First Molecular Characterization of Poxviruses in Cattle, Sheep, and Goats in Botswana

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Abstract

Background

Poxviruses within the Capripoxvirus, Orthopoxvirus, and Parapoxvirus genera can infect livestock, with the two former presenting also zoonotic importance. In addition, they induce similar clinical symptoms in common host species, creating a challenge for diagnosis. Although endemic in the country, poxvirus infections of small ruminants and cattle have received little attention in Botswana, and there was no prior attempt of using molecular tools to diagnose the diseases and characterize the pathogens.

Methods

A high-resolution melting (HRM) assay was used to detect and differentiate poxviruses in samples from four cattle (from Mahalapye, Kasane, and Molepolole), one sheep (from Jwaneng), and one goat (from Kasane). Molecular characterization of capripoxviruses and parapoxviruses was undertaken by sequence analysis of RPO30 and GPCR genes.

Results

The HRM assay revealed lumpy skin disease virus (LSDV) in three cattle samples, pseudocowpox virus (PCPV) in one cattle sample, and orf virus (ORFV) in one goat and one sheep sample. The phylogenetic analyses, based on the RPO30 and GPCR, and the inspection of the multiple sequence alignments showed that the LSDV sequences of Botswana were more like those of common LSDV field isolates encountered in Africa, Asia, and Europe. The Botswana PCPV presented unique features and clustered between camel isolates and cattle isolates of the PCPV group. The Botswana ORFV from goat differed from the ORFV collected in sheep.

Conclusions

This study is the first report on the genetic characterization of pox virus diseases circulating in cattle, goats, and sheep in Botswana. It shows the importance of molecular methods to differentially diagnose pox virus diseases of ruminants.

Background

Poxviruses exist throughout the world, and are responsible for several economically significant and zoonotic diseases affecting human beings as well as wildlife and domestic animals(1, 2). Generally, poxviruses are epitheliotropic and may cause localized cutaneous lesions or generalized lesions involving the skin as well as many organs and tissues (mucosal part of the respiratory and digestive tracts, lungs and kidney). Lesions on the skin of the affected animals can be clinically confused with other cutaneous diseases. Economically, pox virus diseases cause losses due to damage to the skin, decreased milk and meat production and trade restriction in addition to morbidity and mortality (3, 4).
The poxviruses are complex, linear, enveloped, double-stranded DNA viruses with large genomes of 130 – 360 kb in length (5, 6). They belong to the Poxviridae family, which is divided into two subfamilies: Entomopoxvirinae, which infect insects, and Chordopoxvirinae, which infect vertebrates (7). The Chordopoxvirus subfamily comprises ten genera: Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus, Cervidylipoxviruses, Crocodylipoxviruses, and Yatapoxvirus (8).

The genera among the Chordopoxvirinae that affect livestock (especially the ruminants) are Orthopoxvirus - cowpox virus (CPXV) and vaccinia viruses (VACV), Capripoxvirus - goatpox virus (GTPV), sheeppox virus (SPPV), and lumpy skin disease virus (LSDV), and Parapoxvirus – Orf virus (ORFV), Pseudocowpox virus (PCPV), and bovine papular stomatitis virus (BPSV). Some of these poxviruses have similar geographical distribution. They also can infect the same animal species (i.e., ORFV, SPPV, and GTPV cause generalized or localized cutaneous lesions in both sheep and goats), creating a challenge for the clinical diagnosis of these diseases.

Poxviruses are transmitted through several routes such as small abrasions of the skin (e.g., orf), or directly or indirectly from a contaminated environment by aerosol infection through the respiratory tract (e.g., sheeppox and goatpox), or possibly by mechanical transmission by biting arthropods (e.g., lumpy skin disease and sheeppox).

