First Detection and Molecular Characterisation of Pseudocowpox Virus in a Cattle Herd in Zambia

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Abstract

Background: Pseudocowpox virus (PCPV) of the genus *Parapoxvirus* in the family *Poxviridae* causes pseudocowpox in cattle worldwide and presents a zoonotic concern. Most poxviruses produce diseases of similar clinical signs in affected animals, which are impossible to differentiate clinically or by serology. It is, therefore, vital to use molecular assays to identify the causative agents of poxvirus infections rapidly. This study aimed to detect, diagnose, and characterize the causative agent of pox-like skin lesions in a cattle herd in Zambia, initially suspected to be infected with the Lumpy Skin Disease virus.

Methods: We used a high resolution melting (HRM) analysis assay to detect the PCPV genome and sequenced the major envelope protein (B2L) gene for comparative sequence and phylogenetic analysis.

Results: Our field investigations showed cattle presenting atypical skin lesions and high morbidity within the herd. The laboratory diagnosis based on an HRM assay revealed the PCPV genome in the samples. Phylogenetic and comparative sequence analyses confirmed PCPV in the samples. They revealed genomic differences between samples collected in 2017 and 2018 from the same farm.

Conclusion: Our work is the first documented report of PCPV in Zambia. It shows the strength of molecular methods to diagnose pox-like infections in cattle and discriminate between diseases causing similar clinical signs for better veterinary interventions.

Background

Pseudocowpox is a pox-like disease of cattle caused by pseudocowpox virus (PCPV) of the genus *Parapoxvirus* (PPV) within the family *Poxviridae* [1]. The genus *Parapoxvirus* also includes the bovine papular stomatitis virus (BPSV) of cattle, the orf virus (ORFV) of sheep and goats. Additional parapoxviruses affect the red deer of New Zealand (PVNZ), reindeers, seals, and the musk ox [2–5]. Other poxviruses, within the genera *Capripoxvirus* and *Orthopoxvirus*, can also affect cattle.

Parapoxviruses cause papules and erosions on the muzzle, oral mucosa, and udder [6] and may cause high morbidity and loss of productivity [7]. Parapoxvirus infections may, however, also be asymptomatic [8]. Parapoxviruses may also infect humans working in close contact with infected animals [7, 9].

Parapoxviruses infections can be clinically suspected, however, in some forms of the diseases, clinical signs are similar to those of other diseases such as lumpy skin disease (LSD) or bovine herpesvirus infections. In Zambia, LSD is well known and documented since 1929, however, there is no written knowledge on other poxvirus infections of cattle such as PCPV, BPSV, and vaccinia. Cases presenting pox-like lesions are mostly treated as any other diseases endemic to Zambia. They are rarely sent to the laboratory for further diagnosis due to a lack of tools for differential diagnosis. Recent advancements have produced molecular assays that are fast, sensitive, and offer a means to discriminate parapoxviruses from other agents producing similar cutaneous lesions [10, 11],[12].

Here, we report for the first time, pseudocowpox in Zambia from LSD suspected cases. This paper describes the clinical presentation, the molecular detection, and the molecular characterization of the index cases of PCPV from samples collected and follow-up field investigation in a herd of cattle.

Methods

Clinical and epidemiological investigations

In December 2017, the Central Veterinary Research Institute (CVRI) received samples comprising skin nodules and scabs from a herd of cattle, with suspicion of LSD infection. The cattle were of mixed dairy and beef, Zebu-Boran crossbreed in Chiyuni veterinary camp, Chief Chitanda area, Muundu, Chibombo District, Central Province of Zambia.

The affected farm is in the area under zone 3 of the East Coast fever (ECF) control strategy in Zambia [13], an Epidemic area for ECF, which causes high mortalities in animals in Zambia.

The samples tested negative by real-time PCR for LSD. The extracted DNA was stored and subsequently analyzed using a recently developed high resolution melting (HRM) assay for the simultaneous detection and differentiation of eight poxviruses of medical and veterinary importance [12]. A follow-up trip was undertaken on the farm in April 2018 to examine the animals clinically and obtain additional history and samples.

Sample collection and DNA extraction

Skin nodules were collected from the affected cattle (Table 1) and transferred to the CVRI laboratory on ice in a coolbox. The nodules were ground, and 50 mg was homogenized in 2 ml phosphate buffered saline and centrifuged at 2500 rpm for 10 min at 4 ^{o}C. 200 µl of the supernatant was added to 800 µl of RLT Plus and the total nucleic acid extracted using the RNeasy mini kit (QIAGEN) as previously described [14].

Table 1
List of PCPV samples of animals from which specimens were collected for this study, Zambia, 2017–2018.

