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EMERGENCE OF A NEW GENETIC LINEAGE OF NEWCASTLE DISEASE VIRUS IN WEST AND CENTRAL AFRICA - IMPLICATIONS FOR DIAGNOSIS AND CONTROL

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1 **EMERGENCE OF A NEW GENETIC LINEAGE OF NEWCASTLE DISEASE VIRUS IN**
2 **WEST AND CENTRAL AFRICA – IMPLICATIONS FOR DIAGNOSIS AND CONTROL**

3

4 Cattoli G^{1*}, Fusaro A¹, Monne I¹, Molia S², Le Menach A³, Maregeya B⁴, Nchare A⁵, Bangana I⁶,
5 Garba Maina A⁷, N'Goran Koffi J-N⁸, Thiam H⁹, Bezeid O E M A¹⁰, Salviato A¹, Nisi R¹, Terregino
6 C¹, Capua I¹

7

8 **Affiliations**

9 1 OIE/FAO Reference Laboratory for avian influenza and Newcastle disease, Istituto
10 Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD), Italy.

11 2. CIRAD, UPR AGIRs, BP1813, Bamako, Mali.

12 3. Food and Agriculture Organisation (FAO), AGAH Division, Viale delle Terme di Caracalla,
13 Rome, Italy.

14 4. Food and Agriculture Organisation (FAO) Burundi, Avenue du Peuple Murundi, Bujumbura-
15 Burundi.

16 5. Ministère de l'Élevage, des Pêches et des Industries/DSV, BP 2228 Yaoundé, Cameroun.

17 6. Direction de la Santé Animale / Ministère des Ressources Animales, B.P 12091 Niamey, Niger.

18 7. ECTAD Unit, Food and Agriculture Organisation (FAO), United Nations House, Central Area
19 District, Abuja, Nigeria.

20 8. Label Ivoire Elevage (LIVEL), 06 BP 117 Abidjan 06, Côte d'Ivoire.

21 9. Consultant, 01 BP 2266 Ouagadougou 01, Burkina Faso.

22 10. Centre National d'Élevage et de Recherche Vétérinaires (CNERV), BP 167, Nouakchott, Islamic
23 Republic of Mauritania

24

25 *Corresponding author:

26 Giovanni Cattoli

27 OIE/FAO Reference Laboratory for avian influenza and Newcastle disease,
28 Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10,
29 35020 Legnaro (PD), Italy. Tel +39 0498084389 Fax +39 0498084360
30 gcattoli@izsvenezie.it

31

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31 **Abstract**

32 Newcastle disease (ND) is an OIE listed disease caused by virulent avian paramyxovirus type 1
33 (APMV-1) strains, which affect many species of birds and may cause severe economic losses in the
34 poultry sector. The disease has been officially and unofficially reported in many African countries
35 and still remains the main poultry disease in commercial and rural chickens of Africa.
36 Unfortunately, virological and epidemiological information concerning ND strains circulating in the
37 Western and Central regions of Africa is extremely scarce. In the present study, sequence analysis,
38 pathotyping and detailed genetic characterization of virulent ND strains detected in rural poultry in
39 West and Central Africa revealed the circulation of a new genetic lineage, distinguishable from the
40 lineages described in the Eastern and Southern parts of the continent. Several mismatches were
41 observed in the segment of the matrix gene targeted by the primers and probe designed for the
42 molecular detection of APMV-1, which were responsible for the false negative results in the
43 diagnostic test conducted. Furthermore, deduced amino acid sequences of the two major antigens
44 eliciting a protective immune response (F and HN glycoprotein) revealed protein similarities < 90%
45 if compared to some common vaccine strains. Distinct mutations located in the neutralizing
46 epitopes were revealed, indicating the need for detailed assessment of the efficacy of the current
47 vaccines and vaccination practices in Africa. The present investigation provides important
48 information on the epidemiology, diagnosis and control of NDV in Africa and highlights the
49 importance of supporting surveillance in developing countries for transboundary animal diseases.

50

51 Key words: Newcastle Disease, poultry, Africa, genetic characterization

52

52 Introduction

53 Newcastle disease (ND) is a viral disease affecting many species of birds and causing severe
54 economic losses in the poultry sector. The aetiological agent is a single stranded, non-segmented,
55 negative-sense RNA virus belonging to the order *Mononegavirales*, family *Paramyxoviridae*,
56 subfamily *Paramyxovirinae*, genus *Avulavirus* (Lamb et al., 2005). This genus contains the 9
57 serogroups of avian paramyxoviruses (APMV-1 to -9) described so far. According to their virulence
58 in poultry, APMV-1 isolates can be classified as lentogenic, mesogenic or velogenic (i.e. of low,
59 moderate or high virulence for poultry, respectively). In the present paper the term ND virus (NDV)
60 refers to the virulent APMV-1 strains that cause ND.

61 The virulence of an APMV-1 isolate can be determined in the laboratory by an *in vivo* test
62 performed by the experimental inoculation of SPF day-old chicks with the isolate (intracerebral
63 pathogenicity index, ICPI) or it can be predicted molecularly by the analysis of the amino acid
64 sequence at the cleavage site of the fusion glycoprotein (F protein) (Alexander, 2009). Both these
65 methods are officially recognized for APMV-1 pathotyping by the World Organisation for Animal
66 Health – OIE - (OIE, 2008). The ICPI is considered the most sensitive “in vivo” test (Terregino &
67 Capua, 2009) and an index value of 0.7 or greater classifies the isolate as virulent (i.e. velogenic or
68 mesogenic) thus, confirming a diagnosis of ND in the infected hosts. Alternatively, the virulence of
69 ND strains can be assessed by the determination of the presence or not of multiple basic amino
70 acids (e.g. lysine – K; or arginine – R) located at the C-terminus of the F1 protein and phenylalanine
71 (F) at the N-terminus of the F2 protein, which correspond to the cleavage site of the precursor F0
72 glycoprotein (positions 112 to 117) (OIE, 2008).

