J S Afr Vet Assoc. 2025;96:1-6
https://doi.org/10.36303/JSAVA.677

J S Afr Vet Assoc
ISSN 2224-9435 EISSN 1019-9128

ORIGINAL RESEARCH

The prevalence of OvHV-2 in the smegma, nasal secretions and penile environment of rams (*Ovis aries*)

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Ulcerative balanoposthitis (UB) is a venereal disease which has frequently been diagnosed in sheep in South Africa (SA) since 1979, with no conclusive causative agent or treatment available. The purpose of this study was to determine whether ovine herpesvirus 2 (OvHV-2) can be used as biomarker to diagnose the early onset of UB in sheep flocks in SA. The study made use of 10 healthy Dohne Merino rams, two healthy Dorper rams, and 10 UB-affected Dorper rams. Smegma-, blood-, nasal swab- and sheath swab samples, respectively, were collected from all rams, and subjected to a single-tube hemi-nested PCR analysis to determine OvHV-2 status of all the sample types collected. The prevalence of OvHV-2 in all 22 animals was 22.75%, irrespective of breed or health status. The detection rate of OvHV-2 was 33.3% for the 10 UB-positive animals, and 16.7% for the 12 UB-negative animals. In blood, OvHV-2 had a 4.54% detection rate, 8.33% in smegma, 13.64% in nasal samples, and a 0% detection in penile swabs. This study found no significant co-occurrence of UB and OvHV-2, which indicates that OvHV-2 cannot be considered as a biomarker for the early onset of UB or a causative agent of UB.

Keywords: peestersiekte, ovine herpesvirus 2, sheep, venereal

Introduction

In South Africa (SA), with climate regions ranging from semi-arid to extremely arid, the sheep industry is of importance due to this species' exceptional ability to convert forage to meat and wool (Cloete et al. 2014; Courchay 2017; Van der Merwe et al. 2021). The contribution of this industry is modest in monetary terms, but major in terms of regional production and food security, especially in rural sheep production systems (Cloete et al. 2014). Ulcerative balanoposthitis (UB), with the female-related condition known as vaginitis or vulvovaginitis (VV), is a venereal disease occurring in sheep in SA (Trichard et al. 1993; Kidanemariam et al. 2005; Ali 2012; Courchay 2017). Manifestation in a flock is commonly described as ulcerations on the penis surface of the ram, and the vulva and/or vagina of the ewe (Trichard et al. 1993). This disease affects both sexes, with rams more severely affected than ewes (Courchay 2017). Due to the high infection rate of the disease in a sheep flock, and the associated effect on the reproductive output of a flock, UB has been classified as an economically important disease in SA (Trichard et al. 1993). The causative agent is still unknown, although researchers hypothesised possible aetiological agents including *Mycoplasma mycoides mycoides* Large Colony (MmmLC), *Ureaplasma* species, *Acholeplasma* species, or a combination of the respective microbes (Trichard et al. 1993; Kalshingi et al. 2015; Courchay 2017). Kalshingi et al. (2015) suggested that end-stage infections of UB are associated with the presence of *Trueperella pyogenes*.

Pritchard et al. (2008) and Rutten (2012) reported the detection of ovine herpesvirus type 2 (OvHV-2) in enzootic posthitis cases in the UK and Switzerland, respectively. Sheep being a reservoir host for OvHV-2, generally do not show clinical signs, but in the case of OvHV-2 infection, it manifests as a clinical disease similar to orf, with the establishment of balanoposthitis, which is characterised by extensive suppurate lesions covering the glans, fornix and urethral process, and which can cause local necrosis of the affected penile tissue (Gouletsou & Fthenakis 2015; Gieger & Furmaga 2020). Research thus suggested a possible relation between OvHV-2 and UB, for OvHV-2 is the first virus to be associated with UB (Pritchard et al. 2008; Rutten, 2012). Literature on the potential of OvHV-2 as biomarker for the early onset of UB in sheep is lacking, and the potential link to UB has not been established yet in sheep in SA. The study therefore aimed to determine the incidence of OvHV-2 in smegma, the penis sheath environment as well in the upper respiratory system of rams, to establish whether OvHV-2 co-occurs with UB in affected sheep and can be used as a biomarker during the early onset of the disease.

