# Molecular characterisation of Newcastle disease virus isolates from different geographical regions in Mozambique in 2005

**Authors:**
Raul Fringe[1,2]
Anna-Mari Bosman[2]
Karen Ebersohn[2]
Shahn Bisschop[3,4]
Celia Abolnik[4,5]
Estelle Venter[2]

**Affiliations:**
[1]Department of Virology, National Veterinary Research Institute, Mozambique

[2]Department of Veterinary Tropical Diseases, University of Pretoria, South Africa

[3]Avimune, Pretoria, South Africa

[4]Department of Production Animal Studies, University of Pretoria, South Africa

[5]Agricultural Research Council, Onderstepoort Veterinary Institute, South Africa

**Correspondence to:**
Estelle Venter

**Email:**
estelle.venter@up.ac.za

**Postal address:**
Private Bag X04, Onderstepoort 0110, South Africa

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Newcastle disease (ND) is regarded as a highly contagious and economically important disease in poultry and has a worldwide distribution. Viral determinants for Newcastle disease virus (NDV) virulence are not completely understood and viruses of different pathotypes can be found at live-bird markets in different geographical areas. The prevalence of Newcastle disease in village poultry in Mozambique is not well documented and strains of NDV involved in yearly outbreaks are unknown. The fusion (F) protein is an important determinant of pathogenicity of the virus and is used commonly for phylogenetic analysis. Newcastle disease viruses from various geographical regions of Mozambique were sequenced and compared genetically to published sequences obtained from GenBank. Samples were collected in three different areas of Mozambique and NDV was isolated by infection of embryonated chicken eggs. Sequence analysis of the F-protein encoding gene was used to classify 28 isolates from Mozambique into genotypes and compare these genotypes phylogenetically with existing genotypes found in GenBank. The isolates obtained from Mozambique grouped mainly into two clades. In the first clade, 12 isolates grouped together with sequences of isolates representing genotypes from Mozambique that were previously described. In the second clade, 16 isolates group together with sequences obtained from GenBank originating from Australia, China, South Africa and the USA. Eleven of these isolates showed a high similarity with sequences from South Africa. The number of samples sequenced ($n$ = 28), as well as the relatively small geographical collection area used in this study, are too small to be a representation of the circulating viruses in Mozambique in 2005. Viruses characterised in this study belonged to lineage 5b, a similar finding of a previous study 10 years ago. From this data, it merely can be concluded that no new introduction of the virus occurred from 1995 to 2005 in Mozambique.

## Introduction

Newcastle disease (ND) is regarded as one of the most serious diseases in the poultry industry (Alexander 1991). The causative agent, Newcastle disease virus (NDV), is transmitted by ingestion or inhalation and produces a disease with varied clinical manifestations, varying from severe acute to subclinical infection, depending on the virulence of the virus strain (Millar, Chambers & Emmerson 1988).

The virus is classified in the family Paramyxoviridae, genus *Avulavirus* which encompasses a diverse group of single-stranded, negative-sense, non-segmented RNA viruses. Ten distinct avian paramyxovirus (APMV-1 – APMV-10) groups are recognised and have been defined using standard serological tests (Miller *et al*. 2011). Avian paramyxovirus-1 and NDV are synonymous, although the term pigeon paramyxovirus type 1 (PPMV-1) has been used to distinguish the antigenic variant of APMV-1 virus responsible for the continuing panzootic in racing pigeons (Alexander 1998; Office Internationale des Epizooties [OIE] 2004). Both Paramyxovirus (PMV)-2 and PMV-3 viruses have also been isolated from domestic poultry (Alexander *et al*. 1997).

The first reported outbreaks of ND occurred in 1926 on the island of Java, Indonesia. Subsequently, an outbreak of the disease was reported in the same year near Newcastle-on-Tyne, which led to its description the following year (Bruce *et al*. 2002; OIE 2004; Seal *et al*. 2002). From 1926 to 1940, severe cases of the disease were reported near the sea ports of the Indian Ocean. The virus first infected poultry in the tropical rain forests of South-East Asia. Once established in poultry, the worldwide spread of ND was probably the result of trade in live infected poultry. Presently, ND has a worldwide distribution, although, in some countries such as Australia, only viruses of low virulence for chickens have been reported (Alexander *et al*. 1997).