Animal number	Name	Age	Sex	Collection date	host
1.	Lungu	2 year.	M	20.12.17	cattle
2.	Mary	2 year. 2 mo.	F	20.12.17	cattle
3.	Mazete	3 yr	F	20.12.17	cattle
4.	Beenzu	5 yr	F	24.04.18	cattle
5.	Mary	2 year 6 mo.	F	24.04.18	cattle
6.	Mazuba	1yr	M	24.04.18	cattle
7.	Mercy	5 yr	F	24.04.18	cattle
8.	Mutinta	2 yr	F	24.04.18	cattle

HRM assay

We tested the extracted DNA using the HRM assay for the simultaneous detection and differentiation of eight poxviruses. [12]. The method can detect and differentiate members of three different genera of poxviruses *Orthopoxvirus*, *Capripoxvirus*, and *Parapoxvirus* and provides additional genotyping of the viruses within each of the three genera: cowpox virus (CPXV) and camelpox virus (CMLV) [genus *Orthopoxvirus*]; goatpox virus (GTPV), sheeppox virus (SPPV) and lumpy skin disease virus (LSDV) [genus *Capripoxvirus*]; orf virus (ORFV), pseudocowpox virus (PCPV) and bovine papular stomatitis virus (BPSV) [genus *Parapoxvirus*].

The reaction contained 200 nM of each primer (Table 2), 1 X SsoFast™ EvaGreen® Supermix, and 2 µl of the template. Each run included positive control plasmids representing each of the eight pathogens, and a negative control comprising nuclease-free water. The PCR reactions and melting curve analysis were performed on a CFX real-time polymerase chain reaction (PCR) detection system (Bio-Rad), following the conditions earlier described (Gelaye et al., 2017) with slight modifications. Briefly, after an initial denaturation step at 95 °C for 4 min, the products were amplified for 40 cycles of 95 °C for 1 sec, 59 °C for 5 sec and 70 °C for 5 sec. The PCR products were denatured at 95 °C for 30 sec, cooled down at 65 °C for 60 sec, then melted from 65 °C to 85 °C with an increment of 0.2 °C every ten seconds and a continuous data acquisition. We analyzed the data using the CFX Manager Software (Bio-Rad), and the Precision Melt Analysis Software (Bio-Rad).

Table 2
Primers used in this study for the HRM assay and sequencing.

Method	Primers' ID	5′→3′ sequence	Amplicon size (bp)	Target
HRM	OPV-HRM-For	AGGACTAGCCGCGGTAACTTT	56	Orthopoxviruses
	OPV-HRM-Rev	ACAAGATAGAAGCGATGGATACTT		
	CaPV-HRM-For	TCCTGGCATTTTAAGTAATGGT	100	Capripoxviruses
	CaPV-HRM-Rev	GTCAGATATAAACCCGGCAAGTG		
	PPV-HRM-For	TCGAAGATCTTGTCCAGGAAG	112	Parapoxviruses
	PPV-HRM-Rev	CCGAGAAGATCAACGAGGTC		
Sequencing	ORFV-B2Lf-For	GACCTTCCGCGCTTTAATTT	1210	Parapoxviruses
	ORFV-B2Lf-Rev	CCCGCCTGCTAAAAGACT		

Sequencing

The primers used to amplify a fragment of the B2L gene of parapoxviruses [15] are indicated in Table 2. The positive PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced commercially by LGC genomics. The sequences were edited and assembled using Vector NTI 11.5 software (Invitrogen). All sequences were submitted to GenBank under accession numbers MT448677 to MT448684.

Phylogenetic analysis

For comparative study, additional partial B2L gene sequences of other parapoxviruses were retrieved from GenBank and screened to remove short and duplicate sequences. The final data set for phylogenetic analyses comprised 45 sequences, including 8 PCPV sequences of this study, 7 PCPV of cattle and reindeer, 11 camel contagious ecthyma virus (CCEV), 14 ORFV, and 5 BPSV.

After aligning the sequences, using the muscle (codon) option, in MEGA 7, the aligned sequence file was saved in FASTA format, then converted to a nexus format using Seaview. We performed the Bayesian phylogenetic inference with BEAST. First, a BEAST file was produced from the nexus file with the BEAUti module using the TN93 + G nucleotide substitution and a UPGMA starting tree. The Markov Chain Monte Carlo method was run with BEAST, for 10,000,000 generations with a sample taken each 10,000

generations. The TRACER program was used to inspect the log files and determine the optimum number of burn-in based on the Effective Sample Sizes (ESS > 200).

TreeAnnotator was used to generate the Maximum Clade Credibility (MCC) after discarding the 2% burn-in. The tree was visualized with the associated meta-data using the ggtree package in R version 3.5.2 [16].

Results

Clinical and epidemiological investigations

We found about 140 animals at the farm at different stages of infection, from recent to healed lesions.