73 In addition to the gene encoding the structural glycoprotein F, the APMV-1 genome of about 15 kb
74 is composed of 5 other genes encoding 5 structural proteins (nucleoprotein, NP; matrix protein, M;
75 phosphoprotein, P; RNA polymerase, L and haemagglutinin-neuraminidase, HN). Two additional
76 proteins are encoded by the RNA editing of the P protein, namely proteins V and W. The
77 cleavability of protein F is certainly the main determinant for viral virulence, but other proteins such

78 as HN and V are also believed to influence pathogenicity (Huang et al., 2004; de Leeuw et al.,
79 2005; Mebatsion et al., 2001).

80 As for many other RNA viruses, genetic variability of APMV-1 is significant. Recent analyses have
81 revealed the existence of two main distinct genetic clades, termed class I and II (Czeglédi et al.,
82 2006). Class I includes almost exclusively low virulence strains recovered from live bird markets in
83 US or wild waterfowl worldwide. Class II is comprised of the vast majority of viruses of high and
84 low virulence isolated from poultry and wild birds (Czeglédi et al., 2006; Kim et al., 2007).

85 Restriction enzyme and partial sequence analysis of the gene encoding for the F protein (F gene)
86 classified class II APMV-1 isolates in eight main distinct groups (I to VIII) with several sublineages
87 within them (Herczeg et al., 1999). In another study, based on the partial F gene sequence of a large
88 number of APMV-1 collected worldwide (Aldous et al., 2003), the viruses were grouped in 6
89 distinct genetic lineages (1 to 6) with several sublineages within them. The majority of the NDV
90 isolates responsible of the recent outbreaks in Europe, Asia and Southern Africa are placed in
91 lineages 3, 4 and 5 (Alexander et al., 1999; Herczeg et al., 1999; Herczeg et al., 2001; Cattoli et al.,
92 2001; Aldous et al 2003; Abolnik et al., 2004). As an OIE listed disease the notification of ND
93 outbreaks by the OIE member countries is mandatory. APMV-1 is distributed worldwide and ND
94 outbreaks have been notified regularly in all continents since the first description of the disease in
95 1926 (Alexander, 2009). In Africa, outbreaks in poultry caused by NDV have been described in the
96 Eastern and Southern part of the continent (Herczeg et al., 1999; Otim et al., 2004; Abolnick et al,
97 2004). The disease has been officially and unofficially reported in other parts of Africa and,
98 according to the United Nations-Food and Agricultural Organization (UN-FAO), ND still remains
99 the main poultry disease in commercial and rural chickens of West Africa (Bebay, 2006).

100 Unfortunately, virological and epidemiological information concerning ND strains circulating in the
101 Western and Central regions of Africa is extremely scarce and while historically ND has been
102 reported to be widespread (Lancaster & Alexander, 1975) in recent years there has been only a
103 single report (Snoeck et al., 2009). In that investigation, NDVs were detected by PCR in non-

104 commercial farms in Niger, Nigeria and Burkina Faso but viral isolates were not obtained. For this
105 reason, characterization of the detected ND was restricted to the sequence analysis of partial F gene
106 sequences. The authors suggested that NDVs responsible for the outbreaks in the three countries
107 were phylogenetically distinguishable from NDVs circulating in Eastern and Southern Africa and
108 were tentatively grouped in new sublineages within lineage 5, namely 5e, 5f and 5h (Snoeck et al.,
109 2009).

110 In the present investigation we perform a full molecular characterization of ND viruses causing ND
111 outbreaks in poultry in 7 Western and Central African countries. We also discuss the diagnostic
112 implications of the genetic variability detected.

113 **Materials and methods**

114 *Samples included in this study*

115 Virological investigations using real time RT-PCR (rRT-PCR) for APMV-1 and virus isolation (VI)
116 attempts were performed on 50 samples collected from rural chickens in Nigeria, Niger, Ivory
117 Coast, Burkina Faso, Mauritania, Cameroon and Burundi between 2006 and 2008. Samples
118 consisted of internal organs (such as trachea, lungs and intestines) and swabs (cloacal and tracheal)
119 collected during surveillance programs (3,614 samples) or in cases of suspected disease (387
120 samples) and submitted to the OIE/FAO Reference Laboratory for Newcastle disease and Avian
121 Influenza at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in Italy within the
122 framework of diagnostic activities and international collaboration projects to support developing
123 countries.

124 *Laboratory testing*

125 For molecular testing, viral RNA was extracted from the samples using the Nucleospin RNA II Kit
126 (Machery-Nagel, Duren, Germany) and the detection of APMV-1 RNA was performed using a
127 rRT-PCR protocol targeting the M gene (Wise et al, 2004). For virus isolation (VI), samples were
128 prepared and inoculated in SPF embryonated chicken eggs according to the international regulations
129 (OIE, 2008). The allantoic fluid of eggs showing embryo mortality was collected and tested for

130 haemagglutinating activity. Haemagglutinating agents were identified by means of
131 haemagglutination inhibition (HI) tests using standard protocols (OIE, 2008). The virulence of the
132 APMV-1 isolates for chickens was assessed by intracerebral pathogenicity index (ICPI) tests in
133 day-old SPF chickens and by genetic sequencing of the fusion protein cleavage site (Terregino &
134 Capua, 2009).

135 *Nucleotide sequencing and genetic pathotyping*

136 Viral RNA was extracted from the allantoic fluid of APMV-1 positive SPF eggs as described above.
137 For genetic pathotyping a 266-bp hypervariable region of the F gene encompassing the cleavage site
138 (positions 4652 to 4917, with reference to NDV strain La Sota, GenBank accession number
139 AF077761) was RT-PCR targeted (Cattoli & Monne, 2009). Briefly, the APMV-1 F gene segment
140 was amplified with primers NOH-For (5' TACACCTCATCCCAGACAGG 3') and NOH-Rev (5'
141 AGTCGGAGGATGTTGGCAGC 3') using the Qiagen one-step RT-PCR kit (Qiagen, Hilden,
142 Germany). Amplicons were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit
143 (Applied Biosystem, Foster City, CA - USA). The products were cleaned using PERFORMA DTR
144 Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD - USA) and sequenced in a 16-capillary
145 ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA - USA).