In Botswana, pox virus diseases in ruminants seem to be receiving little or no attention. The first publication on pox virus diseases in Botswana was on orf virus in goats (9), without molecularly characterizing the virus. Lumpy skin disease was first observed in 1943 following an outbreak in Ngamiland in northern Botswana. With years, the disease has spread virtually to all the districts in the country (10). In Botswana, LSD is controlled through vaccination with live attenuated vaccines; LSDV Neethling strain (Onderstepoort Biological Products; OBP, South Africa) and attenuated South African LSDV field isolate (Lumpyvax, MSD Animal Health-Intervet, South Africa). Even though LSD has been endemic and widespread in Botswana for many years, there is no genetic information available on LSDVs circulating in Botswana.

This study reports the first confirmed case of pseudocowpox in Botswana, and the first molecular characterization of LSDV, ORFV, and PCPV in the country.

**Methods**

**Study areas and sample collection**

The study was conducted using four archived clinical samples collected from 2010, 2016, 2017 outbreaks, and two samples from a recent outbreak in 2019. These samples were from different regions of Botswana (Figure 1). The samples included three skin biopsies, one skin crust, one lip skin scab as well as one wart. Epidemiological information on the clinical signs and attempted clinical diagnosis were...
collected from sample submission forms that accompanied the samples when they were submitted to Botswana National Veterinary Laboratory (BNVL).

**Sample preparation and DNA extraction**

The samples were cut with a scalpel blade into small pieces and homogenized (10 % w/v) in sterile phosphate buffered saline (PBS), then centrifuged at 2500 rpm for 10 min to collect the supernatant. DNA was extracted from 200 µl of the supernatant using DNeasy Blood and Tissue kit (Qiagen) following the manufacturer’s instructions. The DNA was eluted in 80 µl elution buffer, then kept at -20 °C until further use.

**Molecular detection and genotyping**

The extracted DNA was tested using an HRM multiplex real-time assay for the simultaneous detection and differentiation of eight poxviruses of medical and veterinary importance (11). 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: CPXV and CMLV in the *Orthopoxvirus* genus; GTPV, SPPV, and LSDV in the *Capripoxvirus* genus; ORFV, PCPV, and BPSV in the *Parapoxvirus* genus.

The PCR was set up in a 20 µl reaction volume containing 1x SsoFast™ EvaGreen® Supermix (Bio-Rad), equal concentration (200 nM) of each of the forward and reverse primers (Table 1), and 2 µl of sample DNA. 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 the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories), as earlier described with slight modifications (11). Briefly, an initial denaturation step at 95 °C for 4 min, followed by 40 cycles at 95 °C for 1 s, 59 °C for 5 s and 70 °C for 5 s. The PCR products were then denatured at 95 °C for 30 s, cooled down at 65 °C for 60 s, and melted from 65 °C to 85 °C with an increment of 0.2 °C every ten seconds and a continuous data acquisition. Data was analyzed using the CFX Maestro Software (Bio-Rad) and the Precision Melt Analysis Software (Bio-Rad).

**Sequencing**

The G-protein-coupled chemokine receptor (GPCR) (12), and the 30 kDa DNA-dependent RNA polymerase subunit (RPO30) (13) genes of the *Capripoxviruses* were amplified using primers (14) in Table 1. The PCR reaction was performed in a reaction volume of 20 µl containing 500 nM of each of the forward and reverse primers, 0.2 mM of dNTPs, 1x buffer (Qiagen), 2.5 U of Taq DNA polymerase (Qiagen), and 2 µl template DNA. The amplification consisted of an initial denaturation at 95 °C for 4 min followed by 35 cycles of 95 °C for 40 s, 55 °C for RPO and 56 °C for GPCR for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min.

For the *Parapoxviruses*, the partial B2L gene was amplified by PCR in a reaction volume of 25 µl containing 500 nM of each of the primers (15) in Table 1, two mM dNTPs, 1X PCR Buffer (Qiagen), 2.5 U
Taq polymerase (Qiagen) and five μl template DNA. The cycling conditions were: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 50 s, 52 °C for 60 s and 72 °C for 90 s, and a final extension at 72 °C for 7 min.

