Co-circulation of Peste-des-Petits-Ruminants Virus Asian lineage IV with Lineage II in Nigeria

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Summary

Peste-des-petits-ruminants (PPR), a major small ruminant transboundary animal disease, is endemic in Nigeria. Strains of the causal agent, peste-des-petits-ruminants virus (PPRV), have been differentiated into four genetically distinct lineages based on the partial sequence of the virus nucleoprotein (N) or fusion (F) genes. PPRV strains that were identified initially in Africa were grouped into lineages I, II and III and viruses from Asia were classified as lineage IV and referred to as the Asian lineage. Many recent reports indicate that the Asian lineage is now also present in Africa. With this in mind, this study was conducted to re-assess the epidemiology of PPRV in Nigeria. A total of 140 clinical samples from 16 sheep and 63 goats with symptoms suggestive of PPR were collected from different states of Nigeria during a four year period (2010 – 2013). They were analyzed by the amplification of fragments of the N gene. Results for 33 (42%) animals were positive. The phylogenetic analysis of the N gene sequences with those available in GenBank showed that viruses that were detected belong to both lineage II and IV. Based on an analysis of the N gene sequences, the lineage IV isolates grouped into two clades, one being predominant in

the north-eastern part of the country and the other found primarily in the southern regions of the country. This study reports the presence of PPRV Asian lineage IV in Nigeria for the first time.

KEYWORDS: Morbillivirus, PPR, West Africa, viral spread, nucleoprotein, sheep.

Introduction

Peste-des-petits-ruminants (PPR) is a serious infectious disease of sheep and goats. It is endemic in large parts of Africa (from North Africa to Tanzania), the Middle East, Turkey, Iran and many countries in Asia (Libeau *et al*., 2014). The disease is characterized by pyrexia, depression, anorexia, diarrhoea, respiratory distress, mucopurulent oculo-nasal discharges with matting of the eyelids, necrotic oral lesions with a fetid smell and sometimes abortion in pregnant animals. In Nigeria most outbreaks occur between February and May, the dry harmattan season and also between July and September, mostly in the south-eastern and south-western parts of the country.

This disease is caused by peste-des-petits-ruminants virus (PPRV), which belongs to the genus *Morbillivirus* within the family *Paramyxoviridae,* along with rinderpest virus, measles virus, canine distemper virus, cetacean morbillivirus virus and phocine distemper virus (King *et al*., 2012). They are enveloped viruses with a genome composed of a non-segmented single-stranded negative sense RNA. The genome of PPRV is 15 948 nucleotides in size and encodes for two non-structural proteins C and V, and six structural proteins arranged in the order: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H) and viral RNA-dependent polymerase (L) (Diallo, 2003; Bailey *et al*., 2005; Truong *et al*., 2014). Based on partial sequences of the N and F genes, PPRV has been classified into four genetically distinct lineages (I, II, III and IV) even though the virus is serologically monotypic (Banyard *et al*., 2010; Kwiatek *et al*., 2011). Viruses that were first identified in Africa belong to lineages I, II and III while viruses belonging to lineage IV, also referred to as the Asian lineage, have been isolated in many Asian countries including Iran, Turkey and the Middle East (Banyard *et al*., 2010; Kwiatek *et al*., 2011). However, over the last one to two decades, there have been reports of the presence of the

Asian lineage of PPRV in African countries such as Sudan, Morocco, Egypt, Algeria, Uganda, Gabon, Cameroon and Central African Republic (Banyard *et al*., 2010., Kwiatek *et al*., 2011., Sharawi and Abd-El-Rahim, 2011., De Nardi *et al*., 2012., Luka *et al*., 2012., Maganga *et al*., 2013).

PPRV identified in Nigeria from the 1970s up until 2000 all belonged to lineage II (Shamaki, 2002., Kwiatek *et al*., 2007., Banyard *et al*., 2010). However, following reports of the circulation of lineage IV in Cameroon and the Central African Republic, it has become necessary to re-assess the epidemiological situation of PPRV in Nigeria especially in states at the border with neighbouring countries. Indeed, Cameroon has a long porous border with Nigeria and many inhabitants of north-eastern Nigeria (Taraba, Adamawa, Borno States) have families in that country, thus facilitating animal trade and smuggling and the taking advantage of the limited control over animal movements at the border.

