Genomic comparison of Newcastle disease viruses isolated in Nigeria between 2002 and 2015 reveals circulation of highly diverse genotypes and spillover into wild birds

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Abstract

Newcastle disease virus (NDV) has a wide avian host range and large genetic variability, and virulent strains cause Newcastle disease (ND), a worldwide concern for poultry health. Although NDV has been studied in Nigeria, the genetics of the viruses involved in the endemicity of the disease and the transmission that likely occurs at the poultry-wildlife interface is still largely incomplete. Next-generation and Sanger sequencing was conducted to provide complete (n=73) and partial genomic data (n=38) for the characterization of NDV isolated during 2002-2015 on domesticated and wild birds in Nigeria, including the first genotype IV and sub-genotype VIh complete genome sequences available from the African continent. The phylogenetic analysis revealed that viruses of seven different genotypes circulated in that period, demonstrating high genetic NDV diversity for a single country. In addition, high similarities across NDV isolated from domestic and wild birds were evident, suggesting spillovers, including three novel NDV susceptible species. Furthermore, the first spillover of a mesogenic Komarov vaccine virus is documented, suggesting a previous spillover and evolution of this virus. The similarities between viruses from poultry and multiple bird species and the lack of evidence for host adaptation in codon usage suggest that transmission of NDV between poultry and non-poultry birds is recent. This is especially significant when considering that some viruses were isolated from species of conservation concern. The identified high diversity of NDV in both domestic and wild birds in Nigeria emphasizes the need for active surveillance and epidemiology of NDV in all bird species.

Key Words: Newcastle disease, avian avulavirus-1, Nigeria, phylogenetic analysis, codon usage analysis

INTRODUCTION

Avian avulavirus-1 (AAvV-1), whose isolates are commonly known as Newcastle disease viruses (NDV), used hereafter, is a single-stranded non-segmented, negative sense RNA virus that has high genetic variability [1]. The genetic variability and mobility of NDV are widely studied because virulent strains cause the highly contagious Newcastle disease, which presents high morbidity and mortality in domesticated gallinaceous poultry [2,3]. NDV has been classified into two classes [4] with class II being the more diverse with at least 18 genotypes [5]. NDV in Nigeria was first recorded in the 1950's [6] but molecular characterization of Nigerian isolates has only recently become available (as of 2007 [7]) and members of seven NDV genotypes: I, II, IV, VI, XIV, XVII, and XVIII have been identified in the country [8-10]. The reports of genotypes XIV, XVII, and XVIII are strictly limited to West and Central Africa with sub-genotype XVIIb documented only in Nigeria. One exception is the two genotype XVIII strains that were found in wild birds at a quarantine station in the United States; however, the origin of the birds is unknown [11]. A recent report of NDV in Nigeria demonstrates that most genotypes are widely distributed across the country, but genotype XVII was solely seen in the northern states [8].

NDV has a wide variety of avian hosts and is known to cause disease in many species beyond domestic poultry [12,13]. Interactions between free-ranging wild birds and poultry are likely to occur frequently at the livestock-wildlife interface and exchange of pathogens is highly probable. With the continued documentation of NDV presence in wild avifauna, spillover of virulent and vaccine viruses from poultry is evident [14-17]. Pigeons are known carriers of some sub-genotypes of NDV [18,19], virulent strains of NDV have been isolated from Double-crested Cormorants, and other isolates of low virulence have been identified in different waterbird species [20-22]. These wild bird carriers are of high concern in the movement and transmission of pathogens, including NDV, to and from the poultry sector. It is important to document any other virulent NDV hosts to understand the disease dynamics beyond the poultry industry.

Poultry production is the second most important industry in Nigeria [23] and widespread NDV outbreaks occur in both vaccinated and unvaccinated flocks [24,25]. Chickens raised in a free-range management system rarely have shelter or veterinary care provided and individual movements are not monitored when compared to commercial operations [23]. Thus, interactions with, and potential pathogen transmission between, chickens from neighboring farms and/or other bird species are likely to be common. Furthermore, the local environment has a constant,

uncontrolled input of poultry waste products, which can contaminate soil or water [26]. This is a point of concern considering that NDV has been documented to remain infectious in litter and carcasses for at least a couple of weeks, and in feathers it remains so for several months [27]. NDV also persist in soil or water for up to 90 days in ideal conditions [26]. These factors can easily facilitate pathogen transmission from chicken to chicken and/or from chicken to wild birds, thus contributing to the endemic circulation of NDV. Wild birds have been suggested to be important viral reservoirs [28], but the role of wild birds in circulation of NDV in Nigeria is not fully understood [29]. Furthermore, even with NDV being well documented in Nigeria and phylogenetic analyses published as early as 2007, this study with 111 new isolates further extends the number of species, hosts and locations, while being the first to add complete genomes to the phylogeny of regional strains. Thus, the aim of this study was to use next-generation and Sanger sequencing to obtain complete genomes and complete fusion genes of NDV isolated from domestic and wild birds during active and passive surveillance in Nigeria between 2002 and 2015, as well as two archival strains from 1973 and 1980, and to perform comprehensive phylogenetic and codon usage analyses to help better understand the distribution and genetic variability of NDV in this country.

MATERIALS AND METHODS

Sample collection/processing, virus isolation and identification

Whole carcasses were submitted all year round (between 2002 and 2015) from suspected ND outbreaks in poultry and wild bird species. Post-mortem examinations were conducted at the Central Diagnostic Laboratory, National Veterinary Research Institute (NVRI) Vom, Nigeria, and spleens, lungs, tracheas, and intestines were collected for virus isolation. Similarly, tracheal and cloacal swabs were collected during an active surveillance conducted from June to August 2009 in live bird markets (LBMs) from fifteen of the thirty-six Nigerian States, including the Federal Capital Territory (FCT).

The tracheal and cloacal swabs were collected by trained field officers from sick, apparently healthy, and new batches of bird species supplied to the LBMs. The swabs were collected into virus transport medium containing antibiotics Penicillin (10,000 units/ml), Streptomycin (10,000 mg/ml), Gentamycin (5000 mg/ml), and Amphotericin B (50 mg/ml) with 50% glycerol adjusted to pH 7.2 and transported to the laboratory in a cool box containing ice packs. Virus isolation was conducted at the Regional Laboratory for Animal Influenza and other Transboundary

Animal Diseases (RLAITAD), NVRI, Vom, Nigeria. Tissues and swabs in media were processed and inoculated into 9-11-day-old embryonated chicken eggs from specific antibody-negative flocks by the allantoic sac route following standard procedure [3]. Hemagglutinating agents (HA) from the inoculated eggs were checked for bacterial contamination using blood agar plate.

Viruses from bacteria-free allantoic fluid were identified using the hemagglutination-inhibition (HI) test with NDV-specific antiserum (IZSVe, Italy) according to standard procedure [3]. In addition, all hemagglutinating allantoic fluids were screened for avian influenza using rRT-PCR and were negative [30]. Two archival NDV isolates from 1973 and 1980 were obtained from the RLAITAD repository. Additional viruses isolated between 2002 and 2015 from the passive and active surveillance were included. The wild bird isolates were provided by Dr. John Ibu [31]. The confirmed NDV isolates (n = 101) were shipped to Southeast Poultry Research Laboratory (SEPRL) of the U.S. Department of Agriculture in Athens, GA, USA (n=57), or the University of Pretoria (UP), South Africa (n=44), for further characterization. The available background information for all studied viruses is presented in Table 1.

Virus propagation, RNA isolation, library preparation

The isolates submitted to SEPRL (n = 57) were propagated in 9-day-old specific-pathogen-free embryonated chicken eggs (Table 1). RNA extraction of 35 samples was conducted using Trizol LS reagent (Invitrogen, USA) and QIAamp® Viral RNA Mini Kit (Qiagen, USA) as described previously [32]. The Nextera XT DNA Library Preparation Kit was used for Next-generation sequencing (NGS) library preparation. Sequencing and data assembly were performed as described previously [32]. Sanger sequencing was conducted on 22 isolates after Trizol extraction as described previously [33] using the following primers: CZ8 4008F, 4994R; CZ9 4715F, 5637R; CZ10b 5410F, Z6369R; NG 4311F, 5090R; NG4938F, 5887R; NG 5721F, 6375R (Table 2; 34). Intracerebral pathogenicity index (ICPI) assay was conducted on five samples at SEPRL following established procedures [3].

The isolates submitted to University of Pretoria (n = 44) were shipped inactivated in MagNA Pure 96 External Lysis Buffer (Roche Diagnostics, Manheim, Germany) and total nucleic acids were extracted on a MagNA Pure robotic system (Roche). Libraries were prepared and sequenced on an Illumina HiScanSQ system as described elsewhere [35] (Table 1).