146 *Phylogenetic analysis*

147 To determine the phylogenetic relationships between West and Central Africa viruses and APMV-1
148 viruses previously isolated in Africa and other parts of the world, the sequences of the 266-bp
149 hypervariable region of the F gene were compared to the corresponding region of 332 representative
150 viruses available in GenBank (<http://www.ncbi.nlm.nih.gov/>), for which the genotype was known.
151 Previous studies have demonstrated that sequences of 250 base pairs give meaningful phylogenetic
152 analyses, comparable with those obtained with much longer sequences (Seal et al., 1995; Lomniczi
153 et al., 1998; Snoeck et al., 2008).

154 The lineage-based nomenclature illustrated in a previous study (Aldous et al., 2003) is adopted
155 throughout this paper.

156 To confirm the robustness of the genetic groupings obtained by the phylogenetic analysis of the
157 partial F gene sequences and to detect mutations potentially associated with antigenic and virulence
158 changes, the complete sequences of the fusion (F), haemagglutinin-neuraminidase (HN) and matrix
159 (M) genes of selected African APMV-1 isolates were obtained. For this purpose, RNAs extracted
160 from infective allantoic fluid were reverse transcribed with the SuperScript III Reverse
161 Transcriptase kit (Invitrogen, Carlsbad, CA - USA) and PCR amplification was performed using
162 gene specific primers (Table S1). Using the full length genome positions from the La Sota vaccine
163 strain complete genome (accession number AF077761), the nucleotide regions sequenced were as
164 follows: the complete coding region for the genes F (positions 4544 to 6205; n = 16 isolates), HN
165 (positions 6412 to 8127; n = 17 isolates), M (positions 3290 to 4384; n = 18 isolates). Sequence
166 alignments were constructed and used to develop phylogenetic trees using Bayesian methods
167 available in the computer program MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003).
168 Specifically, we executed a Markov chain Monte Carlo search for one million generations using
169 two runs and four chains (temperature=0.05) and represented the results of the search as a 50%
170 majority rule consensus tree. In each case, the best-fit model of nucleotide substitution was
171 identified using the Akaike information criterion (Posada & Buckley, 2004) as implemented in
172 Modeltest version 3.7 (Posada & Crandall, 2001) (parameter values available from authors on
173 request).

174 Nucleotide diversity was calculated using the Maximum Composite Likelihood method
175 implemented in the MEGA4 software package (Tamura et al., 2007).

176 *Nucleotide sequence accession numbers*

177 GeneBank accession numbers of the sequences analysed in this study are FJ772445 to FJ772495

178 **Results**

179 *Detection of APMV-1 isolates in the samples*

180 The rRT-PCR protocol detected the presence of APMV-1 RNA in 43 of 50 of the clinical samples
181 included in the investigation (Table 1). Forty-one of the samples positive by rRT-PCR were also

182 positive by virus isolation. One sample from Niger and one from Nigeria, both collected in 2008
183 were positive by rRT-PCR but gave a negative result by virus isolation. However, 7/16 of the
184 samples collected in 2006 (3 in Niger and 4 in Mauritania) were negative by the rRT-PCR protocol
185 used but positive by virus isolation in eggs. The M gene rRT-PCR test was carried out on the
186 positive allantoic fluids obtained in the virus isolation tests from these samples in which APMV-1
187 viruses had been confirmed by HA and HI tests, but the rRT-PCR results were also negative. When
188 the rRT-PCR-negative/HI-positive allantoic fluids were tested by the RT-PCR protocol used for
189 molecular pathotyping targeting the F gene positive results were obtained, thus confirming the
190 HA/HI test results and enabling the pathotyping of the isolates.

191 *Phylogenetic analysis of partial F gene sequences*

192 For genetic sequencing, 28 of the 48 APMV-1 isolates were selected, based on the year of isolation,
193 country of origin and outbreak location. The 266 bp region of the fusion gene of these isolates was
194 sequenced and compared phylogenetically to the corresponding region of 332 representative viruses
195 belonging to the six lineages characterized by Aldous et al., (2003). The resulting phylogenetic tree
196 is presented in figure 1.

197 From the topology of the Bayesian tree, presented in figure 1, it can be seen that the two NDV
198 isolates from Burundi were placed in sublineage 5b, a heterogeneous sublineage which is composed
199 of isolates mainly originating from Europe, Africa, Asia and America (Aldous et al., 2003). The
200 sequences of these viruses revealed the highest similarity at nucleotide (nt) level (97.6%) with an
201 isolate from South Africa ZA/71/BA/94 (accession number AF532751). In contrast, the other
202 viruses analysed clustered together in a distinct group showing a mean nt similarity of 88.7% with
203 the sequences of lineage 5 viruses and, more specifically, 89.4% with sublineage 5b viruses. Since
204 the mean nt diversity observed between this new group and lineage 5 (11.3%) falls within the range
205 of nt diversity (10.3% – 43.2%) calculated for the existing 6 lineages, as shown in table 2, we
206 considered this group represented a new lineage, provisionally named lineage 7. At present 33
207 isolates make up lineage 7, all originating from West Africa. Twenty-six isolates were sequenced in

208 the present study and the sequences of seven other viruses were obtained in a previous study
209 (Snoeck et al., 2009). Lineage 7 can be further subdivided into four, statistically supported,
210 sublineages (7a to 7d) with posterior probabilities >91% (figure 2).

211 Sublineage 7a contained 8 isolates, of which 7 were from samples obtained in 2006 in Mauritania
212 and one from a 2008 Ivory Coast sample. The maximum nt divergence within this group was 6.4%.

