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Molecular Characterization of Canine Parvovirus Strains from Domestic Dogs in South Africa and Nigeria

By

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DEDICATION

I dedicate this thesis to:

God almighty the Alpha and the Omega of my life for his goodness and mercy endures forever and ever.

My beloved wife Elizabeth B Dogonyaro Bajehson and daughter Blessing Zugwai Peace Dogonyaro Bajeh for their tireless and endless prayers and kind support. May God almighty in his infinite mercy keep and bless you all exceedingly in Jesus name, amen!

O God you have taught me from my youth; and to this day I declare your wondrous works

Psalm 71:17

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ABSTRACT

Canine parvovirus type 2 (CPV-2), the aetiological agent of haemorrhagic enteritis in dogs emerged in 1978 worldwide. In the mid 1980's, the original CPV-2 had evolved and was completely replaced by 2 variants, CPV 2a and 2b. In 2000, a new variant of CPV (CPV-2c) was detected in Italy and now circulates in other countries. Haemorrhagic enteritis in dogs is a major disease in South Africa and Nigeria. Both infection rates with CPV-2 and case fatality rates in young dogs are high. CPV-2 is a small, negative-sense, single-stranded DNA virus of 5.2kb long and a member of the *Parvoviridae* family, which also includes feline panleukopenia virus (FPV) and mink enteritis virus (MEV). The CPV-2 genome is prone to mutations at the VP2-encoding region. As a result we investigated the genetic composition of the VP2 region in the CPV-2 genome using molecular methods (qPCR) to provide information for comparison of field and vaccine strains of the virus. The conventional PCR detection results yielded 137 (97.85%) of the total of 140 feacal samples screened with diarrhoea positive. One hundred-and-six of 108 samples from South Africa (98.15%) tested positive and two (1.85%) were negative, while 30 (96.77%) from 31 faecal samples from Nigeria were positive and 1 (2.23%) was negative. Results obtained from the genotyping of the CPV-2 strains using CPV-2a/b and CPV-2b/c TaqMan assays employing minor groove binder (MGB) probes, revealed that out of a total of 106 South African samples, 100 (94.34%) were infected with CPV-2b and 6 (5.66%) with CPV-2a**,** while all the Nigerian samples [n=30 (100%)] contained only CPV2a. There was no reported case of CPV-2c.

The VP2 gene of selected DNA samples (n=27), from South Africa (n=19), Nigeria (n=6) and multivalent vaccines (n=2) were amplified and sequenced. These sequences were originally aligned and edited to a total length of 1,750 bp of the CPV-2 VP2 encoding gene. These selected sequences showed 99% maximum identity to the GenBank sequences from the blast results (NCBI BLAST-http://www.ncbi.nlm.nih.gov/BLAST/) and alignment of all the sequences was performed using ClustalX. Two phylogenetic analyses showed most South African field isolates distant from viruses from other parts of the world. A few clustered with Asian and European strains, while Nigerian CPV-2 strains clustered with USA and some European isolates. The results of the protein analysis showed seven changes of amino acids at positions 265, 297, 324, 424, 426, 440 and 475 for most of the South Africans strains while the Nigerian CPV-2 had only one field isolate with an amino acid change.

CHAPTER 1 :
GENERAL INTRODUCTION

Viruses of many families cause disease in companion animals and there is much academic and commercial interest in understanding the pathogenesis, virus transmission in the field and control of these diseases (Patel & Heldens, 2009). Certain viruses, notably canine parvovirus type 2 (CPV-2), canine coronavirus (CCoV) and canine distemper virus (CDV) have been identified as the primary causes of enteritis in dogs. These viruses have been found to be responsible for diarrhoea worldwide (Mochizuki *et al.,* 1993).

Companion animal viral vaccines represent a significant share of the global veterinary vaccines market for which several manufacturers offer products. Some vaccines however, are formulated using viral strains not currently circulating in the environment and which may be in need of updating in view of the possible emergence of new virulent strains in the field.

The importance of dogs as pets to humans is clear. Since ancient times, dogs have served as hunters and trackers, instruments of war, and healers of both the physical and emotional problems of humans (Carmichael, 2003). The dog population in South Africa (SA) was conservatively estimated to be around 4 million in 1999/2000 and it was reported that approximately 1 million dogs visit a veterinarian at least once a year (Leisewitz *et al.,* 2001). In Nigeria, there is no recently updated data available with regards to the dog population but was estimated to be 4,500,000 (Bourn *et al.,* 1994). Animal disease such as viral enteritis which causes diarrhoea in dogs has become an important problem globally. CPV-2 causes severe diarrhoea or fatal disease with a higher mortality rate when CPV-2 and coronaviruses occur concurrently (Pratelli *et al*., 1999; Alicia *et al.,* 1999; Mosallanejad *et al.,* 2008).

Canine parvovirus enteritis was first reported in the USA (Eugster & Nain, 1977), but the identification of the causative virus was first documented as CPV-2 in Canada in June 1978 (Appel *et al.,* 1979). Retrospective serological analysis has since revealed that the first positive sera were collected in 1976 (Koptopoulos *et al.,* 1986). However,

comprehensive independent studies on the rate of CPV-2 molecular evolution has indicated that the virus must have emerged at least ten years earlier (Shackelton *et al.,* 2005), and thereafter fatal cases of enteric disease occurred with increasing frequency (Thomson 1980). Initially, the prevalence of canine parvovirus infection in Ontario, Canada was monitored only through submissions of affected animals for post mortem examination (Carman & Povey, 1984), leading to the conclusion that the morbidity of the virus was low but the case fatality rate high (Eugster *et al.,* 1978; Nelson *et al.,* 1979).

Canine parvovirus is a highly contagious and serious disease caused by a virus that infects the gastrointestinal tract of puppies, dogs, and wild carnivores (Touihri *et al.,* 2009). CPV-2 infections have been reported in all sexes, ages and breeds of dogs (Castro *et al*., 2007; Gombac *et al.,* 2008). It is a member of the genus *Parvovirus* of the family *Parvoviridae* (Berns, 1990; Tattersall & Cotmore, 1990; Touihri *et al.,* 2009), which also includes feline panleukopenia virus (FPV) and mink enteritis virus (MEV) (Parrish *et al.,* 1982).

