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Multi-sampling technique in the diagnosis of *peste des petits ruminants* virus in goats using reverse transcriptase polymerase chain reaction

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Abstract

Peste des petits ruminants (PPR) remains a threat to goat production in Nigeria. The study was designed to evaluate diagnostic efficiencies using different sampling techniques in detecting PPR virus with RT-PCR. A total of 29 samples comprising nasal swabs (NS), ocular swabs (OS), and rectal swabs (RS) were obtained from 13 goats, from suspected outbreaks in three Local Government Areas (LGA), namely Igabi, Sabon Gari and Giwa LGAs, in Kaduna state. More than one sample type (from different routes) here referred to as the multi-sampling technique was obtained per goat in Igabi and Sabon Gari, while a single sample type was obtained per goat in Giwa. The overall detection rate was 31.9%. Only 2 (33.3%) instances existed where only one sample type was positive; whereas on 6 (31.9%) occasions, at least one sample type from the same goat was found positive. The detection rate was 50%, 44.4% and 30% for NS, OS and RS respectively. PPR was not detected in Giwa. In conclusion, using RT-PCR targeting PPRV nucleoprotein gene, NS appeared to have a higher positive detection rate, while RS was the least reliable. Utilizing more than one sample type or multi-sampling technique from different routes per goat enhanced the detection rate of PPRV using RT-PCR.

Keywords: Detection, Goats, Multi-sampling, Nucleoprotein gene, Peste des petits ruminants virus, RT-PCR, Swabs

Introduction

Peste des petits ruminants (PPR) also referred to as "*Kata*" is a highly contagious and fatal disease of goats and sheep caused by PPR virus (Baron *et al*., 2011). It is widespread in Africa, the Middle East and other parts of Asia (CFSPH, 2008; Abubakar *et al*., 2015) and, is currently emerging and re-emerging in different territories of the world (Wu *et al*., 2016). Goat rearing for meat production is a profitable venture in Nigeria. Nevertheless, major production constraints include inadequate credit facilities and disease outbreaks such as PPR (Baruwa, 2013). Case fatality rates have been shown to be higher in young animals (Abubakar *et al*., 2018). There is risk of further maintaining the virus in kids despite vaccination in adults and, this definitely threatens small ruminant population (Kock *et al*., 2015). Serological and antigenic evidences abound of PPR presence in arrays of animals (Balamurugan *et al*., 2012; Diallo, 2012). As with other *Morbillivirus* members such as Canine distemper virus (De Swart *et*

al., 2012), reports suggest that the virus can be found in other atypical hosts such as pigs, camels (Khalafalla *et al*., 2010; Muniraju *et al*., 2014).

Small ruminants morbillivirus, also known as Peste des petits ruminants virus (PPRV), is a linear, single stranded, non-segmented, negative sense RNA virus belonging to the genus *Morbillivirus* of the family Paramyxoviridae. The viral genome is 15,948 nucleotides long and contains six genes encoding six major structural polypeptides: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion (F) protein, hemagglutinin (H), and large (RNA dependent) polymerase protein (L), and two non-structural C and V proteins (Bailey *et al*., 2005). The genome possesses an unusual genomic malleability which confers resistance to it against some therapeutic substances (Holz *et al*., 2012). The four distinct lineages known are I, II and III from Africa; and lineage IV from Asia (Kerur *et al.*, 2008). Following reports of lineage IV in Cameroon and Central Africa Republic, investigations of outbreaks also revealed the presence of the Asian lineage IV clade-A field strains in Nigeria aside the lineage II strain (Woma *et al*., 2015).

The virus is detectable in swabs obtained from animals as early as 2 - 4 days post-infection (Couacy-Hymann *et al*., 2009; Baron *et al*., 2014) as PPRV antigens are excreted within this period. However, as time progresses antigens may be undetected in nasal and ocular swabs, though detectable in faeces up to 4 months post-infection. These results on the post-infection detection periods of PPR antigen in different sample types are of epidemiological relevance in diagnosis and disease control, since infected animals excrete virus in the environment before clinical signs appear or at early onset of clinical signs (Couacy-Hymann *et al*., 2009). With the highly contagious nature of the disease, it can easily be transmitted to healthy animals via direct contact with the faecal matters, nasal or ocular secretions, and excretions of infected animals (Ezeibe *et al*. 2008).

