Characterization of a new genotype and serotype of infectious bronchitis virus in Western Africa

Mariette F. Ducatez,1† Ana Moreno Martin,2 Ademola A. Owoade,3 Isaac O. Olatoye,4 Bello R. Alkali,5 Issoufou Maikano,6 Chantal J. Snoeck,1 Aurelie Sausy,1 Paolo Cordioli2 and Claude P. Muller1

Correspondence
Claude P. Muller
claude.muller@LNS.ETAT.LU

1Institute of Immunology, National Public Health Laboratory, CRP-Santé, 20A rue Auguste Lumière, L-1950 Luxembourg
2Istituto Zooprofilattico sperimentale della Lombardia e dell’Emilia Romagna, Reparto di virologia e sierologia specializzata, Via Bianchi 9, 25124 Brescia, Italy
3Department of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria
4Food Safety, Drug Residues/Animal Diseases Surveillance and Intervention, Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria
5Department Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Sokoto, Nigeria
6LABOCEL, Direction des Laboratoires Vétérinaires, Niamey, Niger

Between 2002 and 2007, more than 1000 chickens from commercial farms, live bird markets and backyard farms in Nigeria and Niger were tested for the presence of the infectious bronchitis virus (IBV) genome. Phylogenetic analysis of full-length sequences of the spike 1 (S1) gene revealed a new genotype of IBV that we refer to as ‘IBADAN’. The minimum genetic distance to the closest ‘non-IBADAN’ strains (UK/7/93 at the nucleotide level; H120 and M41 at the amino acid level) reached 24 and 32 % at the nucleotide and amino acid levels, respectively. The full genome of the IBADAN reference strain (NGA/A116E7/2006) had a genetic distance of 9.7–16.4 % at the nucleotide level with all available fully sequenced strains. As IBV S1 plays a major role in antigenicity, the antigenic relatedness of NGA/A116E7/2006 was compared with strains of other serotypes. NGA/A116E7/2006 did not cross-react with antisera against IT02, M41, D274, Connecticut or 793/B strains in virus neutralization assays. NGA/A116E7/2006 cross-reacted with the QX-like strain ITA/90254/2005 but only to a low level (antigenic relatedness of 33 %), suggesting that IBADAN also represents a new serotype. A comparison of S1 sequences identified several amino acids that may play a role in IBV antigenicity. Despite the absence of obvious clinical signs in poultry infected by IBADAN strains, it is important to test the cross-protection of current vaccine strains.

INTRODUCTION

The recent emergence of a coronavirus variant causing severe acute respiratory syndrome (SARS) in humans has renewed interest in the virus family Coronaviridae. Coronaviruses comprise three genetic groups, two of which (groups 1 and 2) contain viruses that are pathogenic in humans. Turkeys can be infected by group 2 as well as group 3 turkey coronaviruses (Lai & Holmes, 2001). Group 3 viruses such as infectious bronchitis virus (IBV) (Cavanagh, 2000; Enjuanes et al., 2000; Lai & Holmes 2001), the first coronavirus to be discovered, occur only in birds. So far, group 3 viruses have not been found in humans, but phylogenetic analysis of SARS-coronavirus has shown that its genome contains sequences that seem to be of group 3 origin (Stavrinides & Guttman, 2004). IBV was first found in the USA in 1930 and has since been reported from most countries throughout the four continents of America (Johnson & Marquardt, 1975), Europe (Capua et al., 1994; Cavanagh & Davis, 1993;
Gough et al., 1992), Asia (Wang et al., 1997) and Australia (Ignjatovic & McWaters, 1991; Lohr, 1976). Except for 793/B (or 4/91) (Cavanagh & Naqi, 2003), IBV genotypes are rarely reported to spread from one continent to another (Kusters et al., 1987; Zanella et al., 2003).