Canine parvovirus type 2 was first diagnosed in 1978 with the aid of electron microscopy (EM) (Appel *et al.,* 1978). Other methods of laboratory diagnosis include viral isolation, serological and molecular techniques, the latter methods which were developed more recently.

In this study, mutations of the CPV-2 hypervariable (VP2) gene region were determined from dog samples obtained from both SA and Nigeria. Firstly, the presence of CPV-2 was confirmed using the polymerase chain reaction (PCR) followed by viral characterization with real-time PCR using the TaqMan assay and Minor groove binder (MGB) probes (Decaro *et al.,* 2006b).

1.1 Statement of problem

Although canine parvovirus is widespread, the molecular characterization of genotypes of CPV-2 strains circulating in South Africa has not been determined since 1998. In Nigeria there are no details available of the molecular characteristics of the virus, yet commercial vaccines are sold for vaccination of dogs against CPV-2. In addition, veterinarians are sometimes faced with clinical signs compatible with CPV-2 infection in previously vaccinated dogs.

1.2 Hypotheses

Based on the problem statement, the following hypotheses were proposed:

- The present field strains of CPV-2 within SA and Nigeria are genetically different from the imported vaccine strains.
- The new variant, namely CPV-2c is present in South Africa and Nigeria.

1.3 Aims and objectives

The specific aims and objectives of this research project were:

i) To detect CPV-2 from diarrhoeal faecal samples obtained from different areas in South Africa and Nigeria using conventional PCR.

ii) To genetically characterize the detected CPV-2 strains from South Africa and Nigeria using the qPCR technique (TaqMan assay and the MGB probes technology)

iii) To compare and phylogenetically group the viruses with existing sequencing data.

CHAPTER 2 :
LITERATURE REVIEW

2.1 Classification of canine parvovirus type 2

Canine parvovirus type 2 is classified in the family *Parvoviridae* which comprises two subfamilies known as *Parvovirinae and Densovirinae.* The subfamily *Parvovirinae* infects vertebrates and is further classified into three genera namely, *Parvovirus, Erythrovirus* and *Dependovirus,* while the subfamily *Densovirinae* which infects insects, is also further classified into three genera namely, *Densovirus, Iteravirus* and *Contravirus* (Berns, 1990; Tattersall & Cotmore, 1990). CPV-2 is specifically classified within the feline parvovirus subgroup of the genus *Parvovirus* (Siegl *et al.,* 1985). The virus is very similar to feline panleukopenia virus (FPLV) and is 98% identical, differ in 6-7 amino acids in the viral capsid protein VP2 (Carter & Wise, 2006). It is also closely related to mink enteritis virus (MEV), raccoon parvovirus (RPV), and blue fox parvovirus (BFPV) (Jones *et al.,* 1997). The new genus *Bocavirus* which was first cloned in Sweden as a member of the family *Parvoviridae* in 2005 from pooled nasopharyngeal aspirates (NPA), include the human bocaviruses (Allander *et al.,* 2005). The minute virus of canines and bovine parvovirus (Schwartz *et al.,* 2002), also belong to the family parvoviridae. The early belief was that FPLV mutated into CPV-2, and although this has not been proven, the strong similarity to FPLV makes this the most credible theory. However, it is possible that CPV-2 is a mutant of an unidentified parvovirus of some wild carnivore (Truyen *et al*., 1994; Shackelton *et al.,* 2005).

2.2 Molecular characteristic of canine parvovirus type 2

The unique characteristics of CPV-2 make it an emerging and re-emerging pathogen of dogs and cats worldwide (Decaro *et al.,* 2007). The disease is highly contagious and is spread from dog to dog by direct contact with their faeces (Kennan *et al*., 1976). The virus has undergone mutations from the original CPV-2 to CPV-2a and CPV-2b. A third mutant, the CPV-2c has recently been discovered in Italy (Buonavoglia *et al.,* 2001).

CPV-2 is among the smallest animal DNA viruses, with the virion exhibiting a diameter of between 18-26 nm (Kamol *et al.,* 2001). It is comprised of a negative-sense, single-stranded DNA genome (Parrish *et al.,* 1982). The genome is approximately 5.2kb long and has two open reading frames (ORF) that encode non-structural (NS) and structural viral proteins (VP) (Perez *et al.,* 2007). CPV-2 shows high rates of genomic substitutions similar to those of RNA viruses, with values of about 10^{-4} substitutions per site per year. The reported rate of substitution refers only to the VP2 gene. This high rate of evolution is similar to the rate of nucleotide substitution found with RNA viruses such as influenzavirus A (Shackelton *et al.,* 2005). The CPV-2 mature virions contain two viral proteins formed by alternative splicing from the same DNA (Reed *et al.,* 1988). The viral proteins (VP) are made up of VP1, VP2 and VP3, where the VP2 mainly comprises the non-enveloped icosahedral capsid of CPV-2, and only a few amino acid substitutions in its sequence can alter relevant biological characteristics of the virus (Parrish & Carmichael 1986; Strassheim *et al.,* 1994).

2.2.1 The canine parvovirus type 2 variants

Canine parvovirus was designated type 2 to distinguish it from a previously recognized parvovirus of dogs known as minute virus of canines (Binn *et al.,* 1980). After its emergence CPV-2 spread globally and now CPV-2 viruses are endemic in most populations of domestic and wild canivores (Parrish *et al.,* 1988).