Dataset sequences collection

The complete fusion (F) gene coding sequences (CDS) and the complete genome sequences of all available class II NDV isolates were downloaded from GenBank [36] as of November 30th, 2018, resulting in two preliminary datasets of 2,372 and 498 sequences, respectively, and the sequences obtained in the current study were also added to the datasets. The sequences in each dataset were aligned using Multiple Alignment with Fast Fourier Transformation (MAFFT v.7.221.3) [37]. For phylogenetic and codon usage studies of all complete genomes, the coding sequences of all six genes were trimmed from the genomes and concatenated together.

Phylogenetic analysis

The generated datasets were used to estimate evolutionary distances between viruses and groups. The estimates of evolutionary distances were inferred using MEGA6 [38,39]. Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). A preliminary phylogenetic analysis was performed to infer the evolutionary history in each dataset (data not shown). Smaller datasets of the complete fusion and complete gene coding sequences (n = 288 and n = 120, respectively; Online Resource 3, 4) including viruses closely related to the Nigerian viruses studied here and representative viruses from other genotypes were parsed from the initial datasets and further analyzed. Maximum-likelihood trees based on general time-reversible (GTR) model were constructed by using RaxML version 8.2.11 [40] with 1,000 bootstrap replicates. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Trees were visualized using FigTree (v.1.4.2) [41]. The Roman numerals presented in the taxa names in the phylogenetic trees represent the respective genotype for each isolate, followed by the GenBank accession number, host name (if available), country of isolation, strain designation and year of isolation. The isolates were classified into genotypes and sub-genotypes following the criteria defined by Diel et al. [42].

Codon usage analysis of NDV complete genomes

Genome-wide analyses were completed for all poultry (n=45) and wild bird NDV (n=35) sequences from genotypes II, XIV, and XVII (n=80), as previously described [43]. Briefly, DAMBE v7.0.35 software package [44,45] was used to calculate relative synonymous codon usage (RSCU) [46], effective number of codons (ENC)

[47], nucleotide composition, and codon adaptation index (CAI) [45]. RSCU is the value of the observed codon number divided by the expected codon number and was determined for each sequence. To determine the relationship among samples (r), Pearson correlation analysis was carried out on the RSCU results, using the GraphPad Prism 6 software (GraphPad Software Inc., USA). The r coefficient was used to determine the presence and magnitude of the correlation, and the two-tailed P test ($P < 0.01$) was used to determine significance.

In addition to RSCU, ENC values, a non-directional measurement that observes the unequal use of codons across the genome, were also determined for each sequence to compare among poultry and wild bird virus sequences. Nucleotide composition affects this measurement, so it is imperative to not only determine ENC values, but to also look at GC and GT composition of the genome region of interest [47]. Therefore, 13,725 positions were analyzed with 4575 nucleotides for each of the three different codon positions; values were given in percent with the total nucleotide count divided by the total count of each specific nucleotide. Additionally, CAI was also calculated for each sequence, which is used to explain any deviation from the expected codon usage and to study codon usage preference of the different NDV isolates compared to the *Gallus gallus* reference set (as implemented in DAMBE, the number of used codons is provided in Online Resource 6) [45].

Accession numbers

The complete fusion gene and complete genome sequences of NDV obtained in this study were submitted to GenBank and are available under the accession numbers KC568204, MH392227, MH082804-MH092818, MH092820-MH082824, and MH996905-MH996993.

RESULTS

A total of 101 samples collected in Nigeria from 2002 to 2015 had confirmed NDV and were sent to SEPRL and UP for characterization. Ten submitted samples (7 at SEPRL and 3 at UP) contained mixed infections and two NDV were identified in each of them, resulting in a total of 111 viruses characterized in this study. Of these mixed infections, five contained two virulent viruses, four contained one vaccine-like virus and one virulent virus and the remaining one contained one lentogenic virus and one virulent virus. Sixty-four viruses were confirmed as NDV at SEPRL from which 42 complete or near-complete genomes and 22 complete F gene CDS were obtained. Forty-seven viruses were confirmed as NDV at UP, 31 near-complete genomes and 16 F gene CDS were obtained. In total, 73 complete or near-complete genomes and 38 F gene CDS were sequenced from the studied viruses.

The geographic spread of all NDV isolates sequenced here shows no relationship between genotype density and poultry density in Nigeria (Fig. 1). However, because of the random and irregular sampling, no statistically significant conclusion can be drawn. Genotype II appears to occur in the more densely poultry populated states (central and southern regions), whereas genotype XIV and XVII are more evenly dispersed throughout the country. This may be attributed to the regular use of live genotype II vaccines in commercial poultry productions in these areas (Shittu et al 2016). Three states had more viruses isolated from them compared to the other states, possibly because locations were nearest to veterinary laboratories.

NDV virulence and/or pathogenicity markers were deduced for all 111 sequenced viruses. Ninety-nine viruses had cleavage site motifs ($_{113}$RQKR↓F$_{117}$, $_{113}$RQRR↓F$_{117}$, and $_{113}$RRKR↓F$_{117}$) specific for virulent viruses based on criteria utilized by the World Organisation for Animal Health (OIE) to assess virulence of NDV [3]. The remaining 12 viruses had cleavage sites specific to viruses of low virulence ($_{113}$RQGR↓L$_{117}$ and $_{113}$KQGR↓L$_{117}$) (Table 1). Pathogenicity evaluation was done for 5 viruses using ICPI. Four of the viruses were selected from genotypes XIV and XVII which are the most widely spread in Africa NDV. The fifth virus was selected because it belongs to genotype VI (typically isolated from Columbiform birds) but was isolated from a quail (a Galliform species). All the viruses tested using the ICPI assay presented values ranging from 1.76 to 1.86, which characterizes them as virulent NDV based on OIE standards [3] and is in agreement with the deduced amino acid cleavage site sequences (Table 1). ICPI values above 0.7 are defined as virulent and above 1.5 are indicative for velogenic NDV [48]. The results of the expanded F-gene phylogenetic are displayed in the figure (Online Resource 1) and demonstrate that viruses from Nigeria sequenced in this study are classified into seven different NDV genotypes. The complete genome coding sequences tree displayed similar topology confirming the phylogenetic classification of the viruses studied here and their relation to other NDV viruses (Online Resource 2).

Sub-genotype Ia. Two viruses sequenced for this study clustered in sub-genotype Ia together with the I-2 vaccine strain (AY935499, nucleotide identity 99.8-99.9%). Both viruses were isolated from chickens in 2009 in Plateau state. Live genotype I vaccines are used in Nigeria [9, pers. comm. from AT Laleye] and the high nucleotide identity of the two new isolates to the vaccine likely reflects isolation of vaccine strain or vaccine spillover. The viruses isolated in this study are not closely related to the sub-genotype Ia virulent viruses causing ND outbreak in Australia in 1998-1999 (nucleotide distance 5.1 %) (Fig. 2.a).

Genotype II. Twelve viruses obtained in this study were classified as members of genotype II in the central and southern regions that have more commercial poultry operations (and subsequently higher use of live genotype II vaccine [9]) (Fig. 2a). Nine viruses were almost identical to the LaSota vaccine strain (AF077761), with nucleotide identities between 99.82 and- 99.94 %. Three of these viruses were isolated from wild birds from 2002 to 2003: a free-flying House Sparrow (*Passer domesticus*) in Benue state, a free-flying White-backed Vulture (*Gyps africanus*) in Plateau state, and a captive African Hawk-eagle (*Aguila spilogaster*) in Plateau state. One virus isolated from a chicken in Ebonyi state in 2013 was similar to the B1 vaccine (JN872151, identity 99.88 %). The remaining two studied viruses from genotype II were related to the mesogenic Komarov strain (KT445901) (Fig. 2a), displaying nucleotide identities of 99.32 and 99.69 %. The first was isolated from a chicken sample collected in 2015 in the FCT, while the second was isolated from an apparently healthy free-flying African Black Kite (*Milvus migrans*) in Plateau state in 2006.

Genotype IV. Two historical NDVs isolated in Oyo and Plateau states sequenced in this study were identified as members of the probably extinct genotype IV. The viruses were isolated from a chicken in 1973 and from a duck in 1980, respectively. The duck virus (duck/Nigeria/Vom/1980/N55/1980) grouped in a separate branch within genotype IV with three more viruses isolated from chickens in Nigeria in 1973. The chicken isolate (chicken/Nigeria/Ibadan/Oyo_state/VRD/1973) clustered into a different monophyletic group within genotype IV with poultry viruses from UK in (1933), Italy (1944), and Bulgaria (1959) (Fig.2a). The mean distance between the viruses in both groups was 6.3 % (Fig. 2a). These sequences obtained from the chicken isolate from Oyo state is the first genotype IV complete genome sequence available from the African continent.