Materials and methods

Samples

A total of 140 clinical samples consisting of spleen, trachea, lung, liver and lymph nodes were collected between 2010 to 2013 from 16 sheep and 63 goats with symptoms suggestive of PPRV infection. These animals were from different states representing the six different agro-ecological zones of Nigeria. Samples were transported on ice to the laboratory at the National Veterinary Institute in Vom. The location of each sampling was recorded using the GPSMAP® 76 versatile navigator (Garmin).

RNA extraction and RT-PCR

A 10 % w/v homogenate was made by grinding tissue with sterile glass in DMEM medium and the suspension was then clarified at 1200 x g for 5 min. Total RNA was extracted from the sample supernatant using a Qiagen RNeasy Plus RNA extraction kit (Qiagen, Germany) according to the manufacturers' instructions. Reverse transcription PCR (RT-PCR) was performed with 2 µl

extracted RNA using the Qiagen® one step RT-PCR kit (Qiagen, Germany) to amplify a 351 bp segment of the PPRV N gene with the NP3/NP4 diagnostic primers (Couacy-Hymann *et al*., 2002). PCR products were resolved on a 1.2 % agarose gel stained with ethidium bromide to reveal the expected band size.

Sequencing and phylogenetic analysis

Amplified products were purified for sequencing using the Wizard® SV gel and PCR clean-up system (Promega®, USA) according to the manufacturer's instructions. The purified DNA was quantified by spectrophotometer (NanoDrop® ND-1000, PeQlab Biotech Gmbh). Sequencing was carried out in both forward and reverse directions by Inqaba Biotech (South Africa).

The nucleotide sequences of the N gene amplicons were aligned using the Molecular Evolutionary Genetics Analyses (MEGA) software version 5 (Tamura *et al*., 2011). Phylogenetic trees were constructed by comparisons with unique published sequences available (i.e. in peer review journals) in GenBank. A TIM+G substitution model was determined by Modeltest v3.7 (Posada and Crandall, 1998) for the N gene. Distance (neighbour-joining) and character (Bayesian, maximum parsimony) based phylogenies of the nucleotide sequences were explored using PAUP* 4.0b10 (Farris *et al*., 1994) and Mr Bayes v3.2.2 (Ronquist and Huelsenbeck 2003). The reliability of each tree was estimated using 1,000 bootstrap replicates and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein, 1985). Sequences have been submitted to GenBank under accession numbers KF479408 – KF479444, KJ124726 – KJ124773.

Results and Discussion

During this study, most of the outbreaks of PPR occurred during February to May, which is part of the dry harmattan season, in northern states and July to September, part of the rainy season, in southern states. Most of the affected animals were between 3 and 12 months of age. More goats

than sheep were affected in areas where they grazed together and shared the same drinking sources.

A total of 140 samples from 79 animals were tested by RT-PCR. Samples for 33 animals were positive, i.e. 2 out of 16 sheep (13 %) and 31 out of 63 goats (49 %). The details of the positive samples are summarized in Table 1. The amplified products were sequenced and the data were analysed together with PPRV N gene sequences available in GenBank (Kwiatek *et al*., 2007, 2011; Banyard *et al*., 2010). Three phylogenetic analysis methods were used, which all generated trees with similar topologies. The phylogeny inferred with the distance methods was consistent with those of the character based analysis. Based on the partial N gene sequence, most of the PPRV strains collected in Nigeria between 2010 and 2013 (n = 26) grouped together in lineage IV and only a few grouped in lineage II (n = 7) (Fig. 1). Lineage IV isolates from Nigeria were subdivided into two clades (i.e. IV-NigA and IV-NigB), supported by a bootstrap value of 90 %. Samples from the two positive sheep belonged to IV-NigA. All samples in IV-NigA were collected between February and April 2012 and February 2013. PPRVs lineage IV-NigA were distributed across the entire north-eastern part of the country and present only in one southern state (Ondo) (Fig. 2). PPRVs lineage IV-NigB were present only in the north-east of the country, in Plateau and Adamawa states (Fig. 2). The majority of the lineage IV-NigB viruses occurred in the three southern states of Cross River, Imo and Anambra and were isolated from goat samples collected in April 2010, September 2011, July 2012 and March 2013. Lineage IV-NigB viruses showed 94 % identity with IV-NigA and were related most closely to viruses identified in Cameroon and Sudan in 1997 and 2008, respectively.