Newcastle disease has been endemic in southern Africa causing outbreaks of the disease in poultry (Pfitzer *et al*. 2000) and entry through the port of Durban was first recorded during 1944

(Kaschula *et al*. 1945). In Kenya, the first recorded entry of the disease was through the port of Mombassa during 1934 (Valadão 1950). Although the virus was first isolated in 1946, the disease had already been clinically diagnosed in Mozambique in 1938 (Valadão 1950). The disease is one of the major constraints of rural poultry production which could otherwise play an important role in poverty alleviation. Today it is responsible for high mortality in chickens in both traditional and commercial sectors throughout the country (Fringe & Dias 1991; Valadão 1950).

The Paramyxovirus genome consists of a negative-sense, single-stranded ribonucleic acid (RNA) that encodes for six genes: the nucleoprotein, phosphoprotein, matrix protein, fusion protein (F), haemagglutinin-neuramidase (HN) protein and polymerase protein, respectively. The HN protein mediates attachment of the virion to sialic acid-containing receptors, whilst the F protein mediates fusion of the viral envelope with cellular membranes (Lamb & Kolakofsky 2001; Pantua *et al*. 2005; Scheid & Choppin 1974).

Analysis of the nucleotide sequence data from the F gene, encoding for the fusion protein of NDV, has allowed differentiation of closely related viruses resulting in epidemiological evidence of the virus origin (Alexander *et al*. 1997). Newcastle disease viruses were previously classified into nine genetic groups (I–IX), with genetic groups VI and VII divided into several subtypes (Ballargy-Pordany *et al*. 1996; Tsai *et al*. 2004). More recent studies of the F and L (RNA-dependent, RNA polymerase gene) genes revealed two distinct classes: I and II within ND serotype 1. Phylogenetic analysis revealed that class I and II viruses each consist of at least nine genotypes, designated 1–9 and I–IX, respectively (Lui *et al*. 2003). Only class II viruses are responsible for fatal infections in poultry and they have been responsible for major outbreaks worldwide. Newcastle disease viruses have been subsequently re-classified broadly into six lineages (1–6), which correspond to the existing groups (I–IX), and these lineages are divided into 13 sub-lineages (Aldous *et al*. 2003). Avirulent viruses are grouped in lineages 1 and 6, whilst virulent viruses are grouped in lineages 3, 4 and 5; lineage 2 contains both virulent and avirulent viruses. It seems that some lineages tend to group together geographically whilst others circulate worldwide (Aldous *et al*. 2003). For example, isolates obtained from South Africa and the Middle East region group together in lineage 5 (genotype VII) and those from the Far East and Western Europe group in lineage 3d (genotype VIII) and 5b (VIIb) (Lee *et al*. 2004).

Two novel genetic lineages, namely 5b (VIIb) and 3d (VIII) were described by Herczeg *et al*. (1999). They claimed that these groups were responsible for the 1990 and 1995 outbreaks in southern Africa, including Mozambique. This classification system (Ballargy-Pordany *et al*. 1996) made it possible to detect the origin of the virus and evidence exists that the ND strains circulating in southern Africa have a common ancestor in the Far East and southern Europe (Abolnik *et al*. 2004; Liang *et al*. 2002).

The prevalence of ND in village poultry in Mozambique is not documented and only one report revealed the molecular characterisation of NDV isolates circulating in Mozambique between 1990 and 1995 (Herczeg *et al*. 1999). In the present study, the aim was to characterise genetically NDV strains occurring during 2005 in various geographical regions of Mozambique using nucleic acid sequences and to compare these results with NDV sequencing results that have been described in South Africa, as well as other published sequencing data from NDV elsewhere in Africa. This will lead to a better understanding of the epidemiology of the disease and will generate useful data that can be applied to adapt or adjust control programmes in Mozambique.

