CHAPTER SIX

THE MOLECULAR EPIDEMIOLOGY OF NDV GENOTYPE 5d/VIId ("GOOSE PARAMYXOVIRUS") IN SOUTH AFRICA FROM 1999-2006

ABSTRACT

Phylogenetic analysis of velogenic Newcastle disease viruses isolated in South Africa showed that outbreaks that affected poultry in the Kwa Zulu-Natal province from 1999 to 2000 were not caused the same genetic type as the pandemic strain of the early and mid-1990's (VIId), but instead by strains of lineage 5d, previously only described in the Far and Middle East and recently Greece, also known as "Goose paramyxovirus". In 2003 lineage 5d re-emerged in a single outbreak in the same area, and almost exactly one year later an epidemic started that swept across the country. 257 Newcastle disease viruses were characterised in this study. Multiple species including chickens (commercial, ornamental and indigenous), peacocks, Hadeda Ibis (*Bostrychia hagedash*) chicks, geese, ostriches, pheasants and doves were found to be susceptible to lineage 5d strains, that had ICPI values ranging from 1.89 to 1.91. This is the first report of isolation of lineage 5d from peacocks, Hadeda Ibis, doves and ostriches. A second sub-genotype, recently introduced from an east-Asian source circulated at the start of the outbreak in 2004, but this strain disappeared after a few months. Epidemiological analysis revealed a close association between outbreaks and the national/regional road system, indicating that translocation of infected poultry was again the major mode of spread. The supply of vaccinated spent birds by the cull buyer industry into rural areas creates a possible reservoir for the maintenance of velogenic NDV strains in South Africa that periodically spill over into commercial flocks, causing outbreaks.

6.1 INTRODUCTION

Generally, APMV-1 strains are infectious to waterfowl such as geese and ducks without causing overt clinical symptoms, and these species therefore usually act only as carriers of the virus (Yin & Liu, 1997; Takakuwa *et al*, 1998; DJ Alexander, 1997a). However, paramyxovirus infection outbreaks have occurred frequently in Chinese geese since 1997 (Liu *et al*., 2003). In 1999, an outbreak with an incidence of 50-70% occurred in Shanghai goose flocks. Clinical signs varied from ruffled feathers and slight depression to severe systemic infection, resulting in high mortality and characterized by very rapid spread (Jinding *et al*., 2005). Although the mortality rates in adult geese were only 10 to 20%, up to 100% mortalities were observed in young geese under two weeks of age. A novel virus isolate, SF02, was determined to be the etiological agent of these outbreaks and was named "Goose paramyxovirus" (GPMV) (Zou *et al*., 2002; Liu *et al*., 2003). GPMV has been confirmed to be avian paramyxovirus-1 (Newcastle disease virus) by genomic and serotype analyses, and all but one of the strains of goose origin fell into genotype VIId (Liu *et al*., 2003). GPMV is also highly pathogenic to chickens, pigeons, partridges and ducks (Zou & Gong, 2003). MDTs of 45.6 – 60 h and ICPIs of 1.80-1.94 have been recorded (Liu *et al*., 2003) but chickens inoculated with either live or inactivated LaSota vaccines were fully protected from disease and death against challenge with VIId (Liu *et al*., 2003). The phylogeny of NDV was recently revised, and genotype VIId was renamed as lineage 5d (Aldous *et al*., 2003).

At the genomic level, GPMV contains an extra 6nt fragment in the un-translated region (UTR) between the NP and P genes, but this insertion also occurs in NDVs in genotypes VI, VII, VIII and IX while it is absent from NDVs in genotypes I, II, III and IV. The insertion was not only limited to contemporary lineages (VI onwards) as it was also detected in viruses from the 1940s and 1960s (Huang *et al*., 2004). The 3' leader of GPMV genome shares high identity with APMV-6 and other APMV-1 viruses, whereas its 5' trailer is more variable (Zou *et al*., 2005). The HN protein is six amino acids shorter compared to avirulent strains, but this trait is also shared by other virulent strains (Huang *et al*., 2004).

From 1999 to 2000, an outbreak of velogenic viscerotropic NDV occurred in a single commercial flock and village chickens in a relatively small geographic area of the

KwaZulu/Natal province. PCR and sequencing identified the causative strain as a genotype VIId/ lineage 5d (Goose Paramyxovirus) strain (Abolnik *et al*., 2004a). For almost three years thereafter there were no reports of NDV outbreaks nor were any virulent viruses isolated by national laboratories. Then, towards the end of September 2003, a virulent ND virus was isolated from village chickens near Pietermaritzburg, close to the locations of the outbreaks in 1999 and 2000, however, the infection did not seem to spread. Almost exactly one year later in October 2004, a paramyxovirus outbreak started in commercial flocks of the Camperdown/ Richmond districts. Although the index case was a commercial farm, workers reported that a disease with similar symptoms had been killing the fowl in their villages for several weeks already, and a sample was obtained from the village for testing. The infection continued to spread throughout KZN commercial and backyard flocks, and to and throughout most provinces in 2005 and 2006. The purpose of this chapter was to genetically characterize 257 virulent ND viruses collected from outbreaks in South Africa from 1999 to 2006, to describe the epidemiology of the disease, and if possible, to determine the origins of the infections.

6.2 MATERIALS AND METHODS

6.2.1 Viruses

Velogenic ND viruses (Table 6.1) were grown in 9-to-11 day old specific pathogen free (SPF) embryonated chicken eggs by standard procedures (OIE manual of standards, 2000), at Allerton Provincial Veterinary Laboratory, the Tropical Diseases Division (University of Pretoria), Stellenbosch Provincial Veterinary Laboratory, and Deltammune (formerly Avimmune) Laboratory. ICPI and MDT tests were performed at Allerton Provincial Veterinary Laboratory. Isolates are listed in Table 6.1 along with their collection dates, hosts and regions.

Table 6.1 Virulent ND viruses isolated in South Africa from 1999-2006 (n=257)

Isolate[1]	Collection date	Location	Host[2]	Accession number[3]
CKZA99AL308	12/03/1999	New Hanover/Wartburg	Chickens	AF532150
CKZA99AL328	27/08/1999	Thornville	Village fowl	
CKZA99AL331	02/09/1999	Stanger	Village fowl	AF532739
CKZA99AL337	02/11/1999	Durban	Village fowl	
CKZA00AL344	04/01/2000	Durban	Village fowl	
CKZA00AL377	20/10/2000	Stanger	Village fowl	
CKZA00AL378	20/12/2000	Port Shepstone	Village fowl	AF532745
CKZA03AL482	26/09/2003	Pietermaritzburg	Village fowl	EF030966
CKZA04AL491	30/09/2004	Pietermaritzburg	Broilers Breeders	
CKZA04AL495	08/10/2004	Hopewell Village	Village fowl	
CKZA04AL496[4]	**19/10/2004**	**Estcourt**	**Village fowl**	
CKZA04AL497	22/10/2004	Camperdown	Village fowl	
CKZA04AL498	28/10/2004	Glencoe	Village fowl	
CKZA04AL499	28/10/2004	Howick	Broilers	
CKZA04AL500	**03/11/2004**	**Umzimkulu**	**Village fowl**	**EF030967**
CZA04AL501	03/11/2004	Lower Umfolozi	Village fowl	
CKZA04AL502	**09/11/2004**	**Pietermaritzburg**	**Village fowl**	**EF030968**
CKZA04AL503	12/11/2004	Camperdown	Layers	
CKZA04AL504	12/11/2004	Hluhluwe	Village fowl	
CKZA04AL508	12/11/2004	Camperdown	Layers	
CKZA04AL506	15/11/2004	Camperdown	Village fowl	
CKZA04AM59503	16/11/2004	Pietermaritzburg	Chickens	
CKZA04AL510	18/11/2004	Durban	Village fowl	
CKZA04AL509	22/11/2004	Camperdown	Chickens	
CKZA05AI514	25/11/2004	Witsieshoek	Village fowl	
CKZA04AL511	26/11/2004	Lower Umfolozi	Village fowl	
CKZA04AL512	29/11/2004	Estcourt	Broilers	
CKZA04AL512	**29/11/2004**	**New Hanover**	**Village fowl**	**EF030969**
CKZA04AL514	30/11/2004	New Hanover	Village fowl	
CKZA05N238	11/01/2005	Rustenberg	Chickens	
CKZA05N239	18/01/2005	Pretoria	Chickens	
CKZA05AI600	21/01/2005	Phutadithaba	Village fowl	