There are arrays of diagnostic techniques of varying sensitivity and specificity that can be used in confirming the disease. Some are, however, tedious and time-consuming (Balamuragan *et al*., 2007; Osman *et al*., 2009). Proper sampling technique, type of sample/swab and primer employed are very important in the molecular diagnosis and control of PPR field virus (Luka *et al*., 2012). The right transport media is also essential as well as prompt delivery of samples to the laboratory (Bhuiyan *et al*., 2014). Using Lateral Flow test and antigen Enzyme-linked Immunosorbent Assay, it has been observed that faecal swab is least suitable for antigen detection of PPRV, followed by ocular swab, yielding false-negative results. They were all bested by nasal swab, reflecting the increased viral load in nasal swabs of PPRV infected goats compared to ocular swabs (Halecker *et al*., 2020). Given that the N gene is the most abundantly transcribed gene, sensitivity is better achieved with RT-PCR targeting its synthesized mRNA (Kumar *et al*., 2014). Characterization of PPRV based on the N gene, which is highly conserved, also tends to be better in molecular epidemiology of the virus (Kerur *et al*., 2008).

RT-PCR targeting the N gene of PPR virus has proven to be effective in diagnosis of PPR (Tounkura *et al*., 2018), however, diagnosis of PPR in Nigeria is mainly based on clinical signs, which can be somewhat limited, and control is impeded by poor veterinary management and diagnostic services (Annatte *et al*., 2006). With OIE and FAO eager to control and subsequently eradicate PPR from the globe, as has been achieved for rinderpest (Munir *et al*., 2012), development of easier and effective means of diagnosis is of the essence, especially in endemic countries like Nigeria (Herbert *et al*., 2014). The study was therefore designed to determine the best sample/technique that yields the viral antigen, out of three types of swab commonly utilized in the diagnosis of PPR namely ocular, rectal and nasal swab using RT-PCR.

Materials and Methods

Study area

The study was conducted in Kaduna State, North-western, Nigeria. The state lies between latitudes 6° and 11° North and longitude 7° and 44° East. It is 608m above sea level. It has distinct wet and dry seasons, and is within the Northern Guinea Savannah zone and part of the Sahel Savannah zone of Nigeria. Kaduna state shares geographical boundaries with Katsina and Zamfara States to the North, Plateau and Bauchi States to the East, Nasarawa State and the Federal Capital Territory to the South, Niger State to the West; and Kano State to the Northeast.

Nigeria has a goat population of 65.65 million, about 2.5% of which are found in Kaduna State (NASS, 2012). The state comprises 23 Local Government Areas (LGA), from which Giwa, Sabon Gari and Igabi LGAs were selected based on presence of suspected on-going outbreaks.

Sampling procedure and collection

Thirteen goats with clinical signs suggestive of PPR from homes, pens and animal farms were used for the

study. These comprised five from Igabi, four from Sabon Gari, and fours from Giwa. Employing different sampling strategies commonly employed by field veterinarians, multiple samples, triplicate and duplicate, from different routes (multi-route sampling) were obtained from each goat in Igabi and Sabon Gari, while single sample type per goat was obtained from Giwa. There were 29 samples in total: Nasal swabs (NS) (n = 10), ocular swabs (OS) (n = 9), and rectal swabs (RS) (n = 10).

Swabs were collected using sterile swab sticks and inserted in viral transport media (VTM). The samples were transported to the National Veterinary Research Institute (NVRI), Vom, Plateau State and kept at -20^{o}C until further analysis.

RNA extraction/amplification of PPR nucleic acid using RT-PCR

Total ribonucleic acid (RNA) extraction was performed from the samples using a Qiagen RNeasy Plus RNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, each sample was aliquoted and incubated at room temperature for about 10 min in a micro centrifuge tube containing buffer AVL. Following brief centrifugation, 96 – 100% alcohol was used for precipitation. The mixtures were decanted into QIA amp mini spin column (in 2ml collection tube) for further washing and centrifugation, before RNA elution from the mini spin column into a new collection tube.

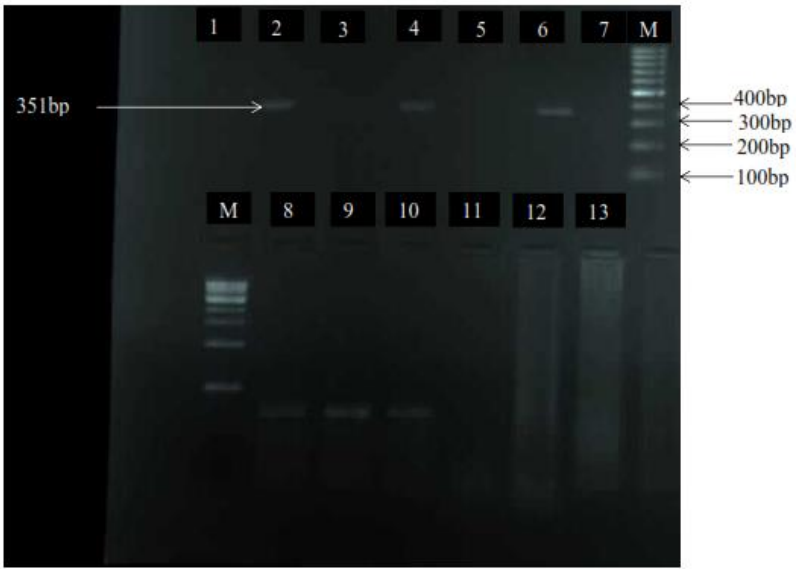

Figure I: Gel pictomicrogram showing the expected 351bp of N gene
M= 100bp Molecular weight marker; Lanes 2, 4, 6, 8, 9 and 10 = positive samples

The positive control was live-attenuated Nigeria 75/1 PPR vaccine produced by NVRI, Plateau state. The extracted RNA was stored at -80^{o}C for RT-PCR.

PPRV nucleoprotein (N) gene was amplified using Titan One Tube One-Step RT-PCR kit (Roche Life Science, Indiana, USA) using PPRV-specific primers. The forward primer: NP3 (5'-TCTCGGAAATCGCCTCACAGACTG-3'), and reverse NP4 (5'-CCTCCTCCTGGTCCTCCAGAATCT-3') previously described by Couacy-Hymann *et al*. (2002) targeted 351bp segment of the N gene. The reaction mixture was set up thus: 12.5µl of nuclease-free water, 6µl of PCR buffer, 0.25µl of forward primer, 0.25µl of reverse primer, 0.5µl of enzyme (taq polymerase) and 5µl of extracted RNA. Nigeria 75/1 vaccine produced by NVRI, Jos, Plateau state, was used as positive control.

Briefly, a reverse transcription was executed at 45°C for 30 minutes before initial denaturation of DNA at 95°C followed by 40 cycles of denaturation at 95°C for 30s. Annealing was at 55°C for 30 s, elongation at 72°C for 30s, and a final extension at 72°C for 10 min. Fragments of cDNA thus amplified were in resolved 1% agarose gel stained with ethidium bromide and the pictomicrogram was visualized accordingly.

Results

Clinical samples from Igabi LGA were obtained in May, 2018. In Sabon Gari, and Giwa LGAs sampling was done in the month of July, 2018. The following clinical signs were observed in the sampled animals: fever, lethargy, coughing, ocular and nasal discharges, dypsnoea, conjunctival congestion, diarrhoea and death. With the commonest clinical signs being abnormal nasal discharge and diarrhoea. The animals were both male and female between 2 months and 1.5 years old. The most severe clinical manifestations were observed in kids or young goats (approximately 2-3 months old).

RT-PCR using NP3 / NP4 primers pair yielded the anticipated 351bp of segment of the N gene as indicated by distinct band picture using 100bp molecular marker on the gel picture (Figure I). The positive detection of PPR from all the sampled locations was found to be 31.9%. In Giwa LGA, where multi-sampling was not employed, evaluating the effectiveness of single sample type in

diagnosis, PPRV was not detected, in nasal, ocular or rectal swabs using RT-PCR (Table 1). 50% of total NS showed positive results when compared with 44.4% for total OS and 30% for total RS. In Igabi and Sabon Gari, one goat from each LGA (22.2%) showed positive result to all three swabs. In two goats (22.2%), NS yielded positive results to PPR, though OS and RS were negative. Similarly, only 4 (44.4%) goats from both LGAs were positive with at least two swabs. NS was one of the samples in both cases.

NS yielded positive results to 3 cases (33.3%) for which OS produced negative results; whereas, only 1 case (11.1%) existed in which OS was positive and NS negative. Also, on 3 (33.3%) occasions, PPRV was detected in NS, and undetected in RS. It is noteworthy that there was no instance when RS was positive and NS was negative concurrently. PPRV antigens were detected in OS on 2 (22.2%) occasions when RS yielded negative results; and undetected in OS on 1 (11.1%) occasion when RS was positive. All the goats sampled in Sabon Gari LGA were positive to at least one swab, except 1 goat (25%) which was negative to all 3 samples (Table 1).

Of all the positive samples, only in two scenarios from both Igabi and Sabon Gari was only one sample found positive representing 33.3% of total positive samples (Table 2).

Discussion

PPR is still an endemic crisis in Kaduna state, Nigeria affecting small ruminant populations in both rural and urban areas, undermining the economy of poor farming communities. Effective diagnosis is of utmost importance in check-mating the menace of this disease.

RT-PCR using NP3/NP4 primers pair detected the presence of PPRV in all the outbreaks except Giwa LGA implying that it remains a very reliable technique in the detection of PPRV, though only available in few laboratories across the country. Its increased availability along with diagnostic techniques like real time RT-PCR will play significant role in the control of the disease. The observed outbreak in Giwa could be due to a different disease such as contagious caprine pleuropneumonia (CCPP), foot-and-mouth disease (FMD) and bluetongue due to presence of pyrexia, nasal discharge, general malaise and death (Balamurugan *et al*., 2014). As previously reported,

Table 1: Comparison of different types of swabs for the detection of PPRV Using RT PCR from various locations of outbreaks in Kaduna State

Location	Animal No.	Age (month)	NS	OS	RS
Igabi	1**	≤ 6	+	-	+
	2***	≤ 6	+	+	+
	3	≥ 12	-		-
	4	≥ 12	-		-
	5*	≤12	-	+	-
Sabon Gari	1**	≤12	+	+	-
	2	≤ 6	-	-	-
	3*	≤ 6	+	-	-
	4***	≤ 6	+	+	+
Giwa	1	≤ 6	-		
	2	≤ 6		-	
	3	≤ 6			-
	4	≤ 6		-	
n/N (x%)			5/10 (50.0)	4/9 (44.4)	3/10 (30.0)

*= 1 positive sample; ** = 2 positive samples; *** = 3 positive samples; + = Nucleic acid detected; - = Nucleic acid undetected; n = Number of positive; N = Number of samples; % = Percentage

Table 2: Positive cases from the outbreaks comparing effectiveness of single-sampling and multi-sampling technique

Locaton	Only 1 sample type positive	At least 1 sample type positive	Total positive
Igabi	1	3	3
Sabon Gari	1	3	3
Total (%)	2 (33.33)	4 (100)	6

% = Percentage

negative finding, however, may be related to the timing of sample collection, as sampling was done weeks after the onset of clinical signs, as well as small sample size (Ezeibe *et al*., 2008). Most of the goats with positive results were between three months old and one year old. This is in agreement with Woma *et al*. (2015).

Sampling in Igabi was done in May, 2018. In Sabon Gari, the positive samples were sourced in July, 2018. Kaduna state has two distinct seasons: Dry harmattan season (April – November) and Rainy season (May – October) (Ajala *et al*., 2008). This is indicative that PPR also affects goats during the wet season in Kaduna state, Northern Nigeria. This contravenes the position of the study conducted in South-eastern Nigeria. It had been reported previously that the disease mainly affects ruminants during harmattan season in Southern Nigeria (Wosu, 1994).

The most severe clinical manifestations were observed in goats not older than three months, similar to that of Abubakar *et al*. (2018) who believed that case-fatality rates were higher in young animals. This may be due to poor immune status especially in kids from unvaccinated does that have inadequate maternal antibodies against the disease.

A nasal swab was found to have a higher positivity rate in the confirmation of PPR using RT-PCR targeting the N gene, followed by an ocular swab. This may be directly related to the pathogenesis of the disease, as inhalation is a very common mode of transmission, and the respiratory tract is probably the site of primary viraemia. Due to tropism, a large amount of the virus undergoes replication at this site within the epithelial and immune cells. Rectal swabs are the least reliable of the three, which could be due to the relatively low levels of SLAM and PVRL4 receptors in the intestine. Parida *et al*. (2019) also reported that detection in faecal samples is intermittent. This is in line with Mahajan *et al*. (2012) who reported nasal swabs as first among the three, closely followed by ocular, and ahead of rectal swabs using c-ELISA.

In conclusion, RT-PCR confirmed PPRV to be responsible for the outbreaks in goats in Igabi LGA and Sabon Gari LGA of Kaduna state. In Giwa LGA, PPR was not confirmed. With RT-PCR, nasal swab was observed to have a higher PPRV nucleic acid detection rate (50.0%) than ocular swabs (44.4%), while rectal swabs (30.0%) were the least reliable of the three. From the variability observed among swab samples, a more efficient protocol for diagnosing PPR using RT-PCR is to utilize multiple swabs from different routes from a given animal (multi-sampling technique), this enhances PPR detection rates and reduce the incidence of false negative conclusion which may be deleterious to poor farmers as accurate diagnosis is key to accurate therapy and care.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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