213 Sublineage 7b was the most heterogeneous sublineage in terms of country of origin of the viruses
214 included in this study. This lineage consisted of 14 viruses originating from four different countries,
215 namely Niger(n=4), Nigeria (n=4), Cameroon (n=2) and Burkina Faso (n=4) obtained from samples
216 collected in 2006 and 2008. The maximum nt divergence within this group was 4.7%.

217 Sublineage 7c consisted exclusively of three isolates from Burkina Faso obtained in 2006, the
218 partial sequences of which were recently published in GenBank (Snoeck et al., 2009). The
219 maximum divergence within this group was 1.5%.

220 The isolates in sublineage 7d were grouped in two further distinguishable clusters: the first (group I)
221 contained five isolates exclusively from Niger, collected in 2006 and 2007 and the second cluster
222 (group II) was composed of three viruses obtained in 2006, two from Niger and one from Nigeria.
223 The maximum divergence within this group was 7.7%.

224 *Pathotyping of APMV-1 isolates*

225 For the APMV-1 isolates selected, the virulence was determined by molecular sequencing and ICPI
226 tests . The deduced amino acids at the F0 protein protease cleavage site revealed that all 28 of the
227 selected isolates had motifs that are typical of velogenic strains (OIE, 2008). Three possessed the
228 motif ¹¹²RRRKR/F¹¹⁷ and the other 25 had the motif ¹¹²RRQKR/F¹¹⁷ . In order to reduce the use of
229 experimental animals, ICPI tests were limited to representative isolates for each country that
230 possessed the different deduced amino acid motifs at the cleavage site of the F protein (Table 3). As
231 shown in table 3, the ICPI values were similar for the two cleavage site motifs and confirmed the
232 virulence of these viruses. The lineage 5b isolate from Burundi produced the highest ICPI value of
233 1.97. Viruses placed in the new lineage 7 gave ICPI values ranging from 1.7 to 1.87.

234 *Phylogenetic analyses of complete F, HN and M gene sequences*

235 For lineage 7, the topology of the phylogenetic tree created using 266-bp fusion gene fragments was
236 confirmed by analyses of the complete coding regions of the F, M and HN genes of 16, 18 and 17
237 representative viruses, respectively (figure 3). By this analysis it was possible to confirm the results
238 obtained for three of the four sublineages, 7a,7b and 7d. For sublineage 7c containing viruses that
239 were not available for sequencing in the present study, only partial F gene sequences had been
240 deposited in GenBank (Snoeck et al., 2009) and therefore sublineage confirmation was not possible.
241 Sequences of F, HN and M genes show comparable ratios of non-synonymous to synonymous
242 substitutions per site ($K_a/K_s=0.086, 0.109$ and 0.053 respectively) indicating the absence of positive
243 selection.

244 Complete nucleotide sequences of the HN genes of viruses belonging to lineage 7 showed the
245 highest nucleotide diversity (12%) compared to the complete sequences of F and M genes of the
246 same viruses for which 11.5% and 10% diversity was recorded respectively.

247 However, in the M gene several mismatches were observed in the region targeted by the primers
248 and probe designed for the rRT-PCR detection of APMV-1 (Wise et al; 2004) (Figure 4).
249 Additional rRT-PCR testing (data not shown) allowed us to establish that the presence of at least 3
250 mismatches in the probe was presumably responsible for the false negative results in the diagnostic
251 molecular test.

252 *Analysis of amino acid sequences*

253 At the F protein cleavage site, sublineages 7a, 7b and 7d-group I possessed four basic amino acids
254 (RRQKR/F), as do most of the viruses belonging to lineage 5. Sublineages 7c and 7d-group II
255 showed five basic amino acids (RRRKR/F). The amino acid arginine at position 114 was codified
256 by two different codons (CGA for sublineage 7c and CGG for sublineage 7d) suggesting that this
257 point mutation occurred independently in the two groups of viruses.

258 Potential N-glycosylation sites at positions 85, 191, 366, 447, 471, 541, characteristic of APMV-1,
259 were conserved in the F glycoprotein of all the NDV isolates belonging to lineage 7. The HN

260 glycoprotein sequence of lineage 7 viruses contained the five potential N-glycosylation sites
261 (residues 119, 341, 433, 481, 508) that are conserved among APMV-1 viruses. One isolate from
262 Nigeria and one from Niger contained an additional site at position 538. However, a previous study
263 has shown that two of these sites (at positions 508 and 538) are not truly glycosylated (McGinnes *et*
264 *al.*, 1995).

265 Residues within the HN protein, which is essential for receptor binding, neuraminidase (NA) and
266 haemagglutination (HA) activity of APMV-1 viruses, (Seal, 2004) were conserved in all the isolates
267 analysed.

268 For 13 of the 14 viruses for which the complete HN and F sequences were obtained at least one
269 amino acid substitution was detected within the known virus neutralizing epitopes of the HN and F
270 proteins (table 4). Specifically, mutations E347D, Y350H and D569G were detected in three
271 neutralizing epitopes of the HN protein (Iorio, et al., 1991), while amino acid substitutions K78R,
272 D170S and L343P were detected within three epitopes located in the F protein (Yusoff et al., 1989).
273 Comparison of the nt and amino acids sequences available for some common vaccine strains, such
274 as La Sota (Genbank accession number AF077761) and V4 (Genbank accession number
275 AY225110), revealed HN protein similarity ranging between 85.5% and 87.7% with the La Sota
276 strain and between 85.1% and 87.4% with the V4 strain . For the F protein, similarity ranged from
277 86.4% to 88.8% with the La Sota strain and from 86.2% to 88.5% with the V4 strain, while for M
278 protein similarity ranged from 87% to 89.5% with the La Sota strain and from 88.8% to 92.2% with
279 V4 strain.

280 The HN proteins of each of the analysed viruses had a length of 571 amino acids, which is the
281 length most commonly observed for the virulent NDV isolates and possessed a cysteine at position
282 123, which is required for the formation of disulphide-linked HN homodimers. A previous study
283 demonstrated that the amino acid cysteine at this position has an influence on both attachment
284 activity and fusion promotion activity and increased the virulence and the pathogenicity of NDV
285 viruses (Romer-Oberdorfer et al., 2006).