CKZA05AL516	31/01/2005	New Hanover	Broilers	
CKZA05N241	16/02/2005	Frankfort	Village fowl	
CKZA05AL517	22/02/2005	New Hanover	Broilers	
CKZA05AL518	22/02/2005	New Hanover	Broilers	
CKZA05N242	23/02/2005	Zeerust	Chickens	
PHZA05N243	02/03/2005	Middleburg	Pheasant	
CKZA05N2343	02/03/2005	Middleburg	Chickens	
CKZA05UP300	03/03/2005	Hartebeeshoek	Village fowl	
CKZA05N246	04/03/2005	Randfontein	Chickens	
CKZA05N244	07/03/2005	Middleburg	Chickens	
CKZA05AL521	14/03/2005	Port Shepstone	Broilers	
CKZA05AL522	17/03/2005	Camperdown	Broilers Breeder	
CKZA05AL525	22/03/2005	New Hanover	Layers	
CKZA05AL526	29/03/2005	New Hanover	Layers	
CKZA05UP408	05/04/2005	Bapsfontein	Chickens	
CKZA05AL527	06/04/2005	Camperdown	Broilers	
CKZA05N255	07/04/2005	Kroonstad	Chickens	
CKZA05N257	08/04/2005	Kroonstad	Chickens	
CKZA05AL530	13/04/2005	Pietermaritzburg	Village fowl	
CKZA05AL531	14/04/2005	Port Shepstone	Broilers	
CKZA05N259	14/04/2005	Kroonstad	Chickens	
CKZA05UP483	15/04/2005	Halfway House	Layers	
CKZA05N265	25/04/2005	Lichtenburg	Chickens	
CKZA05AL532	03/05/2005	Ixopo	Broilers	
CKZA05N272	09/05/2005	Lehurutshe	Village fowl	
CKZA05N275	12/05/2005	Kroonstad	Chickens	
CKZA05UP675	17/05/2005	Kuruman	Chickens	
CKZA05N278	18/05/2005	Vryburg	Chickens	
CKZA05N279	20/05/2005	Vryburg	Chickens	
CKZA05N280	23/05/2005	Ellisras	Chickens	
CKZA05UP754	01/06/2005	Honeydew	Layers	
CKZA05N285	07/06/2005	Vrede (Kroonstad)	Chickens	
CKZA05N300	07/06/2005	Moumong (Brits)	Village fowl	
CKZA05AL536	08/06/2005	Camperdown	Layers	
CKZA05N284	10/06/2005	De Aar	Chickens	
CKZA05N286	10/06/2005	Stutterheim	Chickens	
CKZA05N290	10/06/2005	De Aar	Chickens	EF030971
CKZA05UP803	12/06/2005	Mokopane	Chickens	
CKZA05N287	15/06/2005	Warden (Kroonstad)	Village fowl	
CKZA05N288	15/06/2005	Kudumane (Kroonstad)	Village fowl	
CKZA05M102	17/06/2005	Sibasa	Chickens	
CKZA05AM66479	20/06/2005	Lanseria	Chickens	
CKZA05AL537	23/06/2005	New Hanover	Broilers	
CKZA05AL538	24/06/2005	New Hanover	Layers	
CKZA05N289	28/06/2005	Clarens (Kroonstad)	Chickens	
CKZA05N302	28/06/2005	Vredefort (Kroonstad)	Chickens	
CKZA05AM66832	28/06/2005	Delmas	Chickens	
CKZA05N292	29/06/2005	Warden (Kroonstad)	Village fowl	
CKZA05N294	01/07/2005	Puthaditjhaba (Kroonstad)	Village fowl	
CKZA05M115	04/07/2005	Vryburg	Village fowl	
CKZA05N293	05/07/2005	Hoopstad (Kroonstad)	Village fowl	
CKZA05UP931	05/07/2005	Honeydew	Layers	
CKZA05N301	07/07/2005	Marapong (Ellisras)	Village fowl	
CKZA05AL540	07/07/2005	Port Shepstone	Village fowl	

CKZA05AL541	13/07/2005	Umzimkhulu	Village fowl	
CKZA05UP976	15/07/2005	Noordheuwel	Broilers	
CKZA05UP1008	20/07/2005	Tiegerpoort	Broilers	
CKZA05N305	20/07/2005	Kimberly	Chickens	
CKZA05N306	20/07/2005	Amatole (East London)	Chickens	
CKZA05N304	21/07/2005	Heilbron (Kroonstad)	Village fowl	
CKZA05UP1063	24/07/2005	Makapaanstad	Village fowl	
CKZA05AL543	25/07/2005	Camperdown	Broilers	
CKZA05AL545	25/07/2005	Camperdown	Broilers	
CKZA05N310	25/07/2005	Kwelera (East London)	Chickens	
CKZA05UP1049	26/07/2005	Kameeldrif East	Layers	
CKZA05UP1050	26/07/2005	Newcastle	Layers	
CKZA05AL547	26/07/2005	Mount Currie	Village fowl	
CKZA05AM67794	27/07/2005	Pietermaritzburg	Chickens	
CKZA05AL548	27/07/2005	Mooirivier	Village fowl	
CKZA05AL549	27/07/2005	Underberg	Village fowl	
CKZA05N309	28/07/2005	Vaalwater (Ellisras)	Chickens	
CKZA05N311	28/07/2005	Hartebeespoortdam	Chickens	
CKZA05UP997	29/07/2005	Rustenburg	Layers & Fowl	
GOZA05N317	29/07/2005	Smithfield (Bloemfontein)	Goose	
CKZA05UP1085	01/08/2005	Magaliesburg	Layer chicks	
CKZA05UP1099	02/08/2005	Siyabushwa	Village fowl	
CKZA05UP1097	02/08/2005	Magaliesburg	Broilers	
CKZA05UP1098	02/08/2005	Makapaanstad	Village fowl	
CKZA05UP1111	03/08/2005	Queenstown	Village fowl	
CKZA05UP1134	05/08/2005	Pretoria	SPF Chickens	
CKZA05AL550	05/08/2005	Ixopo	Layers	
CKZA05UP1133	05/08/2005	Soshanguve	Village fowl	
CKZA05UP1139	05/08/2005	Germiston	Village fowl	
CKZA05N312	11/08/2005	Mmabatho	Village fowl	
CKZA05N327	11/08/2005	Kimberly	Chickens	
CKZA05UP1151	11/08/2005	Rustenburg	Layers	
CKZA05N315	12/08/2005	Kgotsong (Bothaville)	Village fowl	
CKZA05AL551	15/08/2005	Ixopo	Layers	
CKZA05AL560	15/08/2005	Camperdown	Broilers	
CKZA05UP1180	16/08/2005	Lichtenburg	Village fowl	
CKZA05UP1181	16/08/2005	Lichtenburg	Village fowl	
CKZA05UP1178	17/08/2005	Warrenton	Layers	EF030975
CKZA05N328	18/08/2005	Grootrietvlei	Chickens	
CKZA05N340	18/08/2005	Witsieshoek	Village fowl	
CKZA05AL552	19/08/2005	Pietermaritzburg	Village fowl	
CKZA05N349	19/08/2005	Ventersdorp	Village fowl	
CKZA05UP1207	22/08/2005	Pretoria	Village fowl	
CKZA05UP1219	22/08/2005	Pretoria North	Layers	
CKZA05N336	22/08/2005	East London	Village fowl	
CKZA05N337	22/08/2005	Butterworth	Village fowl	
CKZA05N336	22/08/2005	East London	Village fowl	
CKZA05UP1221	23/08/2005	Bronkhorstspruit	Broilers breeders	
CKZA05UP1216	23/08/2005	Delmas	Broilers	
CKZA05N350	23/08/2005	Standerton	Chickens	
CKZA05AL553	24/08/2005	Camperdown	Broilers	EF030970
CKZA05AL554	24/08/2005	Ixopo	Layers	
CKZA05N386	24/08/2005	Vredefort	Chickens	
CKZA05UP1233	25/08/2005	Cullinan	Broilers	

CKZA05UP1236	25/08/2005	Bronkhorstspruit	Chickens	
CKZA05N334	25/08/2005	Ventersdorp	Village fowl	
CKZA05N347	25/08/2005	Ventersdorp	Village fowl	
CKZA05UP1176	26/08/2005	Pretoria	Village fowl	
CKZA05N338	26/08/2005	Amsterdam	Village fowl	
CKZA05UP1235	26/08/2005	Bronkhorstspruit	Broilers	
CKZA05UP1250	26/08/2005	Brits	Broilers	
CKZA05UP1251	29/08/2005	Olifantsfontein	Layers	
CKZA05N339	29/08/2005	Bethlehem	Village fowl	
CKZA05AL558	29/08/2005	New Hanover	Broilers	
OSZA05N333	30/08/2005	Brits	Ostrich	
CKZA05UP1278	31/08/2005	Bryanston	Layers	
CKZA05N360	31/08/2005	Heilbron	Village fowl	
CKZA05N342	01/09/2005	Heilbron	Village fowl	
DOZA05N341	02/09/2005	Bethlehem	Dove	
CKZA05UP1306	05/09/2005	Middelburg	Broilers	
CKZA05UP1307	05/09/2005	Randfontein	Layers	
CKZA05UP1333	05/09/2005	Kuruman	Village fowl	
CKZA05N348	05/09/2005	Lydenburg	Village fowl	
CKZA05UP1402	05/09/2005	Pretoria	Free range chickens	
CKZA05UP1412	05/09/2005	Makhado	Chickens	
CKZA05UP1314	06/09/2005	Muldersdrif	Chickens	
CKZA05UP1279	08/09/2005	Kudumane	Free range Chickens	
CKZA05N355	08/09/2005	Brits	Village fowl	
CKZA05N356	09/09/2005	Lydenburg	Chickens	EF030972
CKZA05UP1353	09/09/2005	Meyerton	Free range Chickens	EF030976
CKZA05UP1355	12/09/2005	Pretoria	Broilers	
CKZA05UP1364	13/09/2005	Bramley	Free range Chickens	
CKZA05AL563	14/09/2005	Howick	Bantams	
CKZA05M202	15/09/2005	Standerton	Chickens	
CKZA05N357	19/09/2005	Bloemfontein	chicks	
CKZA05UP1411	19/09/2005	Kuruman	Chickens	
CKZA05M203	20/09/2005	Makhado	Chickens	
CKZA05N362	21/09/2005	Knysna	Chickens	
CKZA05AL566	21/09/2005	Eston	Broilers	
CKZA05M207	22/09/2005	Walkerville	Chickens	
CKZA05UP1446	22/09/2005	Randfontein	Free range Chickens	
CKZA05M208	23/09/2005	Lerato	Chickens	
CKZA05N387	27/09/2005	Nelspruit	Chickens	
CKZA05N404	27/09/2005	Klerksdorp	Village fowl	
CKZA05M233	28/09/2005	Thaba Nchu	Chickens	
CKZA05N405	29/09/2005	Port Elizabeth	Chickens	
CKZA05AL568	30/09/2005	Mooi River	Village fowl	
CKZA05N406	03/10/2005	Fouriesburg	Chickens	EF030973
CKZA05N403	04/10/2005	Port Alfred	Chickens	
CKZA05N408	04/10/2005	Kroonstad	Chickens	
CKZA05UP1510	05/10/2005	Bronkhorstspruit	Chickens	
CKZA05UP1514	05/10/2005	Germiston	Village fowl	
CKZA05UP1565	05/10/2005	Kuruman	Chickens	
CKZA05UP1649	05/10/2005	Germiston	Village fowl	
CKZA05N407	06/10/2005	Bloemfontein	Chickens	
CKZA05N402	07/10/2005	Pretoria	Chickens	
CKZA05M239	07/10/2005	Hertzogville	Chickens	
CKZA05AL570	10/10/2005	Camperdown	Broilers breeders	