All PCR products were separated by electrophoresis on a 1.5 % agarose gel at 100 V for 1 hr. The positive PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Promega) according to the manufacturer’s instructions, then sequenced commercially by LGC Genomics (Germany). The sequences were edited and assembled using Vector NTI Advance™11.5 software (Invitrogen, Carlsbad, CA, USA). All sequences were submitted to GenBank.

**Phylogenetic analysis**

For phylogenetic reconstructions of the RPO30, GPCR, and B2L gene trees, multiple sequence alignments of the nucleotides sequences were performed separately for each gene using the muscle algorithm and the codon option implemented in MEGA7 (16). Additional sequences of the RPO30 and GPCR gene for CaPVs (LSDVs, GTPVs, and SPPVs) and the B2L gene for parapoxviruses (ORFV, PCPV, and BPSV), were retrieved from GenBank and included for comparative analyses.

The sequence alignment FASTA file was converted into a Nexus format file using Seaview programme version 4.7 (17). The Bayesian phylogenetic inference was performed with BEAST. First, the BEAUti module was used to generate BEAST files 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 (18). Additionally, for the GPCR tree, the multiple sequence alignment file of the nucleotide sequences was imported and a slice of the alignment, between positions 80 and 120, was visualized together with the tree (18).

**Results**

**Epidemiological information on the clinical signs and attempted clinical diagnosis**

It appeared that most animals had lesions suggestive either of poxvirus infections, and sometimes, papilloma virus infections (Table 2).

**Molecular detection**

As summarised in table 2, PCPV DNA was detected in one sample (BOT_BOV/2010/6389) and LSDV DNA in three samples (BOT_BOV/2016/172, BOT_BOV/2017/1657, and BOT_BOV/2019/246) from cattle using the HRM real-time PCR assay. ORFV DNA was detected in sheep and goat samples, respectively.
Sequencing

Two fragments for the RPO30 gene (554 bp and 520 bp) and three fragments for the GPCR (617 bp, 603 bp, and 684 bp) were successfully amplified and sequenced in all the three LSDV positive samples (BOT_BOV/2016/172, BOT_BOV/2017/1657, and BOT_BOV/2019/246). Similarly, approximately 1210 bp fragment of the B2L gene was amplified and sequenced in the remaining three samples that were positive for PCPV (BOT_BOV/2010/6389) and ORFV (BOT_OV/2017/158, and BOT_CAP/2019/74). The complete RPO30 and GPCR genes and partial B2L gene sequences were submitted to the GenBank database under accession numbers MW748471 to MW748479.

Molecular characterization and phylogenetic analysis

In both GPCR and RPO30 gene trees, all three cattle samples from Botswana (BOT_BOV/2016/172, BOT_BOV/2017/1657, and BOT_BOV/2019/246) clustered within the LSDV group (Figures 3 and 4). On the GPCR gene tree, the LSDV group was further subdivided into two subgroups: the first subgroup included the LSDVs from Botswana, and field LSDVs from Europe, the Middle East and Africa, and LSDV KS1 derived vaccinal viruses. The second subgroup consisted of LSDV Neethling-like viruses, and the historical LSDV Haden 1959 from South Africa (Figure 3).

The multiple sequence alignments of the GPCR gene showed that all the Botswana LSDVs had sequences identical to each other, and presented a 12-nucleotide deletion found in common field LSDVs (Figure 3).

On the RPO30 gene tree (Figure 4), LSDV isolates produced 3 sub-groups: the first one consisted of the Botswana LSDVs and common field LSDVs encountered in Africa, the Middle East and Europe. The second subgroup contained LSDV KS1-like viruses and LSDV NI-2490, while the third consisted of LSDV Neethling like viruses and the historical LSDV Haden 1959 (Figure 4).

The RPO30 gene sequences were identical for all the Botswana LSDVs and identical to common field isolates, but different from the the vaccinal strains derived from KS1 and Neethling viruses. The Botswana isolates differed from LSDVs from Sudan (GU119938) and (GU119944) by a single non synonymous nucleotide difference leading to amino acid substitutions at one position (T¹⁴ ® N and D¹⁰² ® G) respectively.