Material and methods

Experimental animals and husbandry

Ethical clearance for the study was obtained from the Research Ethics Committee: Animal Care and Use of Stellenbosch University (ACU-2022-24709 and ACU-2022-22342). A total of 10 adult Dohne Merino rams and 12 adult Dorper rams were used for sampling purposes. The average live weight of the rams was ± 52 kg. These animals were housed at the Sheep Section on the Welgevallen Experimental Farm of the University of Stellenbosch, prior to slaughter. All rams were reared in individual disinfected pens, received the same diet *ad lib*, and had free access to drinking water in the period prior to slaughter.

Collection and processing of samples

Each ram was restrained for determination of the UB status of each ram. The clinical signs that were used to classify a ram as UB-affected included ulcerations on the mucosal membrane of the glans penis only, or on the prepuce only, or on both the mucosal membrane of the glans penis and the prepuce. The

classification system for UB was developed based on previously described clinical signs (Trichard et al. 1993; Kidanemariam et al. 2005; Ali 2012; Courchay 2017), and the severity was ranked in terms of the ulcerations present on the glans penis and prepuce area. The clinical examination of all rams indicated that 10 of the 12 Dorper rams exhibited clinical signs associated with the early onset of UB, i.e. ulcerations have not yet advanced to the glans penis. No clinical signs of the disease were observed for any of the 10 Dohne Merino rams used in this study.

Blood samples

Blood samples were collected into 5 mL ethylene diamine tetra-acetic acid (EDTA) tubes (Lasec, South Africa) using venipuncture. Blood samples were transported on ice (4 °C) to the laboratory and stored at -20 °C until OvHV-2 analysis was performed.

Smegma samples

Smegma samples were collected by gently introducing an intra-uterine lavage catheter for bovines (~ 20 cm; Lakato (Pty) Ltd, South Africa) up to the deep portion of the penis sheath (Dibarrat et al. 2007). With the use of a disposable syringe, pre-warmed (37 °C) 10 mL sterile phosphate buffered saline (PBS) was introduced into the sheath environment, followed by a gentle massage for about one minute to ensure an even wash of the sheath environment (Dibarrat et al. 2007). After the washing period, the same catheter (i.e. with catheter being retained in sheath during the massage action) was used to extract the smegma sample. The content of each sampling syringe was then transferred to a sterile transportation vial, with each catheter flushed with fresh pre-warmed PBS (37 °C) to ensure that all extracted content was transferred to the transportation vial. Samples were transported on ice (4 °C) to the laboratory and stored at -20 °C until later analysis.

Penile and nasal swab samples

Penile sheath swabs were collected using a regular Copan FLOQswab™ (HCPN519CS01; Lasec, South Africa) by rolling the swab over the entire inner membrane of the penis sheath area for about 20 seconds (Courchay 2017). The swab was then transferred to a conical tube containing 3 mL universal transport medium (UTM™) (HCPN330C; Lasec, South Africa). The UTM tubes containing the swab were transported on ice (4 °C) to the laboratory and stored frozen at -20 °C until the OvHV-2 analyses were performed.

Nasal swabs were collected using a regular Copan FLOQswab™ (HCPN519CS01; Lasec, South Africa) by rolling the swab inside the entire nasal cavity for about 20 seconds (Courchay 2017). The swab was then transferred to a conical tube containing 3 mL universal transport medium (UTM™) (HCPN330C; Lasec, South Africa). The UTM tubes containing the swab were transported on ice (4 °C) to the laboratory and stored frozen at -20 °C until the OvHV-2 analyses were performed.