The variants (CPV-2a and CPV-2b) differ from the original type CPV-2 by a few amino acid changes in the VP2 protein. The latter accounts for an extended host range *in vivo* [the reported rate of substitution refers only to the VP2 gene, and for an increased affinity to canine transferrin receptors (Hueffer *et al.,* 2003)]. The difference between CPV types 2a and 2b is the presence of two single nucleotide polymorphisms (SNPs) in the capsid protein gene sequence, which determines amino acid changes in the major antigenic sites of the viral capsid (Decaro *et al.,* 2005a). Currently circulating type 2a strains possess Val instead of Ile exactly as CPV-2b; thus, the difference is now restricted to a single residue at position 426. SNP alanine/cysteine (A/C) encountered at position 4449 determines the presence of the amino acid isoleucine (Ile) for CPV type 2a or valine (Val) for CPV type 2b at residue 555 (Parrish *et al.,* 1991). Recently, an antigenic variant of CPV-2 has been reported in Italy, with the amino acid substitution aspartic acid-glutamic acid (Asp-Glu) at residue 426 (Buonavoglia *et al.,* 2001). This is

due to the nucleotide change threonine (T) 4064, and alanine with respect to type 2b. This mutation affects the major antigenic region located over the 3-fold spike of the CPV-2 capsid and monoclonal antibodies (MAbs) capable of distinguishing between CPV type 2b and Glu-426 mutant has been developed, and the new virus designated as canine parvovirus type 2c (CPV-2c) (Nakamura *et al.,* 2004). Therefore, the gene region of interest in the characterization of CPV-2 genome is the VP2 hypervariable region.

2.3 Epidemiology

2.3.1 Distribution

In the late 1970s, a new infectious disease of puppies characterized by either gastroenteritis or myocarditis or both was observed worldwide (Appel *et al.,* 1979). Within 12 months, CPV-2 was identified as the aetiological agent of severe haemorrhagic gastroenteritis in dogs (Kelly, 1978; Appel *et al.,* 1979) and spread rapidly all over the world. Almost simultaneously, the disease was also reported by other authors in Canada (Thompson & Gagnon 1978; Gagnon & Povey. 1979), Australia (Kelly, 1978), United Kingdom (Jefferies & Blackmore, 1979; McCandlish *et al.,* 1979), New Zealand (Gumbrell, 1979), and Belgium (Burtonboy *et al.,* 1979). These outbreaks were shown to be due to a novel, pathogenic canine parvovirus, totally unrelated to the minute virus of canines, an apparently non-pathogenic parvovirus of dogs, which was first described in 1970 (Binn *et al.,* 1970) and designated canine parvovirus type 1 (CPV-1).

In 1979 and 1980, an antigenic variant of CPV-2 was identified in several different countries using monoclonal antibodies and the variant was termed canine parvovirus type 2a (CPV-2a) (Carman & Povey, 1984). In the mid to 1980s, the virus underwent a further antigenic change, and the new variant was referred to as canine parvovirus type 2b (CPV-2b) (De Ybanez *et al.,* 1995).

CPV-2 enteritis was reported in Nigeria in 1985 (Kamalu, 1985). The occurrence of CPV-2 in Nigerian mongrel dogs raised questions as to the source of the infection for Nigerian dogs (Kamalu, 1985). Canine parvovirus type 2 has been described genetically and serologically in South Africa (Steinel *et al.,* 1998). Currently, the prevalence of CPV-2a and CPV-2b are at varying levels in different countries worldwide

(Truyen *et al,* 2000). Canine parvovirus type 2b is the predominant antigenic type in the United States of America (USA) and southern Africa (Parrish *et al.,* 1991; Steinel *et al.,* 1998), Turkey (Yilmaz *et al.,* 2005), whereas CPV-2a is more common than CPV-2b in Italy (Sagazio *et al.,* 1998), and other European countries (Buonavoglia *et al.,* 2000; 2001; Martin *et al.,* 2002; Mochizuki *et al.,* 2001).

A new antigenic variant has been reported in dogs in Europe and Southern Asia (Buonavoglia *et al.,* 2001; Nakamura *et al.,* 2004; and Decaro *et al.,* 2006a). This new antigenic variant is currently co-circulating together with CPV types 2a and 2b in Europe and South America (Martella *et al.,* 2004; Pérez, 2007). These CPV-2 mutants previously designated as Glu-426 mutants and now named canine parvovirus type 2c (CPV-2c), has also been detected in Vietnam (Nakamura *et al*., 2004) and its pathogenicity has been investigated (Decaro *et al.,* 2005b). There are presently no documented cases of CPV-2c infection from Africa.

2.3.2 Susceptible hosts/reservoirs of CPV-2

All sexes, ages and breeds of dogs have been found to be susceptible to CPV-2 infections (Castro *et al*., 2007; Gombac *et al.,* 2008). One study in Slovenia showed that 83.3% of dogs that died due to canine parvovirus infection were males, and 16.7% were females. The difference was statistically significant (Gombac *et al*., 2008). In another study completed in Rio de Janeiro, CPV enteritis within the sexes of the dogs was not found to be significantly different (Castro *et al*., 2007).

Studies on the ages of dogs prone to canine parvovirus infection in Brazil showed that, most of the diarrhoeal samples were obtained from 2-4 month-old puppies (Cubel Garcia *et al.,* 2000; Castro *et al.,* 2007). In Slovenia the highest percentage (67.6%) of deaths was noticed in dogs younger than six months, the lowest, 6.8%, in dogs older that one year and from six months to one year, 25.7% of dogs died due to canine parvovirus infection. Furthermore, comparison between the age groups and death due to canine parvovirus infection showed statistical significance in the Slovenia studies (Gombac *et al.,* 2008).

It has also been reported that Doberman Pinscher, Rottweiler and German Shepherd dogs appear to be under greater risk of developing parvoviral enteritis (Glickman *et al.,* 1985; Houston *et al.,* 1996). The Slovenia report revealed that mixed breeds experienced 23.8% mortality due to canine parvovirus infection, followed by German Shepherds with 16.7%, Rottweilers 10.5%, Labrador Retrievers 7.1%, Poodles 5.9% and low numbers of CPV-2 infection were diagnosed in 21 other breeds. The differences between different breeds and the number of deaths due to canine parvovirus infection were not statistically significant (Gombač *et al.,* 2008). In another study, approximately 50% of mixed-breed puppies and 44% of purebred puppies were CPV-2 positive, with 31.8% of purebred puppies from susceptible breeds such as German shepherd, Rottweiler and Doberman Pinschers showing no statistically significant differences between the breeds (Castro *et al*., 2007). Several species of wild canivores, for example coyotes, raccoons, red foxes and wolves, are also susceptible to canine parvovirus infection (Baker *et al.,* 1993; Truyen *et al.,* 1998). Canine parvovirus infection has also been reported in the bat eared fox, honey badger, cheetah, African wild cat and Siberian tiger (Steinel *et al.,* 2000).