IBV is an enveloped, positive-strand RNA virus with a genome of about 27 kb. The nucleocapsid protein is the most conserved gene of IBV, whilst the spike 1 (S1) subunit of the spike protein gene is the most variable (Cavanagh & Naqi, 2003). This subunit is responsible for inducing neutralizing and serotype-specific antibodies. Mutations within this genome region may therefore result in the emergence of new variants against which vaccines are no longer protective (Moore et al., 1998). IBV causes significant economic losses, mostly because of reduced productivity rather than bird mortality (Cavanagh & Naqi, 2003). The virus primarily infects the respiratory tract, kidneys and oviduct (Cavanagh & Naqi, 2003). Recent reports suggest a shift in tissue tropism (Liu & Kong, 2004; Zhou et al., 2004) and an extended host range into new bird species reared close to domestic poultry in Europe and in China: Italy-02 (IT02) and other reference strains, reciprocal VN assays, with a fixed concentration of virus and serial dilutions of serum, were carried out. The field strains used included NGA/A116E7/2006 and ITA/90254/2005 (97.9% nucleotide identities to QX IBV for the S1 hypervariable region). The reference strains were IT02, D274, 793/B, M41 and Conn. VN tests were performed as described by Thayer & Beard (1998). Two-fold serial dilutions of each antisera were mixed with an equal volume of virus dilution containing 100 EID50 in 0.1 ml and incubated for 1 h at room temperature. Each serum–virus mixture was then inoculated in SPF chicken embryonated eggs by the allantoic sac route. Viruses were back-titrated in each VN test to confirm that 100 EID50 virus per 0.1 ml had been used. Chicken embryos were evaluated 24 h after inoculation for non-specific mortality and 1 week after inoculation to evaluate the presence of specific lesions, as an indication that the virus had not been neutralized by sera. End points corresponded to the serum dilutions that neutralized 50% of the virus. End-point titres were calculated by the method of Reed & Muench (1938).

The VN end-point titres were used to calculate the percentages of antigenic relatedness, r, by the method of Archetti & Horsfall (1950). The r value is equivalent to the square root of r1 × r2, where r1 is the ratio of the heterologous titre with virus 2 to the homologous titre of virus 1, and r2 is the ratio of the heterologous titre with virus 1 to the homologous titre of virus 2. The data were expressed as percentage r values. The r values determined by Archetti & Horsfall (1950) ranged from 0 for isolates that were antigenically unrelated to 100% for isolates that were identical. Isolates with r values between 50 and 100% were considered to be antigenically related (Gelb et al., 1997).

RNA isolation and RT-PCR. RNA was extracted using a QIAamp viral RNA mini kit (Qiagen). RNA was eluted in 60 μl elution buffer. The extracted RNA was first reverse-transcribed with random primers and SuperScript III (Invitrogen). The cDNA was screened for the IBV RNA isolation and RT-PCR. RNA was extracted using a QIAamp viral RNA mini kit (Qiagen). RNA was eluted in 60 μl elution buffer. The extracted RNA was first reverse-transcribed with random primers and SuperScript III (Invitrogen). The cDNA was screened for the IBV

METHODS

Field samples. In the present study, more than 1000 cloacal swabs and 40 lung samples of poultry from Niger (Maradi, Niamey, Tillaberi and Zinder provinces) and Nigeria (Oyo, Ogun, Lagos, Kano, Kaduna and Sokoto states) were analysed, collected between 2002 and 2006. Cockerels, broilers, pullets, layers and breeders were obtained from commercial flocks with 125–200,000 birds (3-weeks to 2-years-old) and from slaughterhouses, as well as from live bird markets in Nigeria and backyard farms in Niger.

Virus cross-neutralization. Monospecific antisera against the viruses NGA/A116E7/2006, ITA/90254/2005 QX-like, M41, D274, Connecticut (Conn) and 793/B were produced following a standard immunization protocol (Gelb & Jackwood, 1998). Briefly, specific-pathogen-free (SPF) chickens were inoculated intratracheally with approximately 10⁵ 50% egg infectious doses (EID50) per bird. At 3 weeks post-inoculation, they received an intravenous injection of the same dose. After another 4 weeks, blood samples were collected and serum was harvested, pooled and inactivated at 56 °C for 30 min before being used in virus neutralization (VN) assays. Serum specific for IT02 was kindly provided by Dr Capua (IZS Padova, Italy).

