High Genetic Diversity of Newcastle Disease Virus in Poultry in West and Central Africa: Cocirculation of Genotype XIV and Newly Defined Genotypes XVII and XVIII

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Despite rampant Newcastle disease virus (NDV) outbreaks in Africa for decades, the information about the genetic characteristics of the virulent strains circulating in West and Central Africa is still scarce. In this study, 96 complete NDV fusion gene sequences were obtained from poultry sampled in Cameroon, Central African Republic, Côte d’Ivoire, and Nigeria between 2006 and 2011. Based on rational criteria recently proposed for the classification of NDV strains into classes, genotypes, and subgenotypes, we revisited the classification of virulent strains, in particular those from West and Central Africa, leading to their grouping into genotype XIV and newly defined genotypes XVII and XVIII, each with two subgenotypes. Phylogenetic analyses revealed that several subgenotypes are found in almost every country. In Cameroon, most strains were related to vaccine strains, but a single genotype XVII strain was also found. Only three highly similar genotype XVII strains were detected in Central African Republic. Subgenotypes XVIIa, XVIIia, and XVIIib cocirculated in Côte d’Ivoire, while subgenotypes XIVa, XIVb, XVIIa, XVIIib, and XVIIIb were found in Nigeria. While these genotypes are so far geographically restricted, local and international trade of domestic and exotic birds may lead to their spread beyond West and Central Africa. A high genetic diversity, mutations in important neutralizing epitopes paired with suboptimal vaccination, various levels of clinical responses of poultry and wild birds to virulent strains, strains with new cleavage sites, and other genetic modifications found in these genotypes tend to undermine and complicate NDV management in Africa.

Newcastle disease, caused by virulent Newcastle disease virus (NDV), is one of the most important diseases in poultry worldwide. This viral pathogen is also a major challenge for the commercial and traditional poultry industries in West Africa (1).

NDV is a member of the Avulavirus genus of the Paramyxoviridae family, subfamily Paramyxovirinae (2). Its single-stranded RNA genome is composed of 6 genes, 3'-NP-P-M-F-HN-L-5', encoding 6 major proteins and a V protein resulting from mRNA editing of the P gene (3). The hemagglutinin-neuraminidase (HN) and the fusion (F) proteins are both glycoproteins expressed at the surface of the enveloped virus. They mediate attachment of the viral particle to sialic acid-containing cell receptors, its fusion with the plasma cell membrane, and the release of progeny virions from the surface of infected cells (4). Both proteins also induce virus-neutralizing antibody responses (5).

NDV strains differ in genome length. The smallest NDV genomes are 15,186 nucleotides (nt) long, but some genomes are longer due to insertions of either 6 nucleotides in the 5' noncoding region of the NP gene (6) or 12 nucleotides in the P gene (7). NDV strains are genetically highly diverse, and their variability continues to unfold (8, 9). Two nomenclature systems have been proposed and are currently used. The first one, separating NDV strains into two classes and several genotypes, was based on restriction site mapping, genome length, and F gene sequences (7, 10). The second, dividing NDV strains into six lineages, was based on phylogenetic analyses performed on partial F gene sequences (11).

Several pathotypes (asymptomatic enteric, lentogenic, mesogenic, viscerotropic velogenic, or neurotropic velogenic) of NDV are recognized depending on the clinical symptoms observed in chickens (12). Avirulent and virulent strains may also be distinguished on the basis of the cleavage site sequence of their F protein. During replication, the fusion gene is translated into a precursor protein, F0, that must be cleaved by host cell proteases into F1 and F2 subunits for viral particles to become infectious (13). Most virulent strains exhibit the consensus sequence 112(R/K)RQ(R/K)R*F117 (asterisks represent the site of cleavage of the precursor protein F0 into its F1 and F2 subunits) at the cleavage site of the F0 precursor, in contrast to 112(G/E) (K/R)Q(G/E)R*F117 in avirulent viruses (12, 14). The additional basic amino acids in the virulent viruses allow the F0 precursor to be cleaved by ubiquitous proteases, such as furin-like enzymes, present in a wide range of...
cells. Thus, virulent viruses have the ability to replicate in a range of tissues and organs, causing fatal systemic infections (15).