CKZA05AL571	13/10/2005	Durban	Chickens	
CKZA05M243	17/10/2005	Delmas	Chickens	
CKZA05N429	17/10/2005	Queenstown	Village fowl	
CKZA05AL575	19/10/2005	Camperdown	Chickens	
CKZA05AL576	20/10/2005	Ezakheni	Chickens	
CKZA05M272	25/10/2005	Mokopane	Broilers	
CKZA05N421	27/10/2005	Murraysburg	Chickens	
CKZA05AL573	28/10/2005	Durban	Chickens	
CKZA05M275	02/11/2005	Nelspruit	Chickens	
CKZA05N516	03/11/2005	Coligny	Broilers	
CKZA05N442	04/11/2005	Bloemfontein	Chickens	
CKZA05M287	04/11/2005	Naboomspruit	Chickens	
HIZA05N500	04/11/2005	Middleburg	Hadeda ibis chicks	
CKZA05UP796	05/11/2005	Tarlton	Chickens	
CKZA05UP1835	05/11/2005	Rynfield	Chickens	EF030977
CKZA05UP1879	05/11/2005	Naboomspruit	Broilers	
CKZA05M279	07/11/2005	Nelspruit	Chickens	
CKZA05M288	09/11/2005	Randfontein	Chickens	
CKZA05N496	10/11/2005	Potchefstroom	Chickens	
CKZA05N517	14/11/2005	Tzaneen	Chickens	
CKZA05N519	14/11/2005	Lindley	Chickens	EF030974
CKZA05AL578	14/11/2005	Dundee	Chickens	
PCZA05N518	23/11/2005	Pretoria	Peacock	
CKZA04AL580	28/11/2005	Howick	Chickens	
CKZA06N515	01/12/2005	Wolmaranstad	Chickens	
CKZA05N540	19/12/2005	Haarlem	Chickens	
CKZA06AM73356	04/01/2006	Klerksdorp	Chickens	
CKZA06UP30	05/01/2006	Pretoria	bantam	
CKZA06UP35	05/01/2006	Kroonstad	Broilers	
CKZA06UP97	13/01/2006	Bramley	Village fowl	
CKZA06UP113	17/01/2006	Kuruman	Village fowl	
CKZA06N553	19/01/2006	Pretoria	Broilers	
CKZA06N555	20/01/2006	Pretoria	Broilers	
CKZA06N556	20/01/2006	Zonderwater	Broilers	
CKZA06AM74097	24/01/2006	Paarl	Chickens	
CKZA06UP171	25/01/2006	Kuruman	Village fowl	
CKZA06UP182	26/01/2006	Viljoenskroon	Broilers	
CKZA06UP184	27/01/2006	Kuruman	Village fowl	
CKZA06UP187	27/01/2006	Skuinsdrift	Broilers	
CKZA06AL585	30/01/2006	Pietermaritzburg	Chickens	
CKZA06AL586	08/02/2006	Uitenhage	Chickens	
CKZA06N573	17/02/2006	Van Wyk's Rivier (Paarl)	Chickens	
CKZA06N574	17/02/2006	Van Wyk's Rivier (Paarl)	Chickens	
CKZA06AL590	03/03/2006	Camperdown	Chickens	
CKZA06N602	07/03/2006	Gedults Rivier (St Albans)	Chickens	
CKZA06AL591	08/03/2006	Pietermaritzburg	Chickens	
CKZA06N607	14/03/2006	East London	Chickens	
CKZA06N616	15/03/2006	Sommerset East	Village fowl	
CKZA06N609	23/03/2006	Worchester	Chickens	
CKZA06N610	23/03/2006	Malmesbury	Chickens	
CKZA06N608	24/03/2006	Rietgat (Brits)	Chickens	
CKZA06UP529	29/03/2006	Bapsfontein/Tiegerpoort	Broilers	
CKZA06N630	31/03/2006	Mokopane	Chickens	
CKZA06N642a	03/04/2006	Calitzdorp	Chickens	

CKZA06N642b	03/04/2006	Plettenberg Bay	Chickens	
CKZA06N628	13/04/2006	Ladismith	Chickens	EF030978
CKZA06N654	20/04/2006	Grahamstown	Chickens	EF030965
CKZA06SB29	01/05/2006	Robertson	Chickens	
CKZA06SB56	03/05/2006	Vredenburg	Chickens	
CKZA06N641	05/05/2006	Stellenbosch	Chickens	EF030979
CKZA06N651	11/05/2006	Stellenbosch	Chickens	EF030980
CKZA06N652	11/05/2006	Stellenbosch	Chickens	EF030981
CKZA06UP736	05/06/2006	Marble Hall	Layers	

[1]Viruses were named according to host, year of isolation and the original lab number. CK=chicken, OS=ostrich, DO=dove, HI=hadeda ibis, GO=goose, PC=peacock, PH=pheasant; N=OVI Biotechnology, M=OVI Virology, SB=Stellenbosch, UP=University of Pretoria, AL=Allerton, AM=Avimmune (Deltammune) e.g. CKZA06AL591= Chicken/South Africa/2006/Allerton lab number 591.

[2]Where available, the type of chicken is specified

[3]Only one representative of each sub-lineage was selected, except for sub-lineage (o)

[4]Sub-lineage (o) viruses are indicated in boldface

6.2.2 RNA Extraction

Viral RNA was extracted from allantoic fluid using TRIzol® reagent (Gibco, Invitrogen), according to the manufacturer's instructions.

6.2.3 RT-PCR

A one-step RT-PCR was performed using the oligonucleotide pair described in Chapter Four (p160), with the addition of MMLV-reverse transcriptase and the following modification to the thermal cycling protocol: incubation at 42ºC for 20 min, followed by 35 cycles of 94ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 1 min. Amplification products were analysed by electrophoresis on 1% agarose gel. Bands of the correct size were excised and DNA purified using the QiaQuick Gel Extraction Kit (Qiagen).

6.2.4 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 ABI3130™ Genetic Analyser. Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) of the 374 nucleotide (nt) region of the 3' end of the F protein, including the F_0 cleavage site, were used to identify closely-related sequences to include in multiple sequence alignments, which were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). The preparation of Fig. 6.2 (variable sites of the multiple nucleotide sequence alignment) was done with MEGA v3.1 software. The region from 61 to 374 was used for the final phylogenetic analysis and multiple alignments, because of shorter reference sequences in Genbank. The results are presented as a rooted neighbour joining tree with 1000 bootstrap trials to assess nodal support values. The majority (n=149) of the South African lineage 5d nucleotide sequences were identical across the 374-bp region analysed, and are represented by a single virus as indicated in Fig. 6.1(a).

6.2.5 Geographic Information System maps

Maps of the distributions of isolates were constructed using ArcGIS 9 (ESRI) software, and are located in Appendix 2.