The animals had nodular lesions, scabs, and dermatitis on the teats, udder, and other parts of the body, including the sternum, limbs, muzzle, and folding of the skin (Fig. 1). Some animals also had enlarged lymph nodes (pre-scapular, parotid, and inguinal). The farmworkers also complained of having itchy nodules on their hands and faces, which cleared on their own within 1–3 weeks.

History revealed that from 2011, the farmer had been taking the cattle for grazing in the Lukenge swamps and mixed with other neighboring cattle. They first observed skin lesions in 2014. Farmworkers were usually taking back infected animals to the farm to treat them with Oximic plus LA (Oxyject 20% plus Sodium diclofenac 0.5%). The farmer reportedly lost close to 300 animals from a herd of 1900 cattle since 2014. Because the farm is an Epidemic area for ECF, it was not possible to rule out the involvement of this disease in the high mortalities reported by the farmer. It was only in 2017 when the disease exacerbated that the farmer consulted the veterinary extension services for further investigation.

Molecular detection

We detected PCPV DNA in all samples using the HRM Assay. Figure 2 shows the amplification curves corresponding to PCPV in all Zambian samples. There was no amplification corresponding to LSDV or Orthopox viruses.

Molecular characterization and phylogenetic analysis

We successfully amplified and partially sequenced the major envelope protein (B2L) gene in all eight samples from Zambia. All sequences of the isolates clustered within the PCPV group in the phylogenetic tree (Fig. 3), confirming that the DNA recovered from infected cattle with pox-like lesions belong to PCPV. The tree showed that within the PCPV group, the isolates collected from camels (CCEV) clustered separately from those collected in cattle and reindeer. All Zambian BL2 gene sequences belonged to the group of cattle/reindeer sequences (Fig. 3).

Interestingly, the first sequences from samples collected in December 2017 produced a separate cluster from those collected in April 2018 during the follow-up investigation of the disease. The only exception

was the sequence of the isolate Mary 2018, closely related to, but different from, the 2017 isolates (Fig. 3). The sequence alignments of the Zambian isolates showed nine polymorphic amino acid sites, seventeen sites at the nucleotide level. Eight of those mutations represented specific changes, sixteen sites at the nucleotide level, between the 2017 and the 2018 isolates. The only exception was the isolate Mary 2018, which presented only two mutations (four at the nucleotide level) as compared to the 2017 isolates (Fig. 4). Mary 2018 and Mary 2017 are from the same animal but collected at two different time points. The mutations seen in the Mary 2018 are among those specific mutations found in the 2018 isolates, showing that the Mary 2018 isolate could be an intermediate variant of the 2017 and 2018 isolates.

Discussion

In this study, we have detected PCPV DNA in samples collected on LSD suspicion in a herd of cattle in Zambia using an HRM assay for differential diagnosis of poxvirus infections. Sequencing and the subsequent phylogenetic analyses confirmed that the DNA in these samples belong to PCPV. In the phylogenetic reconstruction, all Zambian PCPVs clustered with previously published sequences of cattle PCPVs.

Although PCPV in cattle is reportedly present worldwide [17–19], there was neither a record of the disease in Zambia nor in most African countries. Therefore, this marks the first occasion that we have identified PCPV in the country. The absence of a proper means for differential diagnosis of pox-like diseases in cattle and the lack of confirmed cases for PCPV contributed to the initial misdiagnosis. As LSD is endemic in Zambia, the current cases were mistakenly reported as LSD-suspected, based on the sole clinical diagnosis. Similar cases of initial LSD diagnosis have been reported [20]. This underlines the importance of determining the etiology of infections in pox-like skin lesions on cattle.

Our findings highlight the relevance of molecular methods for differential diagnosis and the management of pox diseases in ruminants. This robust HRM assay has enabled differentiation between LSDV and PCPV in a single test and identified PCPV, which otherwise would have gone unnoticed. The correct identification of the disease agent avoids confusion with other critical infectious agents present in Zambia and ensures the implementation of proper interventions.

In the phylogenetic analyses, PCPVs from samples collected in December 2017 clustered independently from those in samples taken on the same farm in April 2018, showing an evolution of the virus during the persistence of the infection on the farm. A close inspection of the sequence alignments showed up to 9 amino acid changes in the partial B2L sequence of isolates collected in April 2108 as compared to those collected in December 2017. Interestingly, the analysis of the PCPV B2L gene in samples collected from the same animal at these two-time points showed that two amino acids mutation occurred.

Such rapid genomic variations in the PCPV suggest a swift ability of this virus to mutate during chronic infections. A previous report revealed a similar evolution in the ATPase gene of the ORF virus during the

persistence of the virus in a sheep herd in Ethiopia [15]. Such alterations in the genome could potentiate the adaptation of the virus to new tissues and promote shedding, thus enhancing its potential spread.

