CHAPTER FOUR[1]

PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN VELOGENIC NEWCASTLE DISEASE OUTBREAK STRAINS OF THE 1990s (GENOTYPES VIII AND VIIb)

ABSTRACT

A previously-published report found that two separate genotypes of exotic NDV circulated in South Africa during the 1990s. In this study, new and existing data was collated to better define the epidemiology of Newcastle disease in South Africa in the 1990s. The results confirmed that genotype VIII/lineage 3d became enzootic in South Africa since it was introduced from south East Asia in the 1960s and 1970s, but that South Africa was not the source for subsequent outbreaks in China, Japan and Italy. Genotype VIII viruses appear to have attenuated, as the most recent isolates were not as virulent as those of the early 1990s. A second genotype, VIIb (lineage 5b), entered South Africa in the early 1990s and swept across the country as part of a panzootic. Although it was previously suggested that the infection was introduced into southern Europe from South Africa, phylogenetic data indicated that instead, strains identical to early South African viruses had been present in Spain and Portugal at least one to two years prior to the outbreaks in South Africa, and that introduction possibly occurred on multiple occasions from Europe. The chronological and geographical separation of South African genotype VIIb strains during the 1990s was consistent with reports from other countries, and the epidemiological pattern suggested that Genotype VIIb was sustained in South Africa throughout the 1990s via the translocation of infected poultry.

[1]Part of the results presented in the chapter were published in Archives of Virology 149(3):603-619 (2004)

4.1 INTRODUCTION

Exotic Newcastle disease has been considered to be enzootic in South Africa since 1971 (L Coetzee, personal communication), and therefore trade in South African poultry and unprocessed poultry products are banned by many countries. After severe outbreaks in the 1970's, only sporadic outbreaks occurred in South Africa over the next decade. Then, in June 1993, a neuro/respirotropic NDV was isolated from an outbreak at a large commercial poultry producer near Hartebeespoort, Gauteng province. Within six months ND had spread throughout southern Africa, causing devastating losses in all types of poultry. An estimated million chickens per week died at the peak of the outbreak. The major outbreak of 1993/94 was eventually brought under control by improved vaccination procedures and bio-security measures (Huchzermeyer & Gerdes 1993; Verwoerd, 1995a; 1995b), but sporadic outbreaks continued to occur for the remainder of the decade (unpublished laboratory data).

The presence of genotypes VIIb (lineage 5b) and VIII (lineage 3d) in southern Africa and their involvement in the NDV outbreaks of the 1990s was first described by Herczeg and colleagues (1999). Genotype VIII which has predominantly been isolated in southern Africa (Herczeg *et al*, 1999), occurred in western China between 1979 and 1985 (Liang *et al*., 2002), Japan in 1991 (Mase *et al*., 2002), Taiwan in 1995 (Tsai *et al*., 2004) and Italy in 1994 (Aldous *et al*., 2003). Phylogenetic evidence has indicated that genotype VIII viruses possibly originated in Southeast Asia (Mase *et al*., 2002; Tsai *et al.,* 2004). Similarly, genotype VII appeared in Taiwan and Indonesia in the 1980's (Yu *et al.* 2001; Lomniczi *et al*., 1998). It was responsible for the fourth pandemic and was the progenitor to the VIIa, VIIb, VIIc and VIId genotypes. Genotype VIIa has been reported in the Far East and Europe, VIIb in the Far East, Middle East, Europe, India and southern Africa, VIIc in the Far East and Europe and VIId in the Far East and South Africa (Lomniczi *et al*., 1998; Jørgensen *et al*., 1998; Alexander *et al*., 1999; Yang *et al.,* 1999; Herczeg *et al*., 2001; Liang *et al*., 2002; Wehman *et al*., 2003a; 2003b; Abolnik *et al*., 2004a; Bogoyavlenskiy *et al*., 2005).

In a previous study (Herczeg *et al*., 1999), six South African genotype VIII, seven genotype VIIb, and six genotype VIIb Mozambican viruses were analysed phylogenetically. Results suggested that genotype VIII viruses were maintained in South Africa by enzootic infections since the 1960s, whereas genotype VIIb was introduced more recently. However, data were insufficient to make accurate inferences about the sources of the two genotypes, nor could it describe the epidemiology of NDV in the region. In 2003, a larger NDV sequence data set was published by the international reference laboratory, VLA Weybridge in the UK. That study contained over 300 strains of exotic NDV submitted by countries across the world, and the authors concurred with Herczeg and colleagues that genotype VIIb had spread from southern Africa to southern Europe in the early 1990s (Aldous *et al*., 2003).

For NDV to be enzootic in South Africa, a source or reservoir must exist from which periodic outbreaks arise. Village chickens have been suspected of being these reservoirs, but wild birds have also been implicated (Verwoerd, 1995b). From 1990 to 2002, 155 ND viruses were collected by national veterinary laboratories across South Africa. These viruses were pathotyped at OVI by nucleotide sequencing of the region at the fusion protein (F_0) cleavage site and phylogenetic analyses. Sixty five of the viruses were identified as being virulent genotype VIIb and VIII viruses. The remaining viruses were typed as vaccine strains or their close derivatives (Abolnik *et al*., 2004a). In this chapter, I collated all available sequence data for genotype VIIb and VIII with sequence data for sixty five additional South African virulent strains isolated during the outbreaks of the 1990s. The objectives were to better define the epidemiology of NDV in South Africa during the 1990s and, if possible, to determine the source of the outbreaks.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

Velogenic ND viruses (Table 4.1) were grown in 9-to-11 day old specific pathogen free (SPF) embryonated chicken eggs by standard procedures (OIE manual, 2000), at Allerton Provincial Veterinary Laboratory, the Poultry Reference Laboratory (University of Pretoria), and Stellenbosch Provincial Veterinary Laboratory. ICPI and MDT tests were performed at Allerton Provincial Veterinary Laboratory according to standard procedures (OIE manual, 2000).

4.2.2 RNA extraction

Viral RNA was extracted from infective allantoic fluid using the QIAamp® Viral RNA Mini Kit (QIAGEN), TriReagent™ (SIGMA) or TRIzol® reagent (Gibco, Invitrogen), according to the manufacturer's instructions.

4.2.3 RT-PCR

Random hexamers were used to generate first strand cDNA according to the method described by Sambrook *et al.* (1989). The following oligonucleotide primers were used to amplify a 1180 base pair fragment spanning the regions between nucleotides 581 of the fusion protein and nucleotides 610 of the matrix protein, which includes the F_0 cleavage site. Reaction mixtures were cycled 35 times at 94ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 1 min.

M610	5'- CTGTACAATCTTGCGCTCAATGTC -3'	(forward primer)
NDVF581	5'- CTGCCACTGCTAGTTGTGATAATCC -3'	(reverse primer)

4.2.4 DNA sequencing and phylogenetic analysis

DNA was sequenced using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions, and was analysed with an ABI377™ automated sequencer. Sequences of as little as 250 base pairs give meaningful phylogenetic analyses for NDV, comparable with much longer sequences (Seal *et al*., 1995; Lomniczi *et al*., 1998). A commonly used region in NDV phylogenetic studies is a 374 nt fragment at the 3' end of the fusion protein, which includes the region encoding the nuclear localization signal sequence and the precursor fusion protein cleavage site (F_0). Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) of the 374 nucleotide (nt) region were used to identify 50 closely-related sequences to include in multiple sequence alignments, which were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Preparation of Fig. 4.4 (variable sites only of the nucleotide sequence alignment) was done with MEGA v3.1. The results are presented as rooted neighbour joining trees with 1000 bootstrap trials to assign confidence values to topology. Virulent viruses were rooted with the lentogenic La Sota/46 virus sequence.