6.3 RESULTS

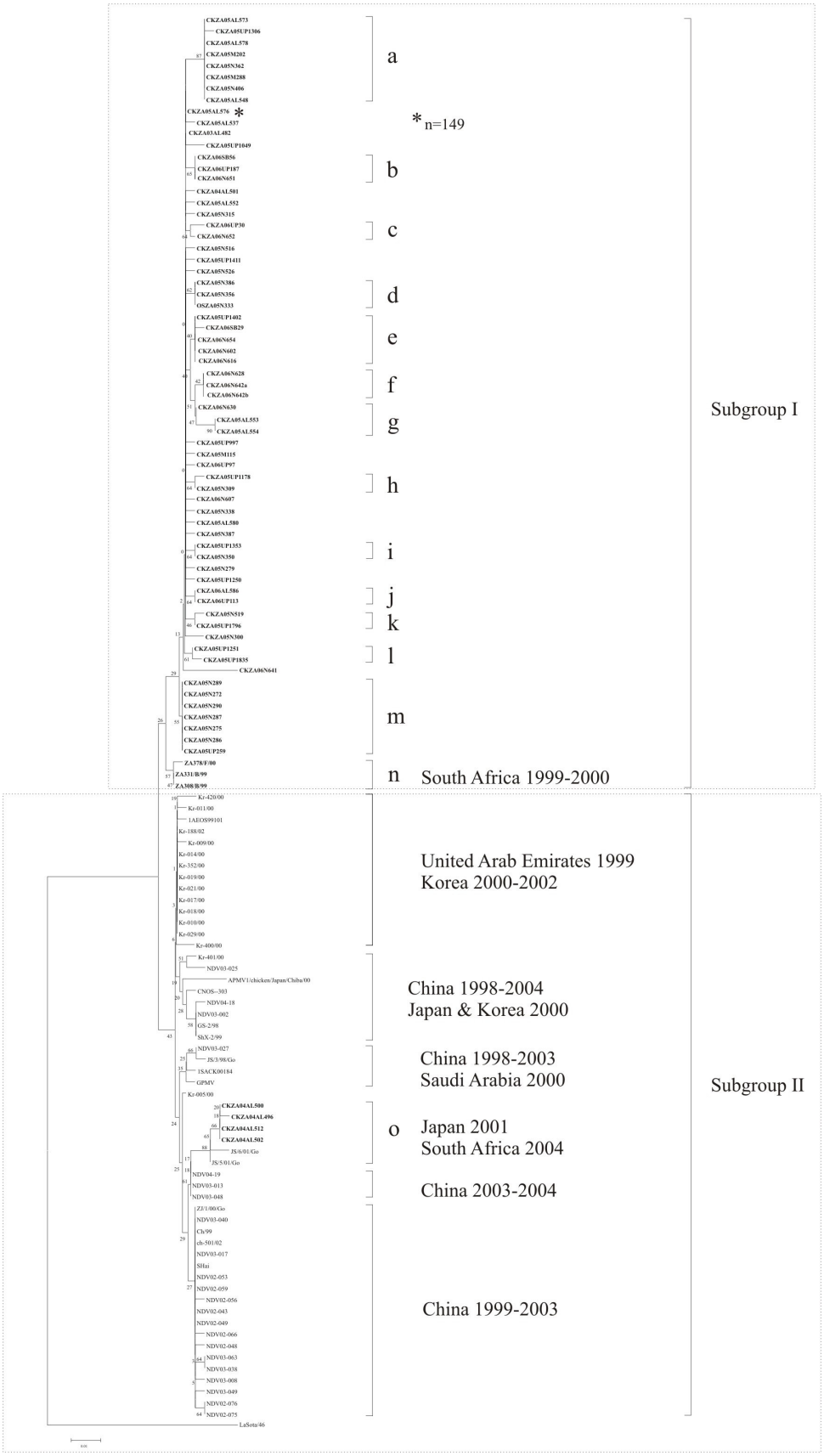


Figure 6.1(a) Phylogenetic tree based on nucleotide sequences of South African lineage 5d viruses (in boldface) and reference strains. The tree is rooted with LaSota/46 virus. Sub-lineages (a) to (o) are indicated. Enlargements of Subgroups I and II are presented in Figs. 6.1(c) and 6.1(d) respectively.

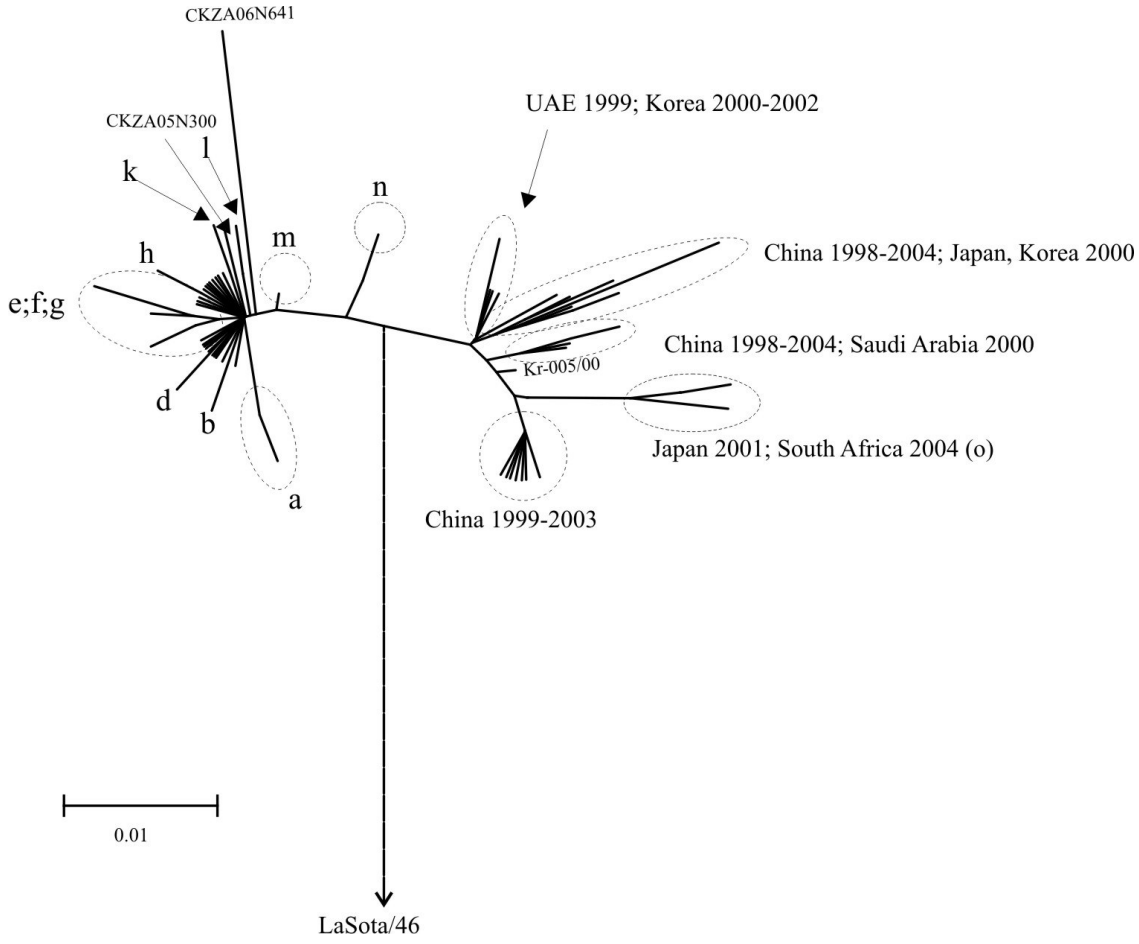


Figure 6.1(b) Radial (unrooted) version of Fig. 6.1(a)