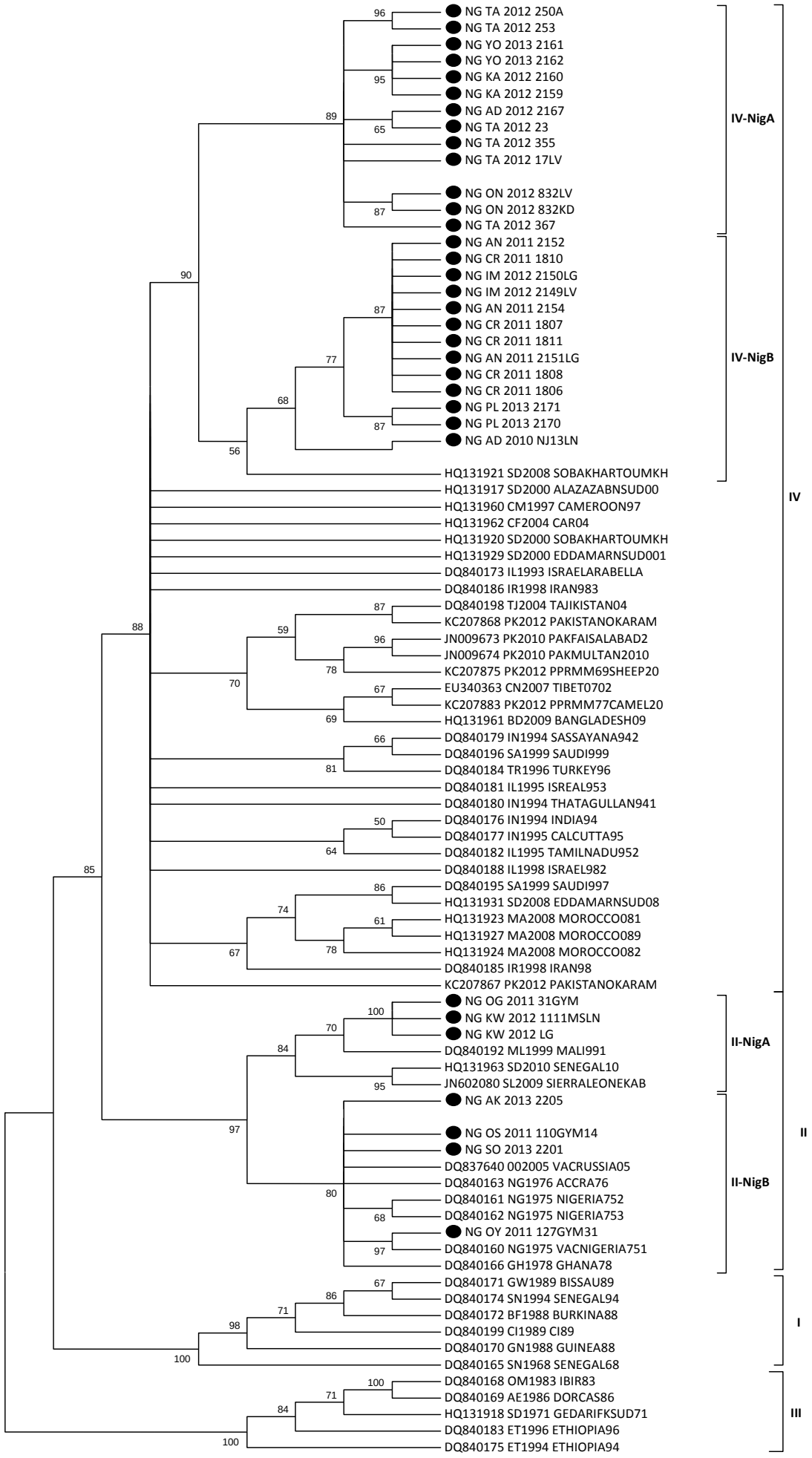

Fig. 1: PPRV N gene phylogenetic analysis. Neighbour-joining unrooted cladogram showing the relationship between the N gene sequences from this study (indicated by black circles, ISO 3166 country code and state code, year of sample collection and sample laboratory number) with unique published sequences obtained from GenBank (indicated by accession number, ISO 3166 country code, year of isolation and name of isolate). The numbers at the nodes are bootstrap values obtained from 1000 re-samplings.