Table 4.1 Velogenic South African ND viruses characterised in this study

NDV Strain	Collection date	District	Genotype	Accession number
ZA11/B/91	22/03/1991	Pietermaritzburg	VIII	AF532140
ZA13/L/91	08/08/1991	Richmond	VIII	AF532142
ZA16/GF/91	12/09/1991	Richmond	VIII	AF532143
ZA19/B/91	10/12/1991	Lion's River/ Merrivale	VIII	AF532752
ZA52/BB/94	03/03/1994	Camperdown area	VIIb	AF532749
ZA53/BB/94	04/03/1994	Camperdown	VIIb	
ZA54/BB/94	07/03/1994	Camperdown	VIIb	
ZA60/B/94	21/03/1994	Camperdown area	VIIb	AF532750
ZA71/B/94	21/04/1994	Camperdown area	VIIb	AF532751
ZA76/L/94	18/05/1994	Lion's River/Merrivale	VIIb	
ZA87/X/94	20/06/1994	Pretoria	VIIb	
ZA108/L/94	15/07/1994	Estcourt	VIIb	AF532139
ZA110/X/94	19/07/1994	Pretoria	VIIb	AF532141
ZA133/B/94	01/09/1994	Camperdown	VIIb	
ZA170/B/95	09/03/1995	Lower Tugela/ Umhlali	VIIb	AF532144
ZA172/B/95	18/04/1995	Lower Tugela/ Umhlali	VIIb	
ZA903/UP/96	21/03/1996	Pretoria	VIIb	
ZA983/UP/96	10/11/1996	Mpumahlanga province	VIIb	
ZA100/L/94	06/07/1997	Durban	VIIb	
ZA256/X/97	22/07/1997	Irene, Pretoria	VIIb	AF532146
ZA825/UP/97	31/11/1997	Mokopane	VIIb	AY210499
ZA839/UP/97	17/10/1997	Kuruman	VIIb	AY210494
ZA842/UP/97	17/10/1997	Pretoria	VIIb	AY210504
ZA837/UP/97	18/10/1997	Wakkerstroom	VIIb	
ZA928/UP/97	05/12/1997	Gabarone, Botswana	VIIb	AY210495
ZA982/UP/97	06/12/1997	Kuruman	VIIb	
ZA96/UP/98	06/02/1998	Tzaneen	VIIb	
ZA148/UP/98	07/03/1998	Gabarone, Botswana	VIIb	AY210507
ZA7351/Rvsdl/98	??/07/1998	Riversdale	VIIb	AY210502
ZA7381/98	??/07/1998	Western Cape	VIIb	AY210512
ZA700/UP/98	12/07/1998	Vorna Valley	VIIb	
ZA699/UP/98	12/07/1998	Johannesburg	VIIb	
ZA704/UP/98	12/07/1998	Rustenburg	VIIb	AY210508
ZA751/UP/98	25/07/1998	Pretoria	VIIb	
ZA753/UP/98	25/07/1998	Ermelo	VIIb	
ZA762/UP/98	25/07/1998	Johannesburg	VIIb	
ZA756/UP/98	25/07/1998	Vereeniging	VIIb	
ZA734/UP/98	25/07/1998	Rustenburg	VIIb	

ZA984/UP/98	27/07/1998	Gabarone, Botswana	VIIb	AF109876
ZA775/UP/98	01/08/1998	Mokopane	VIIb	
ZA798/UP/98	15/08/1998	Cullinan	VIIb	
ZA9357/Moslb/98	??/09/1998	Mosselbaai	VIIb	
ZA933/UP/98	13/09/1998	Pretoria	VIIb	
ZA949/UP/98	13/09/1998	Phalaborwa	VIIb	
ZA959/UP/98	13/09/1998	Mokopane	VIIb	
ZA955/UP/98	13/09/1998	Bronkhorstpruit	VIIb	
ZA917/UP/98	13/09/1998	Witbank	VIIb	
ZA983/UP/98	20/09/1998	Rustenburg	VIIb	AY210509
ZA1003/UP/98	20/09/1998	Makapanstad	VIIb	
ZA296/L/98	20/09/1998	Durban	VIIb	AF532148
ZA922/UP/98	21/09/1998	Johannesburg	VIIb	
ZA444/B/98	??/10/1998	Hammarsdale	VIIb	
ZA3291/Klpmts/99	??/03/1999	Klapmuts	VIIb	AY210503
ZA309/B/99	12/03/1999	Paulpietersburg	VIIb	AF532151
ZA309/B/99	12/03/1999	Paulpietersburg	VIIb	
ZA1320/UP/99	31/07/1999	Pretoria	VIIb	AY210510
ZA1251/UP/99	01/08/1999	Halfway House	VIIb	AY210501
ZA8057/99	??/08/1999	Western cape	VIIb	
ZA922/UP/98	21/09/1999	Johannesburg	VIIb	
ZA335/B/99	26/10/1999	Camperdown	VIIb	AF532740
ZA598/UP/00	02/06/2000	Pretoria	VIII	AY210496
ZA606/UP/00	02/06/2000	Pretoria	VIII	AY210497

4.3 RESULTS

Phylogenetic analysis of the 65 strains isolated in South Africa from 1990 to 2000 confirmed previously published results that two separate genotypes were responsible for outbreaks during this period: genotype VIII (lineage 3d) and genotype VIIb (lineage 5b). Genotype VIII was analysed first:

4.3.1 Genotype VIII (lineage 3d)

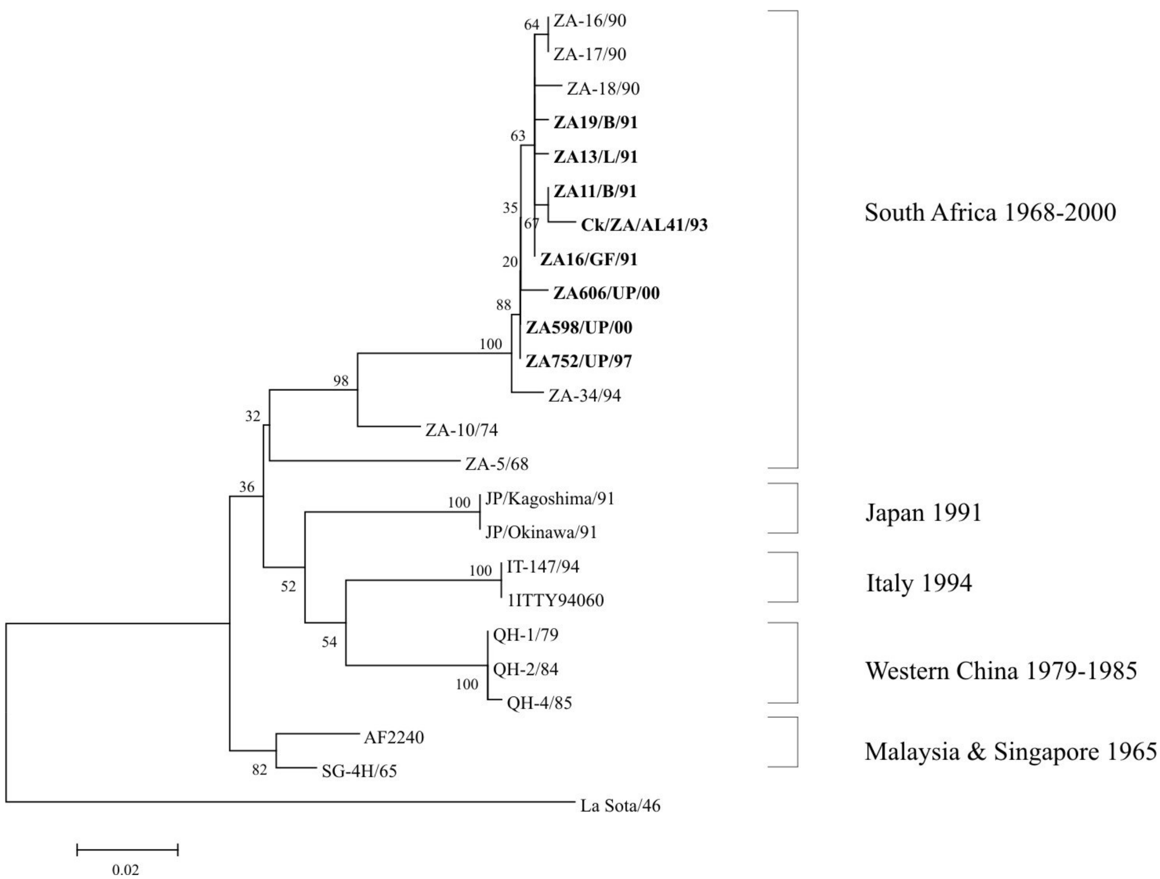

Figure 4.1 Dendogram of a 374 nt 3'-end region of the fusion proteins of South African genotype VIII (lineage 3d) viruses. Viruses whose fusion protein sequences were determined in this chapter are indicated in boldface.