On the phylogenetic tree of the B2L gene, the sequence of sample BOT_BOV/2010/6389 clustered with PCPVs (Figure 5). Within the PCPV group, BOT_BOV/2010/6389 clutered between the B2L sequences of camel isolates and those of cattle/reindeer isolates. The B2L sequences of the two samples BOT_OV/2017/158 and BOT_CAP/2019/74, collected from small ruminants, clustered within the ORFV group (Figure 5).
The BLAST results showed that the nucleotide identities of the Botswana PCPV, BOT_BOV/2010/6389, against published parapoxviruses ranged from 98.63 %-98.97 % with the highest identity (98.97 %) to PCPV strains strain 3/07 (KF478804) isolated from cattle in Germany, and strain VR634 (GQ329670) collected from man infected by cattle in New Zealand.

The B2L sequence of ORFV BOT_CAP/2019/74 was 100 % identical to that of strain OV-SA00 (AY386264) isolated from a goat in the USA. The multiple sequence alignments of the B2L gene revealed 24 nucleotide changes between the B2L gene of BOT_CAP/2019/74 and BOT_OV/2017/158.

Discussion

This study presents the first confirmed case of PCPV in Botswana, and the first molecular characterization of poxviruses affecting sheep, goats and cattle in the country, from animals presenting clinical lesions consistent with poxvirus infections. The DNAs from Lumpy skin disease virus (3 samples), PCPV (1 sample), and ORFV (2 samples) were detected in archived clinical samples collected in 2010, 2016, 2017 outbreaks, and samples from two recent outbreaks in 2019 using an HRM assay for the differential diagnosis of poxvirus infections (11).

It is worth noting that the status of the 2010 sample was previously undetermined, until the availability of this HRM assay which showed the presence of PCPV. Hence, the HRM assay has enabled the correct identification of LSDV and PCPV in cattle and ORFV in small ruminants in a single test as previously stated (11).

The attempted clinical diagnosis of the pox virus diseases were mostly consistent with the results of the HRM assay for LSDV and ORFV. The LSD suspected cattle had typical clinical signs that included skin nodules/bumps/lesions (19, 20) and orf suspected small ruminants had papilloma warts on the udder and/ or lesions on and around the mouth and buccal cavity as described earlier (21, 22). However, in this study, the lesions described for the PCPV positive animal were mostly suggestive for papillomavirus infections, though, the lesions on the udder of cattle are also common in pseudocowpox infections (23, 24).

For all samples, the sequencing data were in full agreement with the HRM data. There was no sequence difference in the RPO30 and GPCR genes of Botswana LSDV samples collected in three separate outbreaks, in 2016, 2017 and 2019, at 3 different geographic locations (Mahalapye, Kasane, and Molepolole), suggesting that the virus is well conserved and that the same strain is responsible for all these outbreaks.

Moreover, the Botswana LSDVs sequences presented similar features to common field isolates of LSDV encountered in Africa, Europe and Asia, including the presence of the 12-nucleotide deletion in their GPCR gene (12, 14, 25-27).
The existence of PCPV in cattle has been reported worldwide, including Japan (28, 29), USA (30), Brazil (31) and Germany (32). In Africa, the disease has recently been reported in Zambia (33) and now in Botswana (this study). Interestingly, the phylogenetic analysis revealed that the newly sequenced PCPV from Botswana was distinct from common cattle PCPVs encountered elsewhere, behaving like an intermediate isolate between cattle isolates of PCPV and camel isolates of PCPV (known as CCEV). This finding is uncommon, as most isolates recovered from cattle usually clustered with the cattle/reindeer isolates. For instance, PCPVs that were recently reported in Zambia presented substantial sequence variation within the same herd and during the same outbreak events in 2017 and 2018, but were all clustering with the cattle isolates of PCPVs (33). The unique genetic feature of the Botswana PCPV suggests that by the time the outbreak occurred in 2010, PCPV was already well established in the country.