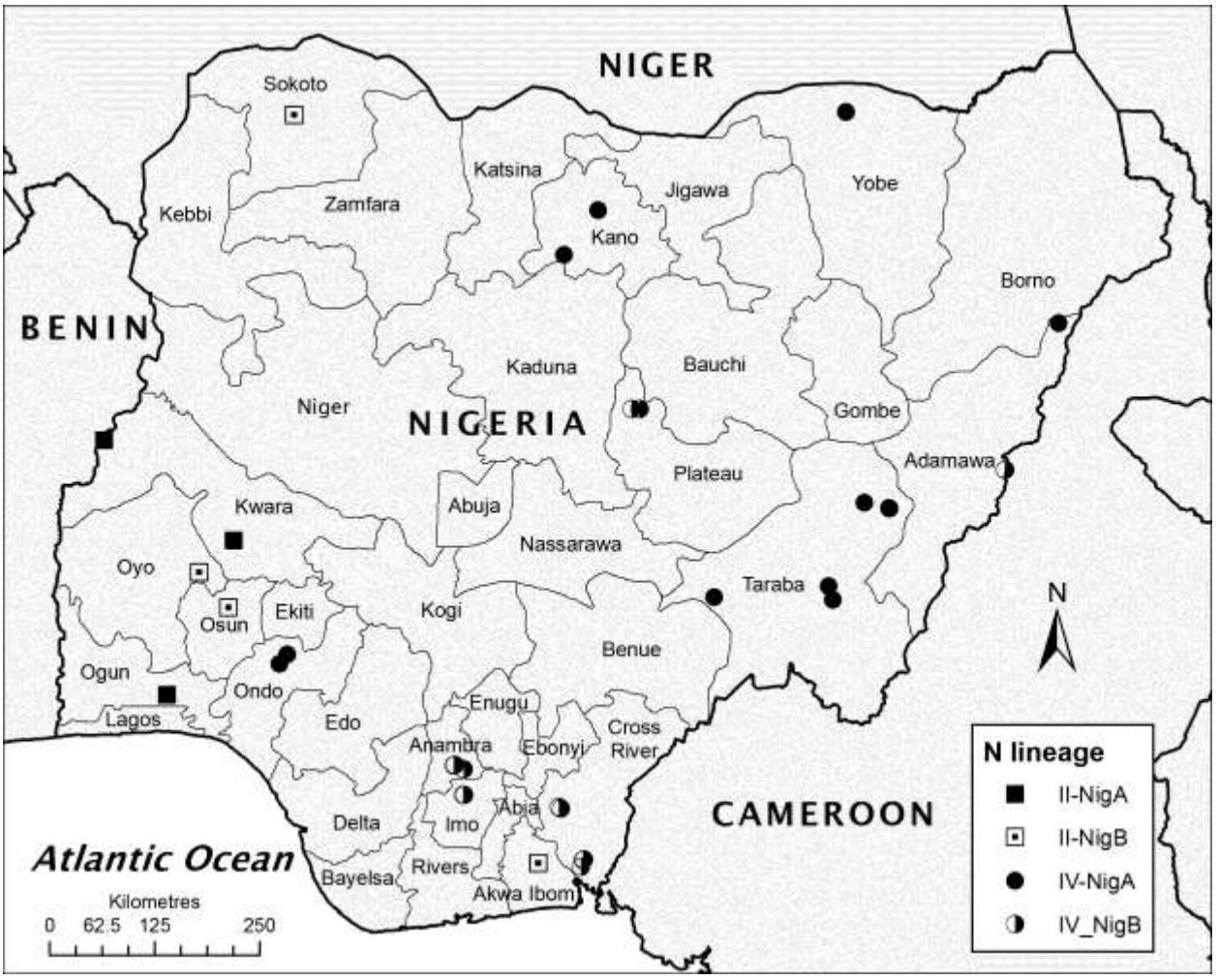

Fig. 2: The distribution of PPRV lineage II (circles) and IV (squares) in different states of Nigeria. The map shows the location of villages where positive PPR samples were collected (see Table 1).

Only seven lineage II virus sequences from the present study were similar to earlier isolates from Nigeria collected in the 1970s and 1990s (Kwiatek *et al*., 2007; D. Shamaki, 2002). They were collected from goats in September 2011, May 2012 and May 2013. The lineage II sequences from this study also formed two separate clades, II-NigA and II-NigB (Fig. 1). Lineage II-NigA samples had a high identity with PPRV strains identified in Mali obtained in 1999 (DQ840198), while those in lineage II-NigB showed 98 % identity with the Nigerian 1975/1 virus (DQ840160) that has been attenuated and is commonly used as a vaccine (Diallo *et al*., 1989; Kwiatek *et al*., 2007).

Table 1. Details of animals in Nigeria positive for PPRV by RT-PCR.

State	Location	Species	Breed	Age*	Anamnesis	Collection date	Sample type	Sample name	Sub-Clade
Adamawa	Njobli	Ca	WAD	7	Diarrhoea, matted eyelids, oral lesions, nasal discharges, anorexia	12.04.2010	Lung	NGAD2010-NJ13LN	IV-NigB
Anambra	Adazi-Ani	Ca	WAD	6	Cough, fever, anorexia, raised hair coat, diarrhoea	21.09.2011	Lung	NGAN2011-2151LG	IV-NigB
Cross River	Akpet Central	Ca	WAD	4	Oculo-nasal discharges, oral lesions, diarrhoea	23.09.2011	Liver	NGCR2011-1807	IV-NigB
Cross River	Ikot-Omin	Ca	WAD	4	Oculo-nasal discharges, oral lesions, diarrhoea	23.09.2011	Liver	NGCR2011-1806	IV-NigB