Sub-genotype VIh. Three viruses sequenced here clustered into sub-genotype VIh with pigeon and quail viruses from Nigeria isolated between 2007 and 2013, a laughing dove virus from Kenya from 2012 and virus isolated from a pigeon in Argentina in 1997 (Fig. 2a) with 4.17% mean evolutionary distance between them. One of the newly sequenced viruses was isolated from a quail in Plateau state in 2008, and the other two from pigeon in Jigawa state in 2008 and Lagos state in 2015. Complete genome was also sequenced from the quail virus.

Sub-genotype VIg. One pigeon virus from 2007 isolated in Jigawa state clustered into sub-genotype VIg together with a Nigerian pigeon virus from 2007 and viruses obtained from pigeons, a dove and a mallard from Nigeria, Russia, Ukraine, Pakistan, Kazakhstan, and Egypt between 2005 and 2016 (Fig. 2a). The two Nigerian viruses were very closely related and shared 99.14 nucleotide identity. They were more distant (4.65 % nucleotide distance) to the rest of the viruses in the sub-genotype.

Genotype XIVb. The most widespread group of viruses (Fig. 1) sequenced in this study was sub-genotype XIVb. Twenty-eight viruses were isolated predominantly from domestic bird species (chickens, pigeons, ducks, and a turkey) between 2006 and 2015 from many states, namely Adamawa, Bauchi, Gombe, Imo, Jigawa, Kaduna, Kano, Katsina, Kebbi, Kogi, Kwara, Nassarawa, Plateau, Taraba, Yobe, and Zamfara. One additional virus from sub-genotype XIVb was obtained from a free-flying raptor (unknown species) that was originally banded in Finland but captured in 2009 by a fisherman in Nigeria (Fig. 2b). Interestingly, some of the viruses within sub-genotype XIVb had nucleotide distances of 2 to 3% although being isolated during the same year suggesting co-circulation of variant lineages.

Genotype XVIIa. The most prevalent group with sixty-two viruses sequenced in this study was sub-genotype XVIIa. Most of these NDV were isolated from domestic species (chickens, ducks, guinea fowl, a turkey, a quail, and a pigeon) between 2002 and 2015 over many states (Adamawa, Bauchi, Borno, Gombe, Imo, Jigawa, Kaduna, Kano, Katsina, Kogi, Nassarawa, Plateau, Rivers, Yobe, Zamfara). Two of the viruses were isolated from wild birds: one Malachite Kingfisher (*Corythornis cristatus*) in Adamawa state in 2010 and one White-backed Vulture (*Gyps africanus;* Critically Endangered [49]) in Plateau state in 2002-2003. Interestingly, this vulture's sample showed a mixed infection with both genotype II and XVIIa viruses. Similarly, to sub-genotype XIVb, there were viruses within sub-genotype XVIIa isolated during the same years but displaying high genetic distance between them (2.5 to 3.5 %). The phylogenetic analysis also demonstrates that there are two sub-groups within sub-genotype XVIIa (Fig. 2c) that are 2.42 % distant from each other.

Sub-genotype XVIIIb. One NDV isolated from a chicken in Ogun state in 2009 and sequenced in this study was characterized as member of sub-genotype XVIIIb. The virus clustered with two other Nigerian chicken NDV from

2011, HF969216 and HF969217 (with nucleotide identity of 97.34 and 97.41 %, respectively), and viruses from Ivory Coast, Togo, and Mali from isolated between 2006 and 2010 from chickens, a duck, a finch and a village weaver (2.94 % nucleotide distance) (Fig. 2b).

Codon Usage Analysis

In order to determine if the isolations from wild birds represent spillover events from poultry or the viruses isolated from wild birds were evolutionary adapted to and possibly maintained in these species, we conducted a complete genome comparison of the codon usage of viruses isolated from poultry and wild birds. RSCU values were determined for 80 of the Nigerian virus sequences (genotypes II, XIV, and XVII) isolated from both poultry and wild birds, and the averages for each are reported in Online Resource 5. RSCU values of >1 have a positive codon usage bias and are considered abundantly used codons. Those that have RSCU values <1 have a negative codon usage bias and are used less. Codons with RSCU values of 1 lack any bias and are used equally within the analyzed sequences, which are AUG (M) and UGG (W) that have only one codon for that amino acid. For all sequences studied here, all codons were used similarly in the complete genome among the Nigerian poultry and wild bird viruses (Fig. 3) with a Pearson r coefficient of 0.9987 that was statistically significant ($P<0.01$). Correlation plots with a Pearson r coefficient of 0.5 to 1 have a high correlation, 0.3 to 0.5 have a medium correlation, 0.1 to 0.3 have low correlation, and < 0.1 have no correlation.

In addition to RSCU, ENC values were also determined for those same viruses (Table 3). ENC values range from 20 to 61, with 20 being exceptionally rare, indicating only one synonymous codon for each amino acid was used, where an ENC value of 61 corresponds to the use of all synonymous codons equally [47]. The average ENC value for both poultry and wild bird viruses was 59.9, indicating that all synonymous codons were used equally, and no bias was observed for either group of viruses. To support this finding, nucleotide composition for each group was also determined to be approximately 46% and 47% for GC and GT content, respectively (Table 3). Although there were no deviations from the expected codon usage, CAI was determined for the virus sequences, as an additional confirmatory test, using *Gallus gallus* as the reference [45], and the average for both poultry and wild bird isolated viruses was 0.74 out of 1, which was expected. These same analyses were repeated using 213 fusion gene sequences, and the results were similar, with no observed biases (data not shown).

DISCUSSION

In this study, we used next-generation and Sanger sequencing to obtain complete genomes and complete F genes of NDV isolated from domestic and wild birds in Nigeria from 2002 to 2015 as well as archived viruses from 1973 and 1980. We performed comprehensive phylogenetic analyses to facilitate the better understanding of the distribution and genetic variability of NDV in this country. We added 38 new complete F gene CDS and 73 new complete or near-complete genome sequences to the GenBank. These sequences were used in our codon usage analyses and will also be useful for future evolutionary studies of NDV circulating on the African continent, specifically for genotypes XVII and XVIII in which only 12 complete genomes were available before this study. Phylogenetic analyses of the viruses showed continued circulation of virulent viruses among both domestic and wild birds in Nigeria. The genotypes of the newly characterized viruses correspond to those previously reported in West Africa and there is no evidence of additional introduction of virulent viruses. Snoeck et al [50] recently classified XIV and XVII as new genotypes but only found them in domestic species; this is the first study in which these genotypes were identified in wild birds. The further delineation of XIVb and XVIIa into two separate groups indicates local divergence and evolution of these viruses over time. Furthermore, the subgroups of viruses in genotype IV indicate that Nigeria was also affected by the first NDV panzootic that occurred during the 1930's and 1940's. Indeed, the phylogenetic distance between the two formed branches within genotype IV suggests that once introduced, these viruses persisted locally and circulated until the 1970's and 1980's in Nigeria. The Nigerian viruses clustered into a possible extinct sub-genotype of genotype IV that cannot be classified and named here due to the lack of enough epidemiologically independent isolates (at least four) as per the classification criteria put forth by Diel et al. [42].

Some genotypes were more widespread (XIVb and XVIIa) and others clustered in the southern or central regions (II and XVIIIb). However, there was no significant relationship between poultry density and number of NDV isolates present in a region. We suspect that this may be an artefact of biased data (e.g., outdated poultry population data and the inconsistent surveillance activities over many years). A recent report [8] suggests that Sokoto state is a potential hotspot of different NDV genotypes, which contradicts our widespread distribution of genotypes across multiple states and Plateau state having the largest diversity of genotypes. The identification of NDV of genotypes XIV and XVII in Nigeria and West Africa over a period of 14 years period is indicative that these viruses became established in the country and the region, but these viruses have not been reported elsewhere in

the continent or in other world regions. The higher nucleotide distances identified between viruses isolated in the same years from within sub-genotypes XIVb and XVIIa and between the two monophyletic branches within sub-genotype XVIIa provide enough evidence to suggest that these viruses continuously evolve locally as a result of active transmission. Despite the identified evolution and cross-species transmission of these viruses, no evidence of codon usage adaptation in wild birds compared to poultry was detected, confirming that codon adaptation in NDV is a slow process [43].