# Materials and methods

## Sampling

Samples from suspected NDV infected and dead chickens were collected from various villages in the southern (72 samples), central (18 samples) and northern (30 samples) regions of Mozambique (Figure 1). Cloacal and tracheal swabs, as well as faeces, were collected from live chickens and samples from the trachea, lung, spleen, intestine, liver, heart, kidney, proventriculus and brain were collected from dead chickens. Swabs were treated separately according to the procedure described by the OIE manual (2004), whilst other organ samples were placed in isotonic phosphate buffered saline (PBS), pH 7.0 – pH 7.4, until processing.


**FIGURE 1:** Map of the geographical areas in Mozambique where samples were collected during the study period.

### Virus isolation

Virus isolation was performed at the Virology Department of the National Veterinary Research Institute (NVRI), in Maputo, Mozambique. Suspensions of organs and/or swabs were first centrifuged in a bench-top centrifuge at 4000 rpm for 5 min. Antibiotics (penicillin [2000 units/mL], streptomycin [0.01 mL/1 mL], gentamycin [50 µg/mL] and mycostatin [1000 units/mL]) were added to the supernatants and incubated for 1 h – 2 h at room temperature (OIE 2004). A volume of 0.2 mL of the supernatant was then inoculated into the allantoic cavity of five 9–11-day-old, specific pathogen free (SPF) embryonated chicken eggs according to the procedure described in the OIE Manual (2004). Eggs were then incubated at 37 °C for 4–7 days. Allantoic fluid was harvested and tested for the presence of a haemagglutinating virus by the haemagglutination (HA) test. Samples that tested positive with the HA test were tested for the presence of NDV using polyclonal anti-NDV serum in a haemagglutination-inhibition (HI) test. Aliquots of NDV-positive allantoic fluid were prepared and stored at -70 °C for polymerase chain reaction (PCR) and sequencing analysis (Alexander 1991, 1998; OIE 2004).

### Virus characterisation

Nucleic acid was extracted from allantoic fluid positive for NDV ($n$ = 44/120) using Trizol LS (Invitrogen; Life Technologies™, Carlsbad, USA) and/or using a QIAamp® Viral RNA Mini Kit, according to the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany).

The GeneAmp®Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, USA) was used to generate first strand complementary deoxyribonucleic acid (cDNA). The following reaction was compiled: 2 µL of ± 75 ng of extracted RNA was added to the RT mixture (5 × reaction buffer, 2.5 mM $Mg^{2+}$, 250 µM of each dNTP, 10 units per 20 µL RNAse inhibitor, 10 mM DTT, 1.25 µM Random Hexamer, 15 units per 20 µL MultiScribe Reverse Transcriptase) in a final volume of 20 µL RNase-free water. The cDNA product (10 µL) was amplified at 25 °C for 10 min, followed by 42 °C for 15 min.

Polymerase chain reaction was then conducted to amplify a 1180 base pair fragment spanning the regions between nucleotides 581 of the F and nucleotides 610 of the matrix protein (M), using previously published primers: M610-5'CTG TAC AAT CTT GCG CTC ATT GTC-3' and reverse primer NDVF581-5'CTG CCA CTG CTA GTT GTG ATA ATC C-3' (Abolnik *et al.* 2004). The PCR amplification mixture consisted of 2 µL cDNA, 1 × RT-PCR buffer, 1.75 mM $Mg^{2+}$, 200 µM of each primer, 2.5 units per 25 µL AmpliTaq Gold deoxyribonucleic acid (DNA) polymerase, in a total volume of 25 µL. The PCR was carried out using the same cycling conditions previously described by Abolnik *et al.* (2004). The PCR products were separated by electrophoresis using a 1.5% agarose gel stained by ethidium bromide (0.2 mg/mL). The results were visualised under UV-illumination and documented using a Kodak EDAC gel documentation system (Kodak, Rochester, USA).

