

# Newcastle Disease Virus in Madagascar: Identification of an Original Genotype Possibly Deriving from a Died Out Ancestor of Genotype IV

Olivier F. Maminiaina<sup>1</sup>, Patricia Gil<sup>2</sup>, François-Xavier Briand<sup>3</sup>, Emmanuel Albina<sup>2</sup>, Djénéba Keita<sup>2</sup>, Harentsoaniaina Rasamoelina Andriamanivo<sup>1</sup>, Véronique Chevalier<sup>4</sup>, Renaud Lancelot<sup>2</sup>, Dominique Martinez<sup>2</sup>, R. Rakotondravao<sup>1</sup>, Jean-Joseph Rajaonarison<sup>5</sup>, M. Koko<sup>5</sup>, Abel A. Andriantsimahavandy<sup>5</sup>, Véronique Jestin<sup>3</sup>, Renata Servan de Almeida<sup>2\*</sup>

**1** FOFIFA-DRZV, Antananarivo, Madagascar, **2** CIRAD, BIOS Department, UMR CMAEE, Montpellier, France, **3** Anses-Ploufragan Plouzané Laboratory, VIPAC Unit, Ploufragan, France, **4** CIRAD, ES Department, UPR AGIRS, Montpellier, France, **5** Antananarivo University Madagascar, Antananarivo, Madagascar

## Abstract

In Madagascar, Newcastle disease (ND) has become enzootic after the first documented epizootics in 1946, with recurrent annual outbreaks causing mortality up to 40%. Four ND viruses recently isolated in Madagascar were genotypically and pathotypically characterised. By phylogenetic inference based on the F and HN genes, and also full-genome sequence analyses, the NDV Malagasy isolates form a cluster distant enough to constitute a new genotype hereby proposed as genotype XI. This new genotype is presumably deriving from an ancestor close to genotype IV introduced in the island probably more than 50 years ago. Our data show also that all the previously described neutralising epitopes are conserved between Malagasy and vaccine strains. However, the potential implication in vaccination failures of specific amino acid substitutions predominantly found on surface-exposed epitopes of F and HN proteins is discussed.

**Citation:** Maminiaina OF, Gil P, Briand F-X, Albina E, Keita D, et al. (2010) Newcastle Disease Virus in Madagascar: Identification of an Original Genotype Possibly Deriving from a Died Out Ancestor of Genotype IV. PLoS ONE 5(11): e13987. doi:10.1371/journal.pone.0013987

**Editor:** Anthony R. Fooks, Veterinary Laboratories Agency, United Kingdom

**Received:** June 22, 2010; **Accepted:** October 17, 2010; **Published:** November 15, 2010

**Copyright:** © 2010 Maminiaina et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This study was mainly funded by the French Ministry of Foreign Affairs (MAE) via the FSP project [GRIPAVI 2006-26] and partly by the EU network of excellence project [EPIZONE (016236, 01/06/2006–31/05/2011, <http://www.epizone-eu.net/default.aspx>)]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: [renata.almeida@cirad.fr](mailto:renata.almeida@cirad.fr)

## Introduction

Newcastle disease (ND) is a highly contagious and widespread disease which causes severe economic losses in domestic poultry, especially in chickens [1,2]. In Africa, ND is the major constraint to rural chicken development. This disease is listed as a notifiable disease by the World Organisation for Animal Health (OIE) [3]. The causative agent of the disease is Newcastle disease virus (NDV), also designated avian paramyxovirus serotype 1 (APMV-1), which belongs to the genus *Auulavirus* within the family *Paramyxoviridae* [4,5]. The genome comprises a single stranded negative sense RNA that encodes the RNA-dependent RNA polymerase (L gene), the haemagglutinin-neuraminidase (HN gene), the fusion (F gene) and matrix (M gene) proteins, the phosphoprotein (P gene) and the nucleoprotein (NP gene). The genome is predicted to be 15186, 15192 or 15198 nucleotides (nt) in length, always a multiple of six nt, which fulfils the so-called 'rule of six' for optimised replication [6,7,8,9,10]. Based on the analysis of nucleotide sequence of the F protein gene, 10 different genotypes (I–X) or 6 different lineages (1–6) of NDV have been identified so far. The genotypes VI (lineage 4) and VII (lineage 5) are further divided into eight (a–h) and five (a–e) subgenotypes/sublineages, respectively [11,12,13,14,15,16,17,18,19]. APMV-1 strains can be categorised according to their virulence into highly (velogenic), intermediate (mesogenic) or non virulent (lentogenic).

The pathogenicity of APMV-1 isolates is assessed on the basis of *in vivo* tests including the intracerebral pathogenicity index (ICPI) in 1-day-old chickens, the mean time of death (MDT) of embryonated specific-pathogen-free hen's eggs after inoculation and the intravenous pathogenicity index (IVPI) in 6-week-old chickens [3]. The molecular basis for these different levels of pathogenicity is known to be linked to the sequence of cleavage site of the precursor fusion protein (F<sub>0</sub>). At this position, a pathogenic NDV strain (velogenic and mesogenic) has at least one extra pair of basic amino acids motif <sup>112</sup>X-R-X-R/K-R-F<sup>117</sup> [20] and can be cleaved by a wide range of proteases of the furin family in different host cells [21].

Since ND was first described in 1926, three worldwide panzootics have occurred [20]. The first panzootic (1926 to 1960) was caused by viruses belonging to genotypes II–III–IV and the second (1960 to 1973) and third (1970–1980) ones by genotypes V–VI. Moreover, severe outbreaks in Western and Southern Europe [22], [23], South Africa [24] and Taiwan [25] in the 90's were caused by genotype VII, the currently circulating genotype in Asia, Africa and Europe. In Madagascar, ND was firstly described in 1946 [26] and since then, outbreaks were regularly reported on the whole island mainly in the rural poultry sector [27]. While the vaccination rate is estimated to reach 100% in commercial farms in Madagascar, probably less than 10% of the free-range poultries are duly vaccinated. ND is considered to

induce more than 40% of mortality in such non protected poultries [28]. In spite of the importance and endemicity of ND in Madagascar, no data is available about the virus variants involved in clinical cases and/or maintenance of this disease in the island. In this study, four APMV-1 strains from Madagascar (named here MG group) were isolated in 1992 and 2008 and were molecularly characterised. Two of these strains were fully sequenced while the two other ones had only F and HN genes sequenced. Phylogenetic analyses showed that the MG group strains clustered within a new distinct genotype, closer to old genotypes. This study represents the first molecular characterisation of APMV-1 circulating in Madagascar and provides evidence on the existence of a new genotype close to an old died out genotype.

## Materials and Methods

### Ethics statement

All animal experiments (ICPI tests) were conducted according to internationally approved OIE standards, under authorizations set forth by the director of the veterinary services of Côtes d'Armor on behalf of the Prefect of Côtes d'Armor (N° 22-18) and the Director of the veterinary services of Hérault on behalf of the Prefect of Hérault (N° 34-114). Certificates of authorization are available from the authors upon request.

### Origin and isolation of virus strains

Synthetic information about the Madagascar isolates is provided in Table 1. An "old" strain (MG-1992) was first isolated by the *Centre National de Recherche Appliquée au Développement Rural* (FOFIFA-DRZV) in 1992, after suspicion of avian influenza or ND in a dead fowl assumed to be vaccinated against ND (La Sota vaccine). The three other isolates were collected in Madagascar during a surveillance programme in African wetlands led in 2007 and 2008 by the *Centre de Cooperation Internationale en Recherche Agronomique pour le Développement* (CIRAD), Montpellier, France and the FOFIFA-DRZV, Antananarivo, Madagascar. The MG-725/08 strain was recovered both from cloacal and tracheal swabs from an unvaccinated apparently healthy chicken. Another strain (MG39-04/08) was isolated from a dead chicken in a commercial farm: this chicken was initially vaccinated with the HB1 strain and boosted with the La Sota vaccine. The last isolate (MG-Meola/08) was recovered from a non vaccinated backyard poultry.

Samples of brain tissues or cloacal/tracheal swabs positives in the APMV-1 specific PCR (protocol based on M gene recommended by the reference laboratory of the OIE) were further processed for virus isolation by inoculation into 9-day old chicken embryonated eggs. All isolates were grown in less than 3 passages on eggs and tested by PCR before molecular sequencing.