The path of introduction of the PCPV strain to Botswana is unknown and needs to be further investigated. Further genetic analysis of the PCPV strain is required to determine if it is genetically distinct enough to be classified as cattle and reindeer isolate or camel variant of PCPV virus.

The phylogenetic tree based on the B2L gene sequence, and the scrutiny of the multiple sequence alignment showed that the sample collected in sheep in 2017 in northern Botswana differed from that of the sample collected in goat in 2019 in the southern part of the country, suggesting that at least two different strains of ORFV are circulating in the country.

The caprine ORFV B2L sequence was fully like a sequence from an ORFV OV-SA00 (AY386264) collected from a goat in the USA, in 2000. The ovine ORFV sequence differed from most ovine sequences, but displayed 99.6 % amino acid similarity level to isolate FJ-ZX (KC568400) collected from a goat in China.

**Conclusion**

This paper reports the first molecular detection and characterization of pox virus diseases circulating in sheep, goat and cattle in Botswana. It shows the importance of molecular methods for the differential diagnosis of pox virus diseases in ruminants (the presence of LSDV and PCPV in cattle and ORFV in small ruminants). As PCPV is a well-known zoonotic disease transmissible through direct contact (e.g. milkers nodules), the information provided here is also of public health relevance.

**Abbreviations**

BNVL – Botswana National Veterinary Laboratory

BPSV – Bovine papular stomatitis virus

CMLV – Camelpox virus

CPXV – Cowpox virus
Declarations

Ethics approval and consent to participate

Samples used were for routine diagnosis at the Botswana National Veterinary Laboratory.

Consent for publication

The study was approved for publication by the Ministry of Agricultural Development and Food Security, Botswana.

Availability of data and materials

DNA sequences generated and analyzed under the current study are available in GenBank under accession numbers MW748471, MW748472, and MW748473 (B2L gene), MW748474, MW748475, and MW748476 (RPO30 gene), MW748477, MW748478, and MW748479 (GPCR gene).

Competing interests

The authors declared no conflicts of interest.

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**Authors’ contributions**

Conceived and designed the study: BMM, and CEL.

Performed the experiments: BMM, TBKS, TK, DX, KN, KK, JFN, CT.

Analyzed the data: BMM, DX, CEL.

Contributed reagents/materials/analysis tools: IN, GJV, GC, CEL.

Wrote the paper: BMM.

Supervised the study: KNM, CMR, GJV, GC, CEL.

Edited the final manuscript: TBKS, JFN, CMR, GJV, GC, CEL.

All authors read and approved the final manuscript.

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Tables

Table 1. Primers used in this study for the HRM assay and sequencing.
| Method | Primer name | Primer sequence | Amplicon size | Target & References |
|--------|-------------|----------------|--------------|---------------------|
| HRM    | CaPV-HRM-For | TCCTGGCATTTTAAGTAATGGT | 100          | Capripoxviruses (1) |
|        | CaPV-HRM-Rev | GTCAGATATAAACCAGCAAGTG |             |                     |
|        | PPV-HRM-For  | TCGAAGATCTTTGTCAGGAAG | 112          | Parapoxviruses (1)  |
|        | PPV-HRM-Rev  | CCGAGAAGACTACAGGGTC |             |                     |
|        | OPV-HRM-For  | AGGACTAGCCCGGTAACTTT | 56           | Orthopoxviruses (1) |
|        | OPV-HRM-Rev  | ACAAGATAGAAGCGATGGGACTT |         |                     |
| Sequencing | CpGPCR-OL1F | TGAATAATATCCATTCTTCTAAACA | 617         | Capripoxviruses (2) |
|        | CpGPCR-OL1R | TCATGTATTTTATAACGATAATGCAA |     |                     |
|        | CpGPCR-OL2F | TTAGCGGTATAATCATTCCAATA | 603         |                     |
|        | CpGPCR-OL2R | GCGATGATTATGATATTGGAAGTG |             |                     |
|        | CpGPCR-OL3F | CACAATTATATTCCAATAATCCAA | 684         |                     |
|        | CpGPCR-OL3R | TGTACATGTGTAATTATAGTTGCTA |     |                     |
|        | CpRPO30-OL1F | CAGCTGTTTTGTTACATTGGTTT | 554         |                     |
|        | CpRPO30-OL1R | TCGATAGAAACAGCCTTTAATGAG |     |                     |
|        | CpRPO30-OL2F | TTTGAACACATTTTATCC AAAA | 520         |                     |
|        | CpRPO30-OL2R | AACCTACATGCATAAACAGAAGC |             |                     |
|        | ORFV-B2Lf-For | GACCTCGCGCTTTAATTT | 1210         | Parapoxviruses (3)  |