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                          10        20        30        40        50        60        70
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ZA16/GF/91       ..........A....V......................................................
ZA-18/90         ..........A....V.............................................T........
ZA19/B/91        ..........A....V......................................................
ZA-16/90         ..........A....V......................................................
ZA-17/90         ..........A....V......................................................
ZA13/L/91        ..........A....V......................................................
ZA11/B/91        ..........A....V......................................................
Ck/ZA/AL41/93    ..........A....V.Q....................................................
ZA606/UP/00      ..........A....V...........................A..........................
ZA725/UP/97      ..........A....V......................................................
ZA598/UP/00      ..........A....V......................................................
ZA-34/94         ..........A....V......................................................
ZA-10/74         ............PM........................................................
ZA-5/68          ..P..........L....TT..................................................
IT-147/94        ...R....YP..P....Q.........P.......................................I..
1ITTY94060       ...R....YP..P....Q.........P.......................................I..
QH-1/79          ........FL..SM.............P.G........................................
QH-2/84          ........FL..SM.............P.G........................................
QH-4/85          ........FL..SM.............P.G........................................
JP/Kagoshima/91  ...E....F....M.........R..................I........V..................
JP/Okinawa/91    ...E....F....M.........R..................I........V..................
AF2240           .........P...M.....T..................................................
SG-4H/65         .........P...M.....T..................................................
Consensus        MGSKSSTRISTPLTLITRIMLILSCICLTSSLDGRPLAAAGIVVTGDKAVNIYTSSQTGSIIVKLLPNMP
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                          80        90       100       110       120
                 ....|....|....|....|....|....|....|....|....|....|....
ZA16/GF/91       ....V......................................R..........
ZA-18/90         ....V......................................R.........I
ZA19/B/91        ....V..................................E...R..........
ZA-16/90         ....V......................................R..........
ZA-17/90         ....V......................................R..........
ZA13/L/91        ....V......................................R..........
ZA11/B/91        ....V......................................R..........
Ck/ZA/AL41/93    ....V......................................R..........
ZA606/UP/00      ....V.................................................
ZA725/UP/97      ....V.................................................
ZA598/UP/00      ....V.................................................
ZA-34/94         ....V.............................................L...
ZA-10/74         ......................................................
ZA-5/68          .......K............................S..........VS.....
IT-147/94        .......K..........................................V...
1ITTY94060       .......K..........................................V...
QH-1/79          ..................................................V...
QH-2/84          ..................................................V...
QH-4/85          ..................................................V...
JP/Kagoshima/91  ......................................................
JP/Okinawa/91    ......................................................
AF2240           .......KP.V.......................................V...
SG-4H/65         .......K..............................................
Consensus        KDKEACARTPLEAYNRTLTTLLTPLGDSIRRIQGSVTTSGGRRQKRFIGAIIGS
```

Figure 4.2 Multiple amino acid alignment of partial fusion (F) protein genes of genotype VIII viruses, including the F_0 cleavage site (underlined). Viruses whose nucleic acid sequences were determined in this study are indicated in boldface.

Genotype VIII/lineage 3d is composed of only 23 isolates from Singapore, Malaysia, Italy, Japan, China and South Africa ranging in years of isolation from 1965 to 2000. Of these, fourteen viruses (60.8%) originated from South Africa, and eight of the fourteen were contributed by the present study. Fig. 4.1 indicates that the South African viruses are distinct from viruses isolated from other regions, but that all contemporary genotype VIII isolates (including South Africa) are likely to be derived from the South East Asian strains isolated in the 1960s. The earliest South African viruses from 1968 (ZA-5/68) and 1974 (ZA-10/74) are basal to the contemporary South African strains, although the 1968 genotype is only supported by a 32% bootstrap value. In contrast, the 1974 virus is supported by a bootstrap value of 98%, supporting suggestions that this genotype was enzootic in South Africa since the 1970s (Herczeg *et al*., 1999; Aldous *et al*., 2003).

The South African genotype VIII viruses sequenced in this study (ZA11/B/91, ZA19/B/91, ZA13/L/91, ZA16/GF/91) were phylogenetically interspersed with four viruses analysed in a previous study (ZA-16/90, ZA-17/90, ZA-18/90 and ZA-34/94) (Herczeg *et al*., 1999). An IVPI value of 2.89 was obtained for ZA/11/B/91, confirming the highly virulent pathotype of genotype VIII viruses. The SA genotype VIII viruses isolated from 1990 to 1993 contained a unique R^{114} for Q substitution (Fig. 4.2). Herczeg and colleagues (1999) suggested that the R^{114} contributed to the high pathogenicity of genotype VIII. After 1994, Genotype VIII viruses went undetected until 1997 (ZA752/UP/97), and were then isolated on two more occasions in 2000 (ZA598/UP/00, ZA606/UP/00). Unlike the viruses of the early-to-mid 1990s (KZN province), these later isolates were obtained from the Pretoria region (Gauteng province). South African genotype VIII viruses isolated from 1994 onwards contained the conventional Q^{114} in the F_0 cleavage site (Fig. 4.2), and did not appear to be as virulent as those isolated in the early 1990s. For example, ZA598/UP/00, which was isolated east of Pretoria, only caused a 2% drop in egg production with no clinical symptoms apart from nasal excretions (unpublished laboratory report data). The phylogenetic results clearly indicate that South Africa was not the source of genotype VIII viruses causing outbreaks in Japan, China or Italy.

4.3.2 Genotype VIIb (Lineage 5b)

Figure 4.3(a) Phylogenetic tree of genotype VIIb isolates. South African viruses whose nucleic acid sequences were determined in this study are indicated in boldface. Ostrich isolates are indicated by arrows. Portuguese and Spanish viruses are boxed. Enlargements of views (a) and (b) are presented in Figs. 4.3(b) and 4.3(c) respectively.