The first records of virulent NDV in West and Central Africa date from the 1950s (16). Since then, several viruses from different outbreaks have been investigated by pathogenicity tests (17, 18), but molecular characterization of these viruses has started only recently (19). Based on partial F gene sequences, we have previously classified West African strains (from Niger, Nigeria, and Burkina Faso) into three new sublineages, 5f, 5g, and 5h (19), while similar full-length F gene sequences from Africa were assigned to a new lineage, lineage 7 (20), creating some confusion. Here, we report 96 additional full-length F gene sequences of NDV detected during surveillance in Nigeria, Cameroon, Central African Republic, and Côte d’Ivoire between 2006 and 2011, providing further insights into the genetic diversity of the circulating NDV strains. With this additional genetic information provided, we revisited all full-length F gene sequences available in public databases and updated the recent classification. Based on objective classification criteria recently proposed by Diel et al. (9), we classified the strains circulating in West and Central Africa into genotypes XIV and newly defined genotypes XVII and XVIII, each with two subgenotypes.

MATERIALS AND METHODS

Sample information. A total of 3,610 domestic birds (mainly chickens but also ducks, geese, guinea fowls, and turkeys) were sampled in a variety of locations, including free ranges, live-poultry markets, and backyard and commercial farms, between 2006 and 2011 in four countries in West and Central Africa (Tables 1 and 2 and Fig. 1). Except in Côte d’Ivoire, where only sick birds were sampled, all other samples were collected during active surveillance where birds were sampled regardless of clinical symptoms. The vast majority of collected material consisted of pooled tracheal and cloacal swabs, but cloacal or tracheal swabs, feces, or organs (lung, liver, intestine, trachea, spleen, and brain) were also included. All samples were shipped on dry ice to Luxembourg, where NDV detection and sequencing were performed.

RNA extraction, PCRs, and sequencing. All swabs and fecal samples were discharged in 500 μl of virus transport medium (VTM) (21). RNA was purified from 140 μl of VTM with the QIAamp viral RNA minikit (Qiagen, Venlo, The Netherlands) or from 50 μl of virus transport medium using the MagMAX-96 AI/ND viral RNA isolation kit (Life Technologies, Merelbeke, Belgium) with Thermo Electron’s KingFisher (Thermo Fisher, Waltham, MA) by following the manufacturers’ instructions. Approximately 30 mg of organs was homogenized with stainless steel beads (Qiagen) and a TissueLyser II (Qiagen) when multiple bands were visible. Sequencing was performed in both orientations using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). Primers used for generating the PCR fragments were listed in Table S2 in the supplemental material. All PCR products are available in Table S2 in the supplemental material, and RT-PCR and PCR conditions are available in Table S1 in the supplemental material. All PCR products were visualized on a 1.5% agarose gel stained with SYBR Safe DNA gel stain (Life Technologies) and purified using the JetQuick PCR purification spin kit (Genomed, Loehne, Germany) or QiAquick gel extraction kit (Qiagen) when multiple bands were visible. Sequencing was performed in both orientations using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies) and ABI 3130 Avant capillary sequencer (Applied Biosystems). Primers used for generating the PCR fragments were used in the sequencing reaction, and for longer PCR products, additional overlapping fragments with one-step RT-PCRs followed by seminested or nested PCRs. All primers used and designed in this study are listed in Table S1 in the supplemental material, and RT-PCR and PCR conditions are available in Table S1 in the supplemental material. All PCR products were visualized on a 1.5% agarose gel stained with SYBR Safe DNA gel stain (Life Technologies) and purified using the JetQuick PCR purification spin kit (Genomed, Loehne, Germany) or QiAquick gel extraction kit (Qiagen) when multiple bands were visible. Sequencing was performed in both orientations using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies) and ABI 3130 Avant capillary sequencer (Applied Biosystems). Primers used for generating the PCR fragments were used in the sequencing reaction, and for longer PCR products, additional

TABLE 1 Distribution of collected and positive samples according to country, year of sampling, and species

<table>
<thead>
<tr>
<th>Country</th>
<th>Yr</th>
<th>Chicken</th>
<th>Duck</th>
<th>Goose</th>
<th>Guinea fowl</th>
<th>Turkey</th>
<th>Not specified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cameroon</td>
<td>2009</td>
<td>4/644</td>
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</tr>
<tr>
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<td>3/88</td>
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</tr>
<tr>
<td>Côte d’Ivoire</td>
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<td>1/7</td>
<td></td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
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<td>0/9</td>
<td>0/15</td>
<td>1/28</td>
<td>0/1</td>
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<tr>
<td></td>
<td>2009</td>
<td>69/576</td>
<td>0/72</td>
<td>3/51</td>
<td>2/42</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>46/464</td>
<td>0/5</td>
<td>1/44</td>
<td>1/1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>147/3,076 (4.8)</td>
<td>1/296 (0.3)</td>
<td>0/9 (0)</td>
<td>4/118 (3.4)</td>
<td>4/79 (5.1)</td>
<td>1/32 (3.1)</td>
</tr>
</tbody>
</table>