Table 2. Summary of clinical signs, attempted clinical diagnosis and molecular findings of the different poxviruses.
| Sample ID         | District         | Type of samples                  | Case history (clinical signs)                                      | Attempted clinical diagnosis | Molecular findings |
|------------------|------------------|----------------------------------|------------------------------------------------------------------|-------------------------------|--------------------|
| BOT_BOV/2010/6389 | Mahalapye        | Skin crust, serum and w/blood    | -Recumbent -Bleeding wounds on the udder, mouth, anus and legs   | Papillomiosis                 | Pseudocowpox positive |
|                  |                  |                                  |                                                                  |                               | Mineral deficiency  |
| BOT_BOV/2016/172 | Kasane (Chobe)   | Skin biopsy                      | Cattle developing skin nodules all over the body                  | LSD                           | Lumpy skin disease positive |
| BOT_OV/2017/158  | Jwaneng          | Lip skin scab, w/blood and sera  | Lesions on and around mouth and buccal cavity                    | Orf                           | Orf positive        |
|                  |                  |                                  |                                                                  | Goitre                        |                    |
|                  |                  |                                  |                                                                  |                               | Blue tongue         |
| BOT_BOV/2017/1657| Molepolole       | Skin biopsy                      | Cow with skin bumps and lameness                                 | LSD                           | Lumpy skin disease positive |
| BOT_BOV/2019/246 | Mahalapye        | Skin biopsy, nasal swab, w/blood and serum | Lesions on the skin, nasal discharge and lacrimation | LSD                           | Lumpy skin disease positive |
| BOT_CAP/2019/74  | Kasane           | Udder with warts                 | Udder of a dead goat with papilloma warts like/ orf infection on udder | Orf                           | Orf positive        |

*Animal species: Bov: bovine; Cap: caprine and Ov: ovine
*Date samples received: 2010, 2016, 2017 and 2019
Figure 1

Map of Botswana showing the location of where samples were collected (red stars). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
Figure 2

HRM detection of pox viral diseases in cattle, goats, and sheep samples from Botswana. The positive control for each of the eight poxviruses displayed a unique melting peak, shown in green color. One cattle and three cattle samples clustering with PCPV and LSDV, respectively, and one goat and one sheep samples clustering with ORFV are shown in purple colour.
Figure 3

Maximum clade credibility (MCC) tree based on the complete GPCR gene sequences of Capripoxviruses. Only the portion of the alignment between positions 80 and 120 is shown. The posterior probabilities are plotted as respective nodes labels. LSDVs from Botswana are highlighted in red and reference sequences are represented with their accession numbers.
Figure 4

Maximum clade credibility tree based on the complete RPO30 complete gene sequences of capripoxviruses. The posterior probabilities are plotted as respective nodes labels. LSDVs from Botswana are highlighted in red and reference sequences are represented with their accession numbers.
Figure 5

Maximum clade credibility tree based on the partial B2L gene sequences of parapoxviruses. The posterior probabilities are plotted as respective nodes labels. The cattle PCPV sequence and sheep and goat ORFV sequences from Botswana, are highlighted in blue.