2.3.3 Transmission of CPV-2

CPV-2 is a highly contagious virus. Its transmission from infected to susceptible dogs takes place mainly by the faecal-oral route, but dogs can also become infected from virus present on fomites such as shoes, clothing, the hands of humans, food bowls and other utensils (Pollock, 1982a; Carmichael, 1994; Decaro *et al*., 2005b). The incubation period of CPV-2 in the field is 4-5 days, but with experimental infections three days.

2.4 Variation in antigenicity and cross-protection among canine parvovirus type 2 strains

Most mutations of CPV-2 that have been identified as crucial for host range or receptor binding can also be defined by antigenic tests. The original antigenic types CPV-2, CPV-2a, and CPV-2b as well as FPLV with the exception of CPV-2c have all been defined by binding of monoclonal antibodies (Mabs) (Parrish & Carmichael, 1983). Most of these MAbs have neutralizing activity. The neutralizing antibodies have been shown to prevent CPV-2 infection, and maternal antibodies protect puppies from infection and disease. Studies with antisera produced against the various antigenic

types and the various earlier CPV-2 viruses have been performed to test the amount of neutralizing activity, specifically against heterologous virus types. These studies have revealed substantial differences in the neutralization titres (Pratelli *et al.,* 2001). Interestingly, cross-neutralizing titres were different when sera raised against CPV-2 were run against CPV-2b virus, as compared to those obtained when sera raised against CPV-2b were run against CPV-2 virus (Pratelli *et al.,* 2001). Other studies have suggested that vaccines containing strains matching the antigenic types of the variants circulating in the local canine population, or polyvalent vaccines could represent an alternative strategy to improving the effective prophylaxis against CPV (Cavalli *et al.,* 2008). These differences may be biologically important when a passively acquired protection is challenged. For instance, a certain titre of maternal antibodies may be enough to resist a challenge infection with the homologous virus, but may not prevent infection after challenge with a heterologous type. The true relevance of the differences between homologous and heterologous neutralizing antibody titres in terms of protection in this virus family is therefore not clear (Truyen, 2006c).

2.5 Diarrhoea in kennel puppies

Since the late 1970s viral enteritis has become recognized as one of the most common causes of infectious diarrhoea in dogs younger than six months, especially in kennel puppies which has resulted in high mortalities in puppies when there is an outbreak of CPV-2 infection.

Diarrhoea in puppies is one of the most common and potentially serious problems encountered in kennel situations today, and occasionally the diarrhoeal disease will affect the entire kennel (Brad & William, 2008). Dogs with CPV-2 infection are at risk of intussusceptions (Ettinger & Feldman, 1995). Fortunately most diarrhoeas are either self-limiting or respond favorably to symptomatic treatments, and the exact cause is rarely investigated. Since the recognition of canine parvovirus in 1978, increased efforts have been made to identify all the causative agents, those factors responsible for the diarrhoea, and to develop improved preventative programmes. The common aetiologies of acute diarrhoea in dogs are complex, often multi-factorial and include diet, medications, stress, and various infectious agents including viruses such as canine parvovirus type 2 (Decaro *et al.,* 2005d), rotavirus (Gouvea *et al.,* 1994), canine adenovirus type 1 (Hu *et al.,* 2001), canine distemper virus (Elia *et al.,* 2006), canid

herpesvirus (Schulze & Baumgartner, 1998) and canine coronavirus (CCoV) (Decaro *et al.,* 2004a), and bacteria (Decaro *et al.,* 2006a) and endoparasites. It is known that not all cases of bloody diarrhoea are caused by canine parvovirus.

In reality, it is common to have more than one "infectious" agent involved in the pathogenesis of the diarrhoea. Such multi-pathogen infections have been identified in the laboratory with the aid of real-time PCR (RT-PCR) (Decaro *et al.,* 2006a; Brad & Williams). Environmental factors such as malnutrition, poor mothering, temperature changes, poor disinfection/sanitation practices, overcrowding, weaning and shipping of dogs from one area to another have all contributed to kennel diarrhoea. The key to managing diarrhoea in kennel situations is a complete diagnostic work-up, and institution of appropriate management strategies. CPV-2 continues to be a major problem in commercial kennel puppies despite improvements in parvovirus vaccines and rigorous vaccination programmes.

CPV-2 infection is easy to diagnose, simple to confirm (pet side diagnostic tests, faecal haemagglutination (HA) or on necropsy), but difficult to eliminate from a kennel environment. There are recent reports of the detection of a strain of CPV-2c with a much higher mortality in Kansas, Oklahoma, and Texas. With this CPV-2c strain high mortality occurred following diarrhea in some cases, notwithstanding adequate CPV-2 vaccination programmes.

2.6 Risk factors for CPV-2 infections

The disease is usually prevalent in unvaccinated dogs due to ignorance of the owners, high costs of vaccines, poor husbandry, and faulty biosecurity practices (Muzaffar *et al.,* 2006). The continuous presence of the pathogens therefore, makes the disease endemic in particular areas. Another serious risk factor for CPV-2 infection is vaccine failure, which has been attributed to interference by maternally-derived antibody (MDA) and is the most important cause of vaccine failure amongst puppies (Pollock & Carmichael, 1982).

2.7 Clinical signs of canine parvovirus type 2 infections

The most common clinical and haematological findings of CPV-2 infection are vomiting, anorexia, depression, dehydration, foul smelling bloody diarrhoea, hypothermia or fever, marked thrombocytopenia and leucopenia (Yilmaz *et al.,* 2005). The initial work on CPV-2 pathogenesis and pathology was carried out using the original CPV-2 viruses. There are only a few published studies that described infections with the newer CPV-2 strains or variants (Decaro *et al.,* 2005b). There are differences in the amount of infectious virus in the affected organs, and there is also uncertainty whether the newer types cause more haemorrhagic signs and lesions than the original CPV-2 virus (Carmichael, 2005).