286 **Discussion**

287 During the period 2005-2008, 38 African countries notified ND outbreaks to the World
288 Organisation for Animal Health (OIE-WAHID interface, Available at:
289 <http://www.oie.int/wahis/public.php>. Accessed on 2nd February 2009). Nineteen of the 38 countries
290 are in West and Central Africa. APMV-1 is clearly widespread in Africa but very limited
291 information is available on the characteristics of the strains circulating in this continent, with the
292 exception of those seen in South Africa (Herczeg et al, 1999; Aldous et al., 2003; Abolnik et al.,
293 2004) and Uganda (Otim et al., 2004). In West and Central Africa, virological data concerning
294 APMV-1 were almost completely absent. Very recently, Snoeck et al (2009) detected NDVs
295 belonging to lineage 3 and 4 in Nigeria. But other APMV-1 viruses detected in Nigeria, Burkina
296 Faso and Niger were tentatively placed in lineage 5 by genetic analysis of a partial fragment of the
297 gene F. In this study Snoeck et al (2009) suggested these viruses formed novel sublineages, namely
298 5g, 5f and 5h or even new genetic lineages. However, isolates were not obtained and the
299 conclusions were considered preliminary by the authors since data were obtained on partial
300 sequence (354 nt) of the F gene only.

301 In our present study the results of the phylogenetic analyses indicate that the majority of the West
302 and Central African strains belong to a novel lineage, here provisionally named lineage 7, which
303 includes the viruses previously assigned to 5g, 5f and 5h sublineages by Snoeck et al., (2009).
304 These findings are supported by the analysis of the complete sequences of the gene F, M and HN.
305 Furthermore, our study indicate that viruses of this newly described lineage are circulating in a vast
306 area of Africa, and are probably endemic in the region between Cameroon in the Central part of
307 Africa and Mauritania in the extreme west. Of the samples from West and Central African countries
308 that were examined in the present study only those from Burundi failed to yield virus of lineage 7.
309 We detected a high degree of genetic variability within lineage 7, which was similar in magnitude
310 to that described for lineage 5 (Aldous et al., 2003).

311 The present study also demonstrated the important impact genetic variability of viruses may have
312 on the diagnosis of this infection, highlighting the limit of the advanced molecular diagnostic tests
313 on viral disease diagnosis. Kim et al., (2007) recently demonstrated that the broad genetic diversity
314 between class I, which includes the genotype 6 described earlier (Aldous et al., 2003), and class II
315 APMV-1 may impair the performances of rapid molecular tests, including those having conserved
316 genes as target for PCR amplification. The results presented in our study on the sequence analysis
317 of the matrix gene demonstrated that genetic variability within class II viruses, may be equally
318 responsible for false negative molecular test results. A broader number of strains are necessary to
319 assess whether this problem is restricted to lineage 7 viruses or extends to other lineages.

320 In a comprehensive study Czeglédy et al., (2006) concluded that the origin of the most recent
321 virulent genotypes was not clear, but it appears that the diversification of the recent epizootic
322 viruses and lineages, has accelerated in the last few decades and suggested that vaccination could
323 have played a role in the generation and spread of the most recent virulent viruses. It has been
324 recently demonstrated (Miller et al., 2007) that genetic diversity in APMV-1 strains influences the
325 efficacy of vaccination control, not necessarily in terms of clinical protection, but mainly in term of
326 virus shedding and subsequent spread of the infection. Miller et al., (2007) showed that virus
327 shedding in chickens experimentally challenged with ND strains correlated with HN and F protein
328 similarities between the challenge and vaccine strains. HN and F protein similarities less than or
329 equal to 90%, as those observed in our study, were significantly related to higher virus shedding. In
330 addition to this, the distinct mutations detected in the African strains analysed in the present study,
331 located in the neutralizing epitopes of the two major antigens (F and HN glycoprotein) targeted by a
332 protective immune response (Seal et al., 2000), present the scientific rationale for deeper
333 investigations on the antigenic properties of these isolates and for detailed assessments of the
334 efficacy of the current vaccines and vaccination practices in Africa.

335 To conclude, the present investigation provides important information on the epidemiology,
336 diagnosis and control of NDV in Africa and highlights the importance of supporting surveillance in

337 developing countries for transboundary animal diseases. It provides evidence on the need to
338 facilitate data and sequence sharing and to monitor the efficacy of validated diagnostic tests
339 continuously. As demonstrated by the present and previous studies (Xing et al., 2008), neglecting
340 traditional assays such as virus isolation may pose serious risks for the correct diagnosis of
341 contagious diseases. As predicted by Aldous et al. (2003), novel genetic lineages and sublineages
342 are emerging as a consequence of the continuous evolution of APMV-1. However, well defined
343 criteria for lineage or sublineage designation are currently not existing for this virus. This study
344 provide additional genetic data which might be useful for the definition of general criteria for
345 lineage designation, similarly to what has been done for the highly pathogenic avian influenza virus
346 A/H5N1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2008).