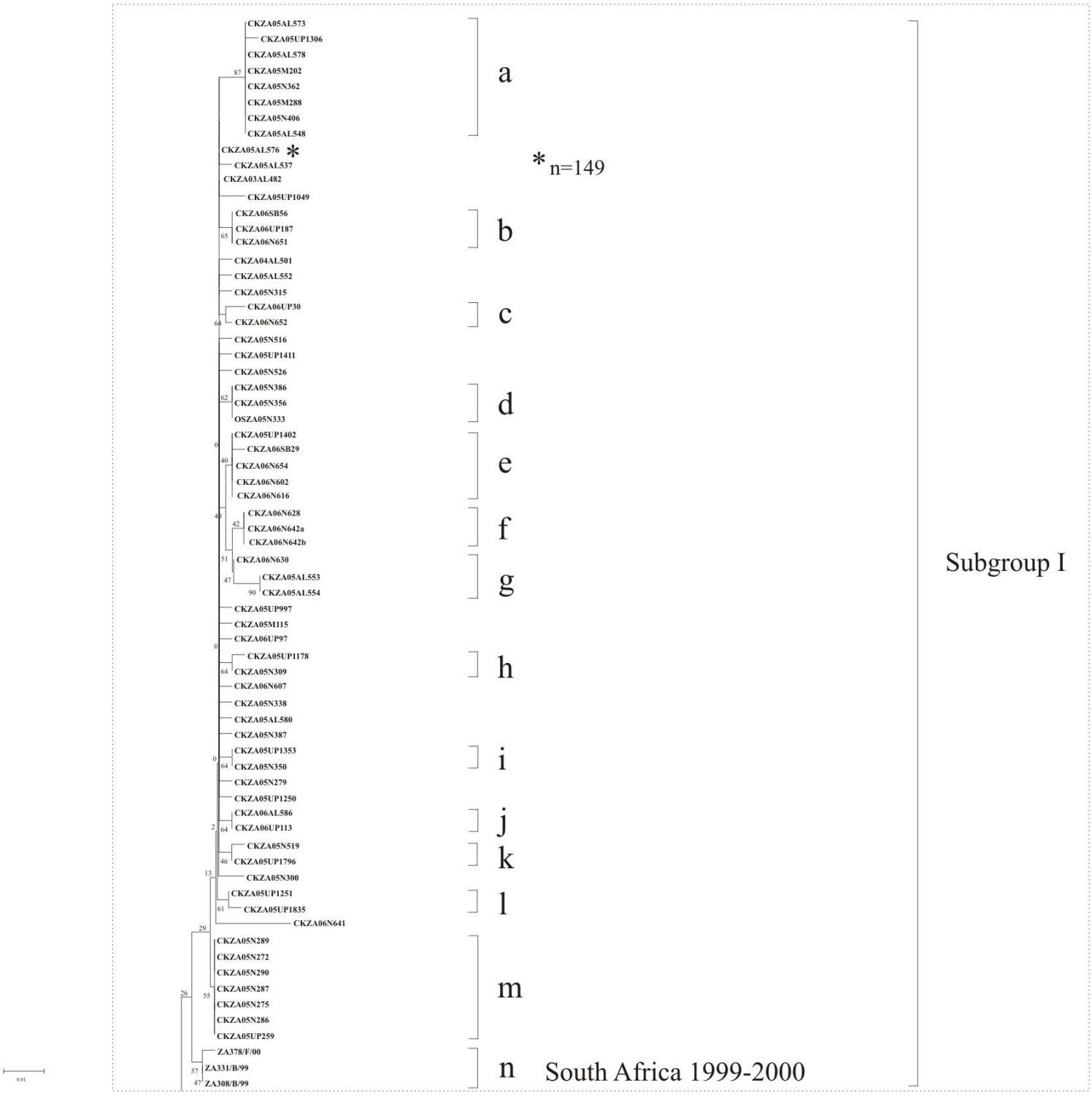


Figure 6.1(c) Enlargement of Fig 6.1(a) depicting Subgroup I phylogeny

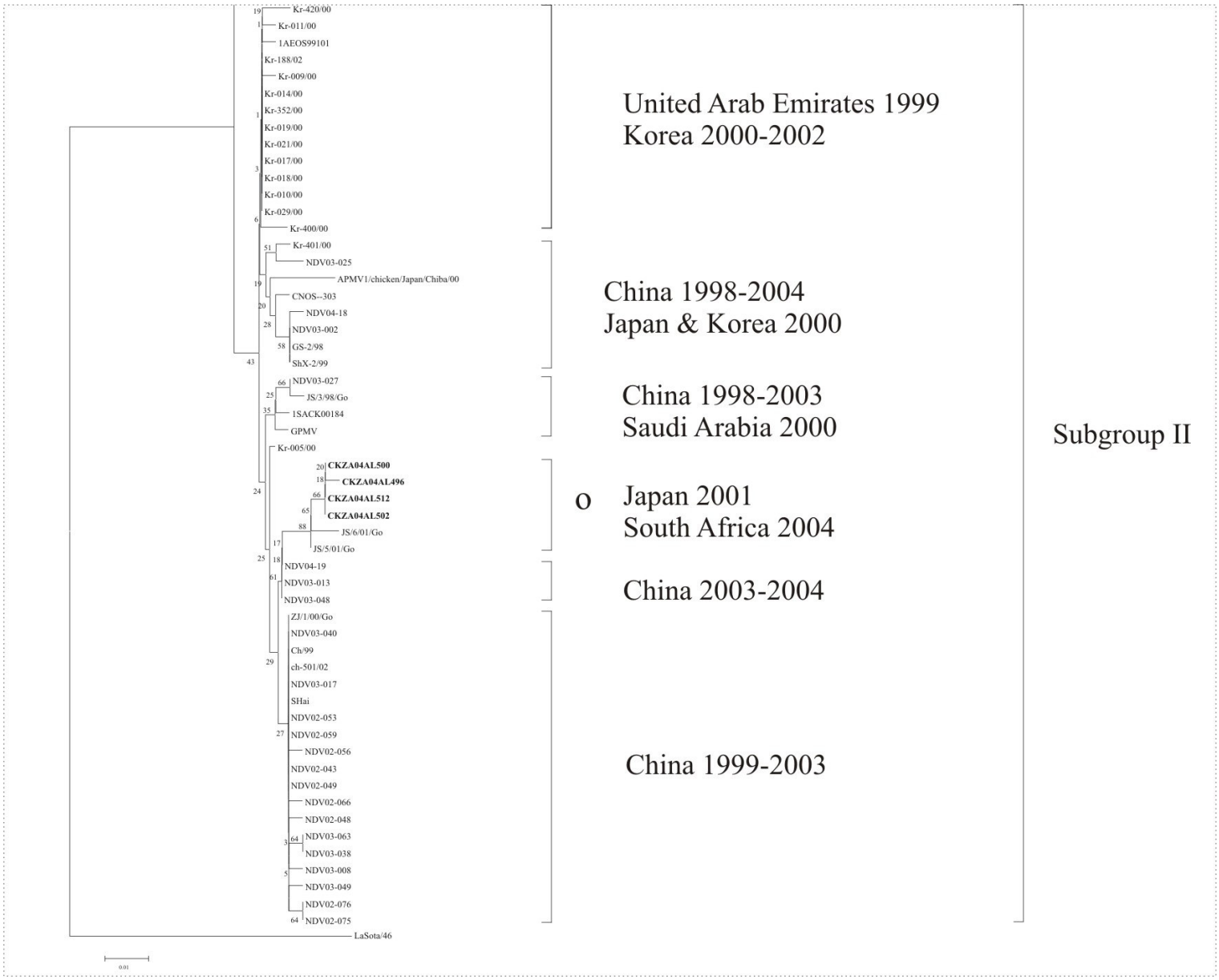


Figure 6.1(d) Enlargement of Fig 6.1(a) depicting Subgroup II phylogeny

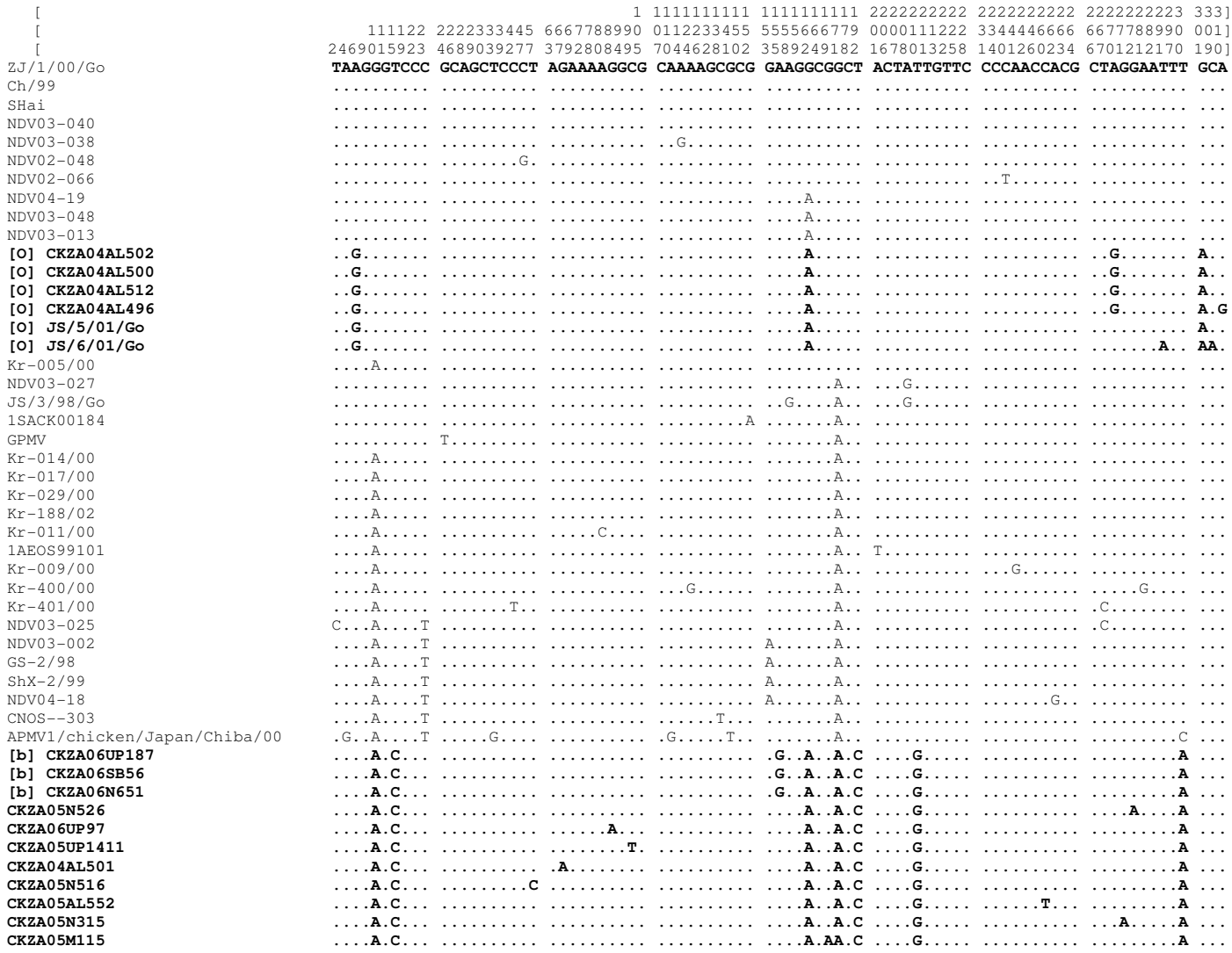
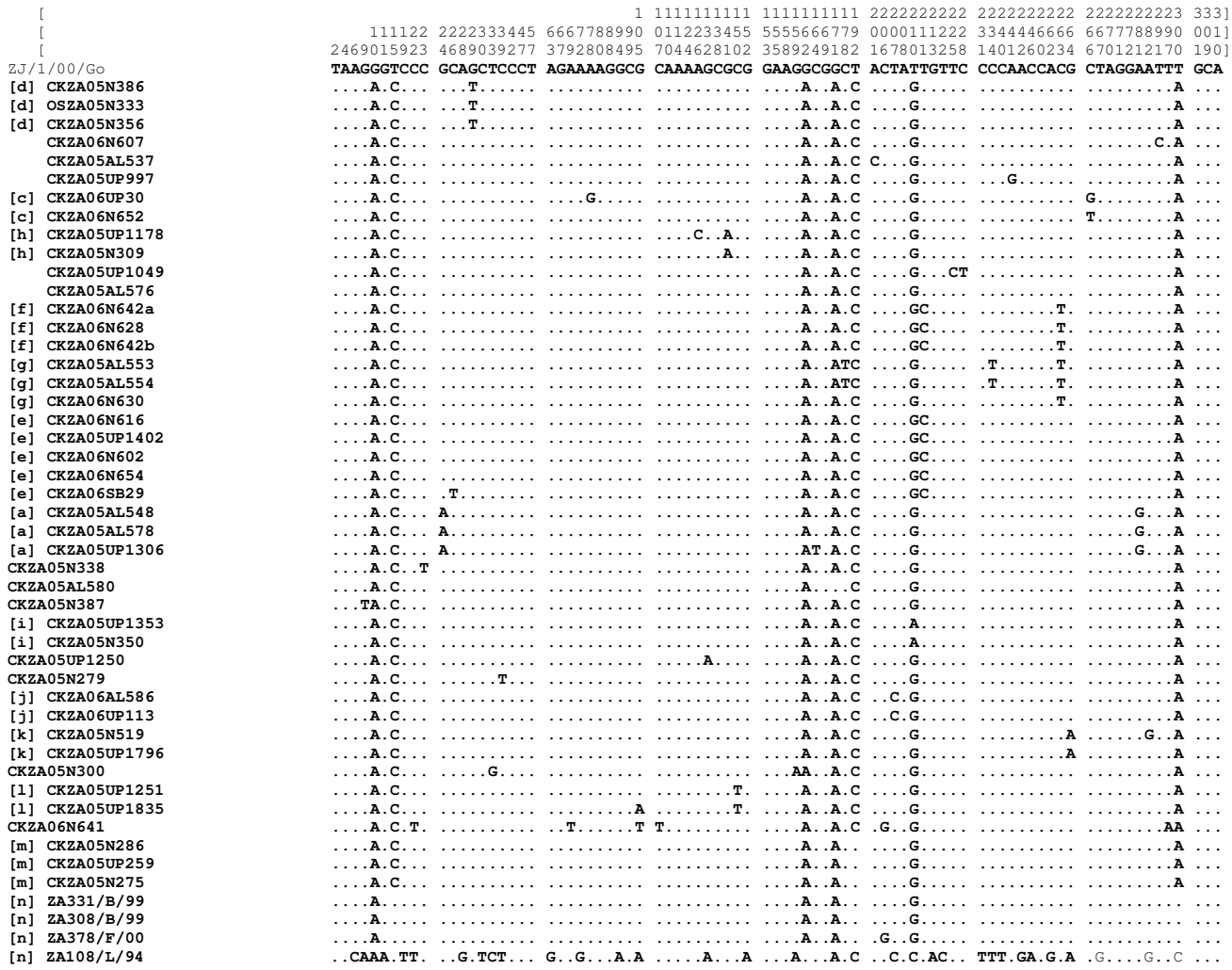