TABLE 2 NDV prevalence in bird species by type of birds in Nigeria

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>No. of positive animals/no. of animals tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken</td>
</tr>
<tr>
<td>Market</td>
<td>99/873</td>
</tr>
<tr>
<td>Backyard farm</td>
<td>11/204</td>
</tr>
<tr>
<td>Commercial farm</td>
<td>6/115</td>
</tr>
<tr>
<td>Free range</td>
<td>5/638</td>
</tr>
<tr>
<td>Unknown</td>
<td>0/22</td>
</tr>
<tr>
<td>Total</td>
<td>121/1,852</td>
</tr>
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</table>
internal primers were used (see Table S1). Contigs were assembled and analyzed using SeqScape version 2.5 (Applied Biosystems).

**Phylogenetic analyses and genotype classification based on complete F sequences.** All complete fusion gene sequences available in GenBank (February 2013) were aligned using ClustalW (23). Sequences with insertions or deletions resulting in frameshifts were cured from the data set. Recombinant sequences were identified with the RDP, Genecov, MaxChi, Chimaera, Bootscan, Sister Scanning, and 3SEQ methods, as implemented in RDP3 software (24). Sequences were identified as true recombinant when P values were <0.001 for at least two tests. All putative recombinant sequences were removed from the data set, as well as the recombinants identified based on complete genome analyses by Chong et al. (25) and Diel et al. (9). The final data set contained 896 previously published sequences and 96 obtained in this study.

Phylogenetic relationships were inferred by comparing the sequences obtained in this study with all complete F gene sequences available in GenBank. The best-fit substitution model was selected based on analyses performed in Topali version 2.5 (26), and the model with the lowest Akaie information criterion 1 (AIC1) and Bayesian information criterion (BIC) was selected. Trees were calculated with the maximum likelihood method, using the general time-reversible (GTR) substitution model with a gamma (G) and invariant (I) site heterogeneity model as implemented in MEGA version 5.03 (27). A total of 992 sequences were included to calculate the tree presented in Fig. S1 in the supplemental material. Figure 2 is a detailed view of genotypes XIV, XVII, and XVIII, as calculated in Fig. S1.

Genotypes and subgenotypes were determined based on (i) tree topology, (ii) bootstrap values, (iii) mean evolutionary distances between (sub)genotypes, and (iv) a minimum of four sequences from at least two distinct outbreaks per (sub)genotype. Between-group distances, i.e., the means of all pairwise distances between two groups in the intergroup comparisons, and the mean interpopulation diversity were calculated with the maximum composite likelihood model and 500 bootstrap replicates (MEGA version 5.03). The mean interpopulation diversity was used to determine the cutoff value between genotypes. For these analyses, class I strains \((n = 118)\) were excluded due to the much higher genetic distance of class I \((between 0.375 and 0.426 mean genetic distance) to all class II strains (MEGA version 5.03). The overall prevalence of NDV in Nigeria between 2006 and 2011 (119/2,427, 4.9%) (27a). The high overall prevalence found in Togo and Benin between 2008 and 2010 (112/2,477, 4.5%) (27a). The high overall prevalence found in Togo and Benin between 2008 and 2010 (112/2,477, 4.5%) (27a) was compared to prevalences of 0.3 to 1.4% in 2010 (27a) and compared to prevalences in the other countries could be explained by differences in sampling methods, i.e., targeting exclusively sick animals or not. However, apparent prevalence rates may be somewhat underestimated due to suboptimal local sample storage before shipment and potential

** RESULTS**

Detection of NDV in samples from West and Central Africa. Between 2006 and 2011, we collected and screened a total of 3,610 samples for the presence of NDV nucleic acids (class II only). In Cameroon and Central African Republic, only chickens were sampled (Table 1). In Côte d’Ivoire and Nigeria, ducks, guinea fowl, and turkeys were also included. In Nigeria, samples from geese were also analyzed. The species origin of 32 samples was not known (Table 1). In Nigeria, chickens were more often infected by NDV (6.5%, \(P < 0.001\)) than turkeys (5.1%) and guinea fowls (3.4%) (Table 2); ducks had a significantly lower risk of infection than all other bird categories \((P < 0.001)\) in Nigeria. Chickens sampled in the Nigerian live-poultry markets were statistically more often infected with NDV (11.3%, \(P < 0.001\)) than those from the other locations. Free-ranging chickens had a lower risk of being infected than chickens from the other locations (0.8%, \(P < 0.001\)). The overall prevalence of NDV in Nigeria between 2006 and 2011 (129/2,342, 5.5%) was higher than the overall prevalence found in Cameroon (16/1,096, 1.5%; 2009 and 2011) and in Central African Republic (3/88, 3.6%; 2008) but was comparable to the overall prevalence found in Togo and Benin between 2008 and 2010 (112/2,477, 4.5%) (27a). The high overall prevalence found in Côte d’Ivoire (8/53, 15%) in 2006 to 2008 compared to prevalences of 0.3 to 1.4% in 2010 (27a) and compared to prevalences in the other countries could be explained by differences in sampling methods, i.e., targeting exclusively sick animals or not. However, apparent prevalence rates may be somewhat underestimated due to suboptimal local sample storage before shipment and potential