2.8 Morbidity and mortality following CPV-2 infection

The morbidity and mortality rates reported for canine parvovirus enteritis in dogs ranged widely, with the highest occurrences in young weaned pups (Eugster *et al.,* 1978; Nelson *et al.,* 1979). Death is usually due to the complications of the severe dehydration and circulatory shock. The virus also suppresses the immune system and depresses bone marrow. In pups between the ages of 2-6 weeks, the virus can also damage the heart muscle (myocarditis). Myocarditis is not seen any more today because of the ubiquitous nature of the virus and the fact that almost all pups now get colostral protection that protects against myocarditis that used to appear in pups younger than eight weeks.

2.9 Laboratory diagnosis of canine parvovirus type 2 infection

Diagnosis of canine parvovirus type 2 infections is very important, especially in kennels and shelters in order to isolate infected dogs and prevent transmission to susceptible contact animals. Diagnosis on the basis of clinical signs is not definitive, since several other pathogenic organisms can cause diarrhoea in dogs. Therefore, a clinical diagnosis of canine parvovirus type 2 infection should always be confirmed with laboratory tests. Various laboratory methods have been developed to detect CPV-2 in the faeces of infected dogs, for example electron microscopy (EM) (Alicia *et al.,* 1999), ELISA, immunochromatographic tests (IC), haemagglutination (HA) tests, viral isolation (VI), haemagglutination inhibition (HI) tests, conventional polymerase chain reaction (C-PCR) and real-time polymerase chain reaction (RT-PCR) (Desario *et al.,* 2005).

2.9.1 Electron Microscopy

Electron microscopy as a means of diagnosis, allows one to visualize minute objects as small as one nanometer. The specimens are not illuminated with light but bombarded by electrons as a source for image formation. Electron microscopy allows the identification and confirmation of CPV-2 viruses based on their size and morphology. Viruses are observed in groups or seen as single particles stained negatively with uranyl acetate, phosphotungstic acid or methylamine tungsten (Alicia *et al*., 1999; Silke *et al*., 2009). The identification of the CPV-2 viruses in faeces can be carried out only during the elimination period of the viruses, which occurs between the 3rd and the 9th day of infection. The sensitivity of electron microscopy is believed to be relatively low due to the large quantities of viruses required for a positive test result (Esfandiari & Klingeborn, 2000).

2.9.2 Immunochromatography test (IC)

The SNAP® Rapid Canine Parvovirus Antigen Kit (BioNote, Korea) for example is only one of the many commercialised IC tests, and is a rapid field diagnostic method used in clinical practice because the test procedure is simple. As a result, it can be performed by veterinarians as well as dog owners (Esfandiari & Klingeborn, 2000). However, a large amount of viral antigen is required to produce a clearly visible band and the interpretation of results may be affected by the subjectivity of the test operator. This is especially common when numbers of viruses are low (Mochizuki *et al.,* 1993; Uwatoko *et al.,* 1995; Esfandiari & Klingeborn, 2000; Desario *et al.,* 2005).

2.9.3 Lateral flow immunoassay (LFA) test

The lateral flow assay (LFA) is a rapid and convenient diagnostic test which may be performed under most conditions and is especially useful for field application. It is easy, simple and rapid to use as a confirmatory test. The lateral flow assay is produced in a dipstick format. Lateral flow tests are a form of immunoassay in which the test sample flows along a solid substrate via capillary action. After the sample is applied to the test it encounters a coloured reagent which mixes with the sample and transits the substrate encountering lines or zones which have been pretreated with an antibody or antigen. Depending upon the analytes present in the sample, the coloured reagent can become bound at the test line or zone.

2.9.4 Haemagglutination test (HA)

The haemagglutination assay (HA) is a method of quantification of viruses by means of haemagglutination. It is an easy, simple and rapid method which can be applied to large numbers of samples. Several viruses from different viral families, including the *Parvoviridae,* possess haemagglutinins on their surfaces that have the ability to agglutinate the red blood cells of several different animal species by binding to receptors on the surface of the red cells. Canine parvovirus is able to agglutinate porcine red cells. Red cells washed in phosphate buffered saline are added to a suspension of parvovirus with a pre-determined titre in a microtitre plate and observed for haemagglutination. The test is regarded as positive when the HA can be blocked by virus-specific antisera.

The advantages of HA are its speed and ease of performance, and the fact that living host systems are not required. Specific haemagglutination activity is detected in the faeces up to nine days post infection.

CPV-2 strains lacking HA activity have been reported (Parrish *et al.,* 1988; Cavalli *et al.,* 2001). However, the HA test carried out in a 96-well plate format allows rapid processing of many samples, in which results can be read after only four hours (Desario *et al.,* 2005).

2.9.5 Viral isolation methods

Isolation of CPV-2 requires cell culture capability, capable and skilled personnel, and also the permissive cell lines to be used. Moreover, viral isolation is time-consuming. It requires a long incubation period (5-10 days) and additional testing by immunofluorescence (IF) assay using an anti-CPV conjugate (Decaro *et al.,* 2006a). Haemagglutination (HA) can also be used in order to detect viral antigens in the cell culture supernatant. The main disadvantage of viral isolation however, is low sensitivity. It has been demonstrated in natural and experimental infections that CPV-2 is detectable by viral isolation only for a few days post-infection (Desario *et al.,* 2005).

2.9.6 Characterization of canine parvovirus type 2 with the haemagglutination inhibition (HI) test

The haemagglutination inhibition (HI) test is mostly used to evaluate maternally derived antibodies and seronversion after CPV vaccination. Moreover, the haemagglutination inhibition (HI) test is also carried out to determine the amount of specific antibody in serum samples. Isolated strains of CPV-2 can be subjected to antigenic characterization in a haemagglutination inhibition assay with a panel of MAbs (Buonavoglia *et al.,* 2001; Nakamura *et al.,* 2004; Desario *et al.,* 2005; Martella *et al.,* 2006). The CPV-2 strains can be typed as CPV-2 (original type), CPV-2a, CPV-2b or CPV 2c on the basis of MAb reactivity (Decaro *et al.,* 2006a).

The value of the HA and HI procedures lies in its applicability to two types of problems:

i) The rapid identification of new virus isolates. If a new isolate can be shown to agglutinate red cells, it can readily be determined whether any known antisera are capable of inhibiting agglutination, and ii) the determination of the presence or absence of antibodies in sera obtained during the course of a disease. The real value of HI for CPV is to confirm the identity of CPV viruses as indicated under the HA section.