### In vivo assay

The intracerebral pathogenicity index (ICPI) was determined according to international OIE standards (OIE, 2009) for two isolates of the MG group (MG-1992 and MG-725/08). Briefly, fresh allantoic fluid with a HA titre  $>1/16$  was diluted  $10^{-1}$  in sterile isotonic saline buffer. Following filtration through 0.22 or 0.45  $\mu\text{m}$  filters this diluted virus was injected intracerebrally (0.05 ml) into 10 one day-old chick hatched from embryonated SPF hens' eggs. Whereas each inoculum was checked for lack of bacterial and fungal contamination following culture on trypto casein soja and sabouraud media, the birds were examined at 24h intervals for 8 days and given a score (0, 1 or 2) according to their status (respectively healthy, sick or dead). The ICPI was calculated as the total of individual scores during 8 days divided by 80 (the number of days x the number of chickens).

### RNA isolation, cDNA synthesis and nucleotide sequencing

For reverse transcription and PCR reactions, RNA was extracted from allantoic fluids by using the Nucleospin<sup>®</sup> RNA Virus kit (MachereyNagel) following the manufacturer's instructions. The cDNA transcriptions were carried out by using the First-strand cDNA synthesis kit (GE Healthcare). Six pairs of oligonucleotide primers previously published [13,29] or designed in this study (Table S1 in supporting material), using BioEdit version 7.0.9.0 and Vector NTI software version 11.0 (©2008 Invitrogen Corporation), were used to amplify six overlapping DNA fragments to generate the complete sequences of F and HN protein genes. All DNA fragments were sequenced in both directions by Cogenics Genome Express S.A. (Meylan-France). For the rest of the genome, 23 overlapping PCR were realized with Platinum Taq DNA polymerase (Platinum<sup>®</sup> Taq DNA Polymerase High Fidelity, Invitrogen). The DNA sequences were determined in both senses using the Big dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions and using the same primers as to PCR reactions.

### Alignment of the F and HN predicted amino acid sequences and phylogenetic analyses

The sequences of the four isolates were compared with previously reported NDV sequences representative of different genotypes available in GenBank. A 374 nucleotide (nt) fragment of a variable portion of the F protein gene (47 to 421), including the F<sub>0</sub> cleavage site and a 1713 nt (92 to 1805) of HN gene were processed by Clustal W [30] in alignX program included into Vector NTI software suite (version 11.0). The nucleotide sequence databank accession numbers of ND viruses used in this study are shown in supporting Table S2 of supporting material. Alignment of complete amino acid sequences of F and HN was processed by

**Table 1.** NDV isolates from Madagascar used in this study.

| Strain                      | Code        | Region/Country           | Statut                   |                 | Sequence  |                       |
|-----------------------------|-------------|--------------------------|--------------------------|-----------------|-----------|-----------------------|
|                             |             |                          | Clinic                   | Vaccine         | size (nt) | Covering full genes   |
| APMV1/Chicken/MG/1992       | MG-1992     | Ivato-Madagascar         | Dead laying chicken      | La Sota         | 15082     | NP, P, M, F, HN and L |
| APMV1/Chicken/MG/725/2008   | MG-725/08   | Mahitsy-Madagascar       | Healthy rural poultry    | NV <sup>a</sup> | 15097     | NP, P, M, F, HN and L |
| APMV1/Chicken/MG/39-4/2008  | MG-39-04/08 | Ivato-Madagascar         | Dead broiler chicken     | La Sota         | 4249      | F and HN              |
| APMV1/Chicken/MG/Meola/2008 | MG-Meola/08 | Tsarahonenana-Madagascar | Sick grower fighter cock | NV              | 3966      | F and HN              |

<sup>a</sup>: not vaccinated.

doi:10.1371/journal.pone.0013987.t001

alignX program in Vector NTI software and finalised using MEGA4 software (version 4.1). Phylogenetic relationships and nucleotide divergence analysis (intra and inter genotype) were established with MEGA4 [31] using the Kimura 2-parameter correction in which the transition/transversion ratio was estimated from the data [32]. The statistical significance of the tree topology generated with the neighbor-joining algorithm was evaluated by 1,000 bootstrap resamplings of the data [33]. To confirm the robustness of the genetic groupings obtained by the phylogenetic analysis on the partial fragment (374 nt) a phylogenetic tree based on full-genome sequences of two isolates of the MG group (MG-1992 and MG-725/08) and 86 other full-genome sequences belonging to different genotypes were further carried out using the same parameters used for F and HN analyses. Putative recombination events in the two full-genomes of MG isolates were examined using the recombination detection program RDP 3.0b [34].

## Results

### Determination of *in vivo* pathogenicity (ICPI test)

ICPI values were determined for the isolates MG-1992 and MG-725/08. These isolates showed a very high ICPI of 1.9 being classified into a velogen-type NDV (Table 2).

### Sequencing MG, genome organisation and phylogenetic analyses

The sequence covering the six open reading frames (ORFs: NP, P, M, F, HN, L), without the complete sequence of the 3'-leader/5'-trailer of two MG strains, MG-725/08 and MG-1992, and the complete nucleotide sequence covering the F and HN genes of two other strains, MG-39-04/08 and MG-Meola/08, obtained in this work were analysed along with those of reference strains retrieved from GenBank.

Class II APMV-1 isolates have two genome lengths. The genotypes considered as "early" (1930–1960; genotypes I to IV, IX and H33(W)) contain 15,186 nt and recent genotypes (that emerged after 1960, genotypes V–VIII and X) contain 15,192 nt (15,186 nt+6 nt in the 5' non-coding region or NCR of NP gene) [12]. As seen in recent genotypes, the MG group has six nucleotides insertion in the 5' non-coding region (NCR) of the nucleoprotein gene between nucleotides 1647 and 1648 increasing its genome size to 15,192 nt (Figure 1).

The phylogenetic analysis of F gene (Figure 2) shows that the MG strains are grouped together. They are closer to the "old" genotype IV but may be distant enough to constitute a new genotype, proposed here as genotype XI or lineage 3g. This phylogenetic topology created using 374 nt fusion gene fragments was confirmed by phylogenetic analyses based on the full NH gene (data not shown) and on the full-genome sequences of two isolates of the MG group (MG-1992 and MG-725/08) and 86 other full-genome sequences belonging to different genotypes (Figure 3).

Based on a partial fragment of F gene (374nt), genetic divergences between the MG group and the other ten genotypes (I to X) are presented in Table 3. The F nucleotide sequences divergence within the MG group was 5%. The maximum intra-genotype divergence in old genotypes was 7% (genotypes I and IV) whereas the maximum intra-genotype divergence in recent genotypes was 9% (genotype VIII). The lower genetic divergence found between the MG group and another genotype (genotype IV) was 12%. In contrast, the higher divergence (25%) was observed with the genotype II that includes the La Sota vaccine strain. Since the maximal intra-genotype divergence ever observed does not exceed the minimal divergence of the MG group with other

genotypes, our data support the hypothesis that MG group constitutes a distinct new genotype. This new genotype is further confirmed by the phylogenetic analysis of the full-genome sequences (Figure 3). Surprisingly, the phylogenetic analysis of whole genome sequences also show that the genotype IV does not cluster with the "old genotypes" (I, II, III, IX), as suggested before (Czegledi et al., 2006), but rather with "recent genotypes" (V,VI,VII).

The analysis of the F<sub>1</sub>/F<sub>2</sub> protein cleavage site sequence of the four MG isolates showed that they share an original cleavage site motif formed by five arginines (R) <sup>112</sup>R-R-R-R-R<sup>116</sup> at the C terminus of F<sub>2</sub> exhibiting an atypical Q/R substitution at position 114 followed by a phenylalanine (↓F<sup>117</sup>) at position 117 of the F<sub>1</sub> amino terminus (Table 2). This motif is considered as a velogenic motif according to OIE [3]. Other virulent-like cleavage motifs with five basic amino acids have been described for genotypes VI, VII and VIII that are formed by a combination of arginine (R) and lysine (K) residues like <sup>112</sup>R-R-K-K-R<sup>116</sup>\*F<sup>117</sup> or <sup>112</sup>R-R-R-K-R<sup>116</sup>\*F<sup>117</sup> [35,36,37,38,39]. The substitution of a glutamine residue by a basic amino acid (R or K), corresponding to nucleotide mutation at positions 4885 or 4886 (CAG→CGG or AAG) is only found in some strains pertaining to the "recent" genotypes VI or VIII.