Figure 4.3(b). Enlargement of view (a)

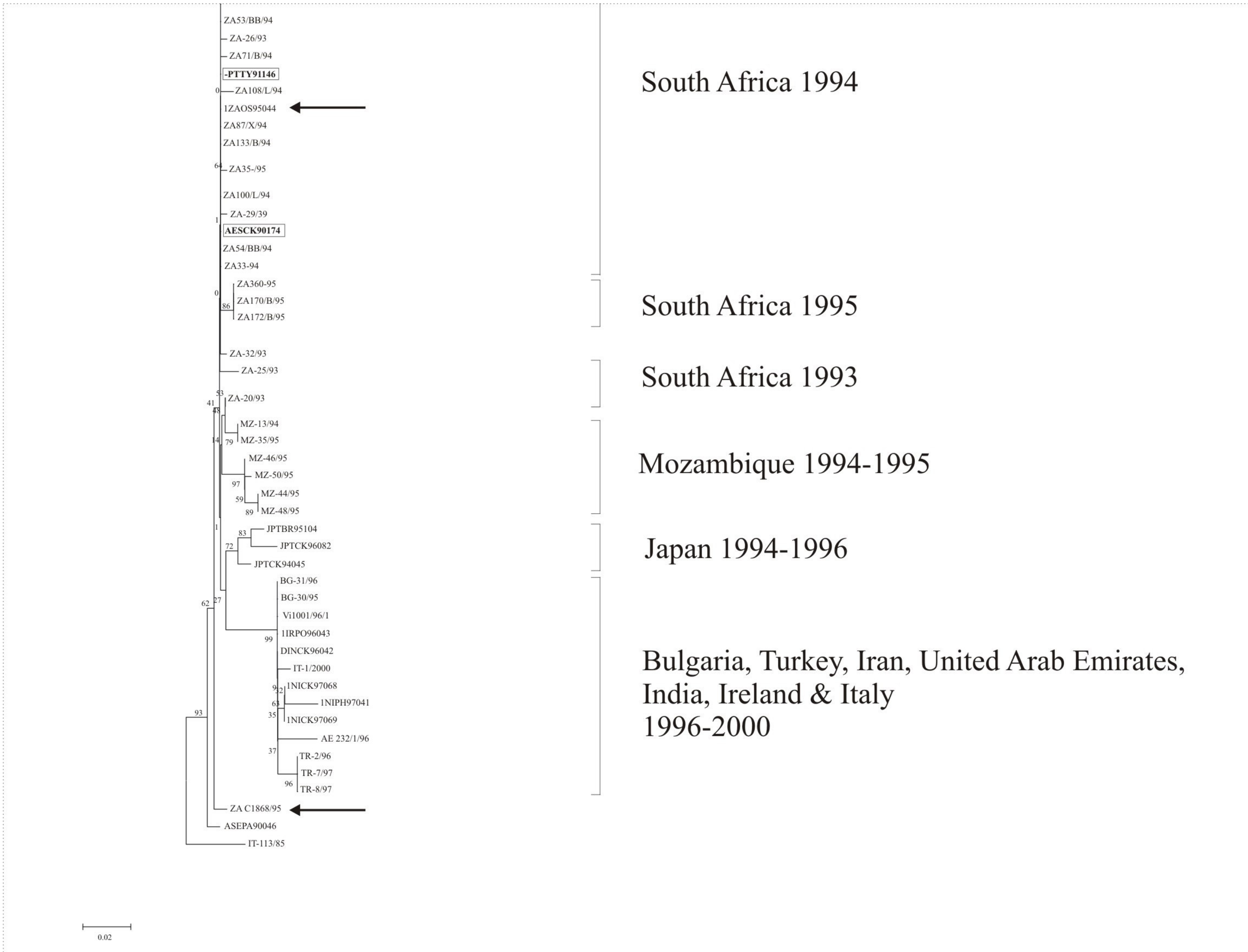

Figure 4.3(c). Enlargement of view (b)

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[                                                                        11111 1111111111 1111111111 2222222222 2222222222 2222222222 2222333333 3333333333 3333333333 33]
[                   11111122 2233333444 4444555555 5566666777 7778888888 9999901222 3333445566 6677789990 0111111223 3333444556 6777777888 8999000011 1122222233 3444555566 66]
[                 6712357847 8912367012 3578012456 7913679012 5692345789 0346977079 2689460325 6824702351 5023569250 1357369284 7013679258 9479023657 8912457902 3458125701 69]
ZA751/UP/98       CTAACTCTAC CTGCACCATG CGTCCTCGGT TTCGCTAAGC TATTCGAAGA TCTTTCTAGA GGAGGCCTCA TTATGAATGA ACAGATAGGC AAGCAGACAG CCTACATCTC GTCGTGGATT GTCAATGGAG AAACCAGTCG TC
ZA982/UP/97       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..
ZA839/UP/97       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ........A. .......... .......... ..
ZA837/UP/97       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ........A. .......... .......... ..
ZA928/UP97        .......... .......... .......... .......... ....T..... .......... .......... .......... .......... .......... ....T..... .......... .......... .......... ..
ZA842/UP/97       ......T... .C.T...... .......... .......... C........G ..A....... .......... .......... .......... T......... .......... .......... .......... ....T..... ..
ZA825/UP/97       ......T... .C.T...... T......... .......... C........G ..C....... .......... .......... .........T T......... .......... .......... .......... ....T..... ..
ZA360-95          ........G. .C.T...... .......... .......T.. .....A...G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA170/B/95        ........G. .C.T...... .......... .......T.. .....A...G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA172/B/95        ........G. .C.T...... .......... .......T.. .....A...G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA60/B/94         ........G. .C.T...... .......... ..G....... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... .G..T..... ..
-PTTY91146        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
AESCK90174        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA110/X/94        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... .G..T..... ..
ZA100/L/94        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA133/B/94        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA53/BB/94        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA54/BB/9         ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA76/L/94         ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA87/X/94         ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA-33/94          ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA52/BB/94        ........G. .C.T...... .......... ..G....... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA-35/95          ........G. .C.T...... .......... .......... .........G ..C....... ...A...... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
ZA71/B/94         ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... T......... .......... .......... ....T..... ..
ZA-20/93          ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .G........ .......... .......... .......... ....T..... ..
ZA108/L/94        ........G. .C.T...... .......... ........A. .........G ..C....... .......... ...A.....G .......... .......... .......... .......... ...G...... ....T..... ..
ZA983/UP/98       .......... .C.T...... T......... ......C... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... ....T..... ..
ZA903/UP/96       .......... .C.T...... T......... ......C... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... ....T..... ..
ZA1320/UP/99      .......... .C.T...... .......... .......... .....A...G ..C....... ..C....... .........G .......... .......... ........C. .......... .......... .......... ..
ZA335/B/99        .......... .C.T...... .......... .......... .....A...G ..C....... .......... .........G .......... .......... ........C. .......... .......... .......... ..
ZA1251/UP/99      ......T... .C.T...... .......... .......... .....A...G ..C....... ..C....... .........G .......... .......... ........C. .......... .......... .......... ..
ZA874/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .....A.... .........G .......... .......... .......... .......... .......... .......... ..
ZA549/UP/99       .......... .C.T...... .......... .......... .........G ..C....... .....A.... .........G .......... .......... ..C....... .......... .......... .......... ..
ZA955/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .A........ .........G .......... .......... .......... .......... .......... .......... ..
ZA917/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .A........ .........G .......... .......... .......... .......... .......... .......... ..
ZA922/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .A........ .........G .......... .......... .......... .......... .......... .......... ..
ZA309/B/99        .......... .C.T...... .......... .......... .........G ..C....... .A........ .........G .......... .......... .......... .......... .......... .......... ..
ZA700/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA699/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA704/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA933/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA753/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA762/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA949/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA1003/UP/98      .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA734/UP/98       .......... .C.T...... .......... .......... .........G ..C....... ..C....... .........G .......... .......... .......... .......... .......... .......... ..
ZA798/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA96/UP/98        .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA775/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA959/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA756/UP/98       .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA3291/Klpmts/99  .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA9357/Mslb/98    .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA7351/Rvsdl/98   .......... .C.T...... .......... .......... ......G..G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA256/X/97        .......... .C.T...... ......T... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... .......... ..
ZA296/L/98        .......... .C.T...... .......... .......... .........G ..C....... .........G .........G .......... .......... .......... .......... .......... .......... ..
ZA444/B/98        .......... .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .......... .......... ........T. ..
ZA984/UP/98       .........T .C.T...... .......... .......... .........G ..C....... .......... .........G .......... .......... .......... .....T.... .......... .......... ..
```