Amplification products were submitted to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa for sequencing. The same set of primers was used for sequencing as described for the PCR. Sequencing data of the partial F gene was assembled using GAP4 of the Staden package (Bonfield, Smith & Staden 1995; Staden 1996; Staden, Beal & Bonfield 2000). Sequence data for the $F_0$ cleavage site was assembled and edited to a total length of 382 base pairs using GAP 4 of the Staden package (Version 1.6.0 for Windows; 2006) (Bonfield *et al*. 1995). Sequences were deposited in GenBank under accession numbers, as summarised in Table 1. To compare sequences obtained in this study with previously published sequences, a basic local alignment search tool (BLAST) search was performed using the BLASTN algorithm. The assembled sequences were aligned with related sequences obtained from GenBank using ClustalX (Version 1.81 for Windows; 2000). The alignment was truncated manually to the size of the smallest sequence. Similarity matrices were constructed using the two-parameter model of Kimura (Kimura 1980) and the Jukes and Cantor (1969) correction model for multiple base changes. Phylogenetic trees were constructed using neighbour-joining (Saitou & Nei 1987) and the maximum parsimony methods by using the MEGA 3.0 software package (Kumar, Tamura & Nei 2004). To assign confidence values to topologies, bootstrapping (Felsenstein 1985) was applied: 1000 replicates per tree for distance methods.

Analysis of the deduced amino acid sequences of the collected samples and data obtained from GenBank was performed by construction of a phylogenetic tree using the maximum parsimony tree. MEGA 4 software was used and subjected to 1000 replicates of bootstrap.

### Ethical considerations

Samples were sent mainly to the National Veterinary Research Institute in Mozambique for diagnostic purposes. Other samples were collected by a veterinarian (Dr Raul Fringe) under the authority of the National Veterinary Research Institute, Mozambique.

## Results

### Confirmation of virus isolation

Samples ($n$ = 120) were collected from the northern, central and southern regions of Mozambique. Newcastle disease virus isolates ($n$ = 39) from embryonated chicken eggs were confirmed by HA and HI testing.

### Characterisation of the Newcastle disease virus

Twenty-eight PCR amplicons were used for sequencing (Table 1). Sequences from these samples, together with sequencing data obtained from GenBank, were aligned and phylogenetic analysis was performed. Nucleic acid sequences were analysed by constructing a neighbour-joining tree (Figure 2) (Saitou & Nei 1987). Similar results were obtained

**TABLE 1:** Samples included in the phylogenetic analysis.

| Sample | Location | Sequence analysis of partial F genes | | | |
|---|---|---|---|---|---|
| | | $F_0$ cleavage site sequence | OIE-defined pathotype | Lineage | GenBank accession number |
| NDV 3 | Maputo Province – Sabie locality[S] | GKQGRL | Avirulent | 1 | HQ702437 |
| NDV 7 | Maputo Province – Sabie locality[S] | RRQKRF | Virulent | 5b | HQ702438 |
| NDV 10 | Maputo Province – Magude district[S] | RRQKRF | Virulent | 5b | HQ702439 |
| NDV 12 | Nampula Province – Monapo district[N] | GKQGRL | Avirulent | 1 | HQ702440 |
| NDV 14 | Niassa Province – Lichinga city[N] | GKQGRL | Avirulent | 1 | HQ702441 |
| NDV 16 | Manica Province – peri-urban zone[C] | GKQGRL | Avirulent | 1 | HQ702442 |
| NDV 17 | Inhambane Province – Homoine district[S] | GKQGRL | Avirulent | 1 | HQ702443 |
| NDV 18 | Inhambane Province – Homoine district[S] | GKQGRL | Avirulent | 1 | HQ702444 |
| NDV 19 | Inhambane Province – Homoine district[S] | GKQGRL | Avirulent | 1 | HQ702445 |
| NDV 20 | Nampula Province – Monape district[N] | GKQGRL | Avirulent | 1 | HQ702446 |
| NDV 21 | Nampula Province – Monape district[N] | RRQKRF | Virulent | 5b | HQ702447 |
| NDV 22 | Sofala Province – peri-urban zone[C] | GKQGRL | Avirulent | 1 | HQ702448 |
| NDV 23 | Sofala Province – peri-urban zone[C] | GKQGRL | Avirulent | 1 | HQ702449 |
| NDV 25 | Inhambane Province – Homoine district[S] | RRQKRF | Virulent | 5b | HQ702450 |
| NDV 26 | Manica Province – peri-urban zone[C] | RRQKRF | Virulent | 5b | HQ702451 |
| NDV 28 | Gaza Province – Chibuto district[S] | RRQKRF | Virulent | 5b | HQ702452 |
| NDV 30 | Maputo Province – Moamba district[S] | RRQKRF | Virulent | 5b | HQ702453 |
| NDV 31 | Inhambane Province – Homoine district[S] | RRQKRF | Virulent | 5b | HQ702454 |
| NDV 32 | Inhambane Province – Homoine district[S] | GKQGRL | Avirulent | 1 | HQ702455 |
| NDV 34 | Maputo Province – Moamba district[S] | GKQGRL | Avirulent | 1 | HQ702456 |
| NDV 35 | Maputo Province – Sabie locality[S] | RRQKRF | Virulent | 5b | HQ702457 |
| NDV 36 | Maputo Province – Sabie locality[S] | RRQKRF | Virulent | 5b | HQ702458 |
| NDV 39 | Inhambane Province – Homoine district[S] | RRQKRF | Virulent | 5b | HQ702459 |
| NDV 40 | Inhambane Province – Homoine district[S] | GKQGRL | Avirulent | 1 | HQ702460 |
| NDV 41 | Inhambane Province – Homoine district[S] | RRQKRF | Virulent | 5b | HQ702461 |
| NDV 42 | Sofala Province – peri-urban zone[C] | GKQGRL | Virulent | 1 | HQ702462 |
| NDV 43 | Maputo Province – Moamba district[S] | GKQGRL | Avirulent | 1 | HQ702463 |
| NDV 44 | Maputo Province – Moamba district[S] | GKQGRL | Avirulent | 1 | HQ702464 |