Our field investigations suggested that the disease started in this herd in 2014, but only drew the attention of farmers when animals' health conditions worsened. The affected cattle had lesions that were not only confined to the teats and udder. The lesions were present on other parts of the animal, including the limbs, mouthparts, ventrum, and skin folds, suggesting a severe form of the disease. PCPV has previously been isolated from atypical sites apart from the teats and udder [18, 19, 21]. As PCPV is a zoonotic disease, humans coming into direct contact with infected animals are at risk of contracting the infection [22]. We could not find an active case of transmission to humans.

Nevertheless, it came to our attention that the farmworkers previously had sores mainly on their hands and forearms, and sometimes the face. Those lesions cleared within a few weeks without treatment. This observation is consistent with previous reports showing that PCPV lesions in humans usually resolve quickly [23]. It is advisable to handle infected animals with caution to reduce the risks.

Conclusion

This first detection and characterization of PCPV demonstrate the power of molecular methods for the differential diagnosis of pox diseases in cattle. The correct identification of the causative agent of pox-like lesions in cattle is essential to discriminate diseases with similar clinical signs and thus allow for proper veterinary intervention. Country-wide surveillance may be crucial to investigate the prevalence of PCPV infections among the cattle population and identify infection risks for other animals and humans in Zambia.

Abbreviations

1. BPSV – Bovine papular stomatitis virus
2. CCEV – Camel contagious ecthyma virus
3. CMLV – Camelpox virus
4. CPXV – Cowpox virus
5. CVRI - Cenral Veterinary Research Institute
6. ECF – East cost fever
7. GTPV – Goatpox virus
8. HRM – High resolution melting
9. LSDV – Lumpy skin disease virus
10. MCC – Maximum clade credibility
11. ORFV – Orf virus
12. PCR – Polymerase chain reaction

13. PCPV – Pseudocowpox virus
14. PPV – Parapoxvirus
15. PVNZ – Parapoxvirus of red deer in Newzealand
16. SPPV – Sheeppox virus

Declarations

Ethics approval and Consent to participate

The study was approved by the Minisrty of Livestock and Fisheries.

Sampling was carried out under the owners consent.

Consent to publication

Publication approved by the Ministry of Fisheries and Livestock, Lusaka, Zambia.

Availabilty of data and materials

DNA sequences generated and analysed under the current study are available in GenBank under accession numbers MT448677 to MT448684.

Competing interests

All authors declared that they have no competing interests

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References

1. Büttner M, Rziha HJ. Parapoxviruses: From the lesion to the viral genome. J Vet Med Ser B. 2002;49:7–16.

2. Vikøren T, Lillehaug A, Åkerstedt J, Bretten T, Haugum M, Tryland M. A severe outbreak of contagious ecthyma (orf) in a free-ranging musk ox (Ovibos moschatus) population in Norway. Vet Microbiol. 2008;127:10–20.
3. Tryland M, Mørk T, Ryeng KA, Sørensen KK. Evidence of parapox-, alphaherpes- and pestivirus infections in carcasses of semi-domesticated reindeer (Rangifer tarandus tarandus) from Finnmark, Norway. Rangifer. UiT The Arctic University of Norway; 2005;25:75–83.
4. Khalafalla AI, Elhag AE, Ishag HZA. Field investigation and phylogenetic characterization of orf virus (ORFV) circulating in small ruminants and Pseudocowpoxvirus (PCPV) in dromedary camels of eastern Sudan. Heliyon. Elsevier Ltd; 2020;6.
5. Becher P, König M, Müller G, Siebert U, Thiel HJ. Characterization of sealpox virus, a separate member of the parapoxviruses. Arch Virol. 2002;147:1133–40.
6. Yaegashi G, Sasaki I, Chiba S, Murakami K. Molecular analysis of parapoxvirus detected in eight calves in Japan. J. Vet. Med. Sci. 2013. p. 1399–403.
7. Mazur C, Ferreira II, Rangel Filho FB, Galler R. Molecular characterization of Brazilian isolates of orf virus. Vet Microbiol. Elsevier; 2000;73:253–9.
8. Roess AA, Mccollum AM, Gruszynski K, Zhao H, Davidson W, Lafon N, et al. Surveillance of Parapoxvirus Among Ruminants in Virginia and Connecticut. Zoonoses Public Health. 2013;60:543–8.
9. Abrahão JS, Silva-Fernandes AT, Assis FL, Guedes MI, Drumond BP, Leite JA, et al. Human Vaccinia virus and Pseudocowpox virus co-infection: Clinical description and phylogenetic characterization. J Clin Virol. 2010;48:69–72.
10. Friederichs S, Krebs S, Blum H, Wolf E, Lang H, Von Buttlar H, et al. Comparative and retrospective molecular analysis of Parapoxvirus (PPV) isolates. Virus Res. 2014;181:11–21.
11. Inoshima Y, Morooka A, Sentsui H. Detection and diagnosis of parapoxvirus by the polymerase chain reaction. J Virol Methods. 2000;84:201–8.
12. Gelaye E, Mach L, Kolodziejek J, Grabherr R, Loitsch A, Achenbach JE, et al. A novel HRM assay for the simultaneous detection and differentiation of eight poxviruses of medical and veterinary importance. Sci Rep [Internet]. Nature Publishing Group; 2017 [cited 2018 Dec 21];7:42892. Available from: http://www.nature.com/articles/srep42892
13. Livestock MOF, Development F. Government of the Republic of Zambia Department of Veterinary Services East Coast Fever (Ecf) Control Strategy. 2010;
14. Settypalli TBK, Lamien CE, Spergser J, Lelenta M, Wade A, Gelaye E, et al. One-step multiplex RT-qPCR assay for the detection of Peste des petits ruminants virus, Capripoxvirus, Pasteurella multocida and Mycoplasma capricolum subspecies (ssp.) capripneumoniae. PLoS One. 2016;11.
15. Gelaye E, Achenbach JE, Jenberie S, Ayelet G, Belay A, Yami M, et al. Molecular characterization of orf virus from sheep and goats in Ethiopia, 2008-2013. Virol J. Virology Journal; 2016;13:1–12.
16. Yu G, Smith DK, Zhu H, Guan Y, Lam TTY. Ggtree: an R Package for Visualization and Annotation of Phylogenetic Trees With Their Covariates and Other Associated Data. Methods Ecol Evol. 2017;8:28–