Like other NDV strains, the complete length of F and HN genes of MG group viruses were 1792 nt and 2002 nt, respectively. The F gene of all MG group isolates showed a single open reading frame (ORF) beginning with a unique double start codons <sup>44</sup>AUGAUG<sup>49</sup> and ending at position 1705 followed by the UGA stop codon. Consequently, the open reading frame of the F gene may extend to 1665 nt rather than 1662 nt usually found in other reference strains.

The predicted amino acid sequences of the complete F gene of the four MG isolates was analysed and compared with different strains pertaining to genotypes I to IX (Table 4). This analysis showed that all seven neutralising epitopes critical for both structure and function of the protein positioned at individual residues D<sup>72</sup>, E<sup>74</sup>, A<sup>75</sup>, K<sup>78</sup>, A<sup>79</sup>, L<sup>343</sup> and the stretch of amino acids <sup>157</sup>ILRLKESIAATNEAV-HEVTDG<sup>171</sup>, are conserved [40,41,42]. The twelve cysteines residues placed at positions 25, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424 and 523 [43] and the predicted N-glycosylation sites (Asn-X-Ser/Thr or N-X-S/T) where X is any amino acid except aspartic acid or proline, located at positions <sup>85</sup>N-R-T<sup>87</sup> <sup>191</sup>N-K-T<sup>193</sup>, <sup>366</sup>N-T-S<sup>368</sup>, <sup>447</sup>N-I-S<sup>449</sup>, <sup>471</sup>N-N-S<sup>473</sup> and <sup>541</sup>N-N-T<sup>543</sup> [44,45] are also conserved in the MG group. However, the analysis of predicted amino acid sequence of a F protein fragment (residues 0–553 for MG isolates or 1–553 for the other strains) revealed substitutions in the MG isolates found also in other strains clustered in the old genotypes (I–IV and IX) like T<sup>16</sup>→I, E<sup>104</sup>→G, Q<sup>195</sup>→R as well as substitutions found in recent genotypes (V to VIII) like V<sup>81</sup>→L, A<sup>106</sup>→V, V<sup>118</sup>→I and V<sup>350</sup>→I. In addition, the MG group has fifteen specific amino acid residues in F protein which are not found in any other genotype (Table 4). Twelve (80%) of these amino acids are located in the head of trimeric NDV-F protein M<sup>0</sup>→-, W<sup>8</sup>→R, P<sup>15</sup>→L, S<sup>272</sup>→N, M<sup>289</sup>→L, S<sup>311</sup>→T, R<sup>364</sup>→S, T<sup>371</sup>→M, L<sup>384</sup>→M, I<sup>397</sup>→T, T<sup>409</sup>→S and H<sup>411</sup>→N. The three remaining substitutions S<sup>244</sup>→G, K<sup>476</sup>→N, and I<sup>522</sup>→A are located in the stalk of the F-trimeric structure (Figure 3). Interestingly, the three MG strains recently isolated in 2008 share eight additional amino acids (Q<sup>4</sup>→K, F<sup>12</sup>→P, S<sup>28</sup>→L, R<sup>73</sup>→K, S<sup>79</sup>→A, E<sup>110</sup>→G, V<sup>202</sup>→I, S<sup>258</sup>→N and H<sup>337</sup>→Y) compared with the strain isolated in 1992.

According to the virulence of NDV strains, HN monomeric protein sequences possess different amino acid sequence lengths: 571, 577, 581 and 616 [46]. The ORF of HN gene of MG group strains begins at position 92 and ends at position 1714. This HN

**Table 2.** F gene referential NDV strains used in this study.

| Strain                   | Class or Genogroup | ICPI <sup>a</sup> value | Cleavage site          |     |     |     |     |   |                        |     |   |
|--------------------------|--------------------|-------------------------|------------------------|-----|-----|-----|-----|---|------------------------|-----|---|
| Identification           | Accession number   |                         | ←-----F <sub>2</sub> → |     |     |     |     | * | ←F <sub>1</sub> -----→ |     |   |
|                          |                    |                         | 112                    | 113 | 114 | 115 | 116 |   | 117                    | 118 |   |
|                          |                    |                         | R                      | R   | Q   | R   | R   | * | F                      | I   |   |
| DE R49/99                | DQ097393           | CI I (6)                | nd                     | G   | -   | -   | G   | - | *                      | L   | V |
| GO1US DCKI               | AY626266           | CI I (6)                | nd                     | E   | -   | -   | E   | - | *                      | L   | V |
| Herts33/56 (PEI)         | AY170140           | H33 (w) <sup>b</sup>    | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| Herts33 (L)              | AY170138           | H33 (w) <sup>c</sup>    | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| Ulster/67                | AY562991           | I (1)                   | 0,0                    | G   | K   | -   | G   | - | *                      | L   | - |
| Ethiopie Panvac (2/P2)   | AY175720           | I (1)                   | 0,0                    | G   | K   | -   | G   | - | *                      | L   | - |
| PHY-LMV42/66             | DQ097394           | I (1)                   | 0,0                    | G   | K   | -   | G   | - | *                      | L   | - |
| Hitchner B1              | AF309418           | II (2)                  | 0,2–0,5                | G   | -   | -   | G   | - | *                      | L   | - |
| La Sota                  | AF077761           | II (2)                  | 0,2–0,5                | G   | -   | -   | G   | - | *                      | L   | - |
| Zimbabwe AV862/95        | AY175710           | II (2)                  | nd                     | G   | -   | -   | G   | - | *                      | L   | - |
| Zambia AV 72/95          | AY175708           | II (2)                  | nd                     | G   | -   | -   | G   | - | *                      | L   | - |
| Mukteswar                | EF201805           | III (3a)                | 1,4                    | -   | -   | -   | -   | - | *                      | -   | - |
| Guangxi5/2000            | DQ485259           | III (3a)                | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| Herts/33 (de Leeuw)      | AY741404           | IVea (3b)               | 1,99                   | -   | -   | -   | -   | - | *                      | -   | - |
| BG 60–81                 | AF402129           | IVea (3b)               | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| BG 5–67                  | AF402104           | IVbg (3b)               | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| SIMF/64                  | AJ243390           | IVbg (3b)               | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| Soudan 72 AV 2203        | AY135753           | IVit (3b)               | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| DE-191/77                | AF525378           | IVit (3b)               | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| IT-48/68                 | AF297969           | IVit (3b)               | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| MA-307/77 <sup>c</sup>   | EU604259           | IV (3b)                 | nd                     | -   | -   | -   | -   | - | *                      | -   | V |
| MA-13/02 <sup>c</sup>    | DQ096598           | IV (3b)                 | nd                     | -   | -   | -   | -   | - | *                      | -   | V |
| Tanzania AV 1300/95      | AY175687           | V (3c)                  | nd                     | -   | -   | -   | K   | - | *                      | -   | V |
| Mexico468/01             | EU518685           | V (3c)                  | nd                     | -   | -   | -   | K   | - | *                      | -   | V |
| Brasil AV1769/90         | AY175649           | V (3c)                  | nd                     | -   | -   | -   | K   | - | *                      | -   | V |
| HR-111/01                | AY150162           | VI (4)                  | nd                     | K   | -   | -   | K   | - | *                      | -   | - |
| Soudan SD-4/75           | AY151384           | VI (4)                  | nd                     | -   | -   | -   | K   | - |                        | -   | - |
| Egypte EG-3/87           | AY150111           | VI (4)                  | nd                     | -   | -   | -   | K   | - |                        | -   | - |
| DE 61/93                 | AY150135           | VI (4)                  | nd                     | -   | -   | K   | K   | - | *                      | -   | - |
| Strain NA                | DQ659677           | VII (5)                 | nd                     | -   | -   | -   | K   | - | *                      | -   | - |
| MZ 13/94                 | AF136775           | VII (5)                 | nd                     | -   | -   | -   | K   | - | *                      | -   | - |
| Botswana ZA148/UP/98     | AY210507           | VII (5)                 | nd                     | -   | -   | -   | K   | - | *                      | -   | - |
| South Africa ZA606/UP/00 | AY210497           | VIII (3d)               | nd                     | -   | -   | -   | K   | - | *                      | -   | - |
| Singapore SG-4H/65       | AF136786           | VIII (3d)               | nd                     | -   | -   | -   | K   | - | *                      | -   | V |
| F48E9                    | AY508514           | IX (3e)                 | 1,89                   | -   | -   | -   | -   | - | *                      | -   | - |
| SBD02                    | DQ227252           | IX (3e)                 | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| TJ03                     | DQ227244           | IX (3e)                 | nd                     | -   | -   | -   | -   | - | *                      | -   | - |
| TW/69                    | AF083959           | X (3f)                  | nd                     | -   | -   | -   | K   | - | *                      | -   | - |
| TW/95-3                  | AF083970           | X (3f)                  | 1,68                   | -   | -   | -   | K   | - | *                      | -   | - |
| <b>MG-1992</b>           | HQ266603           | XI (3g)                 | 1,9                    | -   | -   | R   | -   | - | *                      | -   | V |
| <b>MG-725/2008</b>       | HQ266602           | XI (3g)                 | 1,9                    | -   | -   | R   | -   | - | *                      | -   | V |
| <b>MG-39-4/2008</b>      | HQ266605           | XI (3g)                 | nd                     | -   | -   | R   | -   | - | *                      | -   | V |
| <b>MG-Meola/2008</b>     | HQ266604           | XI (3g)                 | nd                     | -   | -   | R   | -   | - | *                      | -   | V |