**Statistical analyses.** Statistical analyses to assess whether there was a correlation between species or sampling location and the outcome of the detection tests were performed using the chi-square test with Yates correction in SigmaPlot software (Systat Software Inc., San Jose, CA). For the variables with a significant correlation according to the chi-square test, the phi coefficient was calculated in order to assess the direction of the correlation.

**Nucleotide sequence accession numbers.** Sequences were submitted to GenBank under accession numbers HF969123 to HF969245. The following strain nomenclature was used: host/country/strain number/year. In the text, the strains obtained in this study are referred to by their strain number.
FIG 2 Detailed view of genotypes XIV and XVIII (A) and genotype XVII (B), as calculated in Fig. S1 in the supplemental material, that shows the phylogeny of 992 complete F gene sequences analyzed with the maximum likelihood method and the GTR + G + I nucleotide substitution model. Sequences obtained in this study are shown in bold. For Nigeria, the states are indicated as follows: Sokoto State, black circle; Yobe State, gray circle; Plateau State, white circle; Benue State, black square; Lagos State, gray square; Oyo State, white square. Accession numbers of previously published sequences available in GenBank are indicated. Only bootstrap values of ≥60% are shown. The scale corresponds to the number of base substitutions per site. GWH, Green Wood Hoopoe.
PCR inhibitors in fecal material, although this seems to be less of an issue in fecal swabs of poultry (28).

Genetic classification based on complete F gene sequences.  
Sequence of the fusion gene was attempted for all positive samples (n = 157), but only 96 full and 27 partial sequences were obtained. These sequences were used for the genetic classification and phylogenetic analyses described below.

Two nomenclature systems are currently used to describe the genetic diversity of NDV strains. The first one divides NDV strains into classes I and II and into several genotypes within each class (7, 10), while the second defines six lineages and multiple sublineages (11). However, the latter system showed limitations, especially by grouping sublineages 3a, 3b, 3c, 3d, 3e, and 3g into a single lineage despite being polyphyletic. In addition, the phylogenetic grouping of recent strains from West Africa was not clear. Based on partial F sequences, they were either classified as three new sublineages of lineage 5 (19), three clusters in genotype VII (29), or as a new lineage, 7, with four sublineages (20). With the additional genetic information provided by 96 new complete F gene sequences from West and Central Africa generated in this study, we revisited all full-length F gene sequences and updated the recent classification based on objective criteria proposed by Diel and coworkers (9).

Each of the 992 complete F gene sequences was assigned to either class I or class II and to genotypes within class II based on (i) tree topology (see Fig. S1 in the supplemental material), (ii) bootstrap values (≥60%), (iii) evolutionary distances (Table 3), and (iv) the recent classification proposed (9). The mean interpopulation diversity, which corresponds to the mean evolutionary distance between genotypes, calculated for 873 sequences (excluding class I strains), was 0.097 (±0.005). A 10% cutoff for evolutionary distance between genotypes was selected, as suggested before (9).

Based on these criteria, class II strains were classified into 17 genotypes (I to XIV and XVI to XVIII) (Table 3; see also Fig. S1 in the supplemental material). Our analyses confirm strain classification into genotypes I to XIII and XVI (8, 9), except for genotype IV strains, which did not constitute a monophyletic group. Excluding all putative recombinant strains left genotype XV empty. On the other hand, only six sequences from West Africa were included at the time by Diel et al. (9), and these were all grouped into genotype XIV.

The availability of 18 additional sequences published recently and the 92 generated in our study warranted a more detailed analysis of all West and Central African strains. In our analyses, sequences from West and Central Africa were assigned to three genotypes, XIV and the newly defined genotypes XVII and XVIII. Mean evolutionary distances between genotypes XIV, XVII, and XVIII ranged from 0.101 to 0.124, whereas mean evolutionary distances to the other genotypes ranged from 0.1 (between XVIII and XVI) to 0.24 (between XIV and XI) (Table 3). One strain from Mali (GenBank accession no. JF966386, chicken/Mali/ML029_07/2007) was not classified into any genotype (Fig. 2; see also Fig. S1 in the supplemental material). Its clustering as an outgroup of genotype XIV and its high genetic distance to genotypes XIV, XVII, and XVIII (from 0.1 to 0.118) suggested that it may belong to putative genotype XIX, but more sequences are needed to validate its classification.

