCEV			KU645553
11	Arero/10/2013	Oromia-Arero	23/12/2013	CCEV			KU645554
12	Chifra/532/2012	Afar-Chifra	15/01/2012	CCEV			KU645555
13	Chifra/H1/2012	Afar-Chifra	16/01/2012	CCEV			KU645556
14	Chifra/H2/2012	Afar-Chifra	16/01/2012	CCEV			KU645557
15	Chifra/H3/2012	Afar-Chifra	16/01/2012	CCEV			KU645558
16	Chifra/H4/2012	Afar-Chifra	16/01/2012	CCEV			KU645559
17	Chifra/H5/2012	Afar-Chifra	16/01/2012	CCEV			KU645560
18	Fafan/03/2012	Somali-Gursum	05/01/2012	CCEV			KU645561
19	Golajo/02/2012	Somali-Gursum	18/01/2012	CCEV			KU645562
20	Hordha/01/2011	Somali-Jijiga	28/12/2011	CCEV			KU645563
1	Fafan/01/2012	Somali-Gursum	05/01/2012	CMLV	KU645564	KU705085	
2	Fafan/02/2012	Somali-Gursum	09/01/2012	CMLV	KU645565	KU705086	
3	Golajo/01/2012	Somali-Gursum	18/01/2012	CMLV	KU645566	KU705087	
4	Golajo/02/2012	Somali-Gursum	18/01/2012	CMLV	KU645567	KU705088	
5	Golajo/03/2012	Somali-Gursum	18/01/2012	CMLV	KU645568	KU705089	
6	Hadow/01/2012	Somali-Gursum	17/01/2012	CMLV	KU645569	KU705090	
7	Hadow/02/2012	Somali-Gursum	17/01/2012	CMLV	KU6455700	KU705091	
8	Hordha/01/2011	Somali-Jijiga	28/12/2011	CMLV	KU645571	KU705092	
9	Hordha/02/2011	Somali-Jijiga	28/12/2011	CMLV	KU645572	KU705093	
10	Miyu/01/2014	Oromia-Miyu	20/02/2014	CMLV	KU645573	KU705094	
11	Miyu/02/2014	Oromia-Miyu	20/02/2014	CMLV	KU645574	KU705095	
12	Miyu/03/2014	Oromia-Miyu	20/02/2014	CMLV	KU645575	KU705096	
13	Miyu/04/2014	Oromia-Miyu	20/02/2014	CMLV	KU645576	KU705097	
14	Miyu/05/2014	Oromia-Miyu	20/02/2014	CMLV	KU645577	KU705098	
15	Miyu/06/2014	Oromia-Miyu	20/02/2014	CMLV	KU645578	KU705099	
16	Miyu/07/2014	Oromia-Miyu	20/02/2014	CMLV	KU645579	KU705100	
17	Miyu/08/2014	Oromia-Miyu	20/02/2014	CMLV	KU645580	KU705101	
18	Yabello/01/2014	Oromia-Yabello	28/01/2014	CMLV	KU645581	KU705102	
19	Yabello/02/2014	Oromia-Yabello	28/01/2014	CMLV	KU645582	KU705103	
20	Yabello/03/2014	Oromia-Yabello	28/01/2014	CMLV	KU645583	KU705104	
21	Yabello/04/2014	Oromia-Yabello	30/01/2014	CMLV	KU645584	KU705105	
22	Yabello/05/2014	Oromia-Yabello	30/01/2014	CMLV	KU645585	KU705106	
23	Yabello/06/2014	Oromia-Yabello	30/01/2014	CMLV	KU645586	KU705107	
24	Yabello/07/2014	Oromia-Yabello	17/02/2014	CMLV	KU645587	KU705108	
25	Yabello/08/2014	Oromia-Yabello	17/02/2014	CMLV	KU645588	KU705109	
26	Yabello/09/2014	Oromia-Yabello	17/02/2014	CMLV	KU645589	KU705110	
27	Yabello/10/2014	Oromia-Yabello	17/02/2014	CMLV	KU645590	KU705111	

the Ethiopian CMLV formed only one cluster together with isolates from Somalia (AF375081) and Syria (DQ853384). As expected, all CMLV isolates showed more genetic relatedness to variola virus strains than to other orthopoxviruses with high bootstrap value (Fig. 3). Multiple alignment analysis of the partial ATIP gene sequences (840bp) of the 27 Ethiopian CMLV isolates with the homologous CMLV genes retrieved from Genbank (NC003391/AY009089/KP768318) revealed only a single nucleotide variation (G:A) at position 477.