OIE, Office Internationale des Epizooties; NDV, Newcastle disease virus; F-genes, fusion protein genes; $F_0$, precursor fusion protein.
[N], northern region of Mozambique.
[C], central region of Mozambique.
[S], southern region of Mozambique.

by the maximum parsimony method. Nucleotide sequences were translated into amino acids and also analysed by the maximum parsimony method (results not shown) (Tamura *et al*. 2007).

The samples obtained from Mozambique in 2005 grouped mainly into two clades (Figure 2). In the first clade, 12 isolates (NDV 7, 10, 21, 25, 26, 28, 30, 31, 35, 36, 39 and 41) grouped together with sequences of isolates representing genotypes VII, VIIb, VIIc and VIId (Abolnik *et al*. 2004; Herczeg *et al*. 1999). Taking the high bootstrap values in account, these samples clearly can be assigned to genotype VIIb. Four of these isolates (NDV 7, 10, 25 and 39) cluster in close relationship with sequences derived from isolates from Mozambique (AF136780, AF136778, AF136777 and AF136779) previously described by Herczeg *et al*. (1999). The four isolates used in this study were collected in the southern region, one (NDV 10) from the Magude district in Maputo Province (Figure 1) and two from Hamoine district in Inhambane Province (NDV 25 and 39), whilst one sample (NDV 7) was collected from the central region (Sofala Province, peri-urban zone). The other eight isolates (NDV 21, 26, 28, 30, 31, 35, 36 and 41) group together and share a node to the previously mentioned group. These samples were collected from the Hamoine district in Inhambane Province (central–southern region) (NDV 31 and 41), the Monape district in Nampula Province (northern region) (NDV 21) and the Chibuto district in Gaza Province (southern region) (NDV 28 and 30), as well as from the peri-urban zone of Manica Province (central region) (NDV 26) and the Sabie location of Maputo Province (NDV 35 and 36) (Figure 1 and Figure 2).

In the second clade, 16 isolates grouped together with sequences obtained from GenBank originating from Australia and South Africa and, to a lesser extent, to isolates from China and the USA (Figure 2). Eleven of these isolates (NDV 14, 16, 17, 18, 19, 20, 23, 32, 34, 43 and 44) showed a high similarity with sequences from South Africa (AF532741 and AF532742, both from KwaZulu-Natal Province, and AF532743, from Gauteng Province) and an isolate from Queensland, Australia (M24693) described by Toyoda *et al*. (1989) (Figure 2). Two isolates (NDV 40 and 42) that share the same node branched together with two isolates from Australia (AY935491 and AY935495). These isolates originated from New South Wales and were published by Kattenbelt, Stevens and Gould (2006). There is no indication that these isolates are vaccine strains. Three isolates (NDV 3, 12 and 22) branched separately but in a close similarity to isolate M24692 from Australia described by Toyoda *et al*. (1989), as well as the grouping of samples in this study mentioned above (Figure 2).