Figure 6.2 Multiple nucleotide sequence alignment of variable sites only within the fusion protein gene (nt 61-374) of selected South African (in boldface) and other lineage 5d strains. Sub-lineages (a) to (o) are indicated in square brackets.

Fig. 6.2 continued



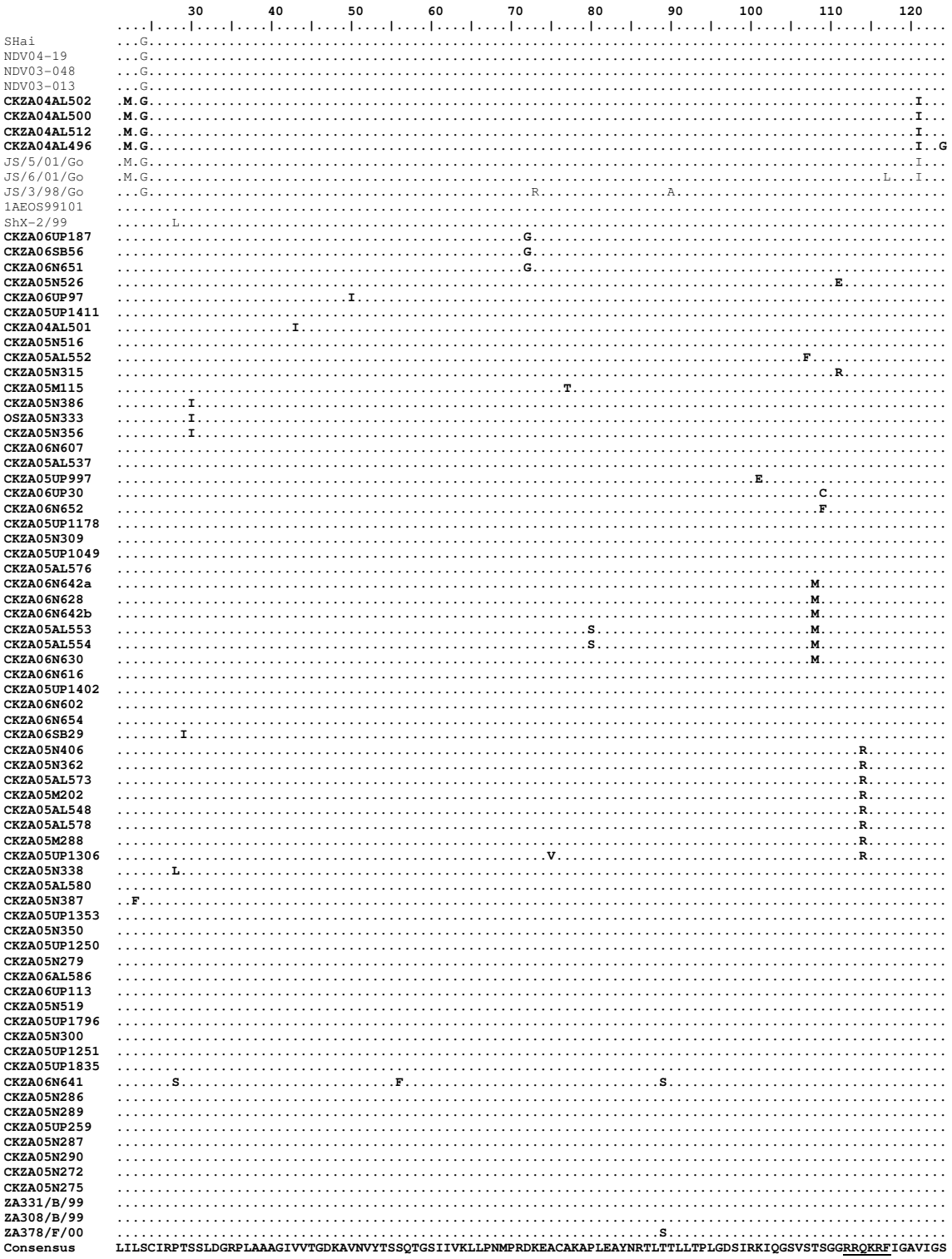


Figure 6.3 Multiple amino acid alignment (residues 21 to 124 of the fusion protein) of South African (in boldface) and other lineage 5d strains. The F_0 cleavage site is underlined.

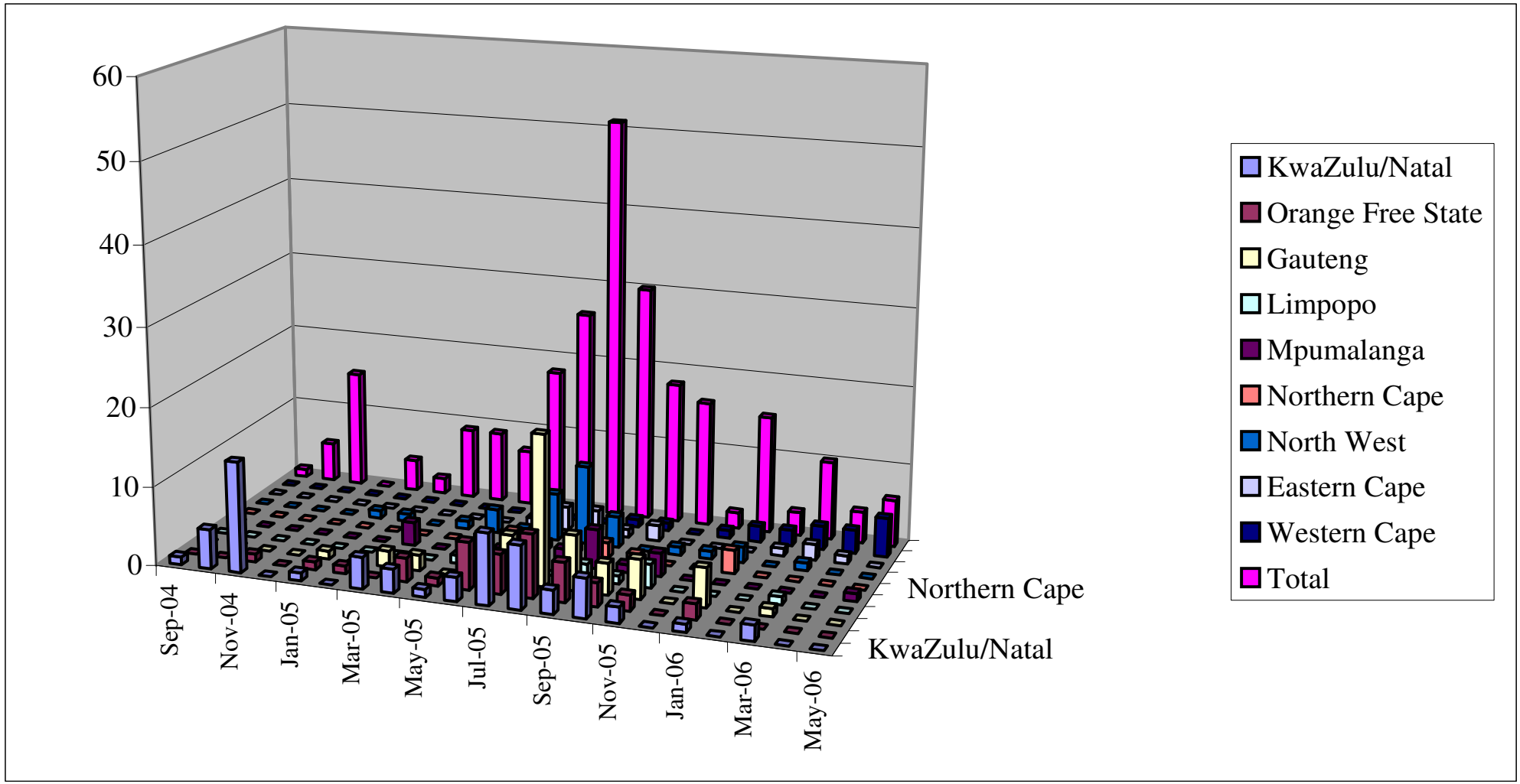


Figure 6.4 Prevalence of NDV GPMV isolates collected from September 2004 to May 2006