36.
17. Cargnelutti JF, Flores MM, Teixeira FRM, Weiblen R, Flores EF. An outbreak of pseudocowpox in fattening calves in southern Brazil. J Vet Diagnostic Investig. 2012;24:437–41.
18. Ohtani A, Yokoyama A, Narushige H, Inoshima Y. First isolation and genetic characterization of pseudocowpox virus from cattle in Japan. Virol J. Virology Journal; 2017;14:1–5.
19. Lederman E, Khan SU, Luby S, Zhao H, Braden Z, Gao JX, et al. Zoonotic parapoxviruses detected in symptomatic cattle in Bangladesh. BMC Res Notes. 2014;7:1–7.
20. Cargnelutti JF, Santos BS, Lebre S das N, Sodré DNA, da Silva RM, Weiblen R, et al. Pseudocowpox and papular stomatitis in cattle in the Rondonia state, Brazil. Cienc Rural. 2014;44:479–85.
21. Blomqvist G, Ullman K, Segall T, Hauzenberger E, Renström L, Persson-Waller K, et al. An unusual presentation of pseudocowpox associated with an outbreak of pustular ulcerative vulvovaginitis in a Swedish dairy herd. J Vet Diagnostic Investig. SAGE Publications Inc.; 2018;30:256–9.
22. Usme-Ciro JA, Paredes A, Walteros DM, Tolosa-Pérez EN, Laiton-Donato K, del Carmen Pinzón M, et al. Detection and molecular characterization of zoonotic poxviruses circulating in the amazon region of Colombia, 2014. Emerg Infect Dis. Centers for Disease Control and Prevention (CDC); 2017;23:649–53.
23. Oğuzoğlu TÇ, Koç BT, Kirdeci A, Tan MT. Evidence of zoonotic pseudocowpox virus infection from a cattle in Turkey. VirusDisease. 2014;25:381–4.