Analysis of the deduced amino acid sequences of the collected samples and data obtained from GenBank did not change the phylogenetic grouping of samples (results not shown).

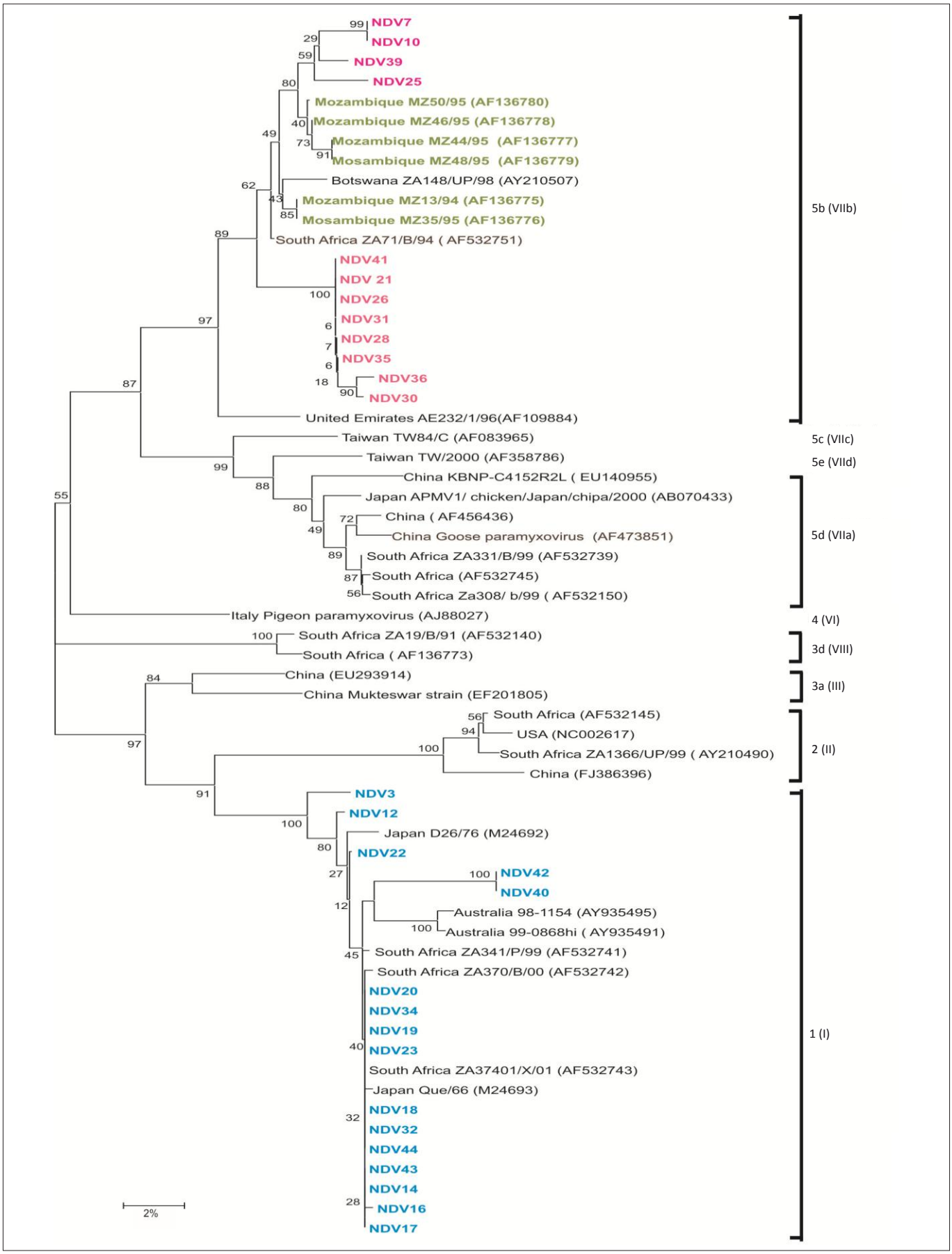


**FIGURE 2:** Results of the neighbour-joining analysis of the sequences of the partial fusion protein encoding gene, showing the phylogenetic relationship of Newcastle disease virus isolates collected in Mozambique in 2005 with other known Newcastle disease virus sequences described in South Africa and other countries worldwide. Different lineages are indicated on the right.

## Trustworthiness

Validated standard operating procedures were used in the laboratory and all the relevant controls were used in all laboratory tests performed. Results obtained can therefore be declared trustworthy.

## Discussion

Outbreaks of ND in Mozambican poultry and the devastation of the infection has been reported for decades, yet only one previous study (Herczeg *et al*. 1999) focused on characterising the causative strains, with samples collected from 1990 to 1995. They concluded that outbreaks in Mozambique and southern Africa were to the result of strains of lineage 5b (VIIb) and 3d (VIII) that were most likely endemic to the region, or were recently introduced. For the present study, 120 samples (mainly swabs) were collected in 2005 from various villages in different geographical areas in Mozambique. We aimed to determine the strains that circulated in the country, 10 years after the first description of lineage 5b in Mozambique.

Of the 28 strains genetically analysed in the present study, 11 were classified as lineage 5b (VIIb) and were phylogenetically closely related to strains described in Mozambique in 1994–1995 (Heczeg *et al*. 1999), as well as from South Africa (1993–1999) and Botswana (1998) (Abolnik *et al*. 2004). These virulent strains were distributed across all regions sampled. Lineage 5d (VIIa), that was first detected in South Africa in 1999 and continues to cause major outbreaks in the region in the past, was not detected in the samples tested here, neither were pigeon paramyxovirus strains (lineage 4) detected in the poultry sampled.