2.9.7 Molecular detection and identification of CPV-2

The diagnosis of CPV-2 infection on the basis of clinical signs alone is inconclusive as mentioned earlier. Molecular methods are the methods of choice for CPV-2 diagnosis because they are based on detection of DNA which has been shown to be highly sensitive (Buonavoglia *et al.,* 2001). The identification and characterization of CPV-2 strains using the TaqMan assay and Minor groove binder probe technology were described by Decaro *et al.,* 2005c; 2006b.

i) Conventional polymerase chain reaction (PCR)

In contrast to the various diagnostic methods discussed above, the conventional polymerase chain reaction (PCR) was demonstrated to be more sensitive for the detection of CPV-2 (Buonavoglia *et al*., 2001; Decaro, *et al* 2005a; Hong *et al.,* 2007). Sequence analysis provides ample information for CPV-2 typing since the fragment amplified by PCR using primers 555 forward and 555 reverse, encodes at least two

informative amino acids (residues 426 and 555 of the VP2 protein are encoded by nucleotides 4062-4064 and 4449-4451, respectively). These primers allows differentiation between CPV-2 (original type), CPV-2a, CPV-2b and the Glu-426 mutant (Desario *et al.,* 2005; Decaro *et al.,* 2005a; 2006b; Hong *et al.,* 2007). By sequence analysis of the short fragment amplified with primers 555 forward and 555 reverse, discrimination between canine parvovirus types 2 and 2a is based only on a single nucleotide polymorphism (G→A) that determines the replacement of the amino acid Val (type 2) with Ile (type 2a) at residue 555 of the VP2 protein (Desario *et al.,* 2005).

ii) Real-time polymerase chain reaction (RT-PCR) assay

The RT-PCR assay, based on the TaqMan and Minor groove binders were demonstrated to be more sensitive than traditional techniques including the conventional PCR. The quantitative real-time PCR is sensitive, specific, and more reproducible and allows the detection and quantification of CPV-2 nucleic acid within a few hours, and it is less time consuming (Decaro *et al.,* 2005c; 2006b; Hong *et al.,* 2007). Also, there is less risk of carry-over contamination than with the traditional and conventional PCR methods (Decaro *et al.,* 2005a; 2006b). In addition, the advantages of the real-time PCR minor groove binder probes™ Technology (Kutyavin *et al.,* 2000).include:

i) The utilization of a minor groove binder (MGB) which attaches to single-stranded DNA probes that enhances the stability of the duplex formed between the probes and the target region of the CPV-2 genome.

ii) Allowing an increase in the melting temperature (Tm) of the DNA duplex, and

iii) Enabling the use of smaller probes capable of detecting short conserved regions of the CPV-2 genome.

However, the molecular assays, especially the real-time PCR method, require expensive equipment, reagents and skilled personnel, thus, their routine use as diagnostic tests for the veterinary practice is limited (Desario *et al.,* 2005). Nevertheless, there are efforts by several companies to adapt molecular methods to clinical practice, taking advantage of microchip technology that would reduce the cost and size of the equipment necessary for testing on site (Desario *et al.,* 2005).

2.10 Vaccination of canine parvovirus type 2 - a global challenge

In the late 1970s and early 1980s, both live and inactivated FPLV vaccines were used to protect dogs against CPV disease due to the shared antigens which stimulated cross-protection. However, the level of protection that they afforded was poor and the duration of the immunity was short. These vaccines were replaced by killed and attenuated CPV vaccines, the latter which provided excellent protection and longer immunity (Spibey *et al.,* 2008). Currently the attenuated vaccines are derived from either CPV-2b isolates or the original type 2 virus.

There have been reported cases of CPV-2 infection after vaccination which poses a challenge to veterinarians and vaccine producers. There is concern that the vaccines used currently to prevent CPV infection in dogs may fail to effectively protect pups against the new canine parvovirus type 2 antigenic variants (Truyen, 2006c). In spite of the fact that the original CPV-2 type was completely replaced by the antigenic types CPV-2a, 2b and 2c, it is still used in most commercial vaccines. Various studies have however, demonstrated that the CPV-2 vaccines are still effective in inducing protection against CPV-2 variants (Greenwood *et al.*, 1995; Carmichael 1994; Yule *et al.*, 1997; Spibey *et al.,* 2008; Larson & Schultz 2008).

Canine parvovirus infection in 6-week-old pups born to vaccinated bitches is likely the result of failure of the maternally derived antibodies (MDA) to protect against CPV-2 due to falling below protective levels (Decaro *et al.,* 2006c). Morbidity and mortality in pups may also be related to inadequate protection against the CPV-2 variants by MDA rather than to a failure in the transfer of MDA from the bitch to its offspring (Decaro *et al.*, 2004b). Due to the physicochemical properties of CPV-2 (high resistance in the environment with long persistence in kennels and shelters), a good vaccine should prevent the disease as well as the viral shedding of the wild strains following infection in dogs. Dogs with HI MDA titres ≥1:80 are considered protected against disease and viral shedding after challenge with virulent CPV-2 (Pollock & Carmichael, 1982). However, more recently it has been observed that pups with HI MDA titres up to 1:160, originally considered protected against CPV-2 infection (Pollock & Carmichael, 1982), were infected by CPV-2b and shed virus in their faeces (Decaro *et al.,* 2005e). Consequently, the minimal MDA level required for protection from CPV-2 infection has to be reconsidered (Decaro *et al.,* 2005e). There have been a number of reports stressing the need to update the CPV-2 vaccines by replacing the original canine

parvovirus type 2 (which has undergone extinction) with the CPV-2 variants currently circulating in local canine populations. Polyvalent CPV vaccines could represent an alternative strategy to improve the effectiveness of the prophylaxis against canine parvovirus (Martella *et al.,* 2005; Truyen, 2006c; Cavalli *et al*., 2008).