In a previous study, active surveillance of wild birds resulted in 13 NDV with varying degrees of virulence based on mean death time (MDT), intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI) and a triple one-step reverse transcription polymerase chain reaction (RT-PCR) [31]. These results showed that the isolate from the White-backed Vulture was mesogenic and the Black Kite, House Sparrow, and African-Hawk Eagle isolates were lentogenic. In our sequencing results, we documented that the White-backed Vulture isolate had two viruses, both virulent and of low virulence, and the Black Kite virus was virulent. These differences may be a result in the mixed infection of two viruses in the vulture sample that may affect the ICPI obtained by Ibu et al. [31] or the advances in technology and higher sensitivity of random deep sequencing used here.

Genotype VI is present in Nigeria with sub-genotypes VIh and VIg continuing to circulate in pigeons. Additionally, this study provides the first isolation of a VIh virus from a quail and the first complete genome available for this sub-genotype. It is known that pigeons may possibly play an important role in the transmission of genotype VI viruses to poultry, which proves to be an interesting case when considering the relatedness of viruses in distant countries, especially considering that pigeons are a non-migratory species [51].

Poultry health is of high concern around the world, thus NDV surveillance is currently more focused on domesticated species than wild birds. Therefore, NDV in wild birds is not well documented as there may be many undocumented cases of wild bird mortalities, morbidity, or clinically healthy individuals that harbor the virus. This study is indicative of a possible diversity of NDV in wild bird species in Nigeria. This highlights the need of conducting a wide-scale NDV surveillance in wild bird populations in the region to identify epidemiological relationships and possible ecological costs of the circulation of NDV in the wild avifauna. White-backed Vultures, African Hawk Eagles, and Malachite Kingfishers have not been previously reported to be infected with any pathotype of NDV. While raptors have been shown to become infected by eating diseased chickens and scavenge off mortalities and this mode of spread to wild birds is a viable option, we have no specific evidence to suggest it for our

isolations. Our study provides the first isolation and complete NDV genomes from these species. Alternatively, NDV has been confirmed in Black Kites in Switzerland [52] and Nigeria [53]; however, the virus obtained here from a Black Kite is the first example of a mesogenic vaccine virus detected in a wild bird. House Sparrows have multiple studies documenting NDV, including experimentally infected birds from Sudan [54] and isolates sequenced of class II, genotypes VII and II in China [55].

Close contact between free-ranging wild birds and backyard poultry regularly occurs at the livestock-wildlife interface [29], thus highlighting an important point for NDV transmission. This study provides evidence for reverse-spillover of a mesogenic vaccine virus (Komarov vaccine) used in Nigeria [8,9] to wild birds (African Black Kite). The identified nucleotide distance between the vaccine and the isolate suggests further circulation and evolution after the spillover indicating that such transmission events may have prolonged effect on wild avifauna. Mesogenic viruses have cleavage site motifs and ICPI values that classify them as virulent according to the definitions outlined by OIE; therefore, some countries have specified that only lentogenic NDV strains can be used as vaccines [3,56]. Spillover of LaSota vaccine virus is also documented, which has been previously documented in other countries [15]. While the lentogenic strains are expected to have low or no adverse effects on wild birds, the effects of the mesogenic vaccine virus (Komarov strain) in wild birds may be of higher cost. Mixed infections in wild birds may be another point of concern, since an infection with two pathogens may increase possibility of disease; however, our study shows a single case where we observed an apparently healthy, free-flying White-backed Vulture (*Gyps africanus*) that showed a mixed infection with genotype II and XVIIa. Although random individuals were sampled, ND may have great effects on and is likely under sampled in wild bird populations, e.g., undetected infected or mortalities or migratory movements out of the study area.

These reverse-spillover events are likely to increase as poultry production and the vaccination against viral threats increase across the globe [14-19]. In Nigeria, like many other countries, free-ranging chickens have no borders or measures protecting themselves against NDV transmission to/from wild birds [57]. Many of these backyard farms may not have as intensive vaccination or biosecurity protocols in comparison to commercial operations [23]. This is especially significant if the lack of complete effectiveness of current vaccines and the fact that workers at commercial poultry farms typically have backyard poultry at their household are considered [31]. Thus, if a pathogen enters the backyard flock it can be devastating; Soos et al [58] showed that backyard chickens are significantly more likely to show clinical signs, which is correlated with active shedding of the disease agent

[59]. We suggest further investigation into the epidemiology of NDV in free-range poultry farms in order to identify points of transmission and to provide information on virus diversity to support planning of optimal disease control.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURES

Fig. 1 Map representing Newcastle disease viruses isolated for this study per state in Nigeria from 1973 to 2015 and the poultry population density for each state (gray shading). The pie charts include the various genotypes isolated from each state and the size being proportional to the total number of viruses from each state. The darkest shaded states indicate the states with the highest poultry population density

Fig. 2 Maximum Composite Likelihood tree constructed with complete fusion gene coding sequences: (2.1) with genotypes XVII, XIV, and XVIII, (2.2) with genotypes XVII, VI, I, II, IV, (2.3) with genotypes XIV, XVIII, VI, I, II, IV collapsed for convenience and imaging purposes. Genotypes and sub-genotypes of viruses are presented with Roman numerals and lowercase letters in each taxa name. Red lettering indicates viruses sequenced for this study, with the asterisk (*) highlighting the birds with mixed infections

Fig. 3 High correlation of relative synonymous codon usage (RSCU) in the complete genome among poultry and wild bird viruses. The average RSCU value for each codon in the complete NDV genome from viruses of genotype II, XIV, and XVII was used to assess the correlation of codon usage among poultry (X axis) and wild bird viruses (Y axis). A Pearson r coefficient of 0.9987 was observed indicating high correlation that was statistically significant ($P<0.01$)

TABLES

Table 1. List of viruses sequenced in this study, their respective genotype, ICPI values (if available), host species, location and year of sample collection and type of sequencing and where it was sequenced (SEPRL = Southeast Poultry Research Laboratory, USA; UP = University of Pretoria, South Africa). Host birds with mixed infections are distinguished with underline text (n=10)

Table 2. Sanger primers used for fusion gene sequencing of 18 samples

Table 3. Codon usage values indicate no significant difference among poultry and wild bird viruses

LIST OF ELECTRONIC SUPPLEMENTARY FILES

Online Resource 1 Maximum Composite Likelihood tree with no collapsed branches constructed with complete fusion gene coding sequences. Genotypes and sub-genotypes of viruses are presented with Roman numerals and lowercase letters in each taxa name.

Online Resource 2 Maximum Composite Likelihood tree constructed with complete genome coding sequences. Genotypes and sub-genotypes of viruses are presented with Roman numerals and lowercase letters in each taxa name. Red lettering indicates viruses sequenced for this study

Online Resource 3 List of sequences used for the maximum composite likelihood tree constructed with complete fusion gene coding sequences (Fig. 2, Online Resource 1). Bolded isolates were sequenced for this study

Online Resource 4 List of sequences used for the maximum composite likelihood tree constructed with complete gene coding sequences (Online Resource 2). Bolded isolates were sequenced for this study

Online Resource 5 Complete genome relative synonymous codon usage (RSCU) values among poultry and wild bird viruses

Online Resource 6 Number of codons used in the reference *Gallus gallus* dataset as implemented in DAMBE