The majority of the viruses we analysed were classified in lineage 1. Lineage 1 not only contains strains that are frequently isolated from wild birds, but also strains that have been adapted for use as vaccines; for example, the Queensland/V-4 ('V-4'), Ulster 2C/67 and I-2 (derived from V-4) that have been used as live vaccines in many countries (Aldous *et al*. 2003). The Avinew-61™ vaccine, a VG/GA derivative is a lineage 1 strain that applied widely in South Africa, and strains that were assumed to be derivates of this live vaccine were isolated frequently in South Africa in a previous study (Abolnik *et al*. 2004). The V-4 vaccine was used in the Macie district, Gaza Province in the 1990s in Mozambique and the I-2 vaccine was introduced in 1998, first in laboratory trials at the National Veterinary Research Institute and then in villages of the Manhiça district, Maputo Province in the southern region of Mozambique. In the following years, the vaccine was used to vaccinate village chickens in five provinces, viz. Gaza, Inhambane, Tete, Zambezia and Nampula. Thus, it is likely that the lineage 1 strains detected in this study are derivates of the V-4 and I-2 vaccines that are widely used in Mozambique.

## Conclusion

In this study, we present a 'snapshot' of the ND situation in Mozambique in 2005, 10 years after a previous study identified lineage 5b that also affected South Africa. But, the latter was affected subsequently by an outbreak of lineage 5d at the time of sampling, and which then completely replaced lineage 5b. This highly virulent strain did not appear to have spilled over to Mozambique by 2005. Lineage 5b therefore persisted in Mozambique from at least 1994 until 2005; however, the current (2012) status remains unknown.

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### Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this paper.

### Authors' contributions

E.V. (University of Pretoria) was the project leader. The laboratory work was conducted by R.F. (National Veterinary Research Institute) and K.E. (University of Pretoria). A.-M.B. (University of Pretoria) also conducted laboratory work and assist in writing the manuscript. C.A. (Onderstepoort Veterinary Institute) and S.B. (Avimune) assist in writing the manuscript and E.V. (University of Pretoria) wrote and submitted the manuscript.

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