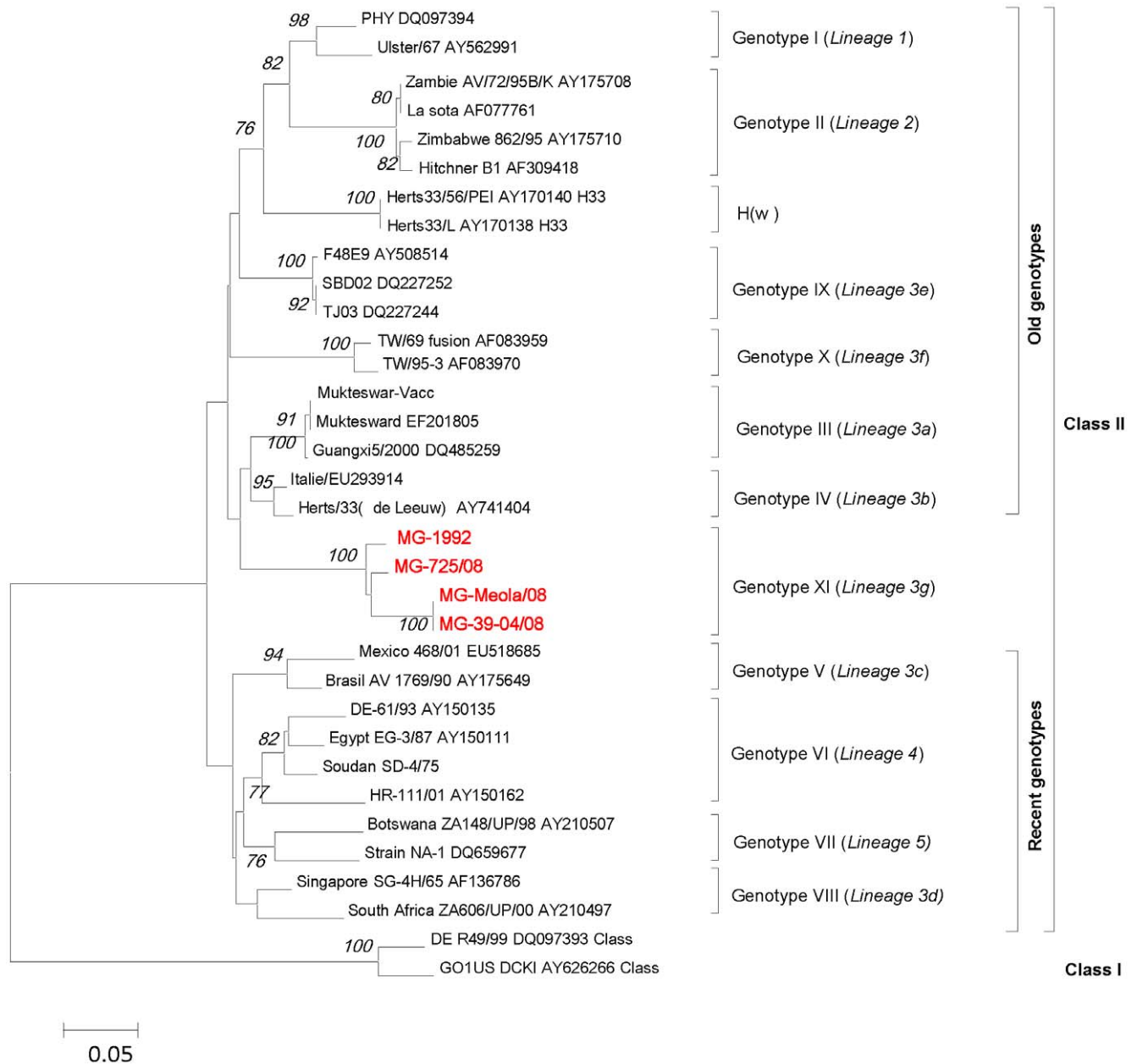
<sup>a</sup>: ICPI intracerebral pathogenicity index;

<sup>b</sup>: Weybridge line [77];

<sup>c</sup>: [8]. The isolates from Madagascar that were subjected to analysis in this work are in bold.