6.3.1 South African lineage 5d strains are closely-related to strains from the Far East

Phylogenetic analysis (Figs. 6.1(a) and (b)) indicates that South Africa ND outbreak strains of 1999-2000, 2003 and 2004-2006 are closely-related and may be classified as lineage 5d by their close sequence homology to viruses isolated in the middle and Far East since 1999. Furthermore, it was evident that two separate genotypes of lineage 5d, or "goose paramyxovirus" were responsible for outbreaks in KZN in late 2004. 253/257 (98.4%) of South African lineage 5d strains form a separate group from other lineage 5d strains from the Far and Middle East and will be referred to as subgroup I (sub-lineages (a) to (n)) in this chapter, whereas other international viruses and the second genotype of South African lineage 5d (sub-lineage (o)) will be referred to as subgroup II (Fig 6.1(a))

6.3.2 The ND outbreak in KwaZulu/Natal from 1999-2000

Seven velogenic viruses (CKZA99AL308, CKZA99AL328, CKZA99AL331, CKZA99AL337, CKZA00AL344, CKZA00AL377 and CKZA00AL378) were isolated between 1999 to 2000 in a relatively small area in KZN (Appendix 2i, p275; Appendix 2 xv, p). In Fig 6.1(a) the group is represented by three viruses, ZA378/F/00, ZA331/B/99 and ZA308/B/99 and form sub-lineage (n). The earliest of the viruses, CKZA99L308, was isolated from 25-day old broilers in the New Hanover/Wartburg area in March 1999, but only village chickens were affected thereafter. Sub-lineage (n) is located at the root of the majority of the SA viruses (Fig. 6.1(b)). This suggests that they gave rise to the later South African lineage 5d viruses (subgroup I). At the nucleotide sequence level (Fig. 6.2), they contain a T^{15} and T^{300} residues that are absent from SA viruses isolated after 2003, but are shared with viruses from the Far and Middle East, supporting the theory of a recent origin in the Far East.

6.3.3 The ND outbreak in KwaZulu/Natal in 2003

CKZA03AL482 was isolated from an outbreak in backyard chickens that killed 17 chickens out of a flock of 18 birds. This single outbreak near Pietermaritzburg occurred almost three years after the initial outbreak in the same geographic area (Appendix 2i,

p275, red dot). At the nucleotide sequence level, CKZA03AL482 is indistinguishable from 149 other subgroup I 2004 outbreak strains (Fig. 6.1), indicating that the same strain re-emerged in 2004 to cause the epidemic.

6.3.4 The rise of the epidemic strain in KwaZulu/Natal

Appendix 2i (p275) maps the locations of the 1999/2000 (sub-lineage (n)), 2003, and initial 2004 isolations. The positions of the three outbreaks overlap, and correspond to a major poultry-producing area in South Africa (Appendix 1) that is also populated with rural villages in the Valley-of-1000-Hills region of KwaZulu/Natal. The index case in the 2004 lineage 5d outbreak was CKZA04AL491, isolated from 8- and 12-week old broiler breeders at a site of a large commercial operation in Hopewell, near Pietermaritzburg on 30 September 2004. A house on each of two sites was affected and a total of 1,901 out of 100,000 birds died (Department of Agriculture (DoA)).

About a week later, on 8 October 2004 the second isolate (CKZA04AL495) was made after reports were received of illness and deaths amongst village chickens at Hopewell Village in the Richmond district, approximately 200 meters from the breeder farm. The villagers reported that chickens had been dying in the township for several weeks prior to the outbreak at the commercial farm. CKZA04AL495 had an ICPI of 1.91, which is characteristic of lineage 5d (Lin *et al*., 2003) and long heat stability.

Newcastle disease also occurred on a broiler rearing site, east/south-east of the breeder site. 5,000 out of 360 000 birds died within three days. The commercial operation suspected that a break in the biosecurity caused the jump from the neighbouring township (DoA).

The next isolate, CKZA04AL496 was the first sub-group II (Fig. 6.1(a), sub-lineage (o)) isolate, from a township near Estcourt. This virus had an ICPI of 1.89. The other sub-lineage (o) isolates were CKZA04AL500, CKZA04AL502 and CKZA04AL512, isolated throughout November 2004. These viruses shared very close genetic relationships (98.7-99.7%) with two Chinese isolates, JS/6/01/Go and JS/5/01/Go, but only 95.2 to 97.1% sequence identities with subgroup I. At the amino acid level (Fig. 6.3), the close genetic relationship to viruses from China is illustrated by shared M^{22}, G^{24} and I^{121} residues. This

suggests that sub-lineage (o) is a recent introduction to SA, rather than having evolved from the 1999/2000 viruses like the sub-group I viruses.

A few other notable cases from the early outbreak cases include CKZA04AL506 that was isolated from a flock of free-range bantams kept by a commercial farmer for ornamental purposes. Although the commercial chickens were well-vaccinated and therefore fully-protected, 180 out of 200 of the bantams died, demonstrating the high pathogenicity of lineage 5d viruses for chickens. A second case involved CKZA04AL509, isolated during an outbreak that destroyed most of 3000 to 4000 pullets on a small chicken farm, but the owner admitted that his vaccination had been poor. Thirdly, the location of CKZA05AL516 (commercial chickens) was geographically very close to that of CKZA04AL514 (village chickens) although the outbreaks were two months apart. Labourers reported that chickens had been dying in large numbers over December and January 2004. All indications are that this is the second recorded case of the strain spreading from village to commercial chickens.

6.3.5 The spread of the outbreak throughout South Africa

From the period September to November 2004, the outbreak remained confined to KZN and was defined by the presence of two separate genotypes, sub-group I and sub-group II. Sub-group II (sub-lineage (o), Appendix 2xvi, p277) disappeared, but by January 2005 (Appendix 2ii, p275) sub-group I had spread northwards to the OFS, Gauteng and North-West provinces. The index case in the north appears to be a large commercial producer, who sourced hatching eggs from KZN that likely were contaminated (S. Bisschop, personal communication). ND seemed to be spreading westward towards Botswana in mid-February. During March, April and May 2005 (Appendix 2iii, p276) the outbreak was still continuing in KZN, and a cluster of outbreaks in Kroonstad village chickens persisted. The disease had also spread eastwards into Mphumalanga, northwards into Limpopo and was progressing steadily through the North West Province (NWP) into the Northern Cape. From June to August 2005 (Appendix 2iv, p276) the main cluster occurred in the Gauteng Province but ND continued to disperse widely throughout the infected regions, appearing for the first time in the Eastern Cape in June 2005. In the period September to November 2005 (Appendix 2v, p277) the Western Cape also became infected and ND moved further eastwards in Mphumalanga towards Mozambique. At this time the infection still continued

in KZN, particularly along the N2 highway. Towards the end of 2005 and beginning of 2006 (Appendix 2vi, p277) the number of cases had diminished dramatically in KZN but clusters of outbreaks still occurred in Gauteng, Northern Free State/southern NWP and on the Northern Cape border. From March to June 2006 (Appendix 2vii, p278) the outbreak was dying out in the northern and eastern regions, but was becoming established in the Eastern and Western Cape provinces. The general distribution is depicted in Fig. 6.4, and indicates that the outbreak peaked around September 2005. It is clear from the figure that in 2006 outbreaks were ceasing in all provinces except the Western Cape, where cases were on the increase heading into the winter months.

6.3.6 Mapping of specific genetic variants to gain insight into how ND is spread in South Africa

The natural tendency of RNA viruses to undergo antigenic drift results in the rise of specific sub-populations during the course of an outbreak. These genetic variants give clues to the spread of viruses in a geographic region, since they are unique and easily traceable. Several sub-lineages ((a) to (m); Appendices 2viii to 2xvi, pp278-282) arose during the course of the South African lineage 5d outbreaks that started in 2004. They are discussed in the order that they appear in the phylogenetic tree, Fig. 6.1(a).

Sub-lineage (a) consists of CKZA05N406, CKZA05N362, CKZA05AL573, CKZA05M202, CKZA05AL548, CKZA05AL578, CKZA05M288 and CKZA05UP1306. This group is defined at the nucleotide sequence level by unique $G^{24}\rightarrow A$ and $A^{281}\rightarrow G$ substitutions (Fig. 6.2), the latter resulting in a unique Q→R substitution within the F_0 cleavage site (Fig. 6.3). The (a) sub-lineage (Appendix 2viii, p278) first arose in KZN (Mooiriver, village fowl) in July 2005 and then appeared in September in Middleburg, Standerton and Knysna only days apart. At the beginning of October 2005 it re-appeared in Fouriesburg in the Free State and again in Durban towards the end of the month. Sub-lineage (a) was finally isolated in November 2005 in Randfontein and Dundee. Generally, the distribution is clustered in the poultry-producing corridor between Durban and Johannesburg (Appendix 1, p) with the single incursion into the Western Cape Province.