Figures

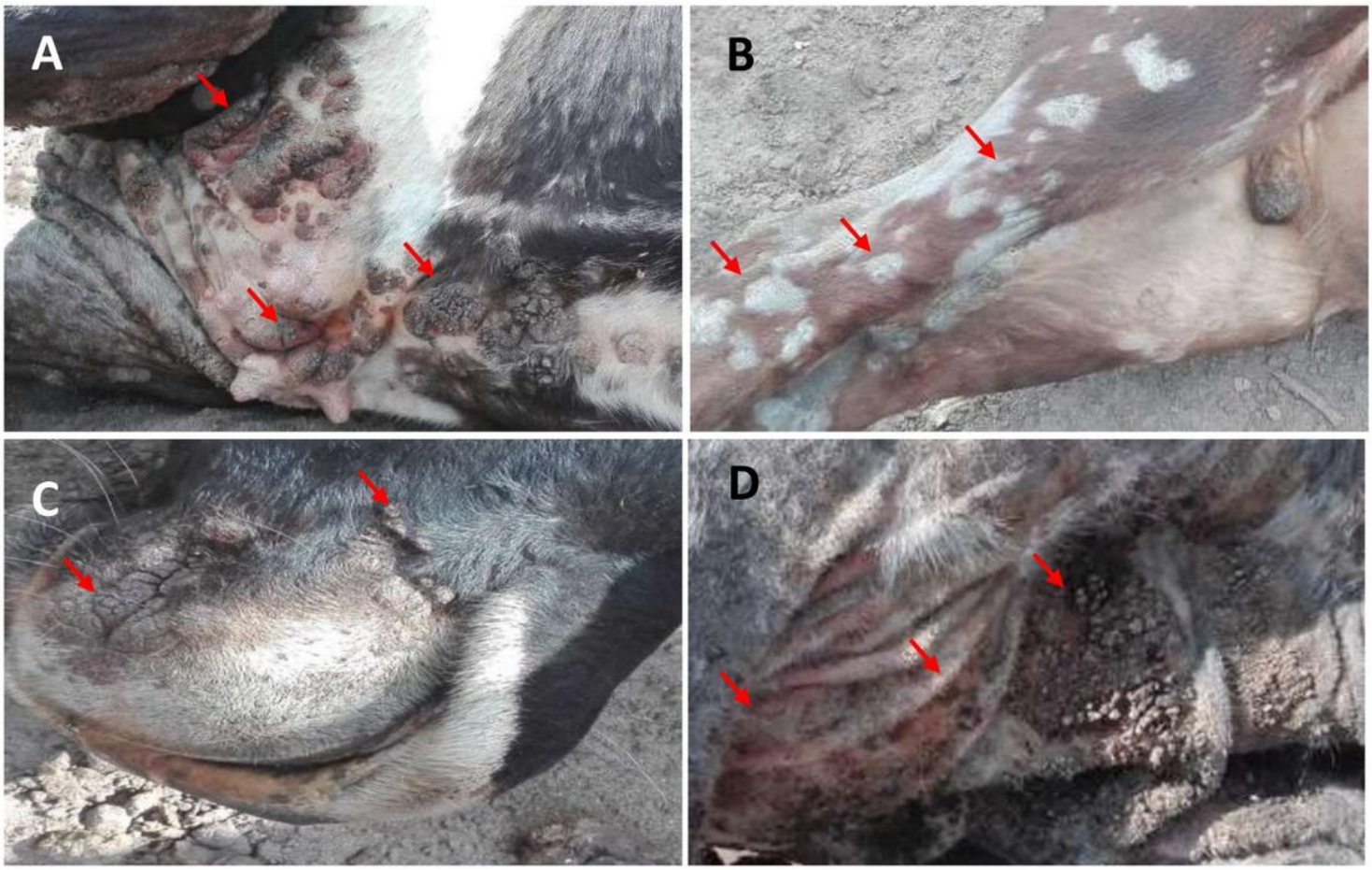

Figure 1

Skin lesions on PCPV infected cattle from Zambia. A) affected cattle with nodules on the skin and udder. B) affected calf with scars around the body C) affected cattle with nodules around the muzzle D) affected cattle with nodules and dermatitis.