As compared to CMLV, the multiple sequence alignments of the complete B2L gene sequences of the Ethiopian CCEV revealed more nucleotide variability. Based on the phylogenetic analysis of the B2L gene, the Ethiopian CCEV isolates grouped into three clusters according to their geographical origin: cluster I (eleven Oromia isolates), cluster II (three Somali isolates) and cluster III (six Afar isolates) as shown in Fig. 4. The local CCEV isolates of cluster I were closely related to one isolate each from India (GQ390365) and Sudan (KR231670). All Ethiopian isolates were more distant to another Indian isolate (GU460370). All CCEV isolates were more closely related to pseudocowpox viruses (PCPV) than other parapoxviruses such as orf viruses (ORFV) and bovine papular stomatitis viruses (BPSV) supported by high bootstrap value (Fig. 4).

3.4. Co-infection

During our preliminary screening of the outbreak samples we used an in house assay (to be published elsewhere) that is able to detect and discriminate between 8 poxviruses of veterinary and medical importance. Two samples (Hordha/01/2011 and Golajo/02/2012) which originated from the Somali region and were found positive for both CMLV and CCEV. We further investigated those two samples by amplifying and sequencing the full HA and partial ATIP genes for CMLV, and the full B2L gene for CCEV. The sequence analysis confirmed that the two samples contained both CMLV and CCEV, suggesting a dual infection by both viruses. The two samples were collected from young male camels of approximately 14–16 months age where nodular lesions had occurred only


Fafan/01/2012
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Hordha/02/2011
Somali isolates
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Yabello/08/2014
Yabello/09/2014
Yabello/10/2014
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CMPV-Syria(DQ853384)
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66
CMPV-India(GQ453436)
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CMPV-CMS(AY009089)
CMPV-Iran(AF375079)
CMPV-Saudi Arabia(AY902251)
84
VARV-Nigeria(AF375138)
100
VARV-Brazil(AF375130)
67
VARV-UK(AF375129)
94
CPXV-France(HQ420894)
CPXV-UK(HQ420900)
100
CPXV-Germany(AY902257)
63
CPXV-Norway(HQ420899)
FPXV-Italy(EF612709)
100
FPXV-Italy(FJ445747)
MPXV-DRC(AF375099)
100
MPXV-Sudan(KC257459)
0.01