The high genetic diversity within certain genotypes justified their subdivision into subgenotypes. Diel et al. (9) proposed the following criteria for the definition of subgenotypes: (i) tree topology, (ii) bootstrap values of ≥60%, and (iii) mean evolutionary distances between subgenotypes of >0.3 and <0.1. According to these criteria, the definitions of the existing subgenotypes Ib, Va, Vb, Vla, Vlb, Vlc, Vle, VIIe, and VIIIf were confirmed in our study, but bootstrap values for subgenotypes VIIb and VIIId were lower (38% to 59%) than the 60% threshold, suggesting suboptimal definitions of these two subgenotypes. However, deduced amino acid sequences showed that subgenotypes VIIb and VIIId could be distinguished based on specific residues: most VIIb strains shared the amino acids S24 (124/126 strains), L28 (115/126 strains), K45 (105/126 strains), H279 (108/126 strains), I513 (124/126 strains), and V520 (110/126 strains) with a few exceptions, whereas most subgenotype VIIId strains had G24 (108/124 strains), P28 (77/124 strains), S28 (40/124 strains), N145 and Q279 (124/124 strains), V531 (117/124 strains), and G520 (123/124 strains) residues. New subgenotypes were defined within genotypes V (Vc) and VI (Vf and Vg), as well as within genotypes XIV (XIVa and XIVb), XVII (XVIIa and XVIIb), and XVIII (XVIIIf and XVIIIb) (see Table S3 and Fig. S1 in the supplemental material).

In order to investigate the classification of other strains from...
Africa that were previously attributed to lineage 5 or genotype VII, phylogenetic analyses were performed on the longest F gene fragment available for each strain, with a subset of 208 representative of all genotypes I to XIV and XVI to XVIII (data not shown). These analyses established that the strains from Burkina Faso (FM200806 to FM200808), previously assigned to sublineage 5h [19], clustered with chicken/Mali/ML029_07/2007, strengthening the hypothesis of the existence of a putative additional genotype XIX present in Burkina Faso and Mali (see Table S4 in the supplemental material). Nevertheless, further classification based on complete F gene sequences is warranted in order to confirm this grouping. On the other hand, previous sublineage 5b or subgenotype VIIb strains from Burundi [20], Mozambique [30, 31], Zimbabwe, and South Africa [31, 32] were grouped into genotype XIII, whereas sublineage 5d or subgenotype VIIId strains from South Africa [32, 33] and Sudan [34] still grouped into subgenotype VIIId in our classification.

**Phylogenetic analyses of the new NDV strains from West and Central Africa.** (i) Vaccine-like strains (based on 375 nt). Two strains from Cameroon (chicken/Cameroon/CAE11-863/2011 and chicken/Cameroon/CAE11-855/2011) clustered in genotype I and were identical to the vaccine strain Queensland V4 (JX524203; Kimura distance of 0%, 375 nt). Strain CAE11-855 had a genetic distance of 0.8% (242 nt) to another isolate from Cameroon (chicken/Cameroon/CS81/2008; FM200839; data not shown). Three strains from 2009 and four from 2011 clustered in genotype II and were similar to the vaccine strain B1 (JN872150; Kimura distance of 0 to 0.5%, 375 nt), suggesting that all genotype I and II strains were related to live vaccine strains (see Fig. S2 in the supplemental material).

(ii) Genotype XIV. Fourteen strains from three Nigerian states ( Sokoto, Yobe, and Lagos States; 2007, 2009, 2011) obtained in our study clustered in subgenotype XIVa (previously named 5f [19] or 7d [20, 35]; see Table S4 in the supplemental material) together with two strains from Niger (2006) and one from Nigeria (2008) (Fig. 2A). Thirty-three strains from Nigeria ( Sokoto, Yobe, and Benue States; 2008, 2009, 2011) sequenced in this study clustered in subgenotype XIVb (previously named 5f [35], 7d [36], or cluster number 1 [29]; see Table S4) together with a strain from Katsina State from 2007 (chicken/Nigeria/VRD07-233/2007) and a strain from Benin (2009). Interestingly, the three strains NIE09-1596, NIE09-1597, and NIE09-1599 were found in a commercial farm in Benue State and represent one of the very few examples of virulent NDV from commercial farms in West and Central Africa (Fig. 2A).