2.11 Control of CPV-2 viruses

2.11.1 Prevention

Prevention is the only way to ensure that a puppy or dog remains healthy because the disease is extremely virulent and contagious. The virus is extremely hardy and has been found to survive in faeces and other organic material such as soil for over a year. It survives extremely cold and hot temperatures. The only household disinfectants that kill the virus are chlorine-based (Ettinger *et al.,* 1995). A dog that recovers successfully from CPV-2 sheds the virus for a few days. Ongoing infection risk is primarily from faecal contamination of the environment due to the virus' ability to survive many months in the environment. The vaccine will take a few days to stimulate effective levels of immunity therefore the contagious individual should remain in quarantine until other animals are protected.

2.11.2 Control

Control of CPV-2 is a global challenge however, the most effective method of control is vaccination. The vaccine based on the original antigenic type CPV-2, have been shown to protect dogs against infection with the new (CPV-2a/2b) antigenic types (Yule *et al.,* 1997), and certain vaccines based on FPLV have been shown to protect cats from being infected with CPV-2b (Chalmers *et al.,* 1999). The ideal is for vaccines to contain the latest antigenic types of a given virus, as this implies the most complete protection, provided the new vaccines are as immunogenic as the old ones (Truyen, 2006). Puppies are generally vaccinated in a series of doses, extending from the earliest time that the immunity derived from the mother wears off until after that passive immunity is definitely gone (Oh *et al*., 2006). Puppies (from 6-weeks-old) are given three vaccinations 3-4 weeks apart (Nelson *et al.,* 1998). The duration of immunity of vaccines for CPV-2 has been tested for all major vaccine manufacturers in the United states and has been found to be at least three years after the initial puppy series and a booster one year later (Schultz 2006).

2.11.3 Treatment

Treatment of CPV-2-mediated enteritis is often unsuccessful in spite of intense efforts by veterinarians. Survival rate depends on how quickly CPV-2 is diagnosed, the age of the animal and how aggressive the treatment is. Treatment for severe cases that are not diagnosed early usually involves extensive hospitalization due to the severe dehydration and damage to the intestines and bone marrow.

Treatment ideally consists of intravenous fluids, suppression of vomiting and antimicrobial drugs (Macintire, 2004). Once the dog can keep fluids down, the IV fluids are gradually discontinued and very bland food slowly introduced. A puppy with mild clinical signs can recover in two or three days if the IV fluids are begun as soon as clinical signs are noticed. If more severe, depending on treatment, puppies can remain ill from five days up to two weeks. It is important to note that the last vaccine dose should be administered at 16 weeks of age. Untreated cases of parvovirosis have a mortality rate approaching 90%, but with aggressive therapy survival rates may approach 80-95% (Prittie & Jennifer, 2004).

CHAPTER 3 :
MATERIALS AND METHODS

3.1 Study areas and samples

3.1.1 South Africa

The majority of faecal samples originated from the greater Gauteng Province, with smaller numbers coming from Mpumalanga, Eastern Cape and Western Cape. Faecal specimens (n=108) from dogs with diarrhoea were received in 2 mL cryovial tubes from the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria, and private veterinary hospitals/clinics in Johannesburg and Cape Town. Some of the samples were confirmed virus positive using the SNAP® Rapid canine parvovirus antigen test kit (Molecular Diagnostic Services, Durban, South Africa) and/or electron microscopy (University of Pretoria). Biodata/history of dogs was recorded using a data capture form designed for this project (Appendix 1). Collected specimens were transported on ice and stored at -20 ℃ until further used.

3.1.2 Nigeria

Faecal specimens (n=31) from dogs with diarrhoea, not confirmed virus positive, were obtained from the National Veterinary Research Institute (NVRI) Vom, National Museum Clinic, Plateau State Veterinary Hospital, Evangelical Church of West Africa (ECWA) Veterinary Clinic, Leo Veterinary Clinic and Yaks & Yaks Veterinary Clinic. All these establishments are located in Jos, Plateau State and in Central Nigeria. Specimens were transported on ice and stored at -20 ℃ until further use.

3.2 Canine parvovirus type 2 controls for PCR

A local field isolate of CPV-2 V01/02, propagated in a Crandall feline kidney (CrFK) cell line, was obtained from the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria, South Africa and three multivalent canine vaccines: Virbac® canigen DHPPi batch number 1909 (Virbac Canigen);

Duramune® Max 5 (Fort Dodge) and Vanguard® Plus 5/CV-L (Pfizer Animal Health Vaccines), were used as positive controls. Lyophilised CPV-2a strain 100/09 and CPV-2c strain 243/07 DNA, kindly provided by Professor Nicola Decaro, Department of Animal Health and Well-being, Faculty of Veterinary Medicine, Bari, Italy, were also used as positive controls. Faecal samples from healthy dog tested negative for CPV-2 using Rapid snap test and Molecular methods diagnosis and as well as PCR graded water were used as negative controls.

3.2.1 Viral isolation

Canine parvovirus type 2, used as a positive control, was propagated on CrFK cells (Mochizuki *et al.,* 1993). Briefly, a confluent monolayer of CrFK cells was rinsed with magnesium- and calcium-free phosphate buffer saline (PBS-) and trypsinised. Cells were suspended in Minimum Essential Medium (MEM) with L-glutamine (200 mM/mL) (Highveld Biological (Pty) Ltd, South Africa). Suspended cells were centrifuged and counted with the aid of a haemocytometer. Cells were seeded in a 25 cm^2 tissue culture flasks (Corning, Scientific Group, South Africa) at a concentration of 200,000 cells/mL, using MEM with 5% foetal calf serum (Highveld Biological (Pty) Ltd, South Africa), with antibiotics (Gentamycin 0.05 mg/mL). A volume of 100 µℓ of the local CPV-2 field isolate V01/02 was inoculated simultaneously with the CrFK cells.

Cells were incubated at 37 ℃ and observed daily for six days for degeneration of CrFK cells due to the replication of CPV-2. To confirm the presence of CPV-2, 200 µℓ of the supernatant was collected and negatively stained for electron microscopy (Section of Electron Microscopy, Department of Veterinary Anatomy, Faculty of Veterinary Science, University of Pretoria). The CPV-2 virus was stored at -80 ℃ until further use.

Shortly before the extraction of the total DNA, virus and cells were thawed and frozen three times, centrifuged at (2,000 rpm or 1,500 x g) for two minutes and the supernatant collected for DNA extraction.