To determine the antigenic relationships between the Nigerian NGA/ A116E7/2006 strain (after seven passages in embryonated chicken eggs) and other reference strains, reciprocal VN assays, with a fixed concentration of virus and serial dilutions of serum, were carried out. The field strains used included NGA/A116E7/2006 and ITA/90254/2005 (97.9% nucleotide identities to QX IBV for the S1 hypervariable region). The reference strains were IT02, D274, 793/B, M41 and Conn. VN tests were performed as described by Thayer & Beard (1998). Two-fold serial dilutions of each antisera were mixed with an equal volume of virus dilution containing 100 EID50 in 0.1 ml and incubated for 1 h at room temperature. Each serum–virus mixture was then inoculated in SPF chicken embryonated eggs by the allantoic sac route. Viruses were back-titrated in each VN test to confirm that 100 EID50 virus per 0.1 ml had been used. Chicken embryos were evaluated 24 h after inoculation for non-specific mortality and 1 week after inoculation to evaluate the presence of specific lesions, as an indication that the virus had not been neutralized by sera. End points corresponded to the serum dilutions that neutralized 50% of the virus. End-point titres were calculated by the method of Reed & Muench (1938).
TAE electrophoresis running buffer and stained with ethidium bromide (15 μg in 100 ml agarose gel). IBV vaccine strain H120, kindly provided by Drs Palya and Penzes (CEVA Phylaxia Veterinary Biologicals, Budapest, Hungary), was used as a positive PCR control and to optimize the different PCRs.

**Sequencing.** PCR products were purified using a Jetquick PCR purification kit (Genomed) following the instructions of the manufacturer. DNA (10–100 ng) was sequenced in both directions with a Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) on a capillary sequencer (model 3100-Avant; Applied Biosystems) using the PCR primers (Eurogentech) as sequencing primers. Strains were designated as three-letter country code/sample ID/year.

**Data analysis.** Sequences were analysed using Seqscape version 2.5 (Applied Biosystems) and BioEdit programs (Hall, 1999). Sequences were aligned with CLUSTAL W (Chenna et al., 2003). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0.2 (Kumar et al., 2001). Phylogenetic analysis of nucleic acid and deduced amino acid sequences was done with the neighbour-joining method using the Kimura two-parameter model and pairwise deletion. Amino acid sequences were also analysed with the neighbour-joining method, with Poisson correction. Bootstrap values (1000 replications) were indicated on the trees. IBV sequences from all relevant reference strains available on GenBank were used for comparison.

## RESULTS

### Virus prevalence and disease symptoms

In Nigeria, 18 % (170/957) of the chickens tested were positive for IBV RNA: 7/40 (18 %) in slaughterhouses in 2002; 161/617 (26 %) in commercial farms in 2006; and 2/300 (1 %) in live birds markets in 2007. The virus was detected in 3–55-week-old commercial broilers and layers, in both chicken and turkey farms. In Niger, only 2 % (1/62) of backyard birds collected in 2006–2007 were IBV positive. In IBV-positive commercial farms, slight losses in productivity were observed, but no obvious clinical symptoms were identified.

When IBV NGA/A116E7/2006 was inoculated intranasally into 6-week-old SPF chickens, the animals developed no clinical symptoms but produced antibodies against the virus. These antibodies were used for cross-neutralization assays, described below.

### Phylogenetic analysis of partial S1 sequences

The hypervariable region of the S1 gene (380 nt) was sequenced from all strains and compared with all relevant IBV sequences available in GenBank (Fig. 1). The 2002 strains clustered with either 793/B-type strains such as UK/7/93 (Z83979) or Massachusetts-type strains such as M41 (DQ664534; Fig. 1). Some Nigerian 2006 strains clustered with 793/B-like strains (NGA/254/2006, NGA/371/2006 and NGA/386/2006; Fig. 1), and others with the Belgian nephropathological strain B1648 (NGA/271/2006, NGA/279/2006 and NGA/324/2006), the D274-like strains (NGA/288/2006 and NGA/295/2006) or with Massachusetts-like strains (NGA/293/2006 and NGA/310/2006; Fig. 1). Interestingly, one farm (SA, sample numbers 288, 293 and 295) was infected with two distinct strains related either to Massachusetts (NGA/293/2006) or to D274 (NGA/288/2006 and NGA/295/2006). The remaining 2006 Nigerian IBV strains (from the north and the south-west) formed a distinct cluster, which was designated ‘IBADAN’ (the name of the Nigerian city where the strain was first detected) (Fig. 1).

**Fig. 1.** Phylogenetic analysis of the hypervariable region of the IBV S1 gene from strains from Nigeria and Niger at the nucleotide level. The tree was constructed with the neighbour-joining method (Kimura two-parameter) with 1000 bootstrap replicates. Only bootstrap values higher than 49 are shown. Filled circles indicate IBV reference strains. Bar, 0.02 nucleotide substitutions per site.