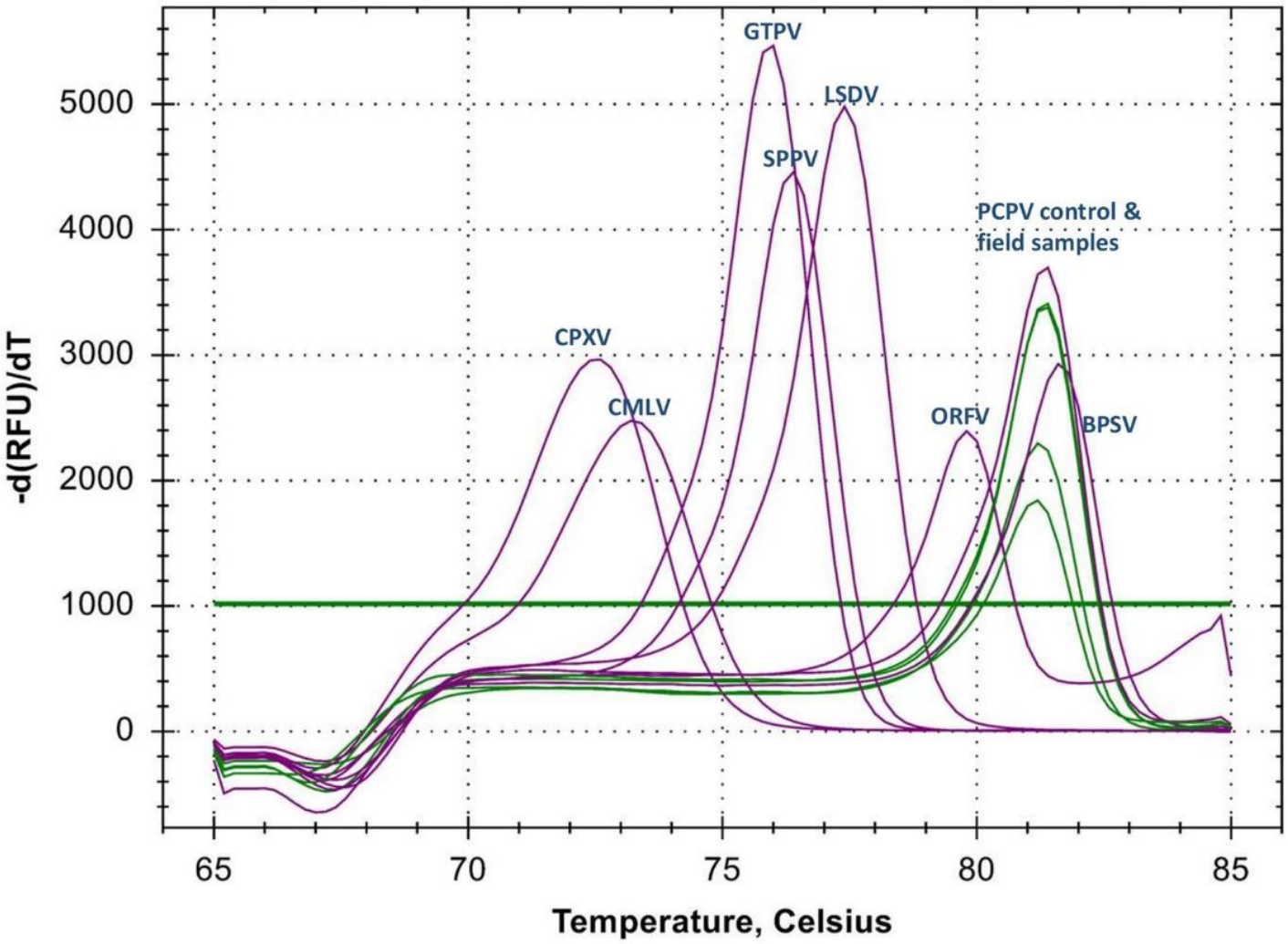

Figure 2

HRM detection of PCPV in selected cattle samples from Zambia. The positive control for each of the eight poxviruses displayed a unique melting peak, shown in purple color. Four samples from Zambia, clustering with PCPV are shown in green color.