Sub-lineage (b) consists of three viruses, CKZA06SB56, CKZA06N651 and CKZA06UP187 (Appendix 2ix, p279), that are defined by A^{155}→G mutations (Fig. 6.2) and result in a non-synonymous D^{72}→G substitution (Fig 6.3). The first location was a broiler operation at Skuinsdrift, NW Province at the end of January 2006, and by May 2006 it had reached Vredenburg and Stellenbosch in the Western Cape.

Sub-lineage (c) consists of only two viruses, CKZA06N652 and CKZA06UP30 that may not be phylogenetically related, but are grouped together on account of containing different mutations at exactly the same position in the F protein. The former contains a C^{166}→T and the latter a C^{266}→G non-synonymous substitutions resulting in unique F and C amino acid residues at position 109, respectively (Fig. 6.3).

Sub-lineage (d) consists of three viruses, CKZA05N386 and CKZA05N356 isolated from chickens, and OSZA05N333 isolated from an ostrich. CKZA05N386 and OSZA05N333 were both isolated near the end of August 2005, from Vredevort in the northern OFS and Brits in NW province, respectively, and CKZA05N356 was isolated in September in Lydenburg (Appendix 2x, p279). This small genetically-distinguished group is defined by a unique G^{29}→T non-synonymous mutation (Fig 6.2) that produced a unique S^{30}→I substitution at the amino acid level (Fig. 6.3).

Sub-lineage (e) viruses (CKZA05UP1402, CKZA06SB29, CKZA06N654, CKZA06N602, CKZA06N616) lack distinguishing amino acid substitutions, but at the nucleotide sequence level (Fig. 6.2) they share a T^{211}→C substitution that is also shared by CKZA06N642a, CKZA06N642b and CKZA06N628 (sub-lineage (f)). This particular variant (Appendix 2xi, p280) first arose in Pretoria in September 2005 before spreading to Gedult's rivier and Sommerset East in the Western Cape in March 2006, Grahamstown in the Eastern Cape in April and Robertson, Western Cape, in May. Therefore, sub-lineage (e) appeared to have originated in Gauteng in 2005 before spreading to and becoming prevalent in the Cape provinces in 2006. Sub-lineage (f) viruses may have shared a common ancestor with sub-lineage (e) as discussed earlier, and were restricted within the Western Cape Province in April 2006 (Appendix 2xii, p280), but the lineage (f) viruses also share a synonymous C^{263}→T mutation with CKZA05AL553, CKZA05AL554 and CKZA06N630 of sub-lineage (g). Sub-lineage (g) consists of viruses isolated in KwaZulu/Natal during August 2005 (CKZA05AL553 and CKZA05AL554) and Mokopane at the end of March 2006

(CKZA06N630) (Appendix 2xiii, p281). Therefore, its possible the ancestral virus of sub-lineages (e), (f) and (g) first arose in the latter part of 2005 in KZN before being spread to the Northern regions and finally down to the Western and Eastern Cape provinces where they continued to spread in 2006.

Sub-lineages (h (i), (j), (k) and (l) only consists of two viruses each, and point mutations are synonymous (Fig 6.2)

Sub-lineage (m) consists of six viruses, CKZA05N272, CKZA05N275, CKZA05N290, CKZA05N286, CKZA05N287 and CKZA05N289. At the amino acid sequence level they are indistinguishable from the majority of SA strains (in the region analysed), but at the nucleotide sequence level they contain a $C^{192}\rightarrow T$ substitution that is shared by the 1999/2000 viruses of sub-lineage (n) and international viruses. Sub-lineage (m) (Appendix 2xiv, p281) was first detected in Lehurutshe near the Botswanan-border village fowl in May 2005 and almost simultaneously in Kroonstad. By June it had spread to de Aar, Northern Cape Province, and Stutterheim in the Eastern Cape Province, but appeared to persist in the Kroonstad area as it was re-isolated near Warden and Clarens in June 2005. Sub-lineage (m) appears to have been restricted to village chickens.

6.4 DISCUSSION

In this chapter the largest amount of NDV sequence data from an outbreak in South Africa thus far was collected and analysed. For the first time we were able to monitor the spread of an outbreak in real-time. I demonstrated that the outbreaks that mainly affected village chickens in KZN from 1999 to 2000 were not caused the same genetic type as the pandemic strain of the early and mid-1990's (VIId), but instead by strains of lineage 5d, previously only described in the Far and Middle East and were therefore probably a recent introduction into South Africa. From 2001-2003 a serological survey of village chickens was conducted to determine whether village chickens could play the role of a reservoir for virulent NDV. The serological test does not distinguish between vaccine and field strains, and village chickens are not usually vaccinated. Only a few sero-positives were found in rural Zulu chickens: about 10% of 166 birds in one district had antibodies against NDV and in another district (n=179) 11% HI positives were detected with titres of up to 2^{10} (unpublished laboratory data). In 2003 lineage 5d re-emerged in a single outbreak in the same area as the 1999/2000 outbreaks, but did not seem to spread. Phylogenetic evidence showed that the 1999/2000 strain was most likely the progenitor of the 2003 virus. Almost exactly one year later a virus identical to the 2003 strain re-emerged in KZN, causing an epidemic that continues to date.

Both commercial and village chickens were affected in the 2004-2006 outbreak and epidemiological and molecular evidence suggests that the outbreak first appeared in villages around the commercial operations. Spillovers were attributed to poor bio-security practices, and outbreaks in commercial operations in KZN were usually associated with poor vaccination practices although sometimes mortalities were reported in well-vaccinated flocks (R Horner, personal communication). The mechanism by which lineage 5d was able to overcome the vaccination barrier in these cases is unknown and requires further investigation. Another interesting phenomenon was the apparently recent introduction of a separate genotype of Lineage 5d into KZN. Four isolations were made of a sub-genotype that shared a very recent common ancestor with two Chinese goose viruses isolated in 2001 (JS5/98/Go and JS6/98/Go). This subgroup circulated concurrently with the subgroup I lineage 5d viruses, but appeared to die out and has not been isolated since. From December 2004, lineage 5d spread northwards along the major poultry-producing corridor between KZN and Gauteng/ North West Provinces, affecting both commercial and backyard flocks.

The index case in the Transvaal region appears to have been contaminated hatching eggs sourced from the supplier in KZN. Over the next few months the disease spread westwards to the Northern Cape, southwards to the Cape provinces, and eastwards into Mphumalanga. The epidemic peaked in September 2005 and by 2006 it had begun to subside in most regions, except for the Western and Eastern Capes where cases were still increasing. The Western and Eastern Cape outbreaks were characterized by at least three separate introduction events from northern regions, as indicated by the mapping of specific genetic variants of lineage 5d. Most large commercial poultry producers have spread their operations across the country (for example, day-old chicks or eggs produced in one province are distributed to layer or broiler operations in another province) (personal communication, Kevin Lovell, South African Poultry Association). GIS mapping also revealed a close association between outbreaks and national/regional roads, which proves that infected chickens and their products are moved extensively and over long distances by road in South Africa. Apart from the large producers, the cull-buyer industry is probably one of the main role-players in this dissemination and this will be discussed in the concluding chapter.

Genotypes VIIc, VIId and VIIe have been predominant among NDV infections in China in recent years, accounting for 78% of the total strains isolated since 1996 (Liu *et al*., 2003). Outbreaks caused by Genotype VIId/lineage 5d were also recently reported in Greece from December 2004 to January 2005 and from July to April 2005 (OIE Disease Information, 8 July 2005 Vol 18, #27; www.oie.int). At the time of writing sequence data was not available for comparison. Goose paramyxovirus is unusual because of its pathogenicity for waterfowl. In the South African epidemic, mortalities were recorded in multiple species including chickens (commercial, ornamental and indigenous), peacocks, hadeda ibis (*Bostrychia hagedash*) chicks, geese, ostriches, pheasants and doves. This is the first report of isolation of lineage 5d from peacocks, hadeda ibis, doves and ostriches. Most of the wild species could have become infected by scavenging off infected dumped chicken waste which is illegal but unfortunately still common (David Allen, personal communication). The molecular determinant of the high virulence of GPMV for waterfowl is not known, but is has been suggested that the sequence differences in the intragenic regions of HN and P genes of GPMV might cause the differences of RNA editing efficiency of P gene and the expression of V protein, which increases virulence (Zou *et al*., 2005).

The reservoir for the maintenance of lineage 5d in South Africa from 2000 to 2004 is unknown. Although village chickens are unvaccinated and highly-susceptible to lineage 5d strains, an infection passed from flock to flock in the district of the Valley-of-1000-Hills in KZN, that lies adjacent to the poultry-producing region, could easily have gone unreported due to the remoteness, and evidence of a low-level infection was indicated by the 2001-2003 serological survey. Layer hens are vaccinated and spent birds sold by cull-buyers into the township and rural regions are able to be infected with and shed virulent NDV strains for up to six months without developing clinical symptoms. The stress of being bundled into cages on open trucks and driven across the country could trigger virus shedding (Shahn Bisschop, personal communication), and these birds are therefore ideal candidates for the reservoirs of virulent NDV in South Africa. The possibility of a wild reservoir cannot be ruled out, but since partridges and pheasants have been found to be highly susceptible to infection with lineage 5d (Zhou & Gong, 2003), the francolin species and guinea fowl both common in KZN are not expected to be the asymptomatic reservoirs. It is also unknown how lineage 5d (on two occasions) was introduced into KZN, and various possibilities are discussed in the concluding chapter.