### Phylogenetic analysis of complete S1 sequences

The full S1 gene sequence was available only from 30 strains from Nigeria and one strain from Niger. The IBADAN-like strains were compared with the ten reference sequences and all complete S1 sequences available in GenBank, as well as with a recent QX strain (ITA/90254/2005), sequenced for the purpose of this study (Fig. 2). The three complete S1 sequences obtained from farm SA (NGA/288/2006, NGA/293/2006 and NGA/295/2006) clustered together with a mean genetic distance between the three strains of 8.3 %, and with the D274 strain (Fig. 2).
The phylogenetic tree of the full-length S1 sequences also confirmed the IBADAN cluster, supported by a bootstrap value of 100 at the main node (Fig. 2). These strains also formed a distinct cluster at the amino acid level (data not shown). Within the IBADAN cluster, the mean genetic distance reached 3.4 % (range 0–8.8 %) at the nucleotide level and 5.2 % (range 0–14.1 %) at the amino acid level. Seven amino acid positions were shared by all IBADAN strains and were not found in any other reference IBV sequence: I414, I463, S475, R491, E493, G512 and Y527 (comparison with the ten reference strains included in the phylogenetic analyses). The minimum genetic distance to the closest ‘non-IBADAN’ strain was 24 % \([\text{between NER/28/2007 and UK/7/93 (Z83979)}]\) or 25 % \([\text{between NGA/A116/2006 and UK/7/93 (Z83979), or between NGA/SOK40-3/2007 and D274 (X15832)}]\) at the nucleotide and amino acid levels, respectively.

As recombinations are relatively frequent in IBV, IBADAN sequence fragments of 100–600 nt were further compared phylogenetically with all available strains. In all cases, IBADAN strains branched separately, excluding any obvious recombination events in S1.

The new IBADAN strains formed location clusters at the nucleotide level (south-western Nigeria; northern Nigeria and Niger; Fig. 3) supported by high bootstrap values. Two farms, A and B, in the south-west of Nigeria were separated by less than 100 km and hosted viruses with 4.6–5.6 % Kimura distances. The genetic distance within the northern Nigerian farm was even higher (6.0 %), whilst it was much lower within farm B strains over a 9 month period (0.6–3.4 %).

After five passages in embryonated eggs, NGA/A116/2006 acquired a single-nucleotide, non-silent mutation (T689C, resulting in the amino acid change V230A). Two more mutations (one silent nucleotide change at A279C; one non-silent nucleotide change at A191G resulting in amino acid change E64G) were acquired after two additional passages.

The new Nigerian strains did not increase the worldwide maximal genetic diversity for the complete IBV S1 genes at either the nucleotide or amino acid level.

**Phylogenetic analysis of the full genome**

The full-genome sequences of both the IBADAN strain NGA/A116E7/2006 and the QX-like strain ITA/90254/2005 were obtained. The phylogenetic analysis of all relevant full-length sequences is presented in Fig. 4. The genetic distance over the full-genome sequences ranged between 4.7 % [strain ArkDPI11 (EU418976) to strain ’serotype California_99’ (AY514485)] and 16.4 % [strain A2 (EU526388) to strain NGA/A116E7/2006 or ’serotype California_99’]. The genetic distances between the IBADAN strain NGA/A116E7/2006 and the ITA/90254/2005 strain reached 10 % at the nucleotide level. The
genetic distance of IBADAN and QX to other genotypes ranged from 9.7 and 10% (ArkDPI11) to 16.4 and 14.1% (A2), respectively.

**Antigenicity of West African IBV**

The antigenic properties of NGA/A116E7/2006 were compared by a virus cross-neutralization assay to ITA/90254/2005 and a number of reference strains (Table 1). Virtually no cross-neutralization of NGA/A116E7/2006 was observed with IT02, D274, 793/B, M41 or Conn antiserum (titres $\leq 1:22$; Table 1). The VN titre of ITA/90254/2005 QX-like antiserum against NGA/A116E7/2006 virus was 1:256. NGA/A116E7/2006 antiserum did not neutralize IT02, D274, 793/B, M41 or Conn (titres $\leq 1:32$), but showed some cross-reactivity with ITA/90254/2005 (VN titre 1:128). Titres against homologous strains were 1:300 for NGA/A116E7/2006 and 1:1024 for ITA/90254/2005 (Table 1). The calculated antigenic relatedness value, $r$, confirmed the absence of a relationship between NGA/A116E7/2006 and IT02, D274, 793/B, M41 or Conn ($r<10$). The value of $r$ when comparing NGA/A116E7/2006 and ITA/90254/2005 was somewhat higher ($r=33\%$), but did not reach the 50% threshold for antigenic relatedness between strains (Table 2).