doi:10.1371/journal.pone.0013987.t002



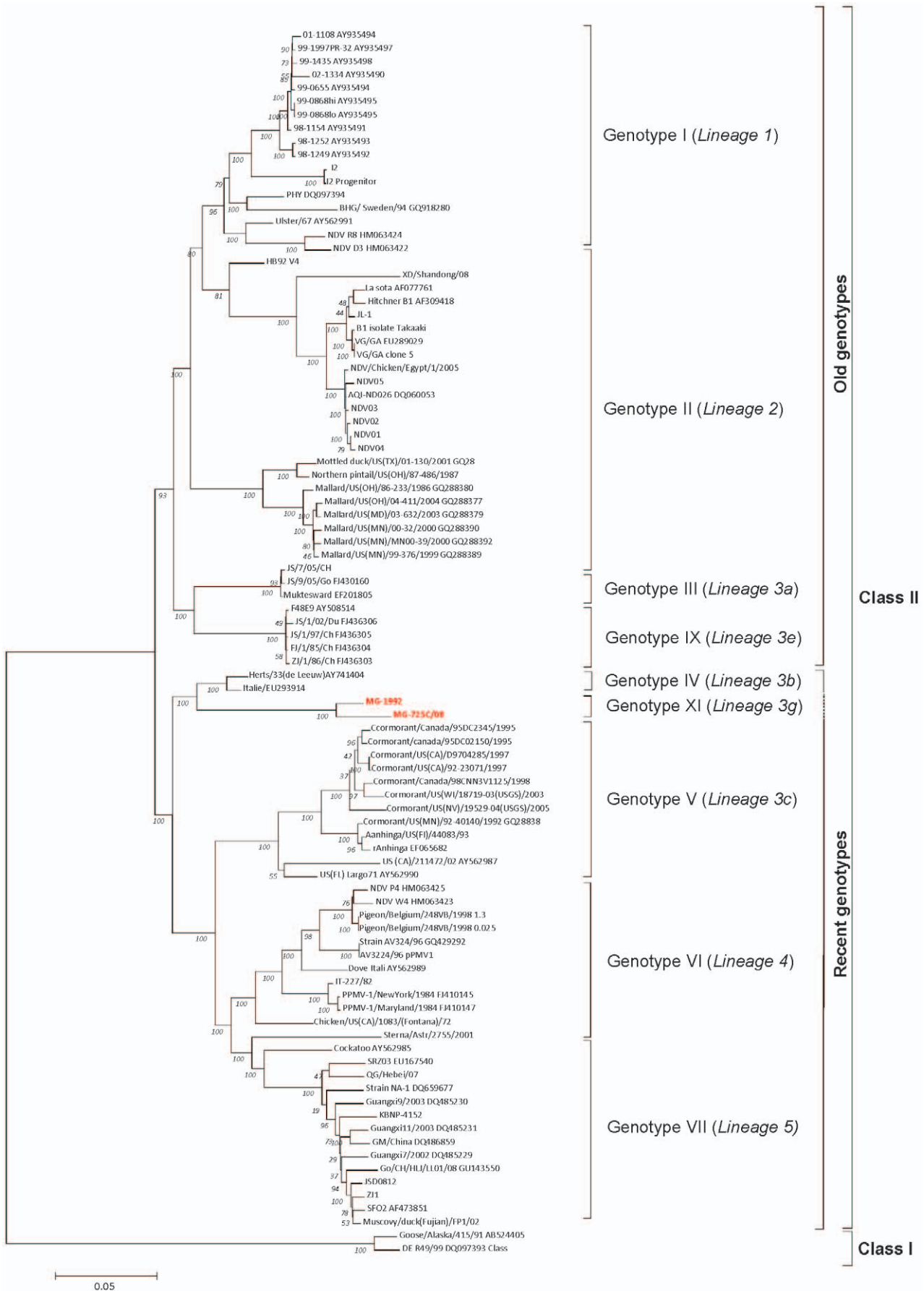


**Figure 2. Phylogenetic tree (unrooted) of nucleotide sequences based on a 374-nt sequence (position 47–421 nt) of the F gene.** Phylogenetic relationships of MG group strains with previously published sequences in Genbank. The evolutionary history was inferred using the Neighbor-Joining method [33]. All results are based on the pairwise analysis. Analyses were conducted using the Kimura 2-parameter method in MEGA4 [31,32] with 1,000 bootstraps [76]. The isolates from Madagascar that were subjected to analysis in this work are in bold and red. Genotype or lineage groupings are indicated on the right. doi:10.1371/journal.pone.0013987.g002

between old and new genotypes or that the current system of genotypes or lineages clustering based on the 374 nt fragment of F gene is probably inadequate to classify NDV isolates.

All MG isolates show the same fusion protein cleavage motif  $^{112}\text{R-R-R-R-R}^*\text{F}^{117}$ . To our knowledge, this atypical virulent-like cleavage site has never been reported before in APMV-1 strains. However, it is found in other paramyxoviruses (*Rubulavirus*) like the simian virus type 5 [45,59]. In addition, the presence of a phenylalanine at position 117 ( $\downarrow\text{F}^{117}$ ) in the MG isolates was previously described as being a possible contributor to neurological effects [10]. Thereafter, the length of amino acid sequence of the

haemagglutinin-neuraminidase protein (571 aa) is also characteristic of virulent strains [46,60,61]. The prediction of virulence based on the F cleavage site pattern was confirmed by *in vivo* tests that has resulted in a high ICPI value (1.9), close to the maximum of 2, with the two strains tested (MG-1992 and MG-725/08). In spite of the established virulence of the MG-725/08 strain, it was recovered both from cloacal and tracheal swabs from an unvaccinated and apparently healthy chicken. However, the possibility that this chicken was sampled during the incubation period of the infection or was partially protected by a previous infection with an avirulent or vaccine strain that circulate in the field cannot be ruled out.



**Figure 3. Phylogenetic tree of the nucleotide sequences based on a 14977nt (NP/P/M/F/HN/L genes).** The evolutionary history was inferred using the Neighbor-Joining method [33]. All results are based on the pairwise analysis. Analyses were conducted using the Kimura 2-parameter method in MEGA4 [31,32] with 1,000 bootstraps [76]. The isolates from Madagascar that were subjected to analysis in this work are in blue [22]. Genotype and the lineage groupings are indicated on the right. doi:10.1371/journal.pone.0013987.g003

Based on neutralising tests and cross-protective analyses, it is accepted that APMV-1 exists as a single serotype [62]. Therefore, the genetic variations of the virus are not expected to result in vaccination failure. However, different levels of cross-protection have been observed in chickens vaccinated with the vaccine strain La Sota belonging to the genotype II and challenged with different wild-type strains [55]. Sporadic cases of ND in commercial farms vaccinated with this vaccine were previously reported in China [7,58], in southern California and adjacent states [63], in Mali [64], Cameroon, Nigeria and Burkina Faso [65]. Whether these observations are related to a reduction of vaccine efficacy or an improper vaccine use in the field remains unclear. It is however possible that NDV strains responsible for ND sporadic outbreaks in vaccinated chickens can escape the immune responses [16,18,66] and thus contribute to the emergence of new genotypes [42]. This vaccine failure has been announced by Alexander et al., [67] during the third panzootic of ND (1981–1983) in Europe in which the pigeons immunized with live NDV B1 vaccine were not well protected against pigeon paramyxovirus isolates (genotype VI). It is also known that the immune pressure imposed by the vaccination may be selecting virulent variant forms of NDV [68]. Many authors have demonstrated that current vaccines prevent disease but cannot stop viral shedding [47,63]. The NDV strains currently circulating (subgenotype VIIId prevalent in Asia, Europe and Africa) and the genotype XI predominant in Madagascar have a significant genetic distance (21% to 25%, respectively), with the widely used vaccine strain La Sota pertaining to the genotype II. These genetic differences and a consequent suboptimal vaccination may be responsible for sporadic cases of ND in vaccinated poultry flocks in Madagascar. In contradiction with

this, multiple sequence alignments of the four MG isolates made with several published sequences including the La Sota strains and HB1, members of genotype II (data not shown) revealed that all the neutralising epitopes identified so far and the predicted attachment receptor sites in the F and HN glycoproteins are conserved. However, all MG group strains possess fifteen and eleven original amino acid substitutions on F and HN proteins, respectively. Some of these substitutions occurred in the globular head of the proteins. Furthermore, 5 out of the 8 mutations in the head of the F protein are concentrated in a short invariable region at position 364–411, suggesting that the observed mutations are actually selected over time by the host immune responses. It is supposed that these substitutions should have resulted in antigenic drift and major changes in the protein conformation [69], particularly the changes in amino acid polarity as T<sup>34</sup>→V, E<sup>62</sup>→V in HN protein and S<sup>244</sup>→G in F protein. The HN/F protein interaction for fusion promotion involves the HN head region (aa 124 to 151) and the F protein heptad repeat 2 (HR2) at position 454 to 492 [70,71]. In these regions, the MG strains show amino acids substitutions at position E<sup>147</sup>→D, K<sup>476</sup>→N of HN and F proteins, respectively. Morrison and Gravel [72] have demonstrated that amino acid substitutions in the head region domain of HN protein as L<sup>133</sup>→I or A<sup>140</sup>→L were responsible for an enhanced or diminished virus attachment activity, respectively. The possibility that other mutations like E<sup>147</sup>→D e/or K<sup>476</sup>→N in the same or related functional region may also contribute to virulence or immune evasion cannot be ruled out. In addition, the MG isolates display other specific mutations, some of them affecting the head of HN. It is tempting to postulate that these modifications (F or HN genes) may play a role in virulence or

**Table 3. Matrix estimate of genetic divergence (%) between nucleotide sequences (374 nt) of NDV strains.**

| Genotypes   | XI        | I        | II       | H(w)     | III      | IV       | V        | VI       | VII      | VIII     | IX       | X        | CI***    |
|-------------|-----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| <b>XI</b>   | <b>5*</b> |          |          |          |          |          |          |          |          |          |          |          |          |
| <b>I</b>    | 20**      | <b>7</b> |          |          |          |          |          |          |          |          |          |          |          |
| <b>II</b>   | 25        | 12       | <b>2</b> |          |          |          |          |          |          |          |          |          |          |
| <b>H(w)</b> | 21        | 15       | 16       | <b>0</b> |          |          |          |          |          |          |          |          |          |
| <b>III</b>  | 15        | 12       | 17       | 15       | <b>1</b> |          |          |          |          |          |          |          |          |
| <b>IV</b>   | 12        | 12       | 17       | 15       | 6        | <b>7</b> |          |          |          |          |          |          |          |
| <b>V</b>    | 19        | 16       | 19       | 18       | 14       | 11       | <b>5</b> |          |          |          |          |          |          |
| <b>VI</b>   | 18        | 16       | 19       | 18       | 14       | 11       | 11       | <b>7</b> |          |          |          |          |          |
| <b>VII</b>  | 21        | 18       | 21       | 21       | 17       | 14       | 14       | 3        | <b>7</b> |          |          |          |          |
| <b>VIII</b> | 22        | 17       | 20       | 17       | 14       | 13       | 11       | 12       | 15       | <b>9</b> |          |          |          |
| <b>IX</b>   | 15        | 13       | 17       | 14       | 10       | 9        | 14       | 14       | 17       | 16       | <b>1</b> |          |          |
| <b>X</b>    | 15        | 18       | 19       | 21       | 15       | 13       | 19       | 19       | 19       | 19       | 15       | <b>3</b> |          |
| <b>CI</b>   | 50        | 46       | 51       | 50       | 48       | 47       | 47       | 46       | 47       | 48       | 49       | 52       | <b>7</b> |

\*Intragenotype or intrasubgenotype: Maximal value of Nucleotide genetic divergence.