PCR processing and DNA quantification

Deoxyribonucleic acid (DNA) was extracted from the blood samples using a Sephadex bead™ livestock DNA extraction kit (LGC, Biosearch Technologies, Hoddesdon, UK), with minor alterations to the manufacturer's protocol (i.e. incubation period for lysis = 20 min at 60 °C, vortexed every five minutes for 10 seconds; lysed samples was centrifuged at 12 000 RPM for five minutes for pellet formation; DNA elution was incubated for 15 minutes at 60 °C, vortexed every five minutes for five seconds; samples were then centrifuged at 12 000 RPM for three minutes clear eluates yields).

For the smegma samples, as well as for the nasal-, penile- and swab samples, DNA was extracted using a Genomic DNA Isolation kit (Norgen Biotek Corporation, Product #24700, #24750, #24770), which was utilised according to the manufacturer's protocol.

After the DNA extraction process, all samples were subjected to validation (NanoDrop 2000 Spectrophotometer with NanoDrop 2001 Ink Software, Thermo Scientific), to quantify nucleic acid concentration. Samples that yielded a good 260/280 ratio (between 1.6–2.5) and an acceptable nucleic acid concentration (i.e. more than 10 ng/µL), were used for the PCR analysis.

Single-tube hemi-nested polymerase chain reaction (PCR) procedure

For the detection of OvHV-2 in the extracted DNA samples, a single-tube hemi-nested PCR approach was used (Bremer 2010). This approach was used for it was validated by Bremer (2010) and improved with an additional amplification step, for improved sensitivity of the procedure. The primers that were used in the protocol are indicated in Table I.

The PCR reactions were performed in a volume of 50 µL consisting of 25 µL TAKARA EmeraldAmp GT PCR Master Mix (RR310A-TAK; Separations (Pty) Ltd, South Africa), 2.5 µL OVR Primer, 2.5 µL MF Primer, 2.5 µL MR Primer (primers manufactured by Inqaba Biotec according to Table I, South Africa), 15 µL double-distilled water, and 2.5 µL DNA template. The PCR amplification cycling conditions were: 25 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds, 72 °C for 30 seconds followed by 45 cycles at 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds (Bremer 2010). A control sample, without any template DNA, was included in each PCR run.

Samples were analysed by gel electrophoresis in a 1.5% agarose gels in 1% Tris/Borate/EDTA (TBE) Buffer (Cat. No: 3-07F01-I; BioConcept, Switzerland). The agarose gels were loaded with

Table I: Primers manufactured and used in the single-tube hemi-nested PCR (Bremer 2010:93–96).

Primer	Sequence	Nucleotide position (5'–3')	Primer length
MF	5'-AAAACAGYAGGCTCCAGGGGGAGG-3'	121133–121156# 433–410*	24
MR	5'-CTGCCMTGCTGGGTCCAGGGCAC-3'	21439–121417# 27–149*	23
OVR	5'-ACGGTCAGTCCAAGAC-3'	121339–121324# 227–242*	16

#The position of the sequence in the complete OvHV-2 gene (NC_007646). *Genbank S64565 sequence from which the primers were originally derived. There is a C in the 3' position of primer MR instead of an A as derived from NC_007646.

1.5 µL of the PCR products and electrophoresed at a constant output voltage (V) of 150 and an output current ($_{M}$A) of 200, for 25 minutes (PowerPac™ Basic, 100-120/220-240V, Bio-Rad). The electrophoresed gels were viewed with the use of a UV transilluminator Gel Doc (Molecular Imager® Gel Doc™ XR+ with Image Lab™ Software), to produce an image which was used for the identification of the OvHV-2 base pairs. The DNA fragment size for OvHV-2 is 207 base pairs (Bremer 2010).

Statistical analysis

Data analysis was performed using Microsoft XLSTAT (Version 2022.1) for Windows. The chi-square test was designed to determine the association between OvHV-2 incidences and the occurrence of UB, breed effect, sample type and location, and UB lesion score, respectively. If 20% or more of the expected cell count of the chi-square table was less than 5, Fisher's exact test was used. Significant differences were noted at a significance level of $p \leq 0.05$.

Results

To establish if OvHV-2 can be considered as a potential biomarker of UB, a single-tube hemi-nested PCR procedure was used to detect OvHV-2 DNA in biological fluids or tissue swabs (i.e. blood, penile sheath environment, smegma, and nasal mucosa) obtained from healthy and UB-affected rams.