(iii) Genotype XVII. Thirty-eight sequences obtained in this study clustered within genotype XVII, most of them from Nigeria ( Sokoto, Yobe, and Plateau States; 2007, 2008, 2009, 2011) but also some from Côte d’Ivoire (2007), Central African Republic (2008), and Cameroon (2009) (Fig. 2B). Based on the analysis of all complete F gene sequences, genotype XVII was divided into two subgenotypes, XVIIa and XVIIb. Subgenotype XVIIa (previously sublineage 5g [19], 7a [36], 7b [20], or cluster number 3 [29]; see Table S4 in the supplemental material) was geographically the most dispersed, as it was found in Nigeria, Côte d’Ivoire, Niger, Cameroon, and Burkina Faso in 2008, in Benin in 2009, and in Mali in 2008 (Fig. 2B). Subgenotype XVIIb (previously named 7b [20]; see Table S4) was constituted exclusively by Nigerian strains. In the future, genotype XVII may need to be further subdivided due to the high genetic distance between strains from Central African Republic (CAF09-014, CAF09-015, CAF09-016; 0.76 and 0.73 mean genetic distances, respectively) and the single virulent strain from Cameroon (CAE08-318; 0.59 and 0.53 mean genetic distances, respectively) to subgenotypes XVIIa and XVIIb. The three strains from Central African Republic were highly similar to each other and originated from a single farm.

(iv) Genotype XVIII. Two strains from Côte d’Ivoire (2007) clustered in subgenotype XVIIa (previously 7a [20]; see Table S4 in the supplemental material), together with one strain from Mauritania (2006) and three strains from Mali (2007, 2008, and 2009) (Fig. 2A). Subgenotype XVIIIb (previously 7a [20] or cluster 2 [29]; see Table S4) was composed of three strains from Côte d’Ivoire (2006 and 2007), two strains from Nigeria (2011) obtained in our study, as well as one strain from Côte d’Ivoire (2008) and one strain from Togo (2009).

An additional set of 22 partial sequences were obtained from Nigeria (n = 21) and Côte d’Ivoire (n = 1) and were assigned to subgenotypes XVIIa or XVIIb (Nigeria) and XVIIIa (Côte d’Ivoire) (data not shown; see Table S4 in the supplemental material). Taken together, these phylogenetic analyses revealed that several (sub)genotypes are found in every country, except in Central African Republic, where only genotype XVII was identified. In Cameroon, nine strains were related to vaccine strains, either B1 or Queensland V4, but a single genotype XVII strain was also found. Subgenotypes XVIIa, XVIIa, and XVIIb circulated in Côte d’Ivoire, while subgenotypes XIVa, XIVb, XVIIa, XVIIb, and XVIIb were found in Nigeria (Fig. 2 and Fig. 3). In Nigeria, which was most extensively sampled, several subgenotypes and several clusters within each subgenotype were found in the same states (Fig. 2). For instance, two clusters of subgenotype XIVa, three clusters of subgenotype XIVb, and four clusters of subgenotype XVIIa were found in Sokoto State. Subgenotypes XIVa, XIVb, and XVIIa were found in live-bird markets in Yobe State (Fig. 2).

**Phylogenetic analyses based on HN sequences.** Phylogenetic analyses performed on 41 complete HN sequences obtained in this study and compared to selected representative strains of each genotype showed that the tree topologies and the genotype assignments of the strains from West and Central Africa were similar for HN and F genes (see Fig. S1 and S2 in the supplemental material). The new genotypes, XIV, XVII, and XVIII, were clearly identifiable and distinct from all other genotypes of class II. Deduced amino acid sequences of the HN gene showed that the HN protein of all strains clustering in genotypes XIV, XVII, and XVIII was 571 amino acids long, a feature shared by many virulent strains. Strain clustering within subgenotypes XIVa-XIVb, XVIIa-XVIIb, and XVIIa-XVIIIb was also similar to that based on F sequences. Nevertheless, phylogenetic analyses based on complete genome sequences of representative strains of each West African subgenotype would be interesting to further investigate and confirm their evolutionary relationship.

**Analyses of deduced amino acid sequences of F and HN proteins.** (i) Cleavage site. Genotype I and II strains carried the fusion cleavage site motif 112GQGRBlack\*117, but the 112GRGRTBlack*117, respectively, both typical of avirulent viruses. Deduced amino acid cleavage site sequences of all genotypes XIV, XVII, and XVIII strains were indicative of virulent viruses. The majority of strains carried the cleavage site sequence 112RRKRBlack*117, but the 112RKKRBlack*117 motif was also observed in subgenotypes XIVb (28/35 strains), XVIIa (6/47 strains), and XVIIb (1/8 strains) (Table 4). Variability was also observed at position 118, and a conservative I118V
substitution was found in all strains of subgenotype XIVb and in three strains of subgenotype XVIIIa (Table 4). Virulent strains were characterized in apparently healthy animals (45/92) and in 13/92 sick animals, and no information was available for 34/92 birds.