347

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468

468 **Table 1.** Detection of APMV-1 viruses in samples by rRT-PCR and virus isolation.
 469

Country of origin	Year of isolation	Number of samples positive for APMV-1 by:	
		rRT-PCR	Virus Isolation
Burkina Faso	2008	4	4
Burundi	2008	2	2
Ivory Coast	2008	1	1
Niger	2006	3	6
	2008	11	10
Nigeria	2006	3	3
	2008	5	4
Cameroon	2008	11	11
Mauritania	2006	3	7
Totals		43/50	48 /50

470
 471

471 **Table 2** - The mean number of base substitutions per site for all sequence pairs between lineages is
472 shown . Analyses were conducted using the Maximum Composite Likelihood method in MEGA4
473 software package.
474

LINEAGE	1	2	3	4	5	6
2	0.126					
3	0.140	0.168				
4	0.145	0.172	0.116			
5	0.159	0.193	0.127	0.103		
6	0.400	0.417	0.442	0.432	0.420	
7	0.203	0.212	0.158	0.140	0.113	0.457

475
476

476 **Table 3.** Pathotyping results of representative African APMV-1 isolates
 477

Isolate	Cleavage site amino acid motif	Lineage	ICPI ^a
Ivory Coast/2601/2008	¹¹² RRQKR/F ¹¹⁷	7a	1.7
Mauritania/1532-1/2006	¹¹² RRQKR/F ¹¹⁷	7a	1.85
Burkina Faso/2415-580/2008	¹¹² RRQKR/F ¹¹⁷	7b	1.8
Burkina Faso/2415-361/2008	¹¹² RRQKR/F ¹¹⁷	7b	1.8
Niger/2602-468/2008	¹¹² RRQKR/F ¹¹⁷	7b	1.7
Nigeria/3724-6/2008	¹¹² RRQKR/F ¹¹⁷	7b	1.8
Cameroon/3490-147/2008	¹¹² RRQKR/F ¹¹⁷	7b	1.87
Niger/1377-14/2006	¹¹² RRQKR/F ¹¹⁷	7d-I	1.78
Niger/1377-8/2006	¹¹² RRQKR/F ¹¹⁷	7d-I	1.8
Niger/1377-9/2006	¹¹² RRRKR/F ¹¹⁷	7d-II	1.7
Burundi/4132-12/2008	¹¹² RRQKR/F ¹¹⁷	5b	1.97

478 ^aIntracerebral pathogenicity in day-old chicks.
 479

479 **Table 4** - Amino acid substitutions located within the neutralizing epitopes of HN and F proteins
 480

Gene	Substitution	Virus
HN	E347D	NDV_ck_2415_580_Burkina Faso_2008
		NDV_ck_2415_577_Burkina Faso_2008
	Y350H	NDV_ck_3490_147_Cameroon_2008
		NDV_ck_3490_149_Cameroon_2008
		NDV_ck_2602_605_Niger_2008
D569G	NDV_ck_2602_625_Niger_2008	
	NDV_ck_1377_1_Niger_2006	
F	K78R	NDV_ck_1377_1_Niger_2006
		NDV_avian_3724_6_Nigeria_2008
	D170S	NDV_ck_2601_Ivory Coast_2008
		NDV_ck_2601_Ivory Coast_2008
		NDV_avian_1532_14_Mauritania_2006
L343P	NDV_ck_2415_361_Burkina Faso_2008	

481
 482

482

483 **Figure 1** – A radial phylogram constructed by Bayesian analysis of 359 NDV isolates belonging to
484 the six lineages identified by Aldous et al., (Aldous et al., 2003). The region analysed was a 264
485 base pair fragment (109 to 374) at the 3' end of the fusion protein gene. Different colours are used
486 to differentiate the viruses from distinct lineages. The new lineage identified in this study is black
487 coloured. * indicates the isolates from Burundi sequenced in this study. The scale indicates the
488 number of substitutions per site. Posterior probabilities of the clades are showed above branches.

489

490 **Figure 2** – A radial phylogram constructed by Bayesian analysis of 33 NDV isolates (26 sequenced
491 in this study) belonging to lineage 7. The region analysed was a 266 base pair fragment (109 to 374)
492 at the 3' end of the fusion protein gene. The circles indicate the four sublineages (7a to 7d)
493 identified in this study. The scale indicates the number of substitutions per site. Posterior
494 probabilities of clades are indicated above branches.

495

496 **Figure 3** – Phylogenetic trees constructed by Bayesian analysis of the complete coding region of
497 HN, M and F genes of viruses analysed in this study. DE/R49/99 and La Sota were selected as
498 representative strains of class I and class II respectively. Blue, red and yellow rectangles indicates
499 the sublineages 7a, 7b and 7d, respectively. The scale indicates the number of substitutions per site.
500 Posterior probabilities of clades are indicated above branches.

501

502 **Figure 4** – Nucleotide sequence alignment of the matrix gene from representative samples tested
503 positive (+) or negative (-) by rRT-PCR. Nucleotide sequence differences between isolates are
504 shown, while identical nucleotides are indicated by a dot. The numbering of nucleotides is relative
505 to the complete genome sequence of the La Sota strain (reference sequence). Primer annealing
506 regions are indicated by two arrows, while probe annealing region is marked by a rectangular.