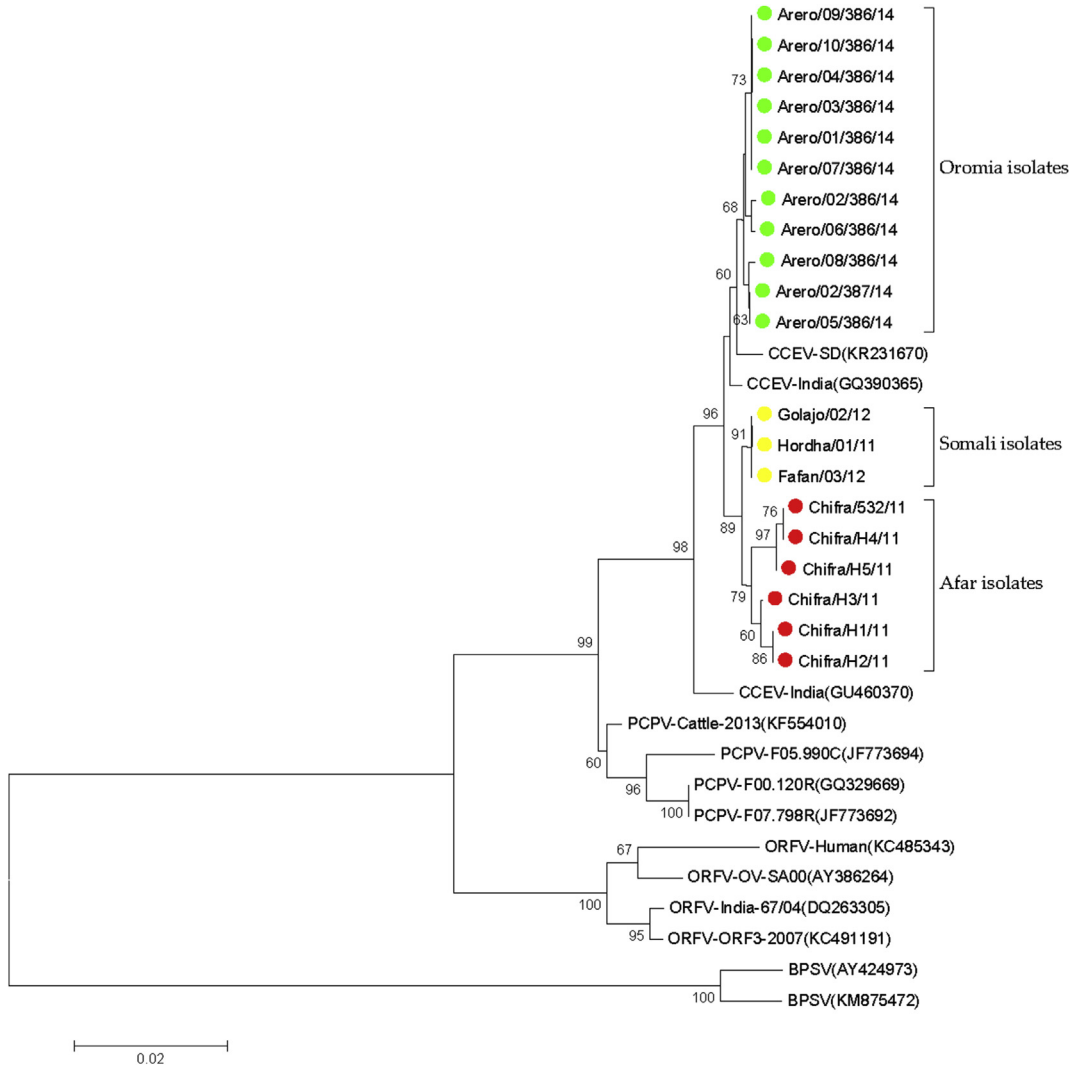

Fig. 4. Phylogenetic analysis of 33 parapoxviruses based on nucleotide sequences of the B2L gene (1137 nt). The B2L gene nucleotide sequences of twenty Ethiopian outbreak isolates and 13 sequences retrieved from the GenBank were used. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was computed using MEGA5.1 software. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The homologue gene sequences from two BPSV strains retrieved from the GenBank were used as out-group. The isolates sequenced in this study are indicated with colored circle.

around the mouth and neck regions; no unique signs were recorded in these two camels as compared to other diseased camels.

4. Discussion

We have isolated poxviruses, CMLV and CCEV, affecting camels from outbreak samples originating from three major camel rearing regions of Ethiopia and analyzed their genetic diversity.

During our investigations, we have observed pox-like skin lesions with similar severity in both sexes and all age groups; however, for the camel contagious ecthyma suspicions, the disease appeared to be more severe in young camels of less than two years old. Khalafalla et al., 2015b also noticed that young camels were at high risk of CCEV infections. Even though camels were reared together with small ruminants and cattle in the visited areas, pox disease was observed only in camels, confirming a narrow host range for both CMLV (OIE, 2012) and CCEV.

We have also noticed that pox disease occurrence in camels was high during the rainy season. Ayelet et al., 2013 and Khalafalla et al., 2015b also recorded highest incidences of CMLV and CCEV infections during rainy season. This could be attributed to an increase in the presence of arthropod vectors during the wet period. Indeed, CMLV was isolated from a camel tick – *Hyalomma dromedarii* taken from infected camels (OIE, 2012), suggesting a possible role of this insect in CMLV transmission.

Analyzing the HA gene of CMLV we have found that the isolates causing camelpox disease in Ethiopia are highly similar to each other and to foreign isolates from Somalia (AF375081) and Syria (DQ853384). Accordingly, all the Ethiopian CMLVs were grouped in

Fig. 3. Phylogenetic analysis of 45 orthopoxviruses based on nucleotide sequences of the HA gene (948 nt). The HA gene nucleotide sequences of twenty-seven Ethiopian outbreak isolates and 18 sequences retrieved from the GenBank were used. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was computed using MEGA5.1 software. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The homologue gene sequences from variola virus (VARV), cowpox virus (CPXV), felinepox virus (FPXV) and monkeypox virus (MPXV) retrieved from the GenBank were also included. The MPXV sequences were used as out-group. The isolates sequenced in this study are indicated with colored circle.

a single cluster in the HA gene phylogenetic tree. Likewise, the analysis of the partial sequence of ATIP gene of CMLV isolates showed high similarity among the Ethiopian isolates and with two foreign isolates of Kazakhstan (NC003391) and Iran (AY009089). Since the sequence of the HA (King, 2012), and ATIP (Meyer et al., 1994) genes are used mainly for species differentiation within the orthopoxviruses, they may not carry enough information for intra-species differentiation. Therefore, the possibility of higher variability among the Ethiopian CMLV isolates cannot be completely ruled out and needs to be confirmed by sequencing the full genome of selected isolates.