(ii) Neutralizing epitopes. The fusion and hemagglutinin-neuraminidase proteins contain several neutralizing epitopes that are known to be important for their structure and function (37, 38). On the F protein, most of our African NDV strains shared the residues D72, E74, A75, K78, A79, and L343 and the stretch 151ILRLKESIAATNEAVHETDG171. However, all genotype XIV strains shared a K78R substitution, and five of 17 subgenotype XIVa strains had an A79G substitution. The three strains from Central African Republic (genotype XVII) shared the A75T and A79T substitutions. Variability was also observed at position 170, where all genotype XVIII strains had a D170S substitution but D170G and D170E were also found in other strains. L343P and L343Q were found twice each (see Table S5 in the supplemental material).

Several amino acid substitutions in neutralizing epitopes were also observed on the HN protein: R197K (n/H110051 strain), R263K (in all but three strains), D287E (n/H110052 strains), R333K (in all but one strain), E347D (n/H110054 strains), D349G (n/H110052 strains), Y350H (n/H110059 strains), R353Q (n/H110054 strains), K356R (n/H110051 strain), R513H (n/H110052 strains), I514V (in all but one strain), S519A (n/H110051 strain), S521G (n/H110051 strain), and D569G (n/H110054 strains) (see Table S5 in the supplemental material).

TABLE 4 Fusion protein cleavage site sequences (positions 112 to 117) and amino acid 118 of all genotype XIV, XVII, and XVIII strains, as well as ICPIs

<table>
<thead>
<tr>
<th>Genotype (no. of strains)</th>
<th>Positions 112–117</th>
<th>Position 118</th>
<th>ICPIa (no. of strains tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgenotype XIVa (n = 17)</td>
<td>RRQKR*F</td>
<td>I</td>
<td>1.8 (n = 1)</td>
</tr>
<tr>
<td>Subgenotype XIVb</td>
<td>RRRKR*F</td>
<td>V</td>
<td>1.65 (n = 1)</td>
</tr>
<tr>
<td>28 strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIE09-1596, NIE09-1599, NIE09-1597, NIE10-034, NIE10-258, NIE10-139, NIE10-325</td>
<td>RRQKR*F</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Genotype XVII</td>
<td>RRKR*F</td>
<td>I</td>
<td>1.51–1.87 (n = 8)b</td>
</tr>
<tr>
<td>50 strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIE10-123, NIE10-124, NIE10-304, NIE10-306, NIE10-310, NIE10-335</td>
<td>RRKR*F</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Subgenotype XVIIIa</td>
<td>RRQKR*F</td>
<td>I</td>
<td>1.65–1.7 (n = 2)</td>
</tr>
<tr>
<td>4 strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIV08-026, CIV08-044, Green Wood Hoopoe/Eastern Hemisphere/S801-22/10</td>
<td>RRQKR*F</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Subgenotype XVIIIb</td>
<td>RRQKR*F</td>
<td>I</td>
<td>1.65 (n = 4)</td>
</tr>
<tr>
<td>7 strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken/Togo/AKO18/2009</td>
<td>RRKR*F</td>
<td>I</td>
<td></td>
</tr>
</tbody>
</table>

a ICPIs are from references 20 and 29.

b All 8 strains tested by ICPI cluster in subgenotype XVIIa.
the supplemental material), suggesting that some of these strains may partially escape antibody neutralization (39).

(ii) N-glycosylation sites. The F and HN proteins both contain several potential N-glycosylation sites [NX(S/T), where X is any amino acid but a proline]. Predicted N-glycosylation sites on the F protein were conserved among genotype XIV, XVII, and XVIII strains (112NRS114, 191NNT193, 366NTS368, 407NIS409 or 447NVT449, 471NNT473, 541NNT543), similarly to all other strains published so far. Thus, there is no expected difference in viral functions such as protein structure, virus replication, or virulence modulated by differential glycosylation (40, 41). On the HN protein, five predicted N-glycosylation sites were conserved among all genotype XIV, XVII, and XVIII strains (139NNS141, 341NNT343, 341NCT343, 341NT543, 341NCT343, 433NKT435, 481NHT483, 508NIS510, or 508NTS510). In addition, 19/20 genotype XIV strains and 2/9 genotype XVII strains for which the HN sequences were obtained had an additional potential N-glycosylation site, 538NKT540, although this site does not seem to be glycosylated (42).