\*\*Intergenotype: Minimal value of genetic divergence.

\*\*\*Class I.

All results are based on the pairwise analysis of 61 NDV sequences including the 4 MG isolates (genotype XI). The nucleic acid analyse were conducted using Kimura 2-parameter method in MEGA4 [31,78]. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 374 nt positions in the final dataset.

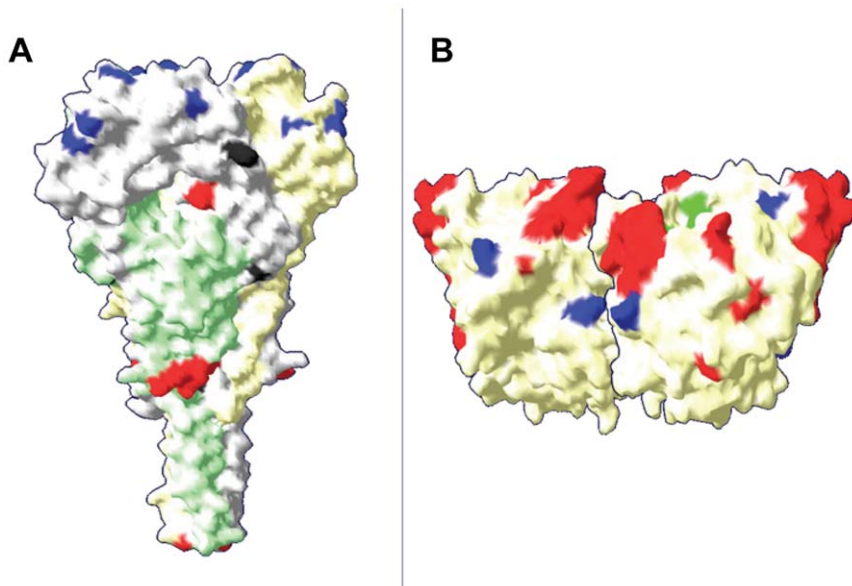
doi:10.1371/journal.pone.0013987.t003



**Table 4.** Residue substitutions specific in deduced F<sub>0</sub> protein and HN protein sequence of the MG group.

|                        |             | <i>Consensus residue and its position</i>        |   |     |     |     |     |     |     |     |     |     |     |     |     |     |    |    |    |     |    |     |     |     |     |     |
|------------------------|-------------|--|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|----|-----|----|-----|-----|-----|-----|-----|
|                        |             | <i>In deduced F<sub>0</sub> protein sequence</i> |   |     |     |     |     |     |     |     |     |     |     |     |     |     |    |    |    |     |    |     |     |     |     |     |
|                        |             | <i>in deduced HN protein sequence</i>            |   |     |     |     |     |     |     |     |     |     |     |     |     |     |    |    |    |     |    |     |     |     |     |     |
|                        |             | 0  | 8 | 15  | 244 | 271 | 311 | 364 | 371 | 384 | 397 | 409 | 411 | 522 | 24  | 34  | 39 | 62 | 78 | 81  | 98 | 147 | 263 | 328 | 430 | 539 |
|                        |             | -  | R | L   | G   | M   | T   | S   | M   | M   | T   | S   | N   | A   | V   | V   | I  | V  | Q  | V   | N  | D   | N   | T   | T   | K   |
| <b>MG-1992</b>         |             | M  | W | P   | S   | T   | S   | R   | T   | L   | I   | T   | H   | I   | A   | T   | L  | E  | R  | M   | S  | E   | R   | I   | I   | R   |
| <b>MG-Meola/08</b>     |             | M  | W | P   | S   | T   | S   | R   | T   | L   | I   | T   | H   | I   | A   | T   | L  | E  | R  | M   | S  | E   | R   | I   | I   | R   |
| <b>MG-725/08</b>       |             | M  | W | P   | S   | T   | S   | R   | T   | L   | I   | T   | H   | I   | A   | T   | L  | E  | R  | M   | S  | E   | R   | I   | I   | R   |
| <b>MG-39-04/08</b>     |             | M  | W | P   | S   | T   | S   | R   | T   | L   | I   | T   | H   | I   | A   | T   | L  | E  | R  | M   | S  | E   | R   | I   | I   | R   |
| <i>Old genotype</i>    | <b>I</b>    | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | I  | A  | -  | -   | -  | -   | -   | -   | -   |     |
|                        | <b>II</b>   | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -/I | -   | -  | R  | -  | -   | -  | -   | -   | -   | -   |     |
|                        | <b>III</b>  | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -/I | -   | -  | A  | -  | -   | -  | -   | -/k | -   | -   |     |
|                        | <b>IV</b>   | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -/I | -   | -  | -  | -  | -   | -  | -   | G/S | -   | -   |     |
|                        | <b>IX</b>   | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -  | A  | -  | -   | -  | -   | -   | -   | -   |     |
|                        | <b>X</b>    | -  | - | L/Q | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?  | ?  | ?  | ?   | ?  | ?   | ?   | ?   | ?   |     |
| <i>Recent genotype</i> | <b>V</b>    | -  | - | -   | -   | -   | -   | -   | -   | -/I | -   | -   | -   | -   | -   | -   | -  | -  | -  | -   | -  | -   | K   | -   | -   |     |
|                        | <b>VI</b>   | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -/N | -   | -/I | -  | -  | -  | -/I | -  | -   | K   | -   | -   |     |
|                        | <b>VII</b>  | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -/G | -/I | -   | -  | -  | -  | -   | -  | -   | K   | -   | -   |     |
|                        | <b>VIII</b> | -  | - | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | ?   | ?   | ?  | ?  | ?  | ?   | ?  | ?   | ?   | ?   | ?   |     |

? : Sequence not available.  
doi:10.1371/journal.pone.0013987.t004



**Figure 4. F-NDV trimer (a) and HN-NDV dimer (b) surface representations of the MG strains.** The location of neutralising epitopes is shown in red, the amino acid substitutions specific to the MG group are in blue in the globular head and in black in the stalk region. The figures were generated by Swiss PDB-viewer, using the X-ray structure of NDV F protein, position 33aa to 454aa for F protein [45] and 124aa to 569aa for HN protein [51].

doi:10.1371/journal.pone.0013987.g004

emergence of escape mutants and finally the apparent lack of vaccine efficacy observed in the field.

The number of unique motifs found in all MG isolates, including the atypical F protein cleavage site R-R-R-R-R\*F-V, also suggests a monophyletic origin of these viruses. Under this hypothesis, the maximum genetic variation observed within MG isolates (5%) is compatible with a divergence from a common ancestor over 50 years, considering that the rate of nucleotide change is approximately 1% per decade under natural field conditions in epizootic periods [12,57]. This scenario is in agreement with the first introduction of an old genotype of NDV in Madagascar in 1946. However, the higher genetic variation (12%) observed between the genotype XI and its closely related genotype IV suggests that the common ancestor of these two genotypes may have emerged several decades before the introduction of its virus progeny in Madagascar. As mentioned before, the accumulation of multiple aa substitutions in F or HN proteins may also result from the immune pressure that can contribute to increase the phylogenetic distance between the MG group and its progenitor genotype. The presence of this selection pressure at specific amino acid sites are recognised as adaptive evolution [47,73]. This abnormal evolution rate of genotype XI with regard to genotype IV may originate from combined characteristics of current poultry production systems including the commercial farm with host genetic homogeneity, intensive and/or improperly executed vaccination programs and backyard poultry breeding with high density of birds (allowing close animal-to-animal contact, and favouring transmission of highly virulent virus over milder forms) [74]. Moreover, high phylogenetic and antigenic distances between vaccines and circulating strains may facilitate the evolution of virulent NDV [75].

## References

- Alexander D (2000) A review of avian influenza in different bird species. *Vet Microbiol* 74: 3–13.
- Sinkovics JG, Horvath JC (2000) Newcastle disease virus (NDV): brief history of its oncolytic strains. *Journal of Clinical Virology* 16: 1–15.