Cross River	Mariam	Ca	WAD	4	Oculo-nasal discharges, oral lesions, diarrhoea	23.09.2011	Liver	NGCR2011-1808	IV-NigB
Cross River	Biase	Ca	WAD	4	Oculo-nasal discharges, oral lesions, diarrhoea	23.09.2011	Liver	NGCR2011-1810	IV-NigB
Cross River	Ikot-Eneobong	Ca	WAD	4	Oculo-nasal discharges, oral lesions, diarrhoea	23.09.2011	Liver	NGCR2011-1811	IV-NigB
Anambra	Umuchi	Ca	WAD	7	Cough, fever, anorexia, raised hair coat, diarrhoea	23.09.2011	Lymph node	NGAN2011-2154	IV-NigB
Anambra	Amada	Ca	WAD	5	Oculo-nasal discharges, oral lesions, diarrhoea	22.09.2011	Liver	NGAN2011-2152	IV-NigB
Osun	Iregba	Ca	WAD	4	Blood tinged diarrhoea, pneumonia, vesicular stomatitis	11.09.2011	Lung	NGOS2011-110GYM14	II-NigB
Ogun	Ijebu-Ode	Ca	WAD	9	Oral lesions, diarrhoea, cough,	29.09.2011	Lung	NGOG2011-31GYM	II-NigA
Oyo	Bodija	Ca	WAD	11	Soiled hindquarters, oral lesions with putrid smell, anorexia, sneezing	29.09.2011	Kidney	NGOY2011-127GYM31	II-NigB
Adamawa	Gulak	Ca	WAD	6	Raised hair coat, depression, diarrhoea	12.04.2012	Lymph node	NGAD2012-2167	IV-NigA
Taraba	Kassa	Ov	WAL	6	Fever, emaciated, pneumonia, anorexia, diarrhoea	27.03.2012	Liver	NGTA2012-17LV	IV-NigA
Taraba	Jalingo	Ca	WAD	15	Weak, depressed, fever, cough, anorexia	01.04.2012	Spleen	NGTA2012-367	IV-NigA
Taraba	Wukari	Ca	WAD	11	Oculo-nasal discharges, oral lesions, diarrhoea, fever	30.03.2012	Lymph node	NGTA2013-23	IV-NigA
Taraba	Maihula	Ov	WAL	5	emaciated, pneumonia, diarrhoea, fever, matted eyelids	17.02.2012	Lung	NGTA2012-250A	IV-NigA
Taraba	Garbabi	Ca	WAD	6	Matted eyelids, emaciated, pneumonia,	31.03.2012	Lung	NGTA2012-253	IV-Nig

