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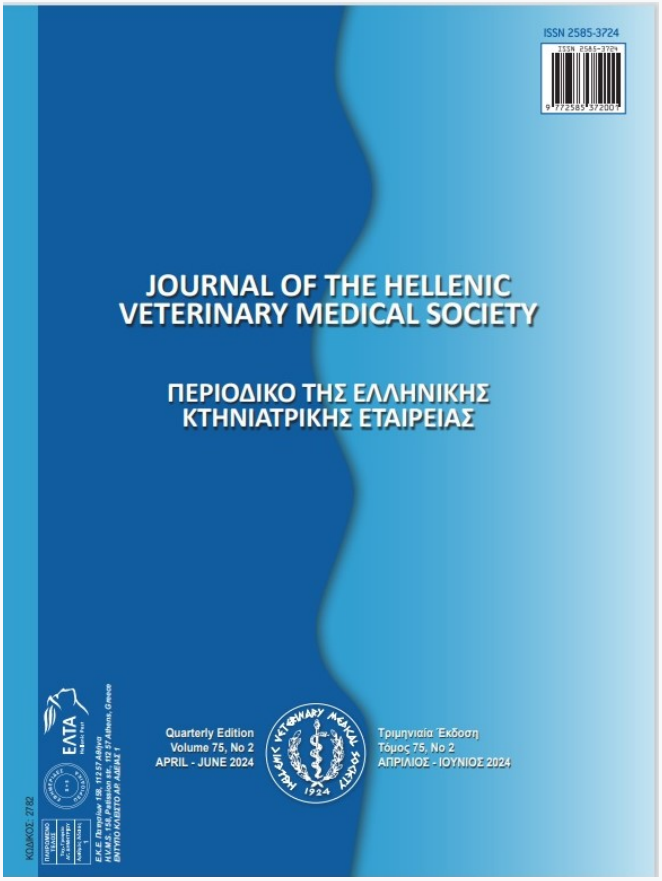

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Research article
Ερευνητικό άρθρο

Cross-sectional survey on bacterial co-infections in Peste des Petits Ruminants virus-infected small ruminants in Enugu State, Nigeria

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ABSTRACT: Small ruminant productivity in Nigeria has been hampered by the Peste des Petits Ruminants Virus (PPRV) and a subsequent bacterial infection. It is vital to understand the most prevalent bacteria that might exacerbate PPRV disease in order to enhance the prognosis and treatment of PPRV patients. The objective of the study was to determine the bacterial co-infections in PPRV infections. Sheep and goats offered for sale at local markets in Nigeria's Enugu state were examined for typical clinical signs of PPR infection and selected for this study. Two hundred and ten ocular, nasal and oral swab samples were collected from 70 sheep and goats. An additional 70 nasal swabs were collected for molecular confirmation by reverse transcription polymerase chain reaction, while the remaining samples were used for bacterial isolation and identification. The detection rate of the PPRV gene was 25.7% (18/70), affecting three sheep (4.3%; 3/70) and 15 goats (21.4%; 15/70). Based on the PPRV-positive animals, 72 bacterial isolates consisting of eight genera and eleven species were obtained. *Staphylococcus aureus* was the most frequently isolated Gram-positive bacterium (31.9%; 23/72), while *Escherichia coli* was the most frequently isolated Gram-negative bacterium (19.44%; 14/72). Staphylococci were the most frequently isolated bacteria from all samples. In this study, it was found that small ruminants infected with PPRV were sold in the study area, providing a potential means of spreading the disease to other animals. In addition, it is highlighted that several bacteria, some of which might be part of the normal flora, might complicate and worsen the clinical presentation of PPRV cases.