In conclusion, all four isolates from Madagascar were clustered in a new genotype XI, presumably deriving from an ancestor close to genotype IV introduced in the 50's and possibly resulting from a self-contained evolution due to geographical and ecological characteristics of this island. The particular evolution of genotype XI in Madagascar reinforces the idea that this island, known for its outstanding flora and fauna biodiversity, is also a unique “natural ecosystem” for micro-organisms. Other genotypes like genotypes I, II, III and VII were also detected in the island (data not shown). These genotypes may have been introduced more recently in the island, probably as a consequence of poultry import or use of live vaccine strains. The co-circulation of different genotypes at the same time and other introductions of new viruses in relation with the worldwide intensification of animal movements, may contribute in the future to dramatically increase the complexity of the situation.

## Supporting Information

**Table S1** Primers used in this study.

Found at: doi:10.1371/journal.pone.0013987.s001 (0.07 MB DOC)

**Table S2** Accession numbers of the sequences used in this study.

Found at: doi:10.1371/journal.pone.0013987.s002 (0.12 MB DOC)

## Author Contributions

Conceived and designed the experiments: OFM PG EA RSdA. Performed the experiments: OFM PG FXB DK RSdA. Analyzed the data: OFM PG FXB EA DK RL DM MK AAA VJ RSdA. Contributed reagents/materials/analysis tools: HRA VC RR JJR MK. Wrote the paper: OFM EA RSdA.

3. OIE (2009) Newcastle disease. Chapter 2.3.14. OIE Manual of Standards for Diagnostic Tests and Vaccines, in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds and Bees. Office International des Epizooties, Paris (May 2009). pp 576–589.
4. Mayo MA (2002) Virus taxonomy. Arch Virol 147: 1071–1076.
5. Mayo MA (2002) A summary of taxonomic change recently approved by ICTV. Arch Virol 147: 1655–1656.
6. Peeters BPH, Gruijthuisen YK, de Leeuw OS, Gielkens ALJ (2000) Genome replication of Newcastle disease virus: involvement of the rule-of-six. Archives of Virology 145: 1829–1845.
7. Cho S-H, Kim S-J, Kwon H-J (2007) Genomic sequence of an antigenic variant Newcastle disease virus isolated in Korea. Virus Genes 35: 293–302.
8. Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, et al. (2006) Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Research 120: 36–48.
9. Huang Z, Panda A, Elankumaran S, Govindarajan D, Rockemann DD, et al. (2004) The Hemagglutinin-Neuraminidase Protein of Newcastle Disease Virus Determines Tropism and Virulence. J Virol 78: 4176–4184.
10. Kattenbelt JA, Meers J, Gould AR (2006) Genome sequence of the thermostable Newcastle disease virus (strain I-2) reveals a possible phenotypic locus. Veterinary Microbiology 114: 134–141.
11. Herczeg J, Wehmann E, Bragg RR, Travassos Dias PM, Hadjiev G, et al. (1999) Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. Archives of Virology 144: 2087–2099.
12. Czeglédi A, Eacute, Di A, Herczeg J, Hadjiev G, et al. (2002) The occurrence of five major Newcastle disease virus genotypes (II, IV, V, VI and VIIIb) in Bulgaria between 1959 and 1996. Epidemiology and Infection 129: 679–688.
13. Aldous EW, Mynn JK, Banks J, Alexander DJ (2003) A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathology 32: 239–257.
14. Tsai H-J, Chang K-H, Tseng C-H, Frost KM, Manvell RJ, et al. (2004) Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. Veterinary Microbiology 104: 19–30.
15. Bogoyavlenskiy A, Berezin V, Prilipov A, Usachev E, Lyapina O, et al. (2005) Molecular Characterization of Virulent Newcastle Disease Virus Isolates from Chickens during the 1998 NDV Outbreak in Kazakhstan. Virus Genes 31: 13–20.
16. Wang Z, Liu H, Xu J, Bao J, Zheng D, et al. (2006) Genotyping of Newcastle Disease Viruses Isolated from 2002 to 2004 in China. Annals of the New York Academy of Sciences 1081: 228–239.
17. Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, et al. (2007) Phylogenetic Diversity among Low-Virulence Newcastle Disease Viruses from Waterfowl and Shorebirds and Comparison of Genotype Distributions to Those of Poultry-Origin Isolates. J Virol 81: 12641–12653.
18. Liu H, Wang Z, Wu Y, Zheng D, Sun C, et al. (2007) Molecular epidemiological analysis of Newcastle disease virus isolated in China in 2005. Journal of Virology Methods 140: 206–211.
19. Lien Y-Y, Lee J-W, Su H-Y, Tsai H-J, Tsai M-C, et al. (2007) Phylogenetic characterization of Newcastle disease viruses isolated in Taiwan during 2003–2006. Veterinary Microbiology 123: 194–202.
20. Alexander DJ (2003) Newcastle disease virus, other avian paramyxoviruses, and pneumovirus infections. In: Disease of poultry, 11th ed. YM Saif, HJ Barnes, JR Glisson, AM Fadly, LR McDougald, DE Swayne. Iowa State University Press Ames, IA. pp 63–87.
21. Gotoh B, Ohnishi Y, Inocencio NM, Esaki E, Nakayama K, et al. (1992) Mammalian subtilisin-related proteinases in cleavage activation of the paramyxovirus fusion glycoprotein: superiority of furin/PACE to PC2 or PC1/PC3. J Virol 66: 6391–6397.
22. Lomniczi B, Wehmann E, Herczeg J, Ballagi-Pordany A, Kaleta EF, et al. (1998) Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). Arch Virol 143: 49–64.
23. Herczeg J, Wehmann E, Bragg RR, Travassos Dias PM, Hadjiev G, et al. (1999) Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. Arch Virol 144: 2087–2099.
24. Abolnik CHR, Bisschop SP, Parker ME, Romito M, Viljoen GJ (2004) A phylogenetic study of South African Newcastle disease virus strains isolated between 1990 and 2002 suggests epidemiological origins in the Far East. Archives of Virology 149: 603–619.
25. Yang CY, Shieh HK, Lin YL, Chang PC (1999) Newcastle disease virus isolated from recent outbreaks in Taiwan phylogenetically related to viruses (genotype VII) from recent outbreaks in western Europe. Avian Dis 43(1): 125–130.
26. Rajaonarison JJ (1991) Production de vaccin contre la maladie de Newcastle à Madagascar. PANVAC Debre Zeit Addis Ababa. pp 135–137.
27. Koko M, Maminiana OF, Ravaomanana J, Rakotonindrina S (2006) Aviculture villageoise à Madagascar : enquête épidémiologique. In Improving farmyard poultry production in Africa: interventions and their economic assessment. TECDOC-1489. AIEA, Vienne. pp 157–163.
28. Maminiana OF, Koko, Ravaomanana J, Rakotonindrina SJ (2007) Epidémiologie de la maladie de Newcastle en aviculture villageoise à Madagascar. Rev Sci Tech Off Int Epi 26: 691–700.
29. Zou J, Shan S, Yao N, Gong Z (2005) Complete Genome Sequence and Biological Characterizations of A Novel Goose Paramyxovirus-SF02 Isolated in China. Virus Genes 30: 13–21.
30. Thompson J, Gibson T, Plewniak F, Jeanmougin F, Higgins D (1997) The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 25: 4876–4882.
31. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 24: 1596–1599.
32. Kimura S, Hatakeyama M, Konagaya A (2004) Inference of S-system Models of Genetic Networks from Noisy Time-series Data. Chem-Bio Informatics Journal 4: 1–14.
33. Saitou, Nei (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425.
34. Martin S, Sambade A, Rubio L, Vives MC, Moya P, et al. (2009) Contribution of recombination and selection to molecular evolution of Citrus tristeza virus. J Gen Virol 90: 1527–1538.
35. Huovilainen A, Ek-Kommonen C, Manvell R, Kinnunen L (2001) Phylogenetic analysis of avian paramyxovirus 1 strains isolated in Finland. Archives of Virology 146: 1775–1785.
36. Meulemans G, Berg TPvd, Decasstecker M, Boschmans M (2002) Evolution of pigeon Newcastle disease virus strains. Avian Pathology 31: 515–519.
37. Terregino C, Cattoli G, Grossele B, Bertoli E, Tisato E, et al. (2003) Characterization of Newcastle disease virus isolates obtained from Eurasian collared doves (*Streptopelia decaocto*) in Italy. Avian Pathology 32: 63–68.
38. Ujvári D, Wehmann E, Kaleta EF, Werner O, Savic V, et al. (2003) Phylogenetic analysis reveals extensive evolution of avian paramyxovirus type 1 strains of pigeons (*Columba livia*) and suggests multiple species transmission. Virus Research 96: 63–73.
39. Lee YJ, Sung HW, Choi JG, Kim JH, Song CS (2004) Molecular epidemiology of Newcastle disease viruses isolated in South Korea using sequencing of the fusion protein cleavage site region and phylogenetic relationships. Avian Pathology 33: 482–491.
40. Toyoda T, Sakaguchi T, Imai K, Inocencio NM, Gotoh B, et al. (1987) Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of newcastle disease virus. Virology 158: 242–247.
41. Yusoff K, Nesbit M, McCartney H, Meulemans G, Alexander DJ, et al. (1989) Location of Neutralizing Epitopes on the Fusion Protein of Newcastle Disease Virus Strain Beaudette C. J Gen Virol 70: 3105–3109.
42. Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB (2003) Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Archives of Virology 148: 1387–1403.
43. Seal BS (2004) Nucleotide and predicted amino acid sequence analysis of the fusion protein and hemagglutinin-neuraminidase protein genes among Newcastle disease virus isolates. Phylogenetic relationships among the Paramyxovirinae based on attachment glycoprotein sequences. Functional & Integrative Genomics 4: 246–257.
44. Panda A, Elankumaran S, Krishnamurthy S, Huang Z, Samal SK (2004) Loss of N-Linked Glycosylation from the Hemagglutinin-Neuraminidase Protein Alters Virulence of Newcastle Disease Virus. J Virol 78: 4965–4975.
45. Chen L, Gorman JJ, McKimm-Breschkin J, Lawrence IJ, Tulloch PA, et al. (2001) The Structure of the Fusion Glycoprotein of Newcastle Disease Virus Suggests a Novel Paradigm for the Molecular Mechanism of Membrane Fusion. 9: 255–266.
46. Romer-Oberdorfer A, Werner O, Veits J, Mebatsion T, Mettenleiter TC (2003) Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. J Gen Virol 84: 3121–3129.
47. Miller PJ, Kim LM, Ip HS, Afonso CL (2009) Evolutionary dynamics of Newcastle disease virus. Virology 391: 64–72.
48. Iorio RM, Glickman RL, Riel AM, Sheehan JP, Bratt MA (1989) Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. Virus Research 13: 245–261.
49. Iorio RM, Syddall RJ, Sheehan JP, Bratt MA, Glickman RL, et al. (1991) Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. J Virol 65: 4999–5006.
50. Yusoff K, Nesbit M, Samson ACR, Emmerson PT (1988) Location of epitopes within the haemagglutinin-neuraminidase (HN) protein of newcastle disease virus by sequencing the HN genes of monoclonal antibody-resistant mutants. Virus Research 11: 12–12.
51. Crennell S, Takimoto T, Portner A, Taylor G (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. Nat Struct Mol Biol 7: 1068–1074.
52. Connaris H, Takimoto T, Russell R, Crennell S, Moustafa I, et al. (2002) Probing the Sialic Acid Binding Site of the Hemagglutinin-Neuraminidase of Newcastle Disease Virus: Identification of Key Amino Acids Involved in Cell Binding, Catalysis, and Fusion. J Virol 76: 1816–1824.
53. Jorgensen ED, Collins PL, Lomedico PT (1987) Cloning and nucleotide sequence of newcastle disease virus hemagglutinin-neuraminidase mRNA: Identification of a putative sialic acid binding site. Virology 156: 12–24.