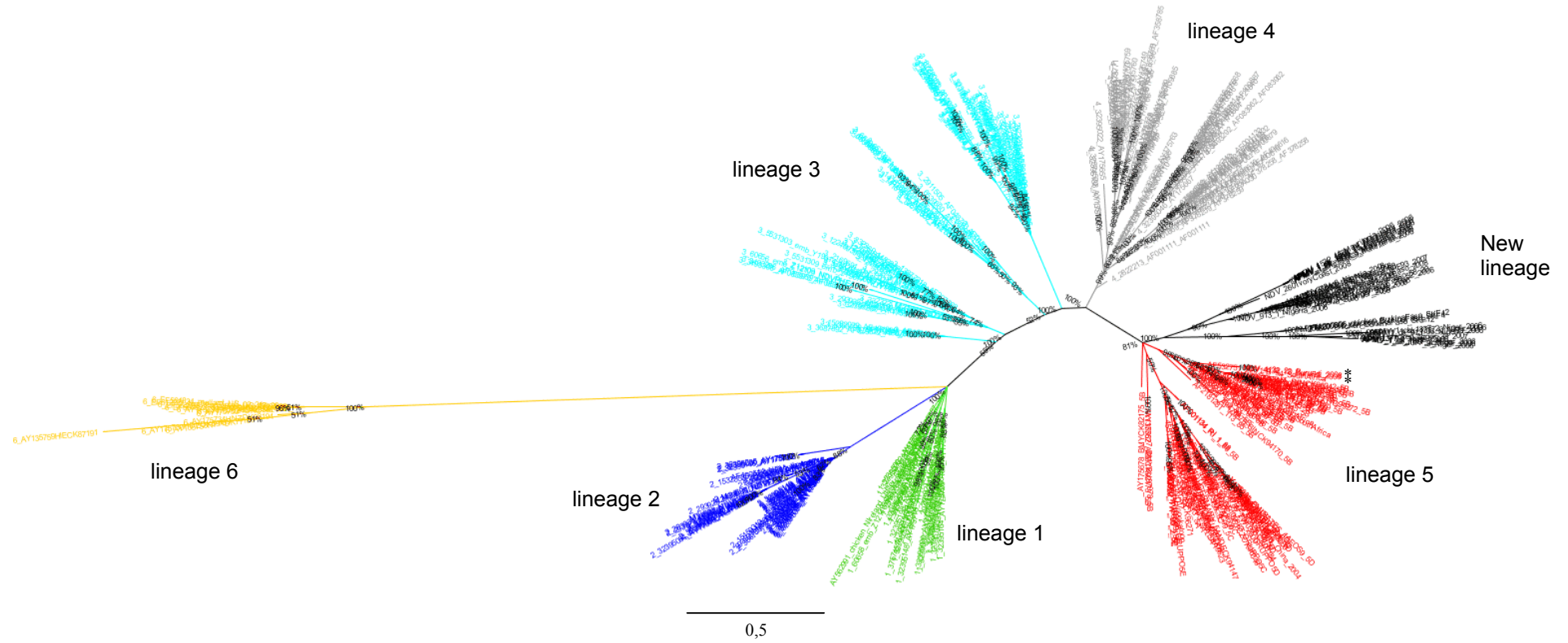
507

508 **Supplemental material**

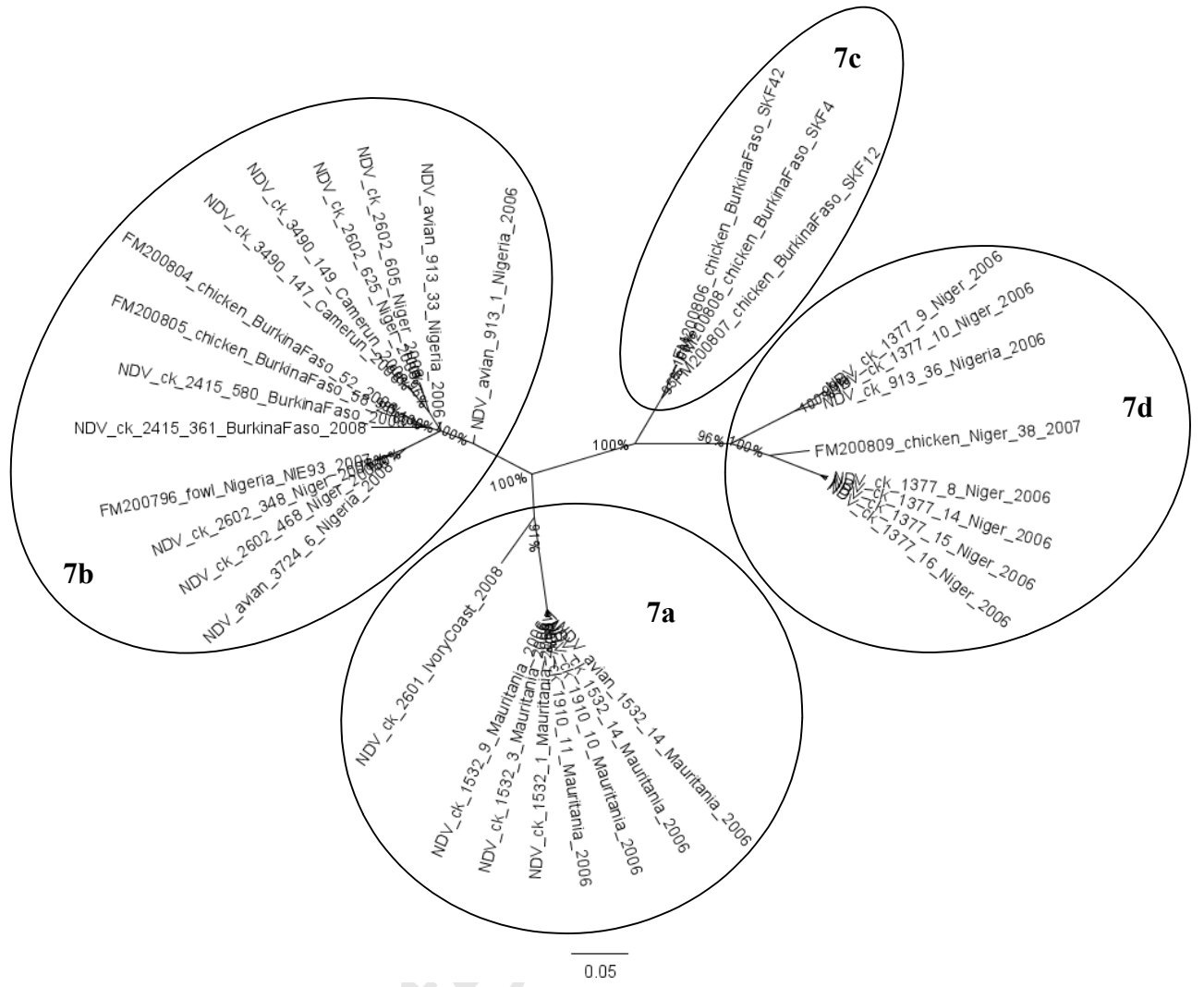
509 **Table S1:** Sequences of primers used for the amplification and sequencing of the F, M and HN gene