Keywords*:* small ruminant; Peste des petits ruminants; bacterial co-infection; reverse transcriptase-polymerase chain reaction; Nigeria

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INTRODUCTION

Mass production of small ruminants is constrained by disease, inadequate nutrition, poor genetic resources of local stocks, marketing, social factors, structural constraints and a lack of highly skilled labor (Yesuf *et al*., 2012). Bacterial respiratory infection can be primary, occurring in healthy individuals or secondary to a variety of diseases that cause immunosuppression (Yesuf *et al.*, 2012). These immunosuppressive disorders could be viral in nature, with the Pest des petits ruminants virus (PPRV) playing an important role. Peste des petits ruminants (PPR) is an acute, highly contagious and devastating disease of small ruminants, a World Organization for Animal Health (OIE) notifiable and economically important transboundary viral disease in sheep and goats, resulting in high morbidity and mortality with devastating economic consequences for livestock farming (Balamurugan *et al.,* 2014; Soltan and Abd-Eldaim, 2014; Kumar *et al*., 2017). Therefore, this disease has devastating effects and significant socioeconomic impact (Torsson *et al*., 2016). The PPRV has a strong affinity for epithelial cells and lymphoid tissue. The epithelial cells of the respiratory tract are damaged by the virus, leading to respiratory and intestinal diseases (Truong *et al*., 2014). It has been suggested that the virus infects immune cells in the mucosa of the respiratory tract and also damages lymphocytes, leading to significant immunosuppression (Sahinduran *et al*., 2012). Secondary bacterial infections occur primarily when the resistance of the airway mucosa is reduced and bacteria growing in the upper airway spread downward (Yesuf *et al*., 2012). PPR, characterized by fever, mouth sores, nasal and ocular discharge, diarrhea, severe gastroenteritis, pneumonia, and death in natural onset, has been complicated by secondary bacterial infections, which have led to an increase in morbidity and mortality rates (Balamurugan *et al* ., 2014; Kumar *et al*., 2014). Some bacteria isolated from sheep and goat pulmonary lungs are *Escherichia coli*, *Klebsiella pneumonia*, *Mannheimia haemolytica*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Pasteurella multocida* (Ugochukwu *et al.,* 2017). Therefore, it is important to isolate and identify the different bacteria involved in order to allow better treatment of PPR cases and thus reduce mortality. This can help reduce the high morbidity and mortality in natural cases.

MATERIALS AND METHODS

Sampling site and inclusion criteria

Between March and June 2018, sheep and goats offered for sale in local markets (Orba and Obollo afor) in Udenu local Government area of Enugu State, Nigeria, were targeted with the consent of the owners. Prior to sampling, the animals were examined for typical clinical manifestations of PPR infection, e.g. mucopurulent nasal discharge, eye discharge, mouth sores, coughing, sneezing, frothy salivation, stiff coat, oral crusts, diarrhea, shortness of breath, stomatitis, dehydration, lethargy (Pope *et al*., 2013; Kumar *et al*., 2014). These small ruminants were raised by local small farmers and small ruminant traders and they received no form of treatment nor vaccination. The predominant breeds in goats are Sokoto Red and West African Dwarf, while sheep are West African Dwarf.

Sample collection and transportation

Nasal (collected in pairs), oral and ocular swabs were collected from 70 small ruminants (sheep and goats) clinically diagnosed with PPR, using sterile swabs after proper restraint of the animals. A total of 210 collected samples (nose, mouth and eye swabs) were immediately taken to the Veterinary Medicine Laboratory, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, for further microbiological processing and analysis, while 70 samples (the second pair of nasal swabs) were properly packed in dry ice and sent to the Biotechnology Centre, National Veterinary Research Institute, Vom, Jos Plateau State, Nigeria for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Reverse transcription polymerase chain reaction (RT-PCR)

Sterile phosphate buffered saline (500 µL) was added to each tube containing the nasal swab sample. This was centrifuged at 10,000xg for 3-5 minutes at 4°C. The swab extract was transferred to a sterile tube. Viral RNA was extracted from 140 µL swab extract using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and RT-PCR was performed using the Qiagen OneStep Ahead RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Amplification was performed under the following thermal conditions: 50°C for 10 minutes to activate reverse transcriptases, 95°C for 5 minutes to activate DNA polymerases, followed by 40 cycles of 95°C for 10 seconds, 55°C for 10 sec, 72 °C for 10 sec sec and a final extension at 72 °C for 2 min. Each amplicon (5 µl) was analysed by electrophoresis, examined and photographed under ultraviolet light using a gel documentation system (BioRAd Gel DocTMxRT model no. Universal HoodII, BioRad, USA) as described by

Chukwudi *et al*. (2020a). PPR positive samples were observed to have bands consistent with the positive control sample (vaccine strain Nig 75/1)

Bacterial isolation and identification

The collected samples were inoculated into various microbiological media such as Nutrient Agar, Blood Agar, MacConkey Agar, Salmonella Shigella Agar (SSA), Eosin Methylene Blue Agar (EMB), Chocolate Agar and Blood Agar, etc. These were incubated at 37°C for 24 hours aerobically. Each sample was properly identified in the different media. Each of the inoculated petri dish plates was thoroughly examined and Gram stained according to the procedures described by Smith and Hussey (2005). Enzymatic and biochemical tests such as catalase test (Reiner, 2010), coagulase test (Becker *et al*., 2014), methyl red test and Voges-Proskauer test (McDevitt, 2009), hemolysis test (Snavely and Brahier, 1960), oxidase test (Isenberg, 2004), indole production test (Oyeleke and Manga, 2008) and urease production test (Beishir, 1991) were performed to subdivide isolated organisms into species.

Statistical analysis

Data were entered into the Statistical Package for Social Sciences (SPSS) version 10. Descriptive data were analyzed.

RESULTS

Detection of PPR viral genome in nasal swabs of sheep and goat using RT-PCR technique

Of the 70 small ruminants clinically diagnosed with PPR, only 18 (25.7%) were positive given the expected band size of 351 bp on the agarose gel (Fig. 1). These were three (4.3%; 3/70) sheep (all West African dwarf breeds) and 15 (21.4%; 15/70) goats (four West African dwarf and 11 Red Sokoto breeds).

Bacterial isolation and identification

The 54 swab samples (18 oral, 18 nasal and18 ocular swabs) collected from the 18 PPR-infected sheep and goats yielded 72 bacterial isolates. Eight genera and eleven bacterial species were isolated (Table 1; Fig 2). Among the Gram-positive bacteria, *Staphylococcus aureus* had the highest isolation rate (23/72, 31.9%) while *Streptococcus pyogenes* had the lowest

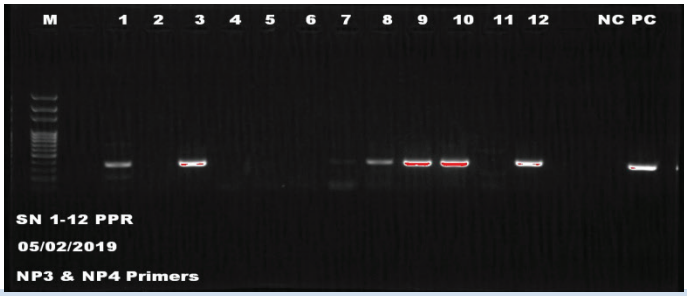
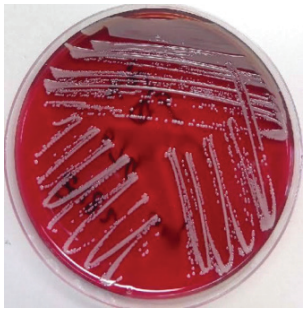


Fig. 1 Gel image of the amplified product prepared from representative samples analyzed by RT-PCR. Lane M = 100 bp DNA molecular weight markers, lanes 1-12 are the test RT-PCR products. Lanes CC and PC are negative and positive controls, respectively

Table 1: Distribution of the different isolates from discharges from PPR-infected sheep and goats

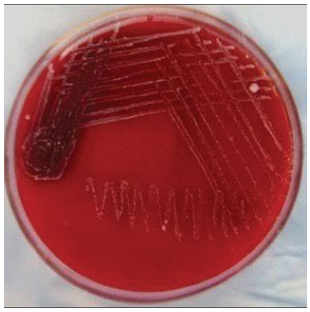
Bacterial isolates	Isolation freq. (%)	*Isolation rate by sample (n=54)
Gram-positive		
Staphylococcus aureus	23 (31.94)	42.59
Staphylococcus epidermidis	8 (11.11)	14.81
Streptococcus viridens	7 (9.72)	12.96
Bacillus spp	2 (2.78)	3.70
Streptococcus pyogenes	1 (1.39)	1.85
Gram-negative		
Escherichia coli	14 (19.44)	25.93
Klebsiella pneumoniae	7 (9.72)	12.96
Proteus mirabilis	4 (5.56)	7.40
Pseudomonas aeruginosa	3 (4.17)	5.56
Pasturellamultocida	2 (2.78)	3.70
Klebsiella oxytoca	1 (1.39)	1.85

Key: *Percentage frequency in terms of the total number of samples.



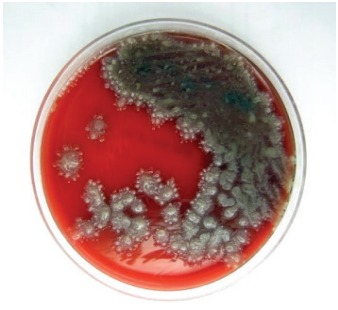
a.*Staphylococcus aureus*

b. *Staphylococcus epidermidis*

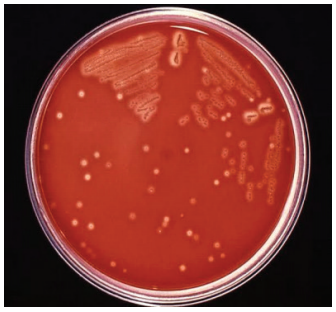


c.*Staphylococcus viridans*

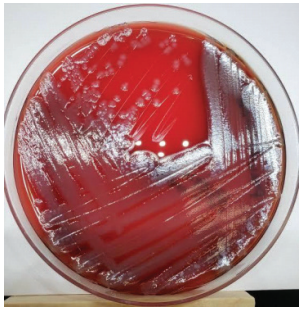
d.*Pastuerella mutocida*



e. Bacillus specie



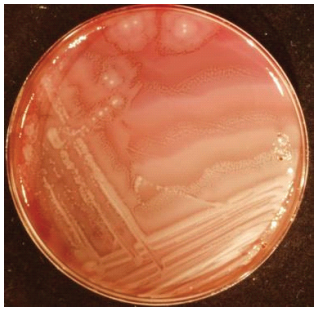
f. *Streptococcus pyogenes*



g. *Pseudomonas aeruginosa*

h. Klebsiella oxytoca

i.*Klebsiella pneumonia*



j. *Proteus mirabilis*

K.*Escherichia coli*

Figure 2. A-K. Pictorial representation of isolated bacteria

(1/72, 1.39%). Among the isolated Gram-negative bacteria, *Escherichia coli* was the most frequently isolated bacterial species (14/72, 19.44%) while *Klebsiella oxytoca* was the least frequent (1/72, 1.39%).

The isolation rates by bacterial species and by sample follow the same pattern (Table 1). However, the result showed that no bacteria were isolated from some samples while others contained at most three types of bacteria (Table 2). The probability distribution showed that 20.37% (11/72 samples) of the samples collected had no bacterial infestation, while only 5.55% (3/72 samples) had involvement of three bacterial species (Table 2).

The bacteria most frequently isolated from oral and nasal and ocular swabs were *Staphylococcus* (Table 3, Fig. 3) while *Bacillus* was only isolated from

nasal and ocular swabs.

Based on the frequency of isolates, nasal swabs yielded the highest isolation rate for *Staphylococcus* (14/31, 45.2%) and Escherichia (9/14, 64.3%), oral swabs yielded the highest isolation rate for *Proteus* (3/4, 75%) and *Pseudomonas* (3/3, 100.0%). However, Oral and nasal swabs showed the same isolation rate for *Klebsiella* and *Pasteurella*.

DISCUSSION

Respiratory, digestive, and lymphatic diseases caused by PPR virus (PPRV) were well described (Truong *et al.,* 2014; Parida *et al.,* 2015). This virus is known to be endemic in Nigeria and many parts of Africa. The resulting economic loss typically results from moderate to high morbidity and mortality, particularly in unvaccinated small ruminants (Bardhan *et al*., 2017). In this survey, we observed a moderately

Table 2: Distribution of bacteria (genus) isolated from oral, nasal and ocular swabs from PPR-positive small ruminant

Bacterial isolate (genus)	Oral swab (%)	Nasal swab (%)	Ocular swab (%)
Staphyococcus	9 (34.6)	14 (43.8)	8 (57.1)
Escherichia	5 (19.2)	9 (28.1)	0 (0.0)
Klebsiella	4 (15.4)	4 (12.5)	0 (0.0)
Proteus	3 (11.5)	1 (3.1)	0 (0.0)
Pseudomonas	3 (11.5)	0 (0.0)	0 (0.0)
Streptococcus	1 (3.8)	2 (6.3)	5 (35.7)
Pasteurella	1 (3.8)	1(3.1)	0 (0.0)
Bacillus	0 (0.0)	1 (3.1)	1 (7.1)
Total	**26**	**32**	**14**

Table 3: Showing the relative distribution of the number of organisms isolated per swab

Number of bacterial isolated	No. of swab	Bacterial isolation rate from swab (%)
0	11	0
1	17	31.48
2	23	42.6
3	3	5.55
Total	**54**	**100**

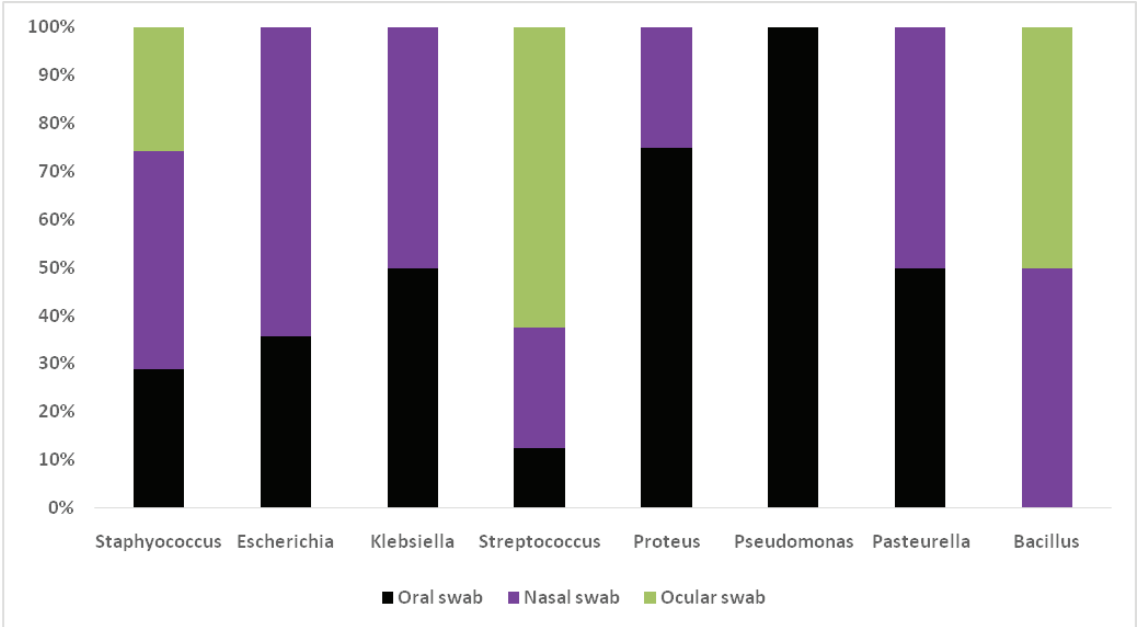


Figure 3: Distribution of bacterial isolates (genus) across swab regions of PPR-infected small ruminants

high PPR infection rate of 25.7% (18/70), which was less than 51.5% reported in North-Central, Nigeria (Luka *et al.,* 2011) and 33.3% in Bangladesh (Nabi *et al.,* 2018). This could be due to variation in sampling time, location, sampling method and management practice (Devi *et al*., 2016). Peste des petits ruminant disease is commonly observed in Nigeria during the harmattan period, which is characterized by dry, cold and dusty weather (Ezeibe *et al*., 2008). In addition, since the PPRV gene has been detected in nasal swabs, this suggests that the virus enters the environment and may be transmitted to other susceptible ruminants that are in close contact. While this study highlights that PPRV-infected ruminants are brought to market for sale, which is an unhealthy practice, it showed that animals purchased at the market and those not sold on the day (since it is a weekly market) are likely to be at high risk of exposure and could eventually develop PPRV if not vaccinated against the virus (Woma *et al*., 2016; Chukwudi *et al.,* 2020b). Transmission of PPRV is commonly via aerosol and faeco-oral routes (Herzog *et al.,* 2020), therefore unvaccinated ruminants exposed to contaminated aerosol or grass/forage are likely to become infected. In addition, due to this active shedding of PPRV (infection rate 27.7%), one could speak of a contamination of the market with the virus.

Secondary bacterial infections often complicate viral diseases and often complicate treatment. Furthermore, the normal bacterial flora can take on a pathogenic role (opportunistic pathogens) if their population is not regulated, as has been observed in healthy animals (Wu *et al*., 2021). A virus-infected animal tends to induce immunosuppression, especially when a virus such as PPRV attacks the immune system. PRRV induces severe lymphocytosis in tonsils, Peyers patches, spleen and lymph nodes, particularly mediastinal and mesenteric lymph nodes (Madboli and Ali, 2012). This PPRV immunosuppression causes metabolic disorders and eventually secondary bacterial infection. However, the isolation of bacteria from PPRV-positive ruminants could not be categorically labelled to as secondary bacteria, as this could also be a co-infection. Several co-infections between viruses and bacteria have been reported in ruminants (Stonos *et al.,* 2017). This study had 72 bacterial isolates from 18 PPRV-positive small ruminants (54 swabs). Although *Staphylococcus aureus* and *Staphylococcus epidermidis* are part of the normal flora, most bacterial isolates from the oral, nasal and ocular cavities that can complicate PPR cases are observed in this study. It has been reported that Staphylococcus species also complicate disease in ruminants or are a major cause of diseases such as mastitis, leading to the formation of pus or giving way to other pathogenic bacteria (Bergonier *et al.,* 2014; CABI, 2022). The isolation of *Streptococcus viridens* and *Streptococcus pyogenes* in PPR diseases with the consequent formation of mucous discharge from the eye and nostrils has also been described (Chakraborty *et al.,* 2014). In this study, the Streptococcus species was the second most common bacterium isolated from mucopurulent ocular discharge from PPRV-positive small ruminants. However, it is not surprising to isolate Bacillus species as they are easily airborne. This survey is limited because the species of Bacillus involved were not determined, although their isolation rate was among the lowest bacterial cultures in the samples collected. *Escherichia coli* were the most abundant Gram-negative bacteria and second-highest bacteria isolated from PPRV-positive small ruminants in this study. While several *E. coli* strains have been isolated from the gastrointestinal tract, some pathogenic and some nonpathogenic (Croxen *et al.,* 2013), their occurrence in nasal cavity swabs is uncommon. However, these *E. coli* isolates from the nasal cavity could have originated from the oral cavity when licking nasal mucus with the tongue, a habit often observed in ruminants. Of course, *Klebsiella pneumonia* and *Pasteurella multocida* have been implicated as the main causes of respiratory infections. The clinical presentation of PPRV-infected ruminants becomes more complex when these bacterial species are superimposed, and these bacteria may still be present even when the virus is eliminated (Hanada *et al*., 2018). The lack of an antibiogram for these isolates limits our discussion of their antibiotic resistance pattern. However, *Klebsiella pneumonia*, *Pasteurella multocida* and *Pseudomonas aeruginosa* have been reported to be resistant to several antibiotics commonly used in ruminant medicine (Eliasi *et al.,* 2020; Awandkar *et al.,* 2022). In addition, we observed that the isolation rate of the bacterial species differed in the caves studied. The reason for this cannot be easily elucidated, but the presence of bacterial species could depend on the characteristics of the bacteria, the animal's immune response and environmental factors, including the presence of other pathogens. In this survey, bacteria were isolated from PPRV-positive ruminants, suggesting a high risk of secondary bacteria and/or co-infection, which can complicate the pathogenesis of the disease owing to the immunosuppressive effect of the virus, and lead

to poor prognosis, high mortality and associated financial losses. Thus, earlier incorporation of antibiotic therapy in the management of PPRV infection will help limit financial losses resulting from death of animal.

In conclusion, Gram-positive and Gram-negative bacteria were isolated from PPRV-infected small ruminants, with Staphylococcus species and E. coli being the most frequently isolated organisms. The research consequently expands the clinical horizon of ruminant clinicians regarding the importance of using broad-spectrum antibiotics in the management of PPRV infection so as to reduce mortality that may occur due to the accompanying bacterial infection.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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