Nanodrop results indicated that usable DNA could be extracted from all blood samples ($n = 22$; 100% extraction success), as well as all the nasal swab samples ($n = 22$; 100% extraction success).

For the smegma samples, a 30% extraction success was reported for the Dohne Merino samples, and an 83.3% extraction success for the Dorper samples, respectively. The two Dorper rams from which OvHV-2 DNA could not be obtained from the smegma samples, both exhibited clinical UB signs. For the penis swab samples, a 20% extraction success was reported for the Dohne Merino rams, whilst a 75% extraction success was reported for the samples obtained from the Dorper rams. Of the three Dorper rams where OvHV-2 DNA could not be extracted from the penis swab samples, one ram was healthy, and two rams exhibited UB clinical signs.

All samples where usable DNA was obtained, were subsequently subjected to a single-tube hemi-nested PCR analysis to determine the prevalence of OvHV-2. Table II presents the prevalence of OvHV-2 in the respective samples obtained from the Dorper and Dohne Merino rams. Sheep breed did not influence the prevalence of OvHV-2 ($p = 0.781$; Table III). An OvHV-2 detection rate of 33.3% and 25.0% was reported for the Dorper and Dohne Merino breeds, respectively.

Table II: The influence of breed on the prevalence of OvHV-2 in biological fluids and tissue obtained from rams

Status for OvHV-2	Sheep breed	
	Dorper	Dohne Merino
Negative	9	8
Positive	3	2

Table III presents the comparison of the different biological fluid and tissue swab samples in terms of OvHV-2 detection. The type of sample as well as sample site did not influence the OvHV-2 detection rate ($p = 0.738$). Although not significant, the highest prevalence of OvHV-2 was recorded for the nasal swab samples ($n = 22$, 15.79%), when compared to the blood samples ($n = 22$, 4.76%), smegma- ($n = 13$, 8.33%) and the penis swab samples ($n = 11$, 0%), respectively.

Table III: The prevalence of OvHV-2 in the different biological fluid or tissue swab samples obtained from Dorper and Dohne Merino rams

Status for OvHV-2	Type of sample			
	Blood ($n = 22$)	Smegma ($n = 13$)	Penis swab ($n = 11$)	Nasal swab ($n = 22$)
Negative	21	12	11	19
Positive	1	1	0	3

Table IV presents the relationship between the co-occurrence of OvHV-2 in UB-positive rams, in terms of the entire sample set (regardless of sample type) and per UB-positive case. The prevalence of OvHV-2 was independent of UB status, regardless of sample type ($p = 0.501$). There was also no significant relationship between the prevalence of OvHV-2 and simultaneous occurrence with each UB case ($p = 0.457$).

Table IV: The co-occurrence of OvHV-2 with ulcerative balanoposthitis in Dorper and Dohne Merino rams respectively, regardless of sample type

Status for OvHV-2	UB status	
	Positive	Negative
Entire sample set		
Negative	37	46
Positive	3	2
Per UB-positive ram		
Negative	7	10
Positive	3	2

Table V presents the relationship between the UB lesion score and the detection of OvHV-2 in Dorper and Dohne Merino rams. When the OvHV-2 positive cases were assessed for their relationship with the assigned UB lesion score, no relationship was observed ($p = 0.614$). When comparing the Dorper and Dohne Merino rams in terms of whether OvHV-2 was detected in rams where UB lesions were observed, there was no significant difference between the respective categories ($p = 1.00$).

Table V: The relationship between the detection of OvHV-2 in Dorper and Dohne Merino rams, and the assigned UB lesion score

Prevalence of OvHV-2	Score 1[a]	Score 2[b]	Score 3[c]	Score 4[d]
No	10	2	4	1
Yes	2	1	1	1

[a] no lesions; [b] very little lesions; [c] moderate lesions; [d] severe lesions

Discussion

The aim of the study was to determine the potential of OvHV-2 as a biomarker for the early onset of UB in South African sheep flocks. Biological samples obtained from sheep that may harbour OvHV-2 included blood, smegma, and penile and nasal swabs, respectively. Samples were obtained from 22 rams, i.e. 10 healthy Dohne Merino rams and 12 Dorper rams (i.e. two healthy and 10 UB-affected) rams. A single-tube hemi-nested PCR was used to detect OvHV-2.