Figure 1

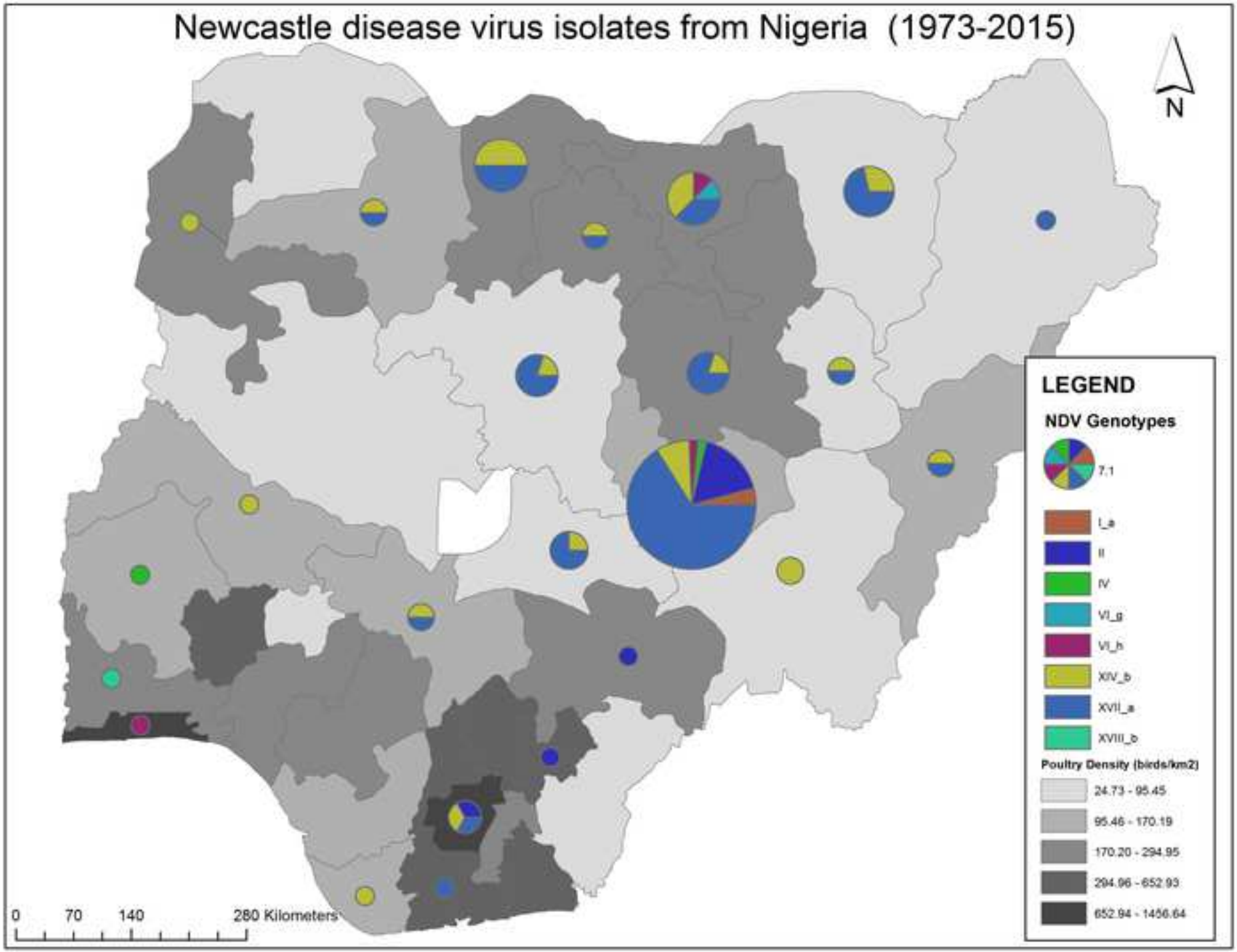
Newcastle disease virus isolates from Nigeria (1973-2015)
N
LEGEND
NDV Genotypes
7.1
I_a
II
IV
VI_g
VI_h
XIV_b
XVII_a
XVIII_b
Poultry Density (birds/km2)
24.73 - 95.45
95.46 - 170.19
170.20 - 294.95
294.96 - 652.93
652.94 - 1456.64
0
70
140
280 Kilometers