Figure 4.4 Muliple nucleotide sequence alignment of partial fusion (F) protein genes of genotype VIIb viruses. Only variable sites are presented, and sequences contributed by the present study are indicated in boldface

Fig. 4.4 continued

```
[                                                                                      11111 1111111111 1111111111 2222222222 2222222222 2222222222 2222333333 3333333333 3333333333 33]
[                   11111122 2233333444 4444555555 5566666777 7778888888 9999901222 3333445566 6677789990 0111111223 3333444556 6777777888 8999000011 1122222233 3444555566 66]
[                 6712357847 8912367012 3578012456 7913679012 5692345789 0346977079 2689460325 6824702351 5023569250 1357369284 7013679258 9479023657 8912457902 3458125701 69]
ZA751/UP/98       CTAACTCTAC CTGCACCATG CGTCCTCGGT TTCGCTAAGC TATTCGAAGA TCTTTCTAGA GGAGGCCTCA TTATGAATGA ACAGATAGGC AAGCAGACAG CCTACATCTC GTCGTGGATT GTCAATGGAG AAACCAGTCG TC
ZA148/UP/98       ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... ...T...... .......... ........C. .......... G......... ..
ZA-5/68           TC..T...G. TCA..T.C.. ...A.C..AC CC..ACG..T ...CT...AG CTCA.....G A...A..C.. ...G..GC.. .......... ..ATG..... TT....C..T .C..C..... A.T...T... G.G.TGA..A CT
ZA-25/93          T.......G. .C.T...... .......... .......... .........G ..C.....A. .......... ...A.....G ........A. .......... .......... .......... .......... ....T..... ..
ZA-26/93          ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T...A. ..
ZA-29/93          ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......A.. .......... .......... .......... .......... ....T..... ..
ZA-32/93          ........GA .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
MZ-13/94          ........G. .C.T...... .......... .......... C........G ..C....... .......... ...A.....G .......... .G........ ..C....... .......... .......... ....T..... ..
MZ-35/95          ........G. .C.T...... .......... .......... C........G ..C....... .......... ...A.....G .......... .G........ ..C....... .......... .......... ....T..... ..
MZ-44/95          ........G. .C.T..T... .......... .......... C........G ..C....... ......T... ...A.....G .T........ .......... .......... .......... .......AG. ....T..... ..
MZ-46/95          ........G. .C.T..T... .......... .......... C........G ..C....... ......T... ...A.....G .T........ .......... .......... .......... .......... ....T..... ..
MZ-48/95          ........G. .C.T..T... .......... .......... C........G ..C....... ......T... ...A.....G .T........ .......... .......... .......... .......AG. ....T..... ..
MZ-50/95          ........G. .C.T..T... .......... .......... C........G ..C....... ......T... ...A.....G .T........ .......... .......... .......... ......A... ....T..... ..
BG-30/95          ........G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... .......... .......... .G.TT..... C.
TR-2/96           ...C....G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... ..T....... .......... .G.TT....A C.
TR-7/97           ...C....G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... ..T....... .......... .G.TT....A C.
TR-8/97           ...C....G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... ..T....... .......... .G.TT....A C.
BG-31/96          ........G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... .......... .......... .G.TT..... C.
IT-1/2000         ........G. TC.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. ........G. .......... .......... .G.TT..... C.
IT-112/84         ........G. .C.T...G.. .......... .......... ...CT...AG C.C.C..... A......... ...A.....G .......... .......... .......... .........C .....CT... ....T..... ..
IT-113/85         ..G.....G. .C.T..T... .......... .......... ...C....AG C.C.....A. A.......T. ...A.....G .......... ......G... .........T AC........ ......T... ....T..C.. ..
RI-1/88           ........G. .C.T...... T.......A. C...A..... ...CT...AG CTC.C..... A......... ...A..G.AG .......... .......... T..G...... .C..CA.C.. .G..G.T... ....T..... ..
ZA_C1868/95       ........G. .C.T...... .......... .......... .......C.G ..C..T.... A......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
BMYCK82175        ........G. .C.TG..... .......... ....A..... CG.CT...AG C.C.C..... A.....T.T. ...AA.G..G .T........ .G........ .....G.... .C.AC..... ......T... ....T..... ..
APECK92173        ........G. ...T...GC. ........A. ....AC.... ...CT...AG C.C.C.C.A. A......... ...A..G..G ........A. .......... ...G.G.T.. .C..C..... ....C.T... ....T..... ..
BINCK94170        ........G. AC.T...... .......... .......... CG.CT...AG CTC.C..... A........G .C.A.....G C.C.G...T. .G........ .......T.. .......... ......T..C ....T...T. ..
1ZAOS99141        .......CG. .C......C. T......... .......... ...C.....G ..C....T.. .......... ...A.....G .......... .......... .......... .......... ..T....... ....T....A ..
JPTBR95104        ........GT .C.T...... .T..T..A.. .......... ...C.....G ..C....... .......... G..A.....G .......... .......T.. .......... .......... .......... ....T..... ..
JPTCK96082        ........GT .C.T...... .T..T..... .......... .......... ..C....... .......... G.TA.....G .......... .....A.T.. .......... ......A... .......... ....T..... ..
BMYBU87078        ........G. ...T....CA ........A. .C..AC.... ...CT...AG C.C.C.C.A. A......... ...A..G..G ........A. ........G. ...G.G.... .C..C..... ....C.T... ....T..... .T
1NICK97069        ........G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .....C.... .......T.. .......... .......... .......... .G.TT..... C.
1NICK97068        ........G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .....C.... .......T.. .......... .......... .......... .G.TT..... C.
ASEPA90046        ........G. .C.T...... .......... .......... ........AG ..C....... A......... ...A.T...G .......... .......... .T........ .......... .......... ....T..... ..
JPTCK94045        ........GT .C.T...... .T........ .......... .........G ..C....... .......... ...A.....G ........A. .......T.. .......T.. .......... .......... ....T..... ..
1ZAOS95044        ........G. .C.T...... .......... .......... .........G ..C....... .......... ...A.....G .......... .......... .......... .......... .......... ....T..... ..
1IRPO96043        ........G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... .......... .......... .G.TT..... C.
DINCK96042        ........G. .C.T...... .......A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .......... .......T.. .......... .......... .......... .G.TT..... C.
1NIPH97041        .....C.CG. .C.T...... ..C....A.. ...A...... C.C.....AG ..C....... .......... ...A.....G .....C.... .......T.. .......... .......... .......C.. .G.TT..... CG
Ck/JP/Niigata/89  ........G. .C.T.T.... ......A... ......G... ...CT...AG C.C.C..... A......... ...A.....G ..GA..G... .........A .......... .......... ......T... ....T..... ..
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```
                          10        20        30        40        50        60        70        80        90       100       110       120
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
ZA-5/68          ..P.S....ST..L....TT.......L.S................................................T......................................VS.I...
ZA-35/95         ..............................................N.............................................................................
ZA-29/93         ............................................................................................................................
ZA53/BB/94       ............................................................................................................................
ZA87/X/94        ............................................................................................................................
AESCK90174       ............................................................................................................................
ZA100/L/94       ............................................................................................................................
ZA108/L/94       .......................N...................................................................................A................
ZA133/B/94       ............................................................................................................................
ZA76/L/94        ............................................................................................................................
1ZAOS95044       ............................................................................................................................
-PTTY91146       ............................................................................................................................
ZA-33/94         ............................................................................................................................
ZA54/BB/94       ............................................................................................................................
ZA71/B/94        ............................................................................................................................
ZA170/B/95       .......................C....................................................................................................
ZA172/B/95       .......................C....................................................................................................
ZA360-95         .......................C....................................................................................................
ZA-26/93         ............................................................................................................................
ZA-32/93         ............................................................................................................................
ZA60/B/94        ....................V.............................................................................................R.........
ZA110/X/94       ..................................................................................................................R.........
ZA52/BB/94       ....................V.......................................................................................................
ZA751/UP/98      .........LA..................D.F............................................................................................
ZA982/UP/97      .........LA..................D.F............................................................................................
ZA928/UP/97      .........LA................L.D.F............................................................................................
ZA839/UP/97      .........LA..................D.F............................................................................................
ZA837/UP/97      .........LA..................D.F............................................................................................
ZA842/UP/97      .....F.........................I............................................................................................
ZA825/UP/97      .....F......................................................................V...............................................
ZA1320/UP/99     ............................................................................................................................
ZA1251/UP/99     .....F......................................................................................................................
ZA874/UP/98      ................................................E...........................................................................
ZA549/UP/99      ................................................E...........................................................................
ZA955/UP/98      .............................................R..............................................................................
ZA917/UP/98      .............................................R..............................................................................
ZA922/UP/98      .............................................R..............................................................................
ZA309/B/99       .............................................R..............................................................................
ZA1003/UP/98     ............................................................................................................................
ZA8057/99        ............................................................................................................................
ZA798/UP/98      ............................................................................................................................
ZA7351/Rvsdl/98  ............................A...............................................................................................
ZA256/X/97       .................W..........................................................................................................
ZA296/L/98       ............................................................................................................................
ZA444/B/99       ............................................................................................................................
ZA984/UP/98      ....................................................................................................M.......................
ZA7381/98        ....................V.......................................................................................................
ZA983/UP/96      ......................F.....................................................................................................
ZA903/UP/96      ......................F.....................................................................................................
1ZAOS9141        ..........A..T.............P............................................................................................I...
ZA148/UP/98      ............................................................................................................................
ZA-25/93         ..........................................I.................................................................................
ZA-20/93         .............................................................................R..............................................
MZ-13/94         .............................................................................R..............................................
MZ-35/95         .............................................................................R..............................................
MZ-44/95         .............................................................................................................E..............
MZ-48/95         .............................................................................................................E..............
MZ-46/95         ............................................................................................................................
MZ-50/95         ............................................................................................................................
BG-30/95         ..........................R..S....................................................................................R.........
BG-31/96         ..........................R..S....................................................................................R.........
Vi1001/96/1      ..........................R..S....................................................................................R.........
1IRPO96043       ..........................R..S....................................................................................R.........
DINCK96042       ..........................R..S....................................................................................R.........
1NICK97069       ..........................R..S....................................................................................R.........
1NICK97068       ..........................R..S....................................................................................R.........
1NIPH97041       ...............T..........R..S...............................................................................A....R.........
TR-2/96          ...N......................R..S....................................................................................R.....I...
TR-7/97          ...N......................R..S....................................................................................R.....I...
TR-8/97          ...N......................R..S....................................................................................R.....I...
IT-1/2000        .........S................R..S....................................................................................R.........
AE 232/1/96      ..........A...........S...R..S................E........................................................E.......K..R.........
JPTBR95104       ................I..........P...........................A....................................................................
JPTCK96082       ................I............D.........................A.S..................................................................
JPTCK94045       ............................................................................................................................
ZA C1868/95      ...................................L........................................................................................
ASEPA90046       .............................S..............................................................................................
IT-113/85        ...R.......................P.S............I.....................................................N...........................
IT-112/84        .............V.............L.S...........................................................................A..P...............
JP/Niigata/89    ...........................L.S........................................R.....................................................
BINCK94170       .........T...............V.L.S......................................LTG......R................................A.............
BMYCK82715       .........................V.L.S...............................................R..............A......H........................
TW/2000          ...RS.....A..T....I.......HL.S.....................V................................................K.......................
TW84/C           ...RS..GSA....QSN.I........L.S......................................................................K.......................
RI-1/88          ..................I........L.S......................................................................K.....A.................
APECK92173       .........L...A....I........L.S............I.................................................A...............................
BMYBU87078       .........L...T....IT.......L.S............I.................................................A...............................
Kuwait 256       ..................I........L.S..........................................................................................I...
Consensus        MGSKPSTRIPVPLMLITRVMLILSCICSTGSLDGRPLAAAGIVVTGDKAVNIYTSSQTGSIIVKLLPNMPKDKEACAKAPLEAYNRTLTTLLTPLGDSIRRIQGSVSTSGGRRQKRFIGAVIGS
```

Figure 4.5 Multiple amino acid alignment of partial fusion (F) protein genes of genotype VIIb viruses, including the F_0 cleavage site (underlined). Viruses whose nucleic acid sequences were determined in this chapter are indicated in boldface.