The DNA extraction success from all 88 samples was characterised by variation between samples, as well as between breeds. The variation in DNA extraction success can potentially be ascribed to animal health status or the freezing of samples prior to analyses. According to the manufacturer guidelines for the kit used to extract the DNA, the frozen storage of samples prior to extraction may have a deleterious effect on the quality and integrity of the DNA that is eventually extracted after storage. Other factors influencing DNA extraction include contamination (proteins, carbohydrates, lipids, and RNAs) and extraction method (it should consider target DNA) (Dilhari et al. 2017). PBS and phenol can also affect DNA isolation rates (Thermo Fisher Scientific 2012; Matlock 2015; Hallmaier-Wacker et al. 2018).

The health status of the animal plays a role in the mucus production of the reproductive tract, and can also influence the successful extraction of DNA in samples obtained from the environment of the reproductive tract. Bacterial infection of the reproductive tract of ruminants causes an immune response. Actions such as immunoglobulin secretions, infiltration of mononuclear cells, plasma cells and lymphocytes of the mucosae and epithelium linings, indicate that reproductive mucosa has the potential of antigen processing and immune response actions against pathogens (Flower et al. 1983; Foster et al. 1988; Acosta-Dibarrat et al. 2014). Acosta-Dibarrat et al. (2014) identified antigen-presenting cells, macrophages, and dendritic cells as well as T-cell receptor lymphocytes, immunoglobulin (Ig) M-containing cells, IgG, and IgA in the preputial mucosa of sheep rams, indicating inductive and effector sites for the mucosal immune response. Researchers found that some diseases (such as cystic fibrosis of the lungs) can cause a higher and stickier mucous production, and that this sticky mucous is caused by a higher concentration of DNA polymers (Yuan et al. 2015). In the current study, a higher DNA extraction rate from smegma and penile swabs samples in the animals identified as UB-positive were observed, suggesting UB causes a mucosal immune reaction leading to higher production of smegma and mucus in the penile sheath environment, with a higher concentration of DNA polymers. This higher concentration will lead to more successful DNA extractions of the samples taken from areas with a higher mucous secretion level.

The detection rate of OvHV-2 in the rams in this study was 22.72%, i.e. an animal was considered OvHV-2-positive if at least one of the four samples detected the OvHV-2 base pair. Previous studies reported varying percentages of OvHV-2-positive cases in sheep flocks, ranging from 24.44% (Southern India), 54% (South-East Asia), 77% (Tenggara), 77.78% (South Africa), 85% (Brazil), to 99% (Northern India), respectively (Bremer 2010; Pinheiro de Oliveira et al. 2019; Kumar et al., 2021; Wiyono et al., 2021). The OvHV-2 detection rate of the current study differs from values reported by Bremer (2010:93–96), Pinheiro de Oliveira et al. (2019) and Wiyono et al. (2021) but agrees the most with the OvHV-2 detection rates in the studies conducted in Southern India (Kumar et al. 2021). Kumar et al. (2021) suggested that geographical regions may influence the detection of OvHV-2 in sheep. In the latter study, differences of up to 60% in detection of OvHV-2 made between the Southern and Northern parts of India (Kumar et al. 2021) were reported.

Bremer (2010) reported that breed did not affect the prevalence of OvHV-2 in sheep. In the study of Bremer (2010:93–96), OvHV-2 was detected in Damara- (75.0%), Dorper- (75.7%), Karakul- (63.6%), and Dohne Merino sheep (75.5%). In the current study, OvHV-2 was detected in both breeds, 25% of the 12 Dorper rams (i.e. three) and 20% of the Dohne Merino rams (i.e. two). was reported for the Dorper and Dohne Merino breeds, respectively.