3.2.2 Reconstitution of lyophilised CPV-2a and CPV-2c DNA and CPV-2 vaccines used as controls

Lyophilised DNA was reconstituted by briefly centrifuging (five seconds) before the re-suspending of the DNA in 50 µℓ of Tris-EDTA (TE) buffer. A further 1:100 dilution of the DNA of each of the isolates was prepared. The commercial vaccines containing CPV-2 were re-suspended using the accompanying diluents prior to extraction of DNA.

3.3 Total DNA extraction

3.3.1 Extraction of total DNA for use as positive controls

The DNA was extracted using the Blood QIAamp® DNA Mini Kit (Qiagen, Southern Cross Biotechnology, South Africa) as described by the manufacturer's instructions with some minor modifications. In summary, 200 µℓ of the vaccine and CPV-2 cell culture supernatant were each added into separate 1.5 mL centrifuge tubes containing 20 µℓ of proteinase K and then 200 µℓ of AL buffer. The solutions were mixed by vortexing for 15 seconds before incubation at 56 ℃ for 10 minutes. The DNA was precipitated by adding 200 µℓ absolute ethanol prior to mixing and centrifugation. The supernatant was collected after centrifuging for one minute at 8,000 rpm (6,000 x g) using an Eppendorf Centrifuge 5430, (Merck, Germany). The mixture was washed with buffers AW1 and AW2 in separate washing steps and centrifuging at 8,000 rpm (6,000 x g) for one minute and 14,000 rpm (20,000 x g) for three minutes, respectively, and each of the DNA was eluted in a total volume of 60 µℓ in AE buffer after centrifuging for one minute at 8,000 rpm (6,000 x g). This was done as follows: 30 µℓ of AE buffer was added for the first elution of the DNA and incubated for three minutes at room temperature, centrifuged for one minute at 8,000 rpm (6,000 x g) and additional 30 µℓ of AE buffer was added to the same tube and centrifuged immediately as above, making a total volume of 60 µℓ to yield more concentrated DNA samples.

DNA, isolated from faecal samples of clinically healthy dogs, was used as negative controls, using the DNA extraction method as described for faecal samples below (section 3.3.2).

3.3.2 Extraction of total DNA from faecal samples

Total DNA was extracted from faecal specimens using the QIAamp® DNA Mini stool kit (Qiagen, Southern Cross Biotechnology, South Africa), according to the manufacturer's instructions (Kapil *et al.,* 2007).

In brief, 200 mg or 200 µℓ of faeces were weighed or aliquoted into 2 mL micro-centrifuge tubes and placed on ice. ASL buffer (1.4 mL) was added to each sample and heated at 70 ℃ for five minutes. The sample-buffer mix was mixed by briefly vortexing and then centrifuged for one minute at high speed (14,000 rpm or 20,000 x g) using the Eppendorf Centrifuge 5430, (Merck, Germany). Inhibitor EX tablets were added to each supernatant and mixed to dissolve the tablets immediately. The suspension was incubated for one minute at room temperature, centrifuged at 14,000 rpm (20,000 x g) for three minutes and all the supernatant was pipetted into a new centrifuged tube and centrifuged at high speed (14,000 rpm or 20,000 x g) for three minutes. The supernatant was treated with 15 µℓ proteinase K and 200 µℓ AL buffer and mixed for 15 seconds before incubation at 70 ℃ for 10 minutes. DNA was precipitated by adding absolute ethanol (200 µℓ) and mixed immediately. The complete lysate of each specimen was washed with buffers AW1 and AW2 and centrifuging for one minute at full speed (14,000 rpm or 20,000 x g) and three minutes at 14,000 rpm or 20,000 x g) respectively after each wash using the Eppendorf Centrifuge 5430, (Merck, Germany), before eluting with 60 µℓ of AE buffer. This was done as follows: 30 µℓ of AE buffer was added for the first elution of the DNA and incubated for five minutes at room temperature, centrifuged for one minute at 8,000 rpm (6,000 x g) and additional 30 µℓ of AE buffer was added to the same tube and centrifuged immediately for another one minute at 8,000 rpm (6,000 x g) using the Eppendorf Centrifuge 5430, (Merck, Germany), making a total volume of 60 µℓ to yield more concentrated DNA samples as a modification of the manufacturer's instructions.

Gel electrophoresis and spectrophotometry (NANODROP® ND-1000, V.3.7 Spectrophotometer, Inqaba Biotechnology, South Africa and Thermo Fisher Scientific) were used to measure the quality and quantity of the extracted DNA prior to the optimization of the conventional PCR.

3.4 Optimization of the conventional PCR

The conventional PCR was first optimized before screening of samples. The PCR, described by Buonavoglia and co-workers in 2001, and amplifies a 583 bp part of the VP2 encoding gene of the CPV-2 virus.

3.4.1 Determination of the optimal DNA concentration

The optimal concentration of extracted DNA obtained from one of the vaccines (Virbac) and the cell cultured CPV-2 was determined. A series of ten fold dilutions were prepared from both the vaccine and the cell culture material using as a starting concentration 28.95 ng/µℓ (vaccine) and 48.35 ng/µℓ (cell culture). Different volumes of 1 µℓ, 2.5 µℓ and 5 µℓ from each tube of the series of dilutions were used as input DNA in a 25 µℓ PCR reaction for optimization of the amplification reaction.

3.4.2 Determination of the optimal primer pair concentration

Primers at a 100 µM stock solution were initially diluted to 20 µM/µl as the working solution. This dilution was used to determine the optimal input DNA concentration in the PCR (3.4.1). In order to obtain the optimum primer concentration, a five fold series of dilutions of the working solution, were prepared. A volume of 0.25 µℓ from the series of dilutions were used as input primer in a 25 µℓ PCR reaction for optimization of the amplification reaction of vaccine and cell culture DNAs (28.95 ng/µℓ and 48.35 ng/µℓ) respectively.

3.4.3 Conventional Polymerase chain reaction

A total reaction volume of 25 µℓ consisted of 1 µℓ of the DNA template from each DNA dilution, 12.5 µℓ Takara premix [1.25 units/25 µℓ Takara Ex Taq™, 2x concentration 0.4 mM dNTP mix, 2x concentration, including 4 mM Mg^{2+} