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Full Research Article

ISOLATION AND MOLECULAR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS IN AN OUTBREAK INVESTIGATION IN BENISHANGUL GUMUZ, ETHIOPIA

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Abstract

Peste des petits ruminants (PPR) is a severe and highly communicable disease. It is viral disease that particularly affects shoats. Its *domino* effects are economic losses through loss of production, deaths, abortions, and the cost of disease control. An outbreak investigation was conducted from May to November 2022 in Bambase district of Benishangul Gumuz regional state to determine the presence of PPR virus, and morbidity, mortality and case fatality rates. Altogether, 180 clinically diagnosed and 32 shoats with typical clinical sign of PPR were identified for laboratory confirmation Nasal and rectal swabs from 32 sheep and goats were collected and cultured on Verodog SLAM (VDS) cell line and tested using then tested using a quantitative real-time polymerase chain reaction (RT_PCR) assay and conventional PCR with 1.5% gel electrophoresis for detection of the N gene. The overall morbidity, mortality and case fatality rate was 17.77%, 2.77% and 15.62% respectively. Meanwhile, goats had a higher morbidity (26.5%), mortality (4.81%) and case fatality rate (18.18%) than sheep (10.3%, 1.03% and 10% respectively). Half (50%; 16/32) of samples were successfully cultivated on Vero dog SLAM cell line, showing cytopathic effects of PPR virus such as rounding cells, foamy vacuolation, aggregation and syncia formation. Out of 32

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animals tested, 17 (53.13%) showed positive results for PPR virus by real-time PCR. Further analysis with conventional PCR revealed the presence of the N gene in all 17 positive samples with a 351bp fragment size. The results showed a higher morbidity rate in caprines than in ovines. Based on these findings, it is recommended to carry out a further phylogenetic study to identify the strain of virus circulating in the study area.

Keywords: cytopathic effect, gel electrophoresis, goats, N gene, sheep

INTRODUCTION

Peste des petitis ruminants (PPR), also known as sheep and goat plague, is an extremely infectious viral disease that gravely affects sheep and goat populations in Ethiopia. This East African nation has a substantial number of these small ruminant animals, with 31.2 million sheep and 29.8 million goats. The sheep and goat sectors play a vital role in Ethiopia's economy, contributing 25% of the country's mutton production, 50% of its wool output, 40% of its skin production, as well as a staggering 92% of its hide and skin exports. Sheep meat production is projected to reach 56,560 tons by 2021, while goat meat production is expected to reach 28,650 tons. Small ruminants are crucial for food security and livelihood resilience both nationally and internationally, making PPR a serious issue (Waret et al., 2008; CSA, 2016/17).

PPR is a significant threat to small ruminants worldwide (Torsson et al., 2017; Hailegebreal, 2018). Caused by the Morbillivirus genus belonging to the Paramyxoviridae family, it is an acute, highly contagious, and frequently fatal viral disease (Zakian et al., 2016). Clinical signs include fever, mucopurulent nasal and ocular discharges, necrotizing and erosive stomatitis, and severe enteritis often leading to death (Gari et al., 2017). Classified as a transboundary animal disease with substantial economic ramifications, PPR ranks among the top ten diseases impacting small ruminants. It spreads mainly through direct contact with infected animals' secretions, resulting in morbidity and mortality rates reaching up to 100% in naive herds. Death typically occurs within 5-10 days of disease onset, although some animals may acquire lifelong immunity post-recovery (Burns et al., 2019).

Ethiopia's initial encounter with PPR is believed to have occurred in 1977, when goat herds in the Afar region exhibited clinical signs suggestive of the disease. However, the causative virus was not confirmed until 1994, with phylogenetic analysis classifying it as lineage III (Roeder et al., 1994). More recently, the country has witnessed the emergence of lineage IV, which has continued to spread among its small ruminant population (Muniraju et al., 2014). This development has spurred additional research efforts focused on molecular characterization and phylogenetic analysis of the circulating PPR virus strains in Ethiopia (Alemu et al., 2019; Rome et al., 2019).

A recent report by Yalew et al. (2019) found that the seroprevalence of PPR in Asossa Zone was 75.7%, leading regional animal health agencies to implement strategies to reduce the disease's prevalence. However, outbreaks still occur in different seasons, and the market connection to North Sudan may facilitate further transboundary

animal disease spread. To ensure proper prevention and control measures for PPR, further molecular-level studies and outbreak investigations are necessary.

Specific Objectives

√ To conduct outbreak surveillance for the presence of PPR virus (PPRV) among small ruminant populations in Bambasi district.

√ To estimate and quantify the PPR disease morbidity rate, mortality rate, and case fatality rate in Bambasi district

MATERIALS AND METHODS

Ethical Clearance Approval

This study design and method has been evaluated and endorsed by the Ethical Committee of Wallagga University in compliance with relevant regulations, humane animal care standards, and the research protocol, as evidenced by the letter of certification labeled with reference number WUSVMRE021/21.

Study Area Description

The study was conducted in Bambasi district, located within the Benishangul Gumuz regional state in western Ethiopia. Geographically, Bambasi district lies at 9°45'N latitude and 34°45'E longitude, with an altitudinal range of 1100-1450 meters above sea level. The area experiences a subhumid climatic condition characterized by slightly warmer temperatures, minimal daily temperature fluctuations, and a reliable high annual rainfall ranging from 1350-1400 mm. The long dry season spans from December to May, during which the average monthly maximum temperatures vary between 29–32 °C. August is the coolest month, with an average minimum temperature of 21 °C. Figure 1 illustrates the specific area within Bambasi district where the PPR outbreak occurred.

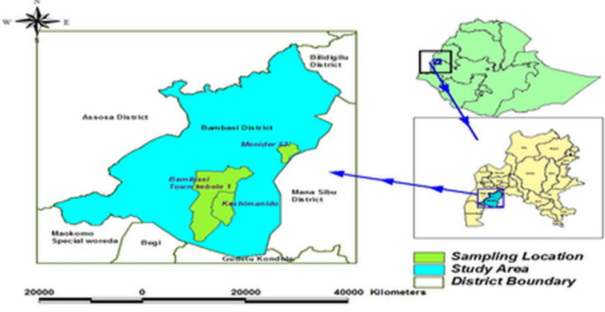

Figure 1: Map showing the study area

Study Animals

The study included both caprine (goat) and ovine (sheep) species, encompassing animals of all ages and sexes. These animals were maintained in an extensive management system, which allowed them to roam freely over large areas. However, herds of sheep and goats that had been vaccinated against PPR or appeared healthy were excluded from the study. Instead, the study focused on herds that had at least one animal exhibiting clinical symptoms consistent with PPR disease, which was a prerequisite for inclusion in the study.

Study Design

The cross-sectional investigation of the PPR outbreak was conducted from May to November 2022.

Sampling Technique

A purposive sampling technique was used in this study, based on clinical diagnosis for typical signs of PPRV disease. Animals exhibiting suspected clinical signs, such as abdominal breathing, depression, anorexia, dry muzzle with erosive or necrotic lesions, and serous oculonasal-to-mucopurulent discharge, were closely monitored. The investigation encompassed a total of 10 herds, comprising 7 sheep herds (n=97) and 3 goat herds (n=83). Among these 10 herds, only 2 sheep herds and 1 goat herd were suspected of experiencing a PPR outbreak, based on the clinical signs observed in the animals. Following the guidelines set forth by the World Organisation for Animal Health (OIE) in its 2022 manual, 32 small ruminants (22 goats and 10 sheep) exhibiting typical clinical signs of PPR disease were selected from these suspected herds for in-depth laboratory confirmation and detection of the PPR virus.

Sample Collection and Transportation

Specimen collection for virus isolation was conducted during the acute phase of the disease when clinical signs were apparent. To ensure the reliability of the PPR samples, specimen collection and transportation were performed according to the SOP Manual (OIE, 2022). These protocols enable both isolated viral detection and molecular detection through clinical evaluation in goats and sheep during early outbreaks.

Nasal and rectal mucosa debris swab samples were collected from small ruminants with clinical signs suggestive of PPR. The samples were collected in cryovials containing 2 milliliters of virus transport medium (VTM) with a pH range of 7.2-7.6. The VTM was formulated with phosphate-buffered saline and included antibiotics (benzylpenicillin) and antifungal agents (ofloxacin hydrochloride), as well as phenol red for identification. Each tube was labeled with information such as animal type, identification number, sample site, purpose, date, and additional notes such as age, sex, vaccination record, eco-zones explored by the animal, and body condition status. The samples were stored

in an icebox and transported to Bambasi District Veterinary Clinic for preservation until transfer to the Animal Health Institute for further analysis.

Virus Isolation on Cell Culture

Nasal swab samples in virus transport media underwent freeze-thaw cycles before homogenization and centrifugation at 3000 rpm for 20 minutes at 4°C. The supernatant was inoculated onto Vero Dog SLAM (VDS) cells for virus isolation, incubated at 37°C with 5% CO2, washed, and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 2% serum. Cytopathic effects indicating virus replication were monitored for seven days, with three passage attempts to confirm the absence of PPR virus (Gomes et al., 2016).

MOLECULAR DETECTION TECHNIQUE

RNA Extraction

Viral RNA was extracted from swabs using a commercial kit (Qiagen) following the manufacturer's instructions and stored at – 80 °C until RT-PCR and conventional PCR analysis (OIE, 2022)

Real-Time (RT) PCR

The real-time RT-qPCR assay was conducted on all extracted viral RNA samples using an Applied Biosystems 7500 thermal cycler. Specific primers and a probe targeting the N gene were employed, and samples with a cycle threshold (Ct) value < 35 were considered positive as described by Batten et al., (2011).

Conventional RT-PCR

Conventional RT-PCR was performed using a standard method described in the OIE Terrestrial Manual (2022), employing the primer pairs NP3 and NP4 (Couacy-Hymann et al., 2002). PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel (Couacy-Hymann et al., 2002).

Data Management and Analysis

The data collected and organized using an Excel spreadsheet. STATA Version 12 were used for statistical analysis. Descriptive statistics were generated to summarize the data. Additionally, key epidemiological parameters, including proportion of animals affected by disease, proportion of animals that died due to the disease, and proportion of infected animals that succumbed to the disease, were calculated. Regarding the laboratory test results, the number of positive samples detected by each diagnostic assay was expressed as a percentage. This allowed for a comparative evaluation of the performance of the different tests in detecting positive cases within the sample set.

RESULTS

An investigation into a PPR outbreak revealed that clinical signs included nasal discharge, fever, erosive lesions on the muzzle and lips, depression, diarrhea, and coughing, observed in 32 out of 180 small ruminants (shoats) (Figure 2).

Figure 2: Clinical signs of Peste des Petits Ruminants (PPR) in affected sheep and goats

The overall morbidity, mortality, and case fatality rates were 17.77%, 2.77%, and 15.62%, respectively. Goats exhibited higher morbidity (26.5%), mortality (4.81%), and case fatality rates (18.18%) compared to sheep (10.3%, 1.03%, and 10%, respectively). Additionally, adult animals were more susceptible than young ones, and males were more susceptible than females in both species (Table 1).

Table 1. Morbidity, mortality and case fatality rate of PPRV.

Factor	Group	No. at Risk	No. Infected	No. of Dead	Morbidity Rate	Mortality Rate	Case fatality rate
Species	Sheep	97	10	1	10.3%	1.03%	10%
	Goat	83	22	4	26.5%	4.81%	18.18%
Age	Young	90	6	1	6.6%	1.1%	16.66%
	Adult	90	26	4	28.8%	4.4%	15.38%
Sex	Male	69	19	3	27.5%	4.3%	15.78%
	Female	111	13	2	11.7%	1.8%	15.38%
Sheep	Young	43	1	-	2.3%	-	-
	Adult	54	9	1	16.7%	1.9%	11.1%
	Male	36	5	-	13.9%	-	-
	Female	61	5	1	8.1%	1.63%	20%
Goat	Young	47	5	1	10.6%	2.12%	20%
	Adult	36	17	3	47.2%	8.3%	17.64%
	Male	33	14	3	42.4%	9.09%	21.42%
	Female	50	8	1	16%	2%	12.5%
Total		180	32	5	17.77%	2.77%	15.62%

Thirty-two samples (28 nasal swabs and 4 rectal swabs) were inoculated into VDS cells for virus isolation and propagation. The cultivation results showed that 53.12% (17/32) of the samples induced cytopathic effects (CPE) in both the first and second passage inoculations, exhibiting characteristic CPE such as cell rounding, foamy vacuolation, aggregation, and syncytia formation. The remaining samples were blind-passaged until the third passage, and if no CPE was observed, they were declared negative for virus isolation. Subsequently, all 32 samples were subjected to molecular testing using real-time RT-PCR and conventional PCR. Both conventional PCR and real-time PCR results confirmed that 53.13% (17/32) of the clinical cases were positive for PPRV.

Out of 17 positive samples, 8 originated from sheep, while 9 came from goats, and all were positive cases. The quantitative RT-PCR analysis revealed cycle threshold (Ct) values ranging from 16.23 to 30.24, with a Ct value of less than 35 being the cutoff for a positive result (Figure 3). The real-time RT-PCR data exhibited an increasing trend for samples with Ct values between 16.23 and 30.24, indicating positive results relative to the positive control. In contrast, samples with Ct values greater than 35 did not show any amplification curve and were considered negative when compared to the negative control.

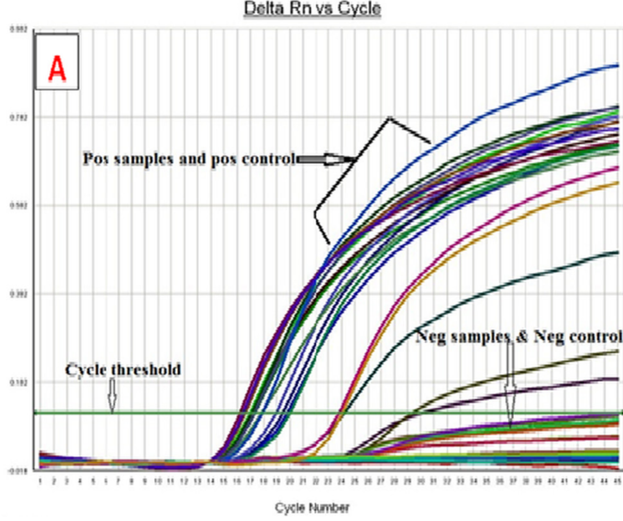

Figure 3: Results of the quantitative RT-PCR analysis for the PPR virus

The conventional PCR method tested 28 nasal swab and 4 rectal swab samples for the presence of the PPR virus. All qRT-PCR positive samples, representing 53.13% (n=17) of the total, also tested positive using conventional PCR. Gel electrophoresis revealed an amplified fragment size of approximately 351 bp, similar to that of a positive control sample (Figure 4). The figure displays the amplified fragment of the N gene of PPRV for the positive control, indicated by the arrowhead. This band lies

between 300-400 base pairs (bp) of the molecular ladder. In contrast, the negative control lane does not exhibit any visible band. Samples that produced bands of similar size to the positive control, approximately 351 bp, were considered positive for the presence of PPRV.

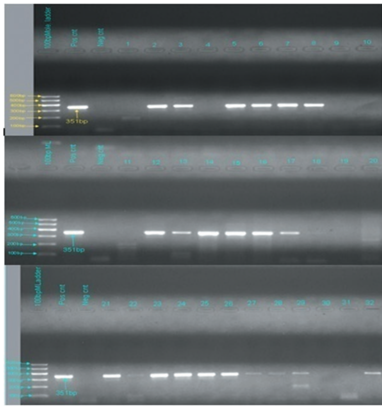

Figure 4: Amplified fragment of the PPR virus from conventional PCR, with gel electrophoresis showing a size of approximately 351 bp

DISCUSSION

PPRV has been widely reported to circulate in various regions of Ethiopia. It results in significant production losses and mortality among small ruminant populations (Roeder et al., 1994; Waret-Szkuta et al., 2008). PPRV is a highly contagious viral disease affecting sheep and goats, characterized by fever, respiratory distress, diarrhea, and potentially fatal outcomes. The disease can have a substantial impact on the livelihoods and food security of farmers and pastoralists who depend on small ruminants for income generation.

The clinical manifestations of PPR observed in the studied area were consistent with those described in previous studies by Kgotlele et al. (2014), Kardjadj et al. (2015), Rahman et al. (2016), and Alemu et al. (2019). These studies have documented common clinical signs associated with PPR, including fever, respiratory distress, diarrhea, and erosive lesions on the muzzle and lips. Furthermore, Rajak et al. (2005) suggested that PPRV may cause more severe immunosuppression in infected goats compared to sheep, possibly due to its ability to inhibit leukocyte proliferation. This could potentially explain the higher susceptibility and severity of clinical manifestations observed in goats compared to sheep in the current study.

In this study of 180 sheep and goats, the morbidity, mortality, and case fatality rates were found to be 17.77%, 2.77%, and 15.625%, respectively. These values were higher for males compared to females, which is consistent with the findings reported by Kardjadj et al. (2015), who found morbidity, mortality, and case fatality rates of 12.2%,

2.5%, and 20.3%, respectively. Another study by Muthuchelvan et al. (2014) reported similar rates of 16.67%, 2.7%, and 16.67% for morbidity, mortality, and case fatality, respectively. These findings suggest that PPRV infection can have a significant impact on small ruminant populations, with males potentially being more severely affected than females.

This study used both conventional and real-time RT-PCR methods to detect PPRV and found that 53.12% (17/32) of samples tested positive for the virus. All 17 positive samples were nasal swabs, while all four rectal swabs tested negative. This result is in line with previous research by Parida et al. (2019), which reported that PPRV is detected later in fecal material than in other body fluids post-infection. This suggests that nasal swabs may be a more reliable method for early detection of PPRV infection, as the virus is more likely to be present in nasal secretions than in fecal material during the early stages of infection. Further research is needed to confirm these findings and to better understand the dynamics of PPRV infection and detection in different body fluids. Nonetheless, this study provides valuable insights into the potential usefulness The current study's finding of a 53.12% positivity rate for PPRV using molecular detection methods from nasal swabs is comparable to results reported by several other studies. Chauhan et al. (2014), Alemu et al. (2019), and Sait and Dagalp (2019) reported positivity rates of 45.8%, 46.4%, and 48%, respectively, while Kwiatek et al. (2011) reported a slightly lower rate of 44.4% in Morocco using similar analyses. However, lower rates have also been reported, such as 25% (8/32) by De Nardi et al. (2012) and 33.3% (7/21) by Anees et al. (2013). These variations in positivity rates may be attributed to factors such as the stage of infection or the type of gene targeted with RT-PCR, as suggested by Luka et al. (2012). Further research is warranted to better understand the factors influencing the detection of PPRV using molecular methods and to enhance the accuracy and reliability of these techniques for diagnosing PPRV infection.

The 351 bp fragment size obtained in this study is consistent with previous studies conducted by Kardjadj et al. (2015) and Rahman et al. (2016), who reported fragment sizes of 351 and 352 bp, respectively, when targeting the N-gene region of PPRV. As evident from the gel image, the N gene product was clearly visible in the positive control lane with a band size between 300-400 bp of the molecular ladder, while no amplification product was observed in the negative control lane. These results confirm the presence of PPRV in the positive samples and validate the reliability of the PCR method for detecting the virus.

The present study found that 53.13% (17/32) of clinical samples tested positive for PPRV isolation on Vero cells. This percentage is higher than the 9.3% (4/43) reported by Zahur et al. (2014) but lower than the 70% reported by Elsheikh et al. (2019). Such discrepancies may arise from variations in sample size, type, or storage and transportation conditions. Nonetheless, the results of this study underscore the need for further investigation in the context of disease control, and molecular diagnosis should be conducted to explore the possibility of a PPR outbreak, as we have done

in Bambasi district. Characteristic CPE observed in cultured samples, including foamy formation, rounding, vacuolation, and cell aggregation/fusion to form syncytia, are outlined by the OIE (2022) as typical features of PPR virus.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the disease outbreak in Bambasi district's sheep and goats was caused by PPRV. Proper diagnosis, awareness among farmers, and better support systems are important to prevent future outbreaks. The recommendation is to investigate the epidemiology and genetic profile of PPRV, research its impact, conduct economic analysis, and improve vaccination coverage and strategies for newly introduced small ruminants.

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Authors' contributions

AM collect data and prepared the initial draft of the manuscript. AA and DS conducted molecular and virological investigation. LY and EH reviewed and contributed to sections and improvement of the manuscript. EH finalized the manuscript for submission.

Competing interests

The authors declare that they have no competing interests.

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IZOLACIJA I MOLEKULARNA DETEKCIJA VIRUSA KUGE MALIH PREŽIVARA U ISTRAŽIVANJU EPIDEMIJE U BENISHANGUL GUMUZU, ETIOPIJA

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Kratak sadržaj

Kuga malih preživara (PPR) je teška i veoma zarazna bolest. To je virusno oboljenje za koje su posebno prijemčive koze i ovce. Domino posledice ove bolesti uključuju ekonomske gubitke izazvanih smanjenjem proizvodnih rezultata, uginućima životinja, pobačajima, kao i troškovima kontrole bolesti. Istraživanje epizootije sprovedeno je od maja do novembra 2022. godine u okrugu Bambase, u regionu Benishangul Gumuz, kako bi se utvrdilo prisustvo virusa PPR, kao i stope morbiditeta, mortaliteta i letaliteta. Ukupno, 180 klinički dijagnostikovanih i 32 životinje sa tipičnim kliničkim znakovima PPR-a identifikovano je za laboratorijsku potvrdu. Uzorkovani su brisevi nosa i rektuma od 32 ovce i koze, koji su potom kultivisani na Vero dog SLAM (VDS) ćelijskoj liniji i testirani kvantitativnom PCR metodom u realnom vremenu (RT_PCR) i konvencionalnom PCR metodom uz elektroforezu na 1.5% gelu u cilju detekcije N gena. Ukupne stope morbiditeta, mortaliteta i letaliteta iznosile su, redom, 17.77%, 2.77% i 15.62%. Koze su imale višu stopu morbiditeta (26.5%), mortaliteta (4.81%) i letaliteta (18.18%) u poređenju sa ovcama (10.3%, 1.03% i 10%). Polovina (50%; 16/32) uzoraka uspešno je kultivisana na Vero dog SLAM ćelijskoj liniji, pri čemu su uočeni citopatski efekti virusa PPR kao što su zaobljene ćelije, vakuole sa penastim sadržajem, agregacija i formiranje sincicija. Od ukupno 32 testirane životinje, njih 17 (53.13%) je pokazalo pozitivne rezultate na virus PPR upotrebom PCR metode u realnom vremenu. Dalja analiza konvencionalnom PCR metodom otkrila je prisustvo N gena u svih 17 pozitivnih uzoraka sa fragmentom veličine 351bp. Rezultati su pokazali veću stopu morbiditeta kod koza nego kod ovaca. Na osnovu ovih nalaza, preporučuje se sprovođenje daljih filogenetskih studija kako bi se identifikovao soj virusa koji cirkuliše u istraživanom području.

Ključne reči: citopatogeni efekat, gel elektroforeza, koza, N gen, ovca