Figure 2a

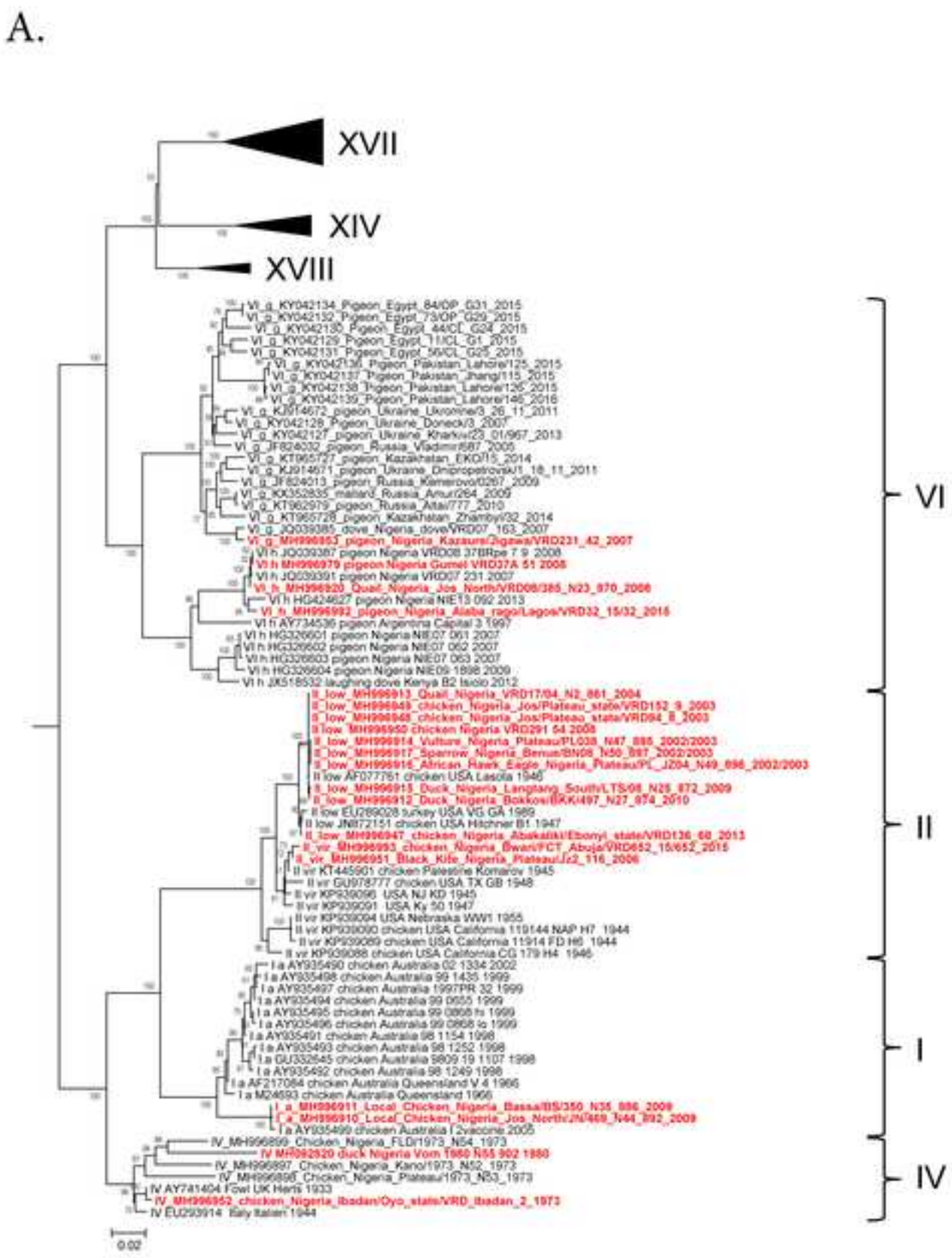

Figure 2b

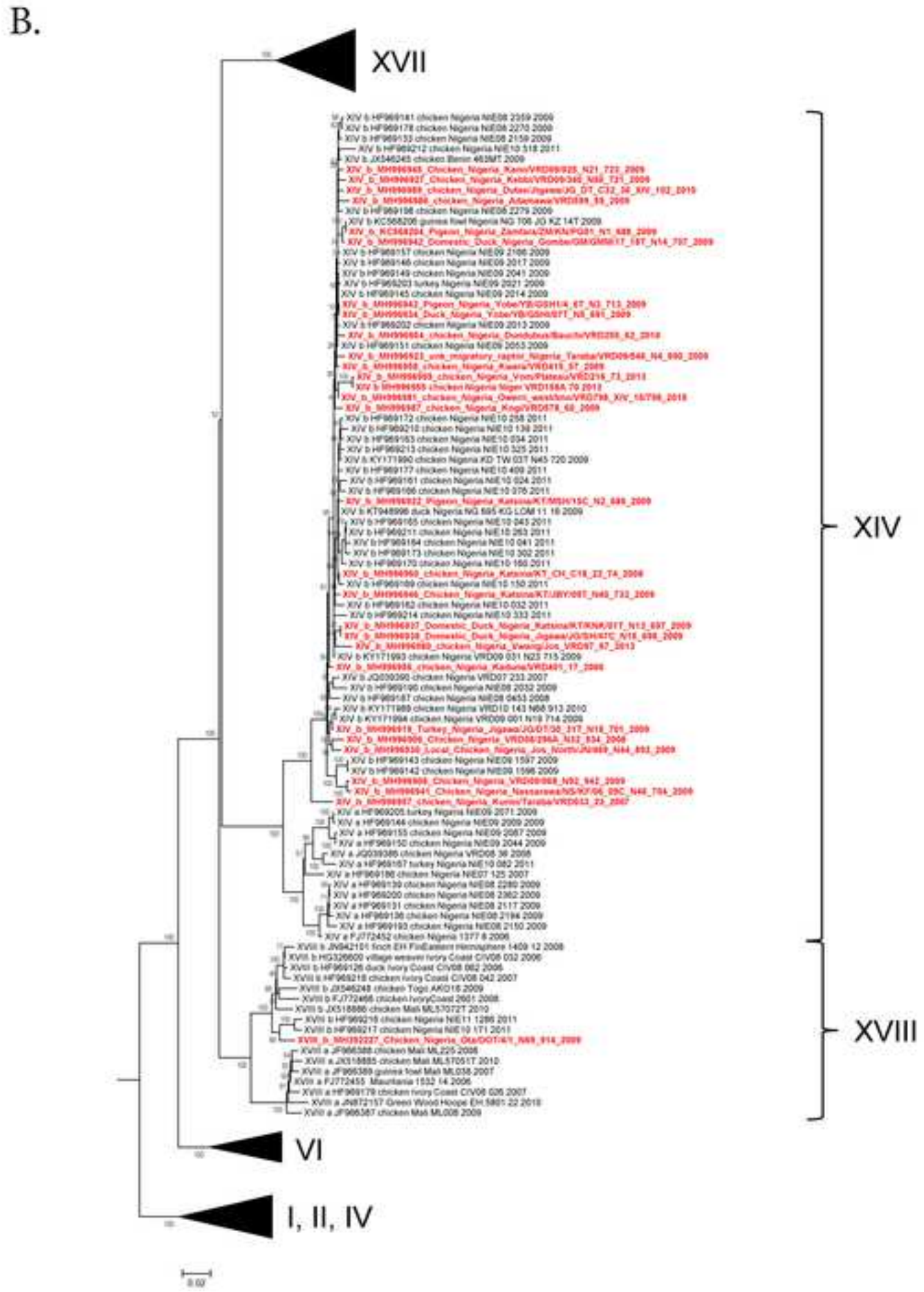

Figure 2c

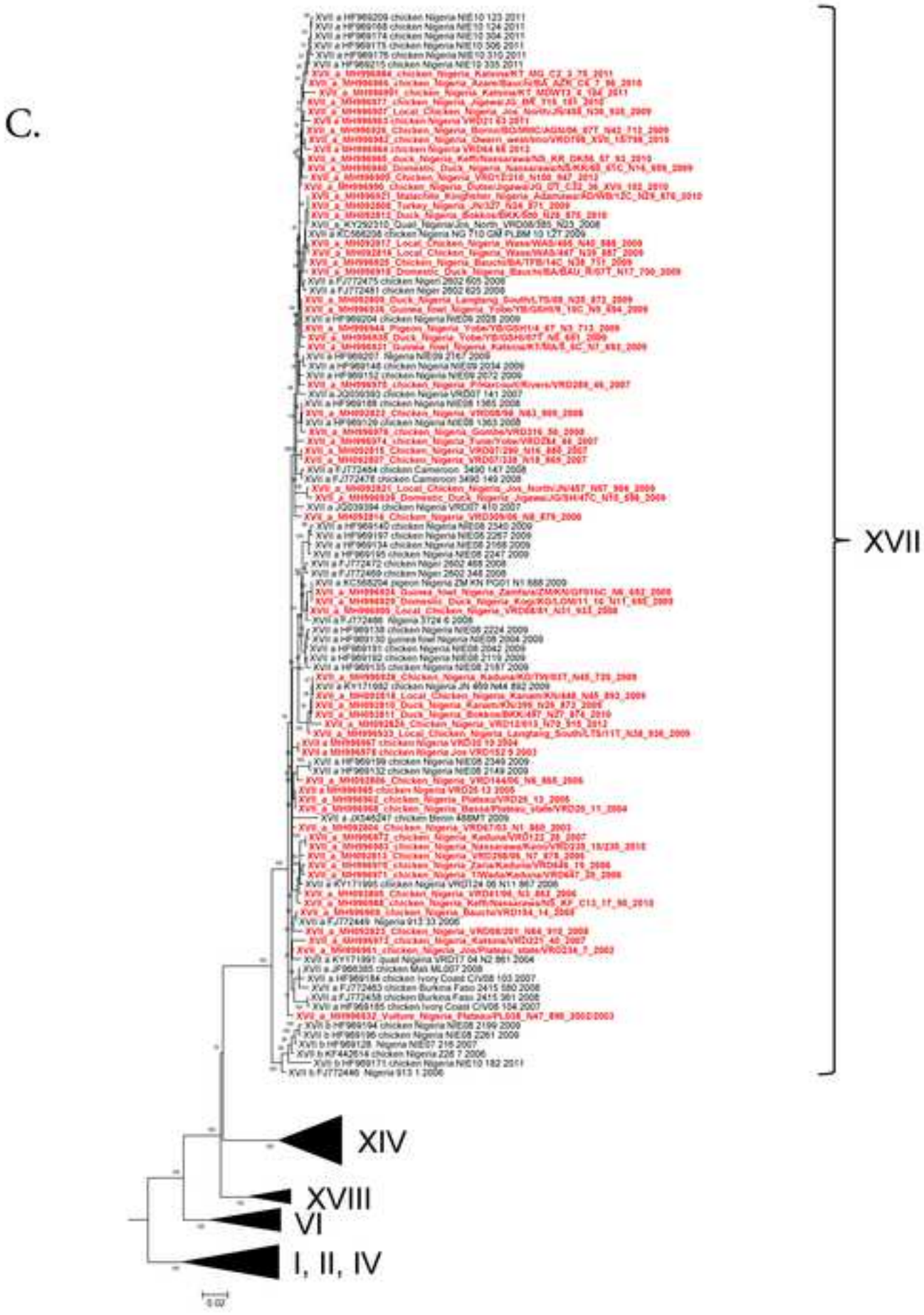

Figure 3

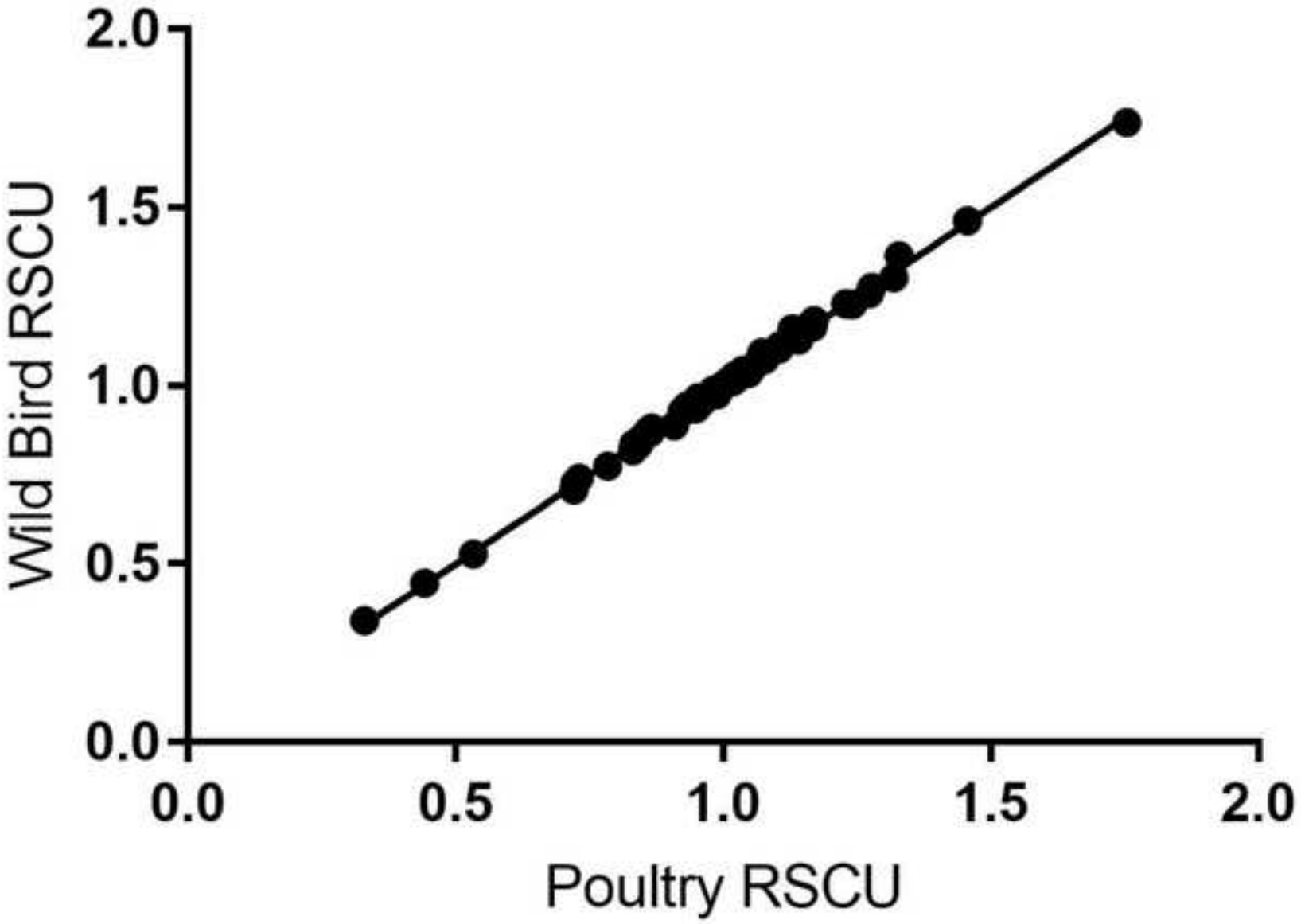

Table 1. List of viruses sequenced in this study, their respective genotype, ICPI values (if available), host species, location and year of sample collection and type of sequencing and where it was sequenced (SEPRL = Southeast Poultry Research Laboratory, USA; UP = University of Pretoria, South Africa). Host birds with mixed infections are distinguished with underline text (n=10)

GenBank #	Sub/ Genotype	Host	Location	Isolate name	Lab ID	Year/date of isolation	Cleavage site	ICPI value	Sequenced at
MH996911	I a	Chicken	Bassa,Plateau state	BS/350 (N35)	886	2009	KQGRL		SEPRL
MH996910	I a	Chicken	Jos North,Plateau state	JN/469 (N44)	892	2009	KQGRL		SEPRL
MH996951	II vir	African Black Kite (*Milvus migrans*)	Plateau state	Jz2	116	13/3/2006	RQKRF		UP
MH996912	II_low	Duck	Bokkos,Plateau state	BKK/497 (N27)	874	2010	RQGRL		SEPRL
MH996917	II_low	House Sparrow (*Passer domesticus*)	Benue state	BN08 (N50)	897	2002/2003	RQGRL		SEPRL
MH996915	II_low	Duck	Langtang South,Plateau state	LTS/08 (N25)	872	2009	RQGRL		SEPRL
MH996916	II_low	African Hawk-eagle *(Aguila spilogaster*)	Plateau state	PL JZ04 (N49)	896	2002/2003	RQGRL		SEPRL
MH996914	II_low	White-backed Vulture (*Gyps africanus*)	Plateau state	PL038 (N47)	895	2002/2003	RQGRL		SEPRL
MH996947	II_low	Chicken	Abakaliki,Ebonyi state	VRD136	68	2/8/2013	RQGRL		UP
MH996949	II_low	Chicken	Jos,Plateau state	VRD152	9	2003	RQGRL		UP
MH996913	II_low	Quail	Nigeria	VRD17/04 (N2)	861	2004	RQGRL		SEPRL
MH996950	II_low	Chicken	Nigeria	VRD291	54	2008	RQGRL		UP
MH996948	II_low	Chicken	Jos,Plateau state	VRD94	8	13/7/2003	RQGRL		UP
MH996993	II_vir	Chicken	Bwari, FCT,Abuja state	VRD652	15/652	27/3/2015	RQKRF		UP