54. Lamb RA, Kolakofsky D (1996) Paramyxoviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM, eds. In *Fundamental Virology*. Philadelphia: Lipincott–Raven. pp 1177–1204.
55. Qin Z, Xu H, Ouyang W, Wang Y, Wang L, et al. (2008) Correlation of the neutralization index in chicken embryo with the homologies of F and HN gene of different Newcastle-disease isolates. *Wei Sheng Wu Xue Bao* 48: 226–233.
56. Lomniczi B, Wehmann E, Herczeg J, Ballagi-Pordány A, Kaleta EF, et al. (1998) Newcastle disease outbreaks in recent years in Western Europe were caused by an old (VI) and a novel genotype (VII). *Archives of Virology* 143: 49–64.
57. Wehmann E, Ujvári D, Mazija H, Vehlner M, Ciglar-Grozdanic I, et al. (2003) Genetic analysis of Newcastle disease virus strains isolated in Bosnia-Herzegovina, Croatia, Slovenia and Yugoslavia, reveals the presence of only a single genotype, V, between 1979 and 2002. *Veterinary Microbiology* 94: 269–281.
58. Yu L, Wang Z, Jiang Y, Chang L, Kwang J (2001) Characterization of Newly Emerging Newcastle Disease Virus Isolates from the People's Republic of China and Taiwan. *J Clin Microbiol* 39: 3512–3519.
59. Millar NS, Chambers P, Emmerson PT (1986) Nucleotide sequence analysis of the haemagglutinin-neuraminidase gene of Newcastle disease virus. *J Gen Virol* 67(Pt 9): 1917–1927.
60. OIE (2004) Newcastle disease.» Chapter 2.1.15. OIE Manual of Standards for Diagnostic Tests and Vaccines, in *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds and Bees*. Office International des Epizooties, Paris (2004). pp 270–282.
61. Ujvári D, Wehmann E, Kaleta E, Werner O, Savic V, et al. (2003) Phylogenetic analysis reveals extensive evolution of avian paramyxovirus type 1 strains of pigeons (*Columba livia*) and suggests multiple species transmission. *Virus Res* 96: 63–73.
62. Alexander DJ, Manvell RJ, Lowings JP, Frost KM, Collins MS, et al. (1997) Antigenic diversity and similarities detected in avian paramyxovirus type 1 (Newcastle disease virus) isolates using monoclonal antibodies. *Avian Pathol* 26: 399–418.
63. Kapczynski DR, King DJ (2005) Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* 23: 3424–3433.
64. Servan de Almeida R, Maminiaina OF, Gil P, Hammoumi S, Molia S, et al. (2009) Africa, a reservoir of new virulent strains of Newcastle disease virus? *Vaccine* 27: 3127–3129.
65. Snoeck C, Ducatez M, Owoade A, Faleke O, Alkali B, et al. (2009) Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. *Archives of Virology* 154: 47–54.
66. Qin Z, Sun L, Ma B, Cui Z, Zhu Y, et al. (2008) F gene recombination between genotype II and VII Newcastle disease virus. *Virus Research* 131: 299–303.
67. Alexander DJ, Russell PH, Parsons G, Elzein EMEA, Ballouh A, et al. (1985) Antigenic and biological characterisation of avian paramyxovirus type I isolates from pigeons - an international collaborative study. *Avian Pathology* 14: 365–376.
68. Miller PJ, Decanini, EduardoLucio, Afonso, Claudio L (2010) Newcastle disease: Evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution* 10: 26–35.
69. Xu M, Chang S, Ding Z, Gao HW, Wan JY, et al. (2008) Genomic analysis of Newcastle disease virus strain NA-1 isolated from geese in China. *Archives of Virology* 153: 1281–1289.
70. Gravel, Morrison (2003) Interacting Domains of the HN and F Proteins of Newcastle Disease Virus. *J Virol* 77: 11040–11049.
71. Zaitsev V, von Itzstein M, Groves D, Kiefel M, Takimoto T, et al. (2004) Second Sialic Acid Binding Site in Newcastle Disease Virus Hemagglutinin-Neuraminidase: Implications for Fusion. *J Virol* 78: 3733–3741.
72. Gravel KA, Morrison TG (2003) Interacting domains of the HN and F proteins of newcastle disease virus. *J Virol* 77: 11040–11049.
73. Han G-Z, He C-Q, Ding N-Z, Ma L-Y (2008) Identification of a natural multi-recombinant of Newcastle disease virus. *Virology* 371: 54–60.
74. Miller PJ, Decanini EL, Afonso CL (2009) Newcastle disease: Evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution*. doi:10.1016/j.meegid.2009.09.012.
75. Miller PJ, King DJ, Afonso CL, Suarez DL (2007) Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. *Vaccine* 25: 7238–7246.
76. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783–791.
77. Czeglédi A, Wehmann E, Lomniczi B (2003) On the origins and relationships of Newcastle disease virus vaccine strains Hertfordshire and Mukteswar, and virulent strain Herts'33. *Avian Pathology* 32: 271–276.
78. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.