HN-L intergenic region. Some strains recently described in Togo and Benin (XVIIIb) showed a 6-nt insertion in the intergenic region between the HN and polymerase (L) genes (29, 43), although insertions are rare in NDV (6, 7). We therefore sequenced this region for 53/92 strains of genotypes XIV, XVII, and XVIII. The insertion was found only in strains of subgenotype XVIIb (NIE11-1286, NIE10-171, C1V08-042, and C1V08-062; this region could not be obtained for C1V08-069) (Fig. 2), suggesting that the insertion of six nucleotides was probably recently acquired by an ancestor of this subgenotype.

DISCUSSION

Adding almost 100 new complete F gene sequences to the 44 African sequences in GenBank, it became necessary to revisit their genotype classification. We propose to classify the new virulent NDV strains into three genotypes, XIV, XVII, and XVIII, each with at least two subgenotypes. Interestingly, no strains related to these genotypes were ever reported from Northern (Egypt [44] and Sudan [34]), Eastern (Uganda [45]; Burundi [20]; Ethiopia, Kenya, and Tanzania [11]; Mozambique [30]; Zimbabwe and Madagascar [46]), and Southern (Botswana and South Africa [31, 32]) Africa, suggesting that their geographic distribution is still restricted to West and Central Africa. Except for in South Africa (31–33), the epidemiology of NDV is still poorly understood in Africa. As surveillance may intensify across West and Central Africa, except perhaps in Nigeria, the genetic diversity of NDV will further increase. For instance, strains from Mali and Burkina Faso clustered outside genotype XIV, and their high genetic distance to genotypes XIV, XVII, and XVIII likely suggests yet another genotype in these countries. The current genotypes XIV, XVII, and XVIII may also need further subdivision in the future, especially genotype XVII.

The detection of vaccine-like strains is due mostly to the use of live vaccines, used mainly in commercial farms in West and Central Africa. In this study, vaccine-related strains were detected only in Cameroon and not in Nigeria, contrasting to our previous study carried out mainly in Nigerian commercial settings (19). On the other hand, subgenotype XIVb strains were found in a commercial farm in Nigeria that had reportedly vaccinated with an unspecified vaccine strain against NDV. Also, genotype XVII strains were found in a commercial farm in Central African Re-
vivo and attenuated pathogenicity in 1-day-old chicks (57). The additional I118V substitution—otherwise found in genotype V (69/73) and XI (4/4) and class I (58/118) strains and a few other exceptions—further reduced pathogenicity (57). This could, however, only partially explain the apparently reduced virulence observed in the field, since Q114R and R114 or I118 and V118 were found in healthy and sick animals, at least in our study. These reverse genetic experiments performed in a Beaudette C backbone (genotype II) may not be directly applicable to genotype XIV, XVII, and XVIII strains due to the high number of amino acid differences of their F and HN proteins (between 10 and 14%). This may also influence the structure of both proteins, their interactions, and conformational changes of the F protein during fusion of the virus with the plasma cell membrane (58). Therefore, additional experiments should be performed in order to assess the role of Q114R and I118V substitutions in genotype XIV, XVII, and XVIII strains.

Outbreaks of NDV in vaccinated flocks have been increasingly reported from Nigeria (35), suggesting a suboptimal protection by vaccination. In general, all vaccine strains (genotypes I, II, and III) are thought to protect against all virulent strains, except for some variant viruses that overcame vaccine protection (59). Challenge experiments with genotype XVIa or XVIIb strains after vaccination with the commonly used LaSota vaccine also confirmed that vaccination conferred efficient protection against clinical disease and death induced by these West African strains, although shedding was not inhibited for all animals (29). Other reasons, such as poor vaccine quality, suboptimal vaccination (60), or coinfections (61), may result in reduced vaccine efficacy. Nevertheless, multiple amino acid substitutions in neutralizing epitopes of F and HN proteins in a number of genotype XIV, XVII, and XVIII strains (see Table S5 in the supplemental material), as well as in other viral proteins that have not been investigated in our study, could contribute to partial vaccine resistance and warrant further vaccine challenge experiments. Vaccines better matching this antigenic diversity of current West and Central African NDV strains are probably required.

In conclusion, we showed that the genetic diversity of virulent NDV strains enzootically circulating in West and Central Africa continues to grow, requiring a continuously updated classification based on rational criteria for (sub)genotypes. While genotype XIV, XVII, and XVIII strains are so far geographically restricted, local and international trade of domestic and exotic birds may lead to their spread beyond West and Central Africa. A high genetic diversity, mutations in important neutralizing epitopes paired with suboptimal vaccination, various levels of clinical response of poultry and wild birds to virulent strains, and strains with new fusion protein cleavage sites and other genetic modifications tend to undermine and complicate NDV management in Africa.

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