MH092820	IV	Duck	Nigeria,Plateau state	Vom_1980_N55	902	1980	RQRRF		SEPRL
MH996952	IV	Chicken	Ibadan,Oyo state	VRD Ibadan	2	1973	RQRRF		UP
MH996953	VI g	Pigeon	Kazaure,Jigawa state	VRD231	42	30/3/2007	RQKRF		UP
MH996920	VI h	Quail	Jos North,Plateau state	VRD08/385 (N23)	870	2008	RRKRF	1.76	SEPRL
MH996992	VI h	Pigeon	Alaba rago,Lagos state	VRD32	15/32	17/1/2015	RRKRF		UP
MH996979	VI h	Pigeon	Gumel,Jigawa state	VRD37A	51	2008	RRKRF		UP
MH996942	XIV b	Duck	Gombe state	GM/GMM/17-18T (N14)	707	2009	RRKRF		SEPRL
MH996919	XIV b	Turkey	Jigawa state	JG/DT/30-31T (N18)	701	2009	RRKRF		SEPRL
MH996938	XIV b	Duck	Jigawa state	JG/SH/47C (N15)	698	2009	RRKRF		SEPRL
MH996989	XIV b	Chicken	Dutse,Jigawa state	JG-DT-C32-36-XIV	102	28/8/2010	RRKRF		UP
MH996930	XIV b	Chicken	Jos North,Plateau state	JN/469 (N44)	892	2009	RRKRF		SEPRL
MH996946	XIV b	Chicken	Katsina state	KT/JBY/09T (N40)	733	2009	RRKRF		SEPRL
MH996937	XIV b	Duck	Katsina state	KT/KNK/01T (N13)	697	2009	RRKRF		SEPRL
MH996922	XIV b	Pigeon	Katsina state	KT/MSH/15C (N2)	689	2009	RRKRF		SEPRL
MH996960	XIV b	Chicken	Katsina state	KT-CH-C18-22	74	12/12/2008	RRKRF		UP
MH996941	XIV b	Chicken	Nassarawa state	NS/KF/06-09C (N46)	704	2009	RRKRF		SEPRL
MH996957	XIV b	Chicken	Kurmi,Taraba state	VRD033	23	14/2/2007	RRKRF		UP
MH996906	XIV b	Chicken	Nigeria,	VRD08/296A (N32)	934	2008	RRKRF		SEPRL
MH996945	XIV b	Chicken	Kano state	VRD09/025 (N21)	723	2009	RRKRF		SEPRL
MH996908	XIV b	Chicken	Nigeria,	VRD09/068 (N92)	942	2009	RRKRF		SEPRL
MH996927	XIV b	Chicken	Kebbi state	VRD09/340 (N50)	721	2009	RRKRF		SEPRL
MH996923	XIV b	Migratory raptor (unknown species)	Taraba state	VRD09/546 (N4)	690	2009	RRKRF		SEPRL
MH996955	XIV b	Chicken	Nigeria,	VRD158A	70	23/8/2013	RRKRF		UP
MH996959	XIV b	Chicken	Vom,Plateau state	VRD216	73	22/8/2013	RRKRF		UP