Figs. 4.3(a) to (c) illustrate the phylogenetic relationships between South African genotype VIIb viruses and the closest relatives in the Genbank database. The early southern African genotype VIIb isolates (1993 to 1995) sequenced in this study are interspersed with previously-published isolates ZA-33/94, ZA-26/93, ZA-35/95, ZA-29/93, ZA-38/94, ZA-25/93 and ZA-20/93 from the same period, and are phylogenetically separated from later isolates. The chronological separation is supported at the nucleotide level by a unique A^{174} substitution (Fig. 4.4) that is also present in a South African ostrich isolate, 1ZAOS91141 isolated in 1991 (Aldous *et al.*, 2003). Within this early group, two smaller sub-lineages are evident. The first sub-lineage is composed of isolates ZA60/B/94, ZA52/BB/94 and ZA110/X/94. The first two viruses were isolated in Camperdown in March 2004, whereas ZA110/X/94 was isolated in Pretoria four months later (Table 4.1). Phylogenetically, ZA60/B/94 appears to be more closely-related to ZA110/X/94 and these two virus share a non-synonymous $A^{344}\rightarrow G$ substitution (Fig. 4.4) that resulted in a $K^{115}\rightarrow R$ mutation within the F_0 cleavage site (Fig. 4.5). The $K^{115}\rightarrow R$ mutation was also found within isolates from Bulgaria, Turkey, Iran, the United Arab Emirates, India, Ireland and Italy between 1996 and 2000 (Fig 4.3; Fig. 4.5). ZA60/B/94 and ZA52/BB/94 shared a non-synonymous G^{61} substitution (Fig. 4.4) that resulted in a $L^{21}\rightarrow V$ mutation (Fig. 4.5). Despite having a virulent F_0 cleavage site motif, ZA60/B/94 had a mean death time value between 60 and 90 hours, which classified it as a mesogenic and not a velogenic virus. The attenuation of undefined virulence determinants elsewhere in the genome of ZA60/B/94 is expected to account for the decreased pathogenicity.

The second sub-lineage within the early genotype VIIb viruses is composed of three isolates, viz. ZA170/B/95, ZA172/B/95 and ZA360-95, which are identical at the nucleotide sequence level. Nucleotide substitutions $A^{70}\rightarrow T$ and $G^{84}\rightarrow A$ were observed (Fig. 4.4). The former resulted in a unique $S^{24}\rightarrow C$ substitution (Fig. 4.5). ZA360-95 was isolated from an ostrich (Alexander *et al.,* 1999) and ZA170/B/95 and ZA172/B/95 from broilers in the Lower Tugela/Umhlali area of KZN. Despite a lack of nucleotide sequence data from provinces other than KZN, the spread of the early major outbreak is well documented (Huchzermeyer & Gerdes, 1993; Verwoerd *et al* 1995a; 1995b; 1997; 1999). From the phylogenetic evidence presented here, it seems likely that ZA-20/93 or a close genetic relative gave rise to the Mozambican outbreak strains. The two most interesting inclusions into the early South African clade were two viruses from Spain and Portugal (AECK90174 and -PTTY91148), that were isolated from poultry in 1990 and 1991, respectively (Aldous *et al*., 2003). These two viruses were identical at the nucleotide sequence level to ZA-29/93, ZA53/BB/94, ZA87/X/94, ZA100/L/94, ZA133/B/94, ZA76/L/94, 1ZAOS95044, ZA-33/94 and ZA54/BB/94, the earliest southern African isolates apart from 1ZAOS9141.

Chronological phylogenetic separation was also evident for viruses isolated in 1996, 1997, and 1998 to 1999 (Fig. 4.3). The 1996 isolates ZA983/UP/96 and ZA903/UP/96 shared a unique non-synonymous $A^{69}{\rightarrow}C$ substitution (Fig. 4.4) that produced a unique F^{24} residue (Fig. 4.5). The 1997 isolates ZA752/UP/97, ZA825/UP/97, ZA839/UP/97, ZA842/UP/97, ZA837/UP/97, ZA928/UP/97, ZA982/UP/97 and a single 1998 isolate (ZA751/UP/98) shared numerous synonymous and non-synonymous nucleotide substitutions (Fig. 4.4), and were distinguished at the amino acid level by unique L^{10}, A^{11}, D^{30} and F^{32} residues (ZA842/UP/97 had an I^{32} mutation) (Fig.4.5).

Within the 1997 sub-lineage, other nucleotide and amino acid residues that indicated recent common ancestors for some of the strains were evident (Fig. 4.4, Fig. 4.5). For example, ZA842/UP/97 and ZA825/UP/97 shared an $S^{6}{\rightarrow}F$ substitution. ZA842/UP/97 was isolated in October 1997 in Pretoria and ZA825/UP/97 in November in Mokopane (Limpopo province).

ZA751/UP/98, ZA982/UP/97, ZA928/UP/97, ZA839/UP/97 and ZA837/UP/97 contained T^{29}, C^{32}, A^{89}, T^{94} and A^{201} substitutions (Fig. 4.4) that indicated a shared ancestral virus. This variant was first detected in Kuruman (Northern Cape province) in mid-October 1997, and almost simultaneously in Wakkerstroom (Mpumahlanga province). Two months later it appeared in Gaborone, Botswana, and was re-isolated in Kuruman, indicating that it persisted there for at least two months. Finally, the 1997 variant was detected in July 1998 in Pretoria.

In the 1998/1999 sub-lineage, ZA955/UP/98, ZA309/B/99, ZA917/UP/98 and ZA922/UP/98 contain a unique $G^{136}{\rightarrow}A$ non-synonymous substitution (Fig. 4.4) that resulted in a unique $G^{46}{\rightarrow}R$ mutation (Fig. 4.5). This variant circulated in a relatively small area of approximately 115 square kilometres between Witbank, Bronkhorstspruit and Johannesburg (all in the Gauteng province) in September 1998 before moving to Paulpietersburg in northern KZN in March 1999.

A genetic variant that arose in 1998 was characterized by a synonymous $A^{138}\rightarrow C$ substitution (Fig. 4.4). These viruses, ZA700/UP/98, ZA699/UP/98, ZA704/UP/98, ZA933/UP/98, ZA983/UP/98, ZA753/UP/98, ZA762/UP/98, ZA949/UP/98, ZA1003/UP/98 and ZA734/UP/98 were identical at the nucleotide sequence level (Fig. 4.4) and are represented by ZA1003/UP/98 in Fig. 4.5. The first isolations occurred near Johannesburg, Vorna Valley (Midrand, Gauteng), and Rustenberg (North West province) almost simultaneously, and then appeared two weeks later in Ermelo (Mpumahlanga) in July 1998. It was also re-isolated in Johannesburg and Rustenberg at the same time that it was detected in Ermelo. Two months later the variant had spread to Pretoria, then to Phalaborwa (Limpopo province), before being isolated in Makapanstad near Pretoria.