NGA/A116E7/2006 and ITA/90254/2005, which cross-reacted antigenically, shared only 71% nucleotide identity or 72% amino acid identity over the complete S1 gene.

**DISCUSSION**

In the south-west of Nigeria, up to 26% of the poultry from commercial farms were infected with IBV. In northern Nigerian live bird markets and in Niger backyard poultry, IBV infections seemed to be less common. In a more systematic prevalence study (Ducatez *et al.*, 2004; Owoade *et al.*, 2006) conducted in Nigeria from 1999 to 2004, 84% of the commercial poultry were positive for IBV antibodies. Most of the IBV strains characterized here grouped together (mean and maximal genetic distances of 3.4 and 8.8%, respectively) and were distinct from all strains reported so far. No clear minimal genetic distance has been proposed to distinguish between IBV genotypes, unlike some other viruses such as measles virus (WHO, 2003) and hepatitis B virus (Norder *et al.*, 1992).

Recently, a new IBV genotype QX was proposed. For the ‘original reference’ strain D388 isolated in The Netherlands, only a short sequence was available (the S1 hypervariable region). Therefore, we sequenced ITA/90254/2005 to generate the only full sequence of a QX-like strain currently available. This virus S1 gene was separated by 14.3% at the nucleotide level and 16% at the amino acid level from its closest relative of a different genotype (Z China, AF140352). IBADAN S1 was separated by 24 and 25% at the nucleotide and amino acid levels, respectively, from UK/7/93 (Z83979), the closest strain of a different cluster.

S1 is the main antigenic protein of IBV, inducing neutralizing and serotype-specific antibodies (Cavanagh & Naqi, 2003). The 25% genetic distance on an amino acid...
level prompted us also to investigate the antigenic cross-reactivity of IBADAN strains with strains of other relevant serotypes. The IBADAN-like strain NGA/A116E7/2006 showed no antigenic cross-reactivity with any of the tested IBV serotypes and only a weak cross-reactivity with ITA/90254/2005 QX-like strain (Table 1). The relatively low antibody titre induced by the IBADAN-like strain partially explains some of the low level of antigenic relatedness (r) calculated for this new virus. Nevertheless, the VN data suggested that QX-like viruses may antigenically be the closest relatives of IBADAN. The comparison of the full genomes of NGA/A116E7/2006 and ITA/90254/2005 showed a diversity of 10% at the nucleotide level. IBADAN was separate from any known full-genome IBV nucleotide sequence with a genetic distance ranging from 9.7 to 16.4%. This very high global molecular diversity therefore further justifies the designation of a new genotype, IBADAN.

A comparison of the amino acid sequence of IBV S1 showed that V70, K95, V211 and S335 were shared by NGA/A116E7/2006 and ITA/90254/2005 but not by any of the other strains included in the VN assay (IT02, M41, D274, Conn and 793/B, which had A/T/I70, T/S/V95, A211 and N/K335). This observation suggests that any of these positions may explain the low level of cross-reactivity between NGA/A116E7/2006 and ITA/90254/2005. However, these amino acid positions are not unique for ITA/90254/2005 and NGA/A116E7/2006 strains – some of them are also found in B1648 and Z and J China (data not shown). These comparisons may provide some guidance for further antigenicity studies.

In Nigeria, breeder farms vaccinate 10-day-old chicks with a single dose of live-attenuated IBV vaccine (e.g. Massachusetts-like strains H120 and H52). Therefore, it cannot be fully excluded that some of the strains detected (e.g. NGA/A1/2002, NGA/A2/2002 and NGA/G4/2002 strains) were vaccine-derived. In one farm, strains were found that clustered with both putative vaccine strains (Massachusetts-like, NGA/293/2006; Fig. 1) and the wild-type strain D274 (NGA/288/2006 and NGA/295/2006), which could be an indication of vaccine failure. However, full S1 sequences of NGA/293/2006 clustered with NGA/288/2006 and NGA/295/2006, which could be indicative of recombination between Massachusetts-like and D274-like strains.

In conclusion, we have presented here a set of new IBV strains from two countries in West Africa, which are genetically and antigenically clearly distinct from all other known IBV strains. We propose to call the new genotype and serotype IBADAN, according to the location where the reference virus was found. It is important to compare the pathogenicity of IBADAN strains with other circulating IBV strains and to test their sensitivity with respect to current vaccines.

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