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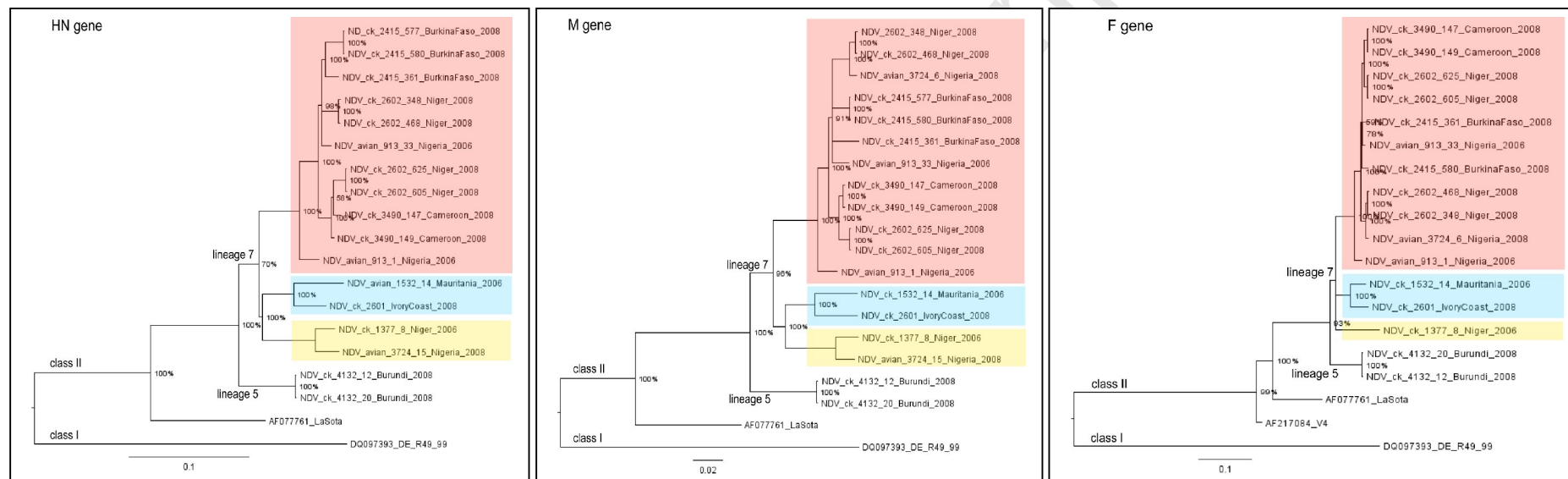


Table S1: Sequences of primers used for the amplification and sequencing of the F, M and HN gene

NAME		SEQUENCE	GENE
M-2996-F	forward	TGAAAACGACGGCCAGTCYAAGCTCCTRAGYAAGCTRGA	M
M-3910-R	reverse	CAGGAAACAGTATGACCCATCAATAGTGACATTGA	M
M-3293-F	forward	TGAAAACGACGGCCAGTAARATGGACTCATCYAGRAC	M
MF-3710-F	forward	TGAAAACGACGGCCAGTCAAAGCTGTADGGTTGTG	M
MF-4650-R	reverse	CAGGAAACAGTATGACCAAGAGGCCTGCCRTCAA	M
F-4514-F	forward	TGAAAACGACGGCCAGTGTAGAAGADTYTGGATCC	F
F-5218-R	reverse	CAGGAAACAGTATGACCGAATACYGTAGTCAAYTCRG	F
F-5443-R	reverse	CAGGAAACAGTATGACCAGGTGGCACGCATATTATT	F
F-5100-F	forward	TGAAAACGACGGCCAGTATGCAGCARTTTGTYAAT	F
F-4544-R	reverse	CAGGAAACAGTATGACCTARGTAATRAGAGCRGATG	F
F-5664-F	forward	TGAAAACGACGGCCAGTAGACYGAAGGCGCACTYAC	F
F-7979-R	reverse	CAGGAAACAGTATGACCAGRGCCACYTGCTTRTATA	F
F-5757-F	forward	TGAAAACGACGGCCAGTAGATRACAACATGTAGRTG	F
F-6449-R	reverse	CAGGAAACAGTATGACCGGCTAACYGCRCCGGTCCAT	F
F-START	forward	TGAAAACGACGGCCAGTATGGGCYCYARAYCTTCTAC	F
F-460-R	reverse	CAGGAAACAGTATGACCAGCAATGCTCTCYTTAAG	F
F-586-R	reverse	CAGGAAACAGTATGACCTTTAYACAGTCCAATTC	F
F-656-F	forward	TGAAAACGACGGCCAGTTACYTAACTGARYTGACTAC	F
F-1258-R	reverse	CAGGAAACAGTATGACCACATTGCATGAWTGTCTRTC	F
HN-6398-F	forward	TGAAAACGACGGCCAGTTCMCAACATCCGTTCTACCGCATC	HN
HN-7040-R	reverse	CAGGAAACAGTATGACCGAATGYGAGTGATCTCTGCA	HN
HN-6731-F	forward	TGAAAACGACGGCCAGTTTATGAAYGCAATAACNTC	HN
HN-6979-F	forward	TGAAAACGACGGCCAGTATGAGYRCTACCCAYTACTG	HN
HN-7549-R	reverse	CAGGAAACAGTATGACCATAGATAAGATGGCYTGCTG	HN
HN-7326-F	forward	TGAAAACGACGGCCAGTGGTGGCAAAYTACCCAGGAG	HN
HN-8072-R	reverse	CAGGAAACAGTATGACCAGGGTATTGGATATTTTCRGCAATGC	HN
HN-7110-F	forward	TGAAAACGACGGCCAGTATCGRAAGTCYTGCAGTGTG	HN
HN-8106-R	reverse	CAGGAAACAGTATGACCATCTCAACTAGTAAVGGGRACGATYC	HN
HN-8015-F	forward	TGAAAACGACGGCCAGTCATACACRACATCRACATG	HN
HN-8509-R	reverse	CAGGAAACAGTATGACCGGTARCCCAGTYAATTTCCA	HN