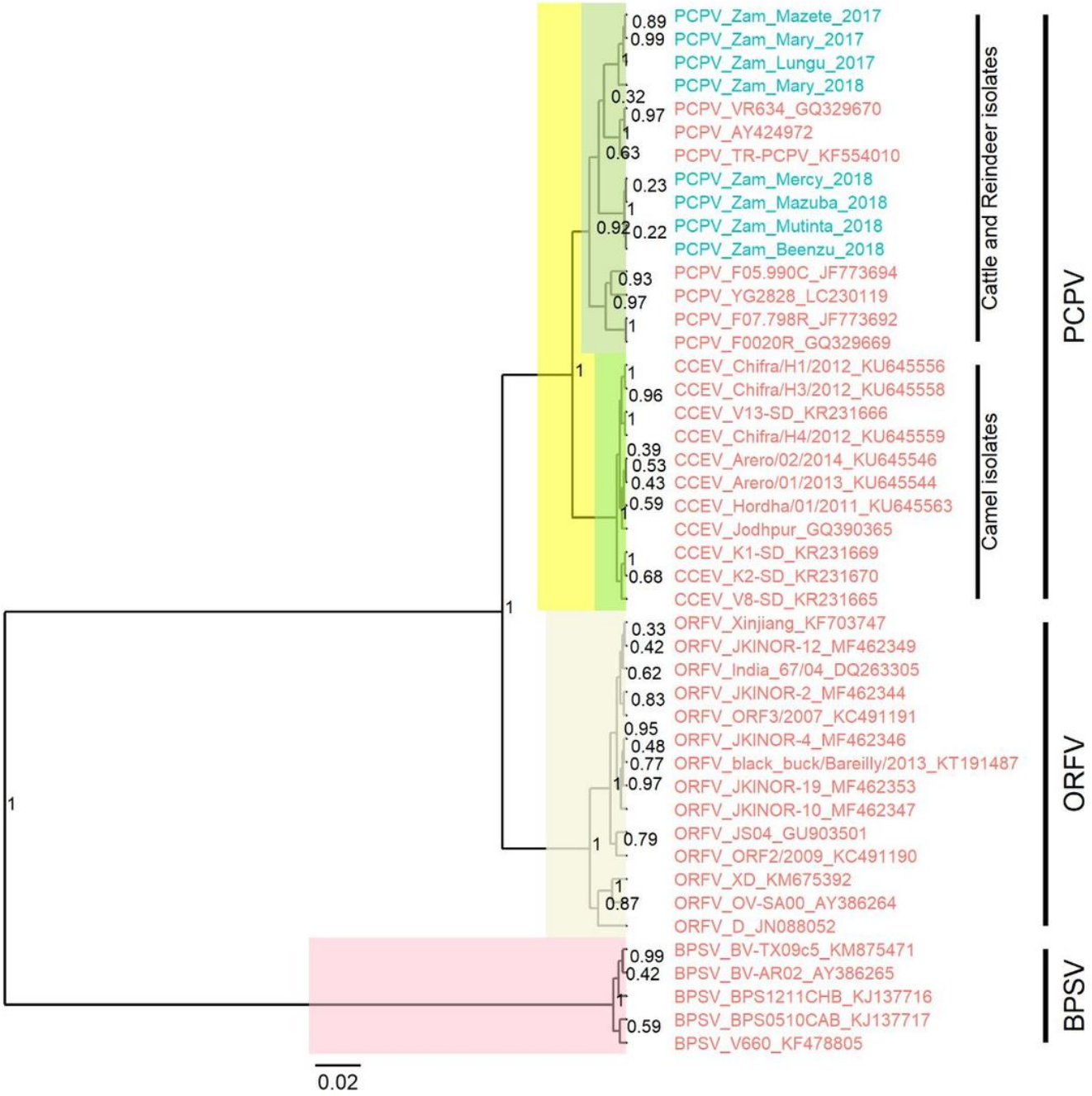

Figure 3

Maximum clade credibility (MCC) tree based on the partial B2L gene sequences of parapoxviruses. The posterior probabilities are plotted as respective nodes labels. The cattle PCPV sequences from Zambia, are highlighted.

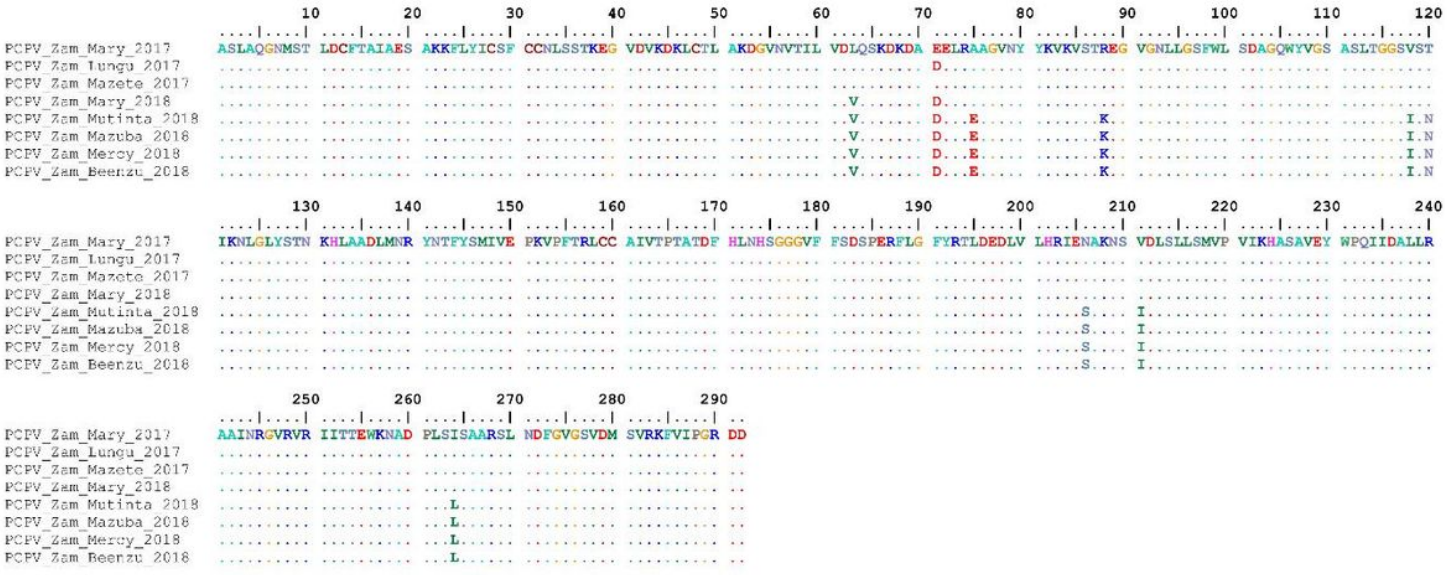

Figure 4

Multiple sequence alignments of the deduced amino acid sequences of the PCPV B2L gene. The PCPV collected in December 2017 and April 2018 in Zambia are compared. Identical amino acids are shown as dots in reference to the first sequence. Note the sequence differences between the 2017and 2018 isolates.