MH996954	XIV b	Chicken	Dundubus,Bauchi state	VRD255	62	23/7/2010	RRKRF		UP
MH996956	XIV b	Chicken	Kaduna state	VRD401	17	15/6/2006	RRKRF		UP
MH996958	XIV b	Chicken	Kwara state	VRD415	57	28/9/2009	RRKRF		UP
MH996987	XIV b	Chicken	Kogi state	VRD578	60	21/12/2009	RRKRF		UP
MH996986	XIV b	Chicken	Adamawa state	VRD599	59	21/12/2009	RRKRF		UP
MH996981	XIV b	Chicken	Owerri-west,Imo state	VRD798-XIV	15/798	4/2015	RRKRF		UP
MH996980	XIV b	Chicken	Vwang, Jos,Plateau state	VRD97	67	30/5/2013	RRKRF		UP
MH996943	XIV b	Pigeon	Yobe state	YB/GSH1/4-6T (N3)	713	2009	RRKRF		SEPRL
MH996934	XIV b	Duck	Yobe state	YB/GSHI/07T (N5)	691	2009	RRKRF	1.84	SEPRL
KC568204	XIV b	Pigeon	Zamfara state	ZM/KN/PG01 (N1)	688	2009	RRKRF		SEPRL
MH996921	XVII a	Malachite Kingfisher (*Corythornis cristatus*)	Adamawa state	AD/WB/12C (N29)	876	2010	RQKRF		SEPRL
MH996918	XVII a	Duck	Bauchi state	BA/BAU-R/07T (N17)	700	2009	RQKRF		SEPRL
MH996925	XVII a	Chicken	Bauchi state	BA/TFB/14C (N38)	711	2009	RQKRF		SEPRL
MH996966	XVII a	Chicken	Azare,Bauchi state	BA-AZR-C6-7	96	3/3/2010	RQKRF		UP
MH092811	XVII a	Duck	Bokkos,Plateau state	BKK/497 (N27)	874	2010	RQKRF		SEPRL
MH092812	XVII a	Duck	Bokkos,Plateau state	BKK/500 (N28)	875	2010	RQKRF		SEPRL
MH996926	XVII a	Chicken	Borno state	BO/MMC/AGN/06-07T (N42)	712	2009	RQKRF		SEPRL
MH996939	XVII a	Duck	Jigawa state	JG/SH/47C (N15)	698	2009	RQKRF		SEPRL
MH996977	XVII a	Chicken	Jigawa state	JG-BR-T15	101	17/3/2010	RQKRF		UP
MH996990	XVII a	Chicken	Dutse,Jigawa state	JG-DT-C32-36-XVII	102	28/8/2010	RQKRF		UP
MH092808	XVII a	Turkey	Nigeria,	JN/327 (N24)	871	2009	RQKRF		SEPRL
MH092821	XVII a	Chicken	Jos North,Plateau state	JN/457 (N57)	904	2009	RQKRF		SEPRL
MH996907	XVII a	Chicken	Jos North,Plateau state	JN/458 (N36)	935	2009	RQKRF		SEPRL
MH996928	XVII a	Chicken	Kaduna state	KD/TW/03T (N45)	720	2009	RQKRF		SEPRL
MH996929	XVII a	Duck	Kogi state	KG/LOM/11-16 (N11)	695	2009	RQKRF		SEPRL
MH092810	XVII a	Duck	Kanam,Plateau state	KN/399 (N26)	873	2009	RQKRF		SEPRL
MH092818	XVII a	Chicken	Kanam,Plateau state	KN/448 (N45)	893	2009	RQKRF		SEPRL
MH996931	XVII a	Guinea fowl	Katsina state	KT/MA/5-6C (N7)	693	2009	RQKRF	1.86	SEPRL
MH996991	XVII a	Chicken	Katsina state	KT-MDWT3-4	104	18/8/2011	RQKRF		UP
MH996984	XVII a	Chicken	Katsina state	KT-MG-C2-3	75	18/8/2011	RQKRF		UP
MH092809	XVII a	Duck	Langtang South,Plateau state	LTS/08 (N25)	872	2009	RQKRF		SEPRL
MH996933	XVII a	Chicken	Langtang South,Plateau state	LTS/11T (N38)	936	2009	RQKRF		SEPRL
MH996940	XVII a	Duck	Nassarawa state	NS/KR/60-61C (N16)	699	2009	RQKRF	1.85	SEPRL
MH996988	XVII a	Chicken	Keffi,Nassarawa state	NS-KF-C13-17	90	4/11/2010	RRKRF		UP
MH996965	XVII a	Duck	Keffi,Nassarawa state	NS-KR-DK56-57	93	4/11/2010	RQKRF		UP
MH996932	XVII a	White-backed Vulture (*Gyps africanus*)	Plateau state	PL038 (N47)	895	2002/2003	RQKRF		SEPRL
MH092815	XVII a	Chicken	Nigeria	VRD07/290 (N16)	880	2007	RQKRF		SEPRL
MH092807	XVII a	Chicken	Nigeria	VRD07/338 (N18)	869	2007	RQKRF		SEPRL
MH092823	XVII a	Chicken	Nigeria	VRD08/201 (N64)	910	2008	RQKRF		SEPRL
MH996905	XVII a	Chicken	Nigeria	VRD08/81 (N31)	933	2008	RQKRF		SEPRL
MH092822	XVII a	Chicken	Nigeria	VRD08/98 (N63)	909	2008	RQKRF		SEPRL
MH092824	XVII a	Chicken	Nigeria	VRD12/013 (N70)	915	2012	RQKRF		SEPRL
MH996909	XVII a	Chicken	Nigeria	VRD12/210 (N100)	947	2012	RQKRF		SEPRL
MH996972	XVII a	Chicken	Kaduna state	VRD122	28	2007	RRKRF		UP
MH092806	XVII a	Chicken	Nigeria	VRD144/06 (N6)	865	2006	RRKRF		SEPRL
MH996978	XVII a	Chicken	Jos,Plateau state	VRD152	9	2003	RQKRF		UP

MH996969	XVII a	Chicken	Bauchi state	VRD154	14	2005	RQKRF		UP
MH996968	XVII a	Chicken	Bassa,Plateau state	VRD20	11	2004	RQKRF		UP
MH996963	XVII a	Chicken	Nigeria	VRD21	63	5/11/2011	RQKRF		UP
MH996973	XVII a	Chicken	Katsina state	VRD221	40	15/3/2007	RQKRF		UP
MH996961	XVII a	Chicken	Jos,Plateau state	VRD234	7	15/5/2002	RQKRF		UP
MH996983	XVII a	Chicken	Nassarawa,Kano state	VRD235	15/235	13/2/2015	RRKRF		UP
MH996985	XVII a	Chicken	Nigeria	VRD25	12	2005	RQKRF		UP
MH996962	XVII a	Chicken	Plateau state	VRD26	13	2005	RQKRF		UP
MH996974	XVII a	Chicken	Fune,Yobe state	VRD284	44	17/4/2007	RQKRF		UP
MH996975	XVII a	Chicken	P/Harcourt,Rivers state	VRD289	46	17/4/2007	RQKRF		UP
MH092813	XVII a	Chicken	Nigeria	VRD298/06 (N7)	878	2006	RQKRF		SEPRL
MH996967	XVII a	Chicken	Nigeria	VRD30	10	2004	RQKRF		UP
MH092814	XVII a	Chicken	Nigeria	VRD309/06 (N8)	879	2006	RQKRF		SEPRL
MH996976	XVII a	Chicken	Gombe state	VRD316	56	11/6/2008	RQKRF		UP
MH092805	XVII a	Chicken	Nigeria	VRD41/06 (N3)	862	2006	RQKRF		SEPRL
MH996964	XVII a	Chicken	Nigeria	VRD64	66	2012	RQKRF		UP
MH996970	XVII a	Chicken	Zaria,Kaduna state	VRD646	19	2/11/2006	RQKRF		UP
MH996971	XVII a	Chicken	T/Wada,Kaduna state	VRD647	20	2/11/2006	RQKRF		UP
MH092804	XVII a	Chicken	Nigeria,	VRD67/03 (N1)	860	2003	RQKRF		SEPRL
MH996982	XVII a	Chicken	Owerri-west,Imo state	VRD798-XVII	15/798	4/2015	RQKRF		UP
MH092816	XVII a	Chicken	Wase,Plateau state	WAS/447 (N39)	887	2009	RQKRF		SEPRL
MH092817	XVII a	Chicken	Wase,Plateau state	WAS/465 (N40)	888	2009	RQKRF		SEPRL
MH996944	XVII a	Pigeon	Yobe state	YB/GSH1/4-6T (N3)	713	2009	RQKRF		SEPRL
MH996935	XVII a	Duck	Yobe state	YB/GSHI/07T (N5)	691	2009	RQKRF		SEPRL
MH996936	XVII a	Guinea fowl	Yobe state	YB/GSHI/9-10C (N9)	694	2009	RQKRF		SEPRL
MH996924	XVII a	Guinea fowl	Zamfara state	ZM/KN/GF01bC (N6)	692	2009	RQKRF	1.86	SEPRL
MH392227	XVIII b	Chicken	Ota,Ogun state	OOT/4/1 (N69)	914	2009	RQKRF		SEPRL

vir = virulent
low = low virulence

Table 2. Sanger primers used for fusion gene sequencing of 18 samples

Primer name	Primer Sequence (5′–3′)
CZ8 4008F	ATA TCG GGC TTA TGT CCA CTG
CZ8 4994R	CTT AAG CCG GAG GAT GTT GGC
CZ9 4715F	TCT CAG ACA GGG TCA ATC
CZ9 5637R	AAG CTG ACG TAT TGC CGC TCA
CZ10b 5410F	GAA TTT GCC CTC AGT CGG GA
CZ10b Z6369R	GTG GCT CCT CTG ACC GTT CTA
NG 4311F	CGT GCT GTY GCA GTG ACY G
NG 5090R	CGT CGG TGA CCT CAT GCA C
NG 4938F	GGC AGT GTA GCT CTT GGG G
NG 5887R	GAG CCT CAG AGT TAT CCC GTC
NG 5721F	CGC CAT ATA TGG CCC TCA AAG G
NG 6375R	CTC YGA YCG TTC TAC CCG

Table 3. Codon usage values indicate no significant difference among poultry and wild bird viruses

	Genomes	ENC Average	ENC SD	GC%	GT%	CAI Average	CAI SD
Poultry	45	59.91	0.1725	46.3	47.7	0.7401	0.0019
Wild Birds	35	59.92	0.1481	46.2	47.7	0.7398	0.0023

ENC = Effective Number of Codons
SD = standard deviation
CAI = Codon Adaption Index