Although the detection rate per breed, in the current study is lower than that of Bremer (2010), no breed differences were observed in the current study, agreeing with the findings of Bremer (2010) in regard to breed effect on OvHV-2 detection.

Rutten (2012) suggested that OvHV-2 may be a possible aetiological agent of ovine ulcerative vulvitis and balanoposthitis due to the isolation of the virus in biopsy samples taken from a ram with UB. Pritchard et al. (2008) reported OvHV-2 detection in blood samples of two ewes severely affected by ulcerative vulvitis, and detection of OvHV-2 from penis ulcers from a UB-affected ram. In the current study, only three (33.3%) of the 10 UB-positive animals were positive for OvHV-2. In the 12 UB-negative animals, two (16.7%) animals tested were indeed positive for OvHV-2. Findings from this study indicate that there is no significant co-occurrence of UB and OvHV-2, which indicates that OvHV-2 cannot be considered as a biomarker or a causative agent of UB.

In the present study, only five OvHV-2 detections were made. OvHV- 2 can cause MCF, a systemic disease, which spreads through the circulatory system to the entire body (Løken et al. 2009). If the virus is present in an animal, OvHV-s detection is expected in the blood. The current study detected one case of (4.54%) OvHV-2 in the blood. Hüssy et al. (2002) demonstrated significantly high levels of OvHV-2 in the semen of rams, suggesting OvHV-2 could be sexually transmitted. The presence of OvHV-2 in the smegma of the current study supports the findings of Hüssy et al. (2002), although only one (8.33%) smegma sample detected OvHV-2. The nasal swab samples showed the highest OvHV-2 detection rate (13.64%) of all the sample types. Replication and shedding of OvHV-2 occurs primarily in and from the nasal cavities, which explains these high detection rates (Kim & Crawford 2003; Li et al. 2004; Bremer 2010; Yildirim et al. 2012; Iman et al. 2015; Pinheiro de Oliveira et al. 2019; Gieger & Furmaga 2020; Wiyono et al. 2021). No OvHV-2 base pairs showed up in the penile swab samples.

Wiyono et al. (2021) detected OvHV-2 with PCR from pooled nasal, ocular, and vaginal samples of 22 sheep and yielded a 54.55% detection rate of OvHV-2. The current study showed a much lower detection rate than that of Wiyono et al. (2021),

however the different samples collected during this study, which were subjected to PCR assay, were not pooled.

The prevalence of UB lesions in cases that tested positive for OvHV-2 was investigated for possible associations between the two diseases. The need to investigate lesion severity and the co-occurrence with OvHV-2 was brought on by studies reporting on skin lesions caused by OvHV-2 in native sheep breeds (Iman et al. 2015). Gouletsou & Fthenakis (2015) reported that OvHV-2 can cause clinical diseases with characteristics similar to orf in sheep, with the clinical signs being extensive suppurative lesions covering the glans, fornix, and urethral process, with the possibility of forming local necrosis. These clinical signs are the same as that of balanoposthitis (Gouletsou & Fthenakis 2015).

Results from this study showed a definite variation in the degree of UB lesions observed, with no significant association between the degree of the lesions and the presence of OvHV-2. The present study indicated that there is no significant association between the degree of the lesions and the presence of OvHV-2 and that the lesions observed in the animals are thus caused by UB itself and not by the virus.

Conclusion

This is the first study to investigate the prevalence of OvHV-2 in UB-affected sheep in SA. The isolation of OvHV-2 in both healthy and UB-affected rams in this study indicates that sheep can be considered natural hosts of OvHV-2. Factors such as breed, UB status, sample type or UB lesion score were found to be not related to OvHV-2 prevalence. Findings from this study indicated that OvHV-2 cannot be considered as a biomarker for the early onset of UB in South African sheep flocks.

Acknowledgements

The authors acknowledge GF Jordaan for guidance and help with the statistical analysis.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Ethical approval for the study was obtained from the Research Ethics Committee: Animal Care and Use of Stellenbosch University (ACU-2022-24709 and ACU-2022-22342.

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