ZA1320/UP/99, ZA1251/UP/99 and ZA335/B/99 shared synonymous $G^{84}\rightarrow A$ and unique $T^{285}\rightarrow C$ substitutions (Fig. 4.4). Furthermore, the two earlier isolates ZA1320/UP/99 and ZA1251/UP/99 shared the synonymous $A^{138}\rightarrow C$ substitution with several 1998 isolates, ZA704/UP/98 and homologues (Fig. 4.4), suggesting that the 1999 variants arose from them. The 1999 variant was first detected in Pretoria at the end of July 1999 before spreading to Halfway House (Gauteng) in August, and to Camperdown in October.

Viruses excluding the distinguishing features discussed above, except for the $T^{351}\rightarrow C$ synonymous substitution (Fig. 4.4) that separates the early from the late VIIb viruses, were grouped together. This sub-lineage consisted of the isolates ZA798/UP/98, ZA96/UP/98, ZA775/UP/98, ZA959/UP/98, ZA756/UP/98, ZA3291/Klpmts/99, ZA9357/Moslb/98 and ZA8057/99. These viruses were isolated from Tzaneen (Limpopo) in February 1998, then Mokopane in June, Vereeniging (Gauteng) in July, Cullinan (Gauteng) in August, back in Mokopane in September and then in Mosselbay (Eastern Cape) in September and Klapmuts (Western Cape) near Paarl in March 1999. This indicated a southward spread, with the persistence in Mokopane for at least three months. ZA8057/99 was also isolated in August 1999 in the Western Cape Province, although the exact location is unknown. ZA7351/Rvsdl/98 and ZA7381/98 which are separate variants, were detected in July 1998 which suggests at least two separate NDV introduction events into the Western Cape region.

Other viruses isolated in 1997 and 1998 were closely related to those discussed above, but contained generally synonymous point mutations at the nucleotide sequence level. ZA444/B/98 only caused about 5-10% mortality. This particular outbreak was not widespread as only a few farms in the district were affected and NDV was only detected on routine virus isolation attempts. It lasted only a few weeks, which was attributed to a heavy ND vaccination program in place on those farms at that time. ZA984/UP/98 was the second isolate of this study that originated from Gaborone, Botswana. The unique $R^{101} \rightarrow M$ substitution (Fig 4.5), and other synonymous substitutions at the nucleotide sequence level (Fig. 4.4) could perhaps be attributed to geographical separation after a probable earlier introduction from South Africa.

4.4 DISCUSSION

In a previous study (Herczeg *et al.*, 1999), it was established that two separate genotypes of NDV circulated in South Africa during the early 1990s. The first, genotype VIII or lineage 3d, had been isolated from South Africa in 1968, 1974 and in the early 1990s up to 1994. It is believed that this genotype was enzootic in South Africa since the 1960s. The results of the current chapter, whereby eight additional viruses were added to genotype VIII, one of the smallest exotic NDV lineages in the world, confirmed that genotype VIII was maintained in an enzootic infection in South Africa since at least the 1970s. Most isolations of genotype VIII were made in the KwaZulu/Natal Province, one of the largest and most intensive poultry-producing areas in South Africa (Appendix 1), but three more viruses were detected in the latter part of the decade. After 1994, genotype VIII did not appear to be as virulent as it initially was (R Horner, personal observation). Whether this was due to improved and widespread vaccination in the 1990s, or attenuation, such as the mutation of the $R^{114} \rightarrow Q$ in the F_0 cleavage site is unknown. Vaccinated chickens are still able to shed viruses but remain clinically healthy, and this could account for the failure to detect genotype VIII between 1994 and 1997. June 2000 was the last time that genotype VIII was isolated in South Africa, or anywhere else in the world according to published data.

The incurrence of genotype VIIb (lineage 5b) into South Africa in the early 1990s is concurrent with the rise of this pandemic strain in many parts of East Asia and Europe in the 1990s. The data presented by Herczeg and colleagues in 1999 was limited. It did however demonstrate that South Africa was the likely source of the pandemic VIIb strain to Mozambique in 1993. They furthermore concluded that genotype VIIb had been introduced to South Africa first, and then to southern Europe, based on a divergence of only 2.4-4.5 % between the South African cluster and one containing strains from the United Arab Emirates, Turkey and Bulgaria. Aldous and colleagues (2003), who published the most comprehensive study on NDV phylogenetics yet, found that another strain, FI 1001/96, had only 2.7% sequence divergence from ZA 33/94, and therefore agreed with Herczeg and colleagues that VIIb had spread from southern Africa to Europe. However, in that extensive study, two pivotal sequences were published: -PTTY91146 isolated in Portugal from a turkey in 1991, and AESCK90174 isolated from a chicken in Spain in 1990. Neither was noted in the phylogenetic tree amongst the South African isolates (Aldous *et al*., 2003) despite the fact that these two European viruses shared 100% nucleotide sequence identities with the early South African epidemic strains, therefore I suggest that the VIIb strain responsible for the outbreaks in South Africa originated in Southern Europe, and not the opposite. Genotype VIIb may even have been introduced from abroad into South Africa on multiple occasions: strain 1ZAOS9141 (Aldous *et al*., 2003) was isolated from a South African ostrich in 1991, long before the index case at Hartebeespoort in June 1993. That strain shared only 97% nucleotide sequence identities with the early South African genotype VIIb viruses compared to the Spanish (1990) and Portuguese (1991) viruses. It is quite possible that day-old chicks, eggs or unprocessed poultry products were imported into South Africa from Europe before the infection was reported there. The chronological and geographical separation of South African genotype VIIb strains during the 1990s is consistent with reports from other countries (Aldous *et al*., 2003). The epidemiological pattern suggests that Genotype VIIb (and probably VIII) was spread throughout South Africa via the movement of infected poultry, rather than by wild birds. Most locations of isolation lie along major transportation routes and fall within or are close to the main poultry-producing areas in South Africa (Appendix 1). Spent hen vendors and their roles in the dissemination of ND will be discussed in greater detail in the final chapter. Furthermore, no evidence was found that indigenous village chickens acted as sub-clinical long-term reservoirs for infection of the formal industry. These birds are usually unvaccinated and are highly-susceptible to virulent NDV strains. In contrast, commercial chickens are vaccinated, and therefore likely to maintain and even shed virulent NDV strains while remaining clinically healthy. Vaccination combined with the transportation of infected birds and equipment across the country could thus have played the critical role in sustaining the initial 1993/94 outbreak throughout the remainder of the decade.

South African poultry infected with exotic NDV strains are clearly a threat to neighbouring trade partners when bio-security measures fail, as indicated by the spread of genotype VIIb from South Africa to Mozambique and Botswana in the 1990s. The initial routes of entry into South Africa of genotypes VIII and VIIb were probably via importation (illegal or otherwise) of infected poultry, although introduction by wild birds cannot be ruled out. Fortunately, genotype VIIb disappeared after 1999 (unpublished laboratory data), but in the same year a new genotype, VIId, arose and this is discussed in Chapter Six.