					diarrhoea				A
Taraba	Kassa	Ca	WAL	17	Oral lesions, matted eyelids, diarrhoea	29.03.2012	Lymph node	NGTA2012-355	IV-NigA
Kano	Kano Municipal Council	Ca	WAL	12	Pneumonia, depressed, fever, oral lesions	15.04.2012	Lung	NGKA2012-2159	IV-NigA
Kano	Dogongora	Ca	WAL	5	Anorexia, emaciated, diarrhoea, weakness	19.04.2012	Lymph node	NGKA2012-2160	IV-NigA
Ondo	Akure	Ca	WAD	14	Diarrhoea, cough, oral lesions, fever, pneumonia	27.04.2012	Liver	NGON2012-832LV	IV-NigA
Ondo	Idanre	Ca	WAD	7	Diarrhoea, cough, oral lesions, fever, pneumonia	27.04.2012	Lung	NGON2012-832KD	IV-NigA
Kwara	Baruten	Ca	WAD	5	Emaciation, diarrhoea, vesicles on feet, weakness	09.05.2012	Lymph node	NGKW2012-1111MSLN	II-NigA
Kwara	Illorin	Ca	WAD	5	Emaciation, diarrhoea, vesicles on feet, weakness	09.05.2012	Lung	NGKW2012-LG	II-NigA
Imo	Eziama-Obaire	Ca	WAD	13	Cough, fever, anorexia, raised hair coat, diarrhoea	16.07.2012	Liver	NGIM2012-2149LV	IV-NigB
Imo	Iho	Ca	WAD	4	Oculo-nasal discharges, oral lesions, diarrhoea	18.07.2012	Lung	NGIM2012-2150LG	IV-NigB
Yobe	Yusufari	Ca	WAL	9	Raised hair coat, weak, depressed, fever, anorexia	15.02.2013	Lymph node	NGYO2013-2161	IV-NigA
Yobe	Yusufari	Ca	WAL	10	Raised hair coat, weak, depressed, fever, anorexia	15.02.2013	Lung	NGYO2012-2162	IV-NigA
Plateau	Angwa Kurma	Ca	WAD	6	Oral lesions, diarrhoea, cough, emaciation	19.03.2013	Lung	NGPL2013-2170	IV-NigB
Plateau	Jos	Ca	WAD	8	Matted eyelids, oculo-nasal discharges, depression, soiled vent	19.03.2013	Liver	NGPL2013-2171	IV-NigB
Akwa Ibom	Uyo	Ca	WAD	14	Matted eyelids, oculo-nasal discharges, depression, soiled vent, emaciated	03.05.2013	Lymph node	NGAK2013-2205	II-NigB
Sokoto	Sokoto	Ca	WAD	14	Matted eyelids, oculo-nasal discharges, depression, soiled vent, emaciated	21.05.2013	Lymph node	NGSO2013-2201	II-NigB

* Age in months, Ca - Caprine, Ov - Ovine, WAD - West African Dwarf, WAL - West African Long-leg.