In contrast to CMLV, the analysis of B2L gene of the Ethiopian CCEVs showed higher variability. The phylogenetic tree reconstructions using the B2L gene sequences of CCEVs revealed 3 clusters formed by the Ethiopian isolates. These clusters perfectly matched to the geographical location of the isolates, with the Oromia isolates clustering separately from Afar and Somali isolates. This suggests that at least 3 variants of CCEV are circulating in the country. The Afar and Somali isolates are more closely related to each other. This is clearly in line with the geographical origin of the samples as Jijiga and Gursum districts in Somali region are relatively closer to the Chifra district in the Afar region. The isolates collected from Afar and Somali are relatively more distant to those collected in Arero, Miyu and Yabello districts in the Oromia region located far away from these two regions. Although, no supportive document is available to propose the possibility of contact between live camels of these two regions, the possibility of contacts either through trade and/or grazing or watering points cannot be ruled out.

It was surprising to see that the eleven Oromia isolates were more genetically related to the Sudanese isolate (KR231670) than to other local isolates, especially, because the Oromia region is located far from Sudan.

The present phylogenetic study also revealed that the local CCEVs were genetically related more closely to PCPVs than ORFVs. This is in agreement with previous reports (Abubakr et al., 2007; Haller et al., 2014) which concluded that CCEV is likely belonging to a sub cluster of PCPV that has adapted to camels. Based on the phylogenetic analysis of the B2L gene, we can deduce that two major genetic clades or lineages of CCEVs have been circulating in camels in Ethiopia; supported by high bootstrap value where population one contained the Oromia isolates together with the Sudanese (KR231670) and Indian (GQ390365) isolates; while population two comprised the Afar and Somali isolates. This statement might need further confirmation through full genome sequencing of the isolates.

This study also revealed the co-circulation of CMLV and CCEV in two of the three major camel rearing regions of the Ethiopia, the Oromia and the Somali region. In the Afar region, only CCEV was detected. Nevertheless, as samples were taken, from only one location in the Afar region, a possible co-circulation of CMLV with CCEV in this region cannot be ruled out.

Since both camelpox and camel contagious ecthyma develop comparable clinical lesions particularly on the face and neck regions, the differential diagnosis of these two diseases, based solely on clinical examination is challenging owing to the co-circulation of both CMLV and CCEV in Ethiopia. For instance, one sample collected from Oromia (Arero/02/2014), one from Somali (Fafan/03/2012) and six from Afar (Chifra isolates) were submitted for diagnosis on suspicion of CMLV infection. However; the PCR and sequencing results revealed that camels were indeed infected by CCEV, not CMLV. Additionally, two samples which were collected on suspicion of CMLV infection from the Somali region (Hordha/01/2011 and Golajo/02/2012) were dually infected by both CMLV and CCEV. This highlights the need to use differential diagnostic methods, such as those based on molecular methods. Prior to this work, there was no laboratory based data on CCEV in Ethiopia available, and all pox-like lesions on camels were mostly considered as CMLV infections. Hence, this report will create awareness among animal health professionals and diagnosticians on the existence of camel contagious ecthyma in the country. The main weakness of the present study is that a cross-sectional sampling method was applied and therefore, it is difficult to see the seasonal dynamics of the disease.

Until this study, all pox-like skin lesions in camels have been diagnosed clinically as camel pox disease in Ethiopia where the existence of CCEV was undocumented. The present study identified CCEV in addition to CMLV as the main agent causing pox diseases in Ethiopian camels. A continuous monitoring of poxvirus infecting camels, by sequencing genes of more virus isolates is needed to better understand the diversity and the dynamic of poxvirus infections of camels in Ethiopia. Furthermore, the availability of multiple pathogen detection methods targeting both CCEV and CMLV in one reaction will add value to the diagnosis and facilitate the management of poxvirus infections of camels in the country and worldwide.

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