The findings of this study relating to PPR surveillance in Nigeria over a four year period (2010 – 2013) have confirmed the widespread distribution of PPRV throughout Nigeria, as has been described since the early 1970s (Isoun and Mann, 1972) and also revealed the presence of PPRV lineage IV in the country for the first time.

Analysis of the sequence data obtained from the samples showed the presence of viruses belonging to two lineages, namely, PPRV Asian lineage IV and PPRV lineage II, the so-called "indigenous" lineage given that it is the lineage to which PPRV isolated in Nigeria between 1975-1976 and also in the late 1990's (Shamaki, 2002; Kwiatek *et al*., 2007) belong. Surprisingly, the number of samples that belonged to this "indigenous" group was small compared to those of the "emerging" lineage IV. Some earlier Nigerian samples collected between 2008 and 2009 were also classified previously as lineage IV (Diallo, A., unpublished data). The Nigerian lineage IV viruses from this study formed two distinct clades, IV-NigA and IV-NigB which were clearly distinct from other lineage IV viruses except for the sequence of a Sudanese isolate (HQ131921) (Fig. 1). Of particular interest are the samples collected in Adamawa state in April 2010. This state borders Cameroon, where a 1997 lineage IV virus (HQ131960, Fig 1) was reported (Kwiatek *et al*., 2011). It is tempting to think that viruses of lineage IV-NigB were first introduced into Nigeria from Cameroon and then evolved into lineage IV-NigA viruses due possibly to genetic drift following in-country transmission, as has been suggested for measles viruses (Santibanez *et al*., 2002; Alla *et al*., 2006; Waku-Kouomou *et al*., 2010). Further in-depth genetic analysis using next generation sequencing is planned in order to confirm this hypothesis. The presence of a lineage IV-NigA virus (NG_ON_2012_832LV) in Ondo State, a region where lineage II and lineage IV-NigB viruses are predominant may be the result of importation of infected animals from northern Nigeria. A lineage II PPRV (NG_AK_2013_2202) was identified in Akwa Ibom state in the far south of the country which is a region where lineage IV-NigB viruses predominate. This may be also as a result of importation from states bordering Benin where PPRV lineage II is predominant e.g. Ogun, Osun, Oyo and Kwara States.

The lineage II isolates subdivided into two distinct clades. One clade was closely related to the Nigeria 75/1 vaccine strain while the second clade was closely related to viruses found in Mali, Senegal and Sierra Leone. At present, there is no information on the PPRV lineages circulating currently in Niger so the isolates collected in Yobe and Sokoto states are of particular interest. These two states have borders with Niger and while the sample collected in Sokoto (NG_SO_2013_72) was identified as belonging to lineage II-NigB the samples collected in Yobe (NG_YO_2013_2161/2162) were of lineage IV-NigB.

This study has shown that Nigerian sheep and goats are infected by viruses of two PPRV lineages: lineage II, the "indigenous lineage", which is being replaced by the Asian lineage. Kwiatek *et al.*, (2011) reported a similar situation in Sudan where PPRV lineage IV is now dominant and has replaced lineage III that was present in the country until 2000. The authors suggested that the lineage IV viruses which were present historically in Asia and parts of the Middle East may have become more virulent and so have spread more easily to Africa. It is possible that these more virulent lineage IV PPRV strains have a selective advantage over lineage III viruses in the case of Sudan and lineage II viruses in the case of Nigeria. This view is supported by the remarkably rapid spread of the lineage IV viruses over the north-eastern part of Nigeria bordering Cameroon. It is unknown whether this change in PPRV lineage distribution in Nigeria has any relationship to pathogenicity or is just a result of geographical speciation. A more widespread analysis of PPRV in other African countries would help to understand the geographic extent of the spread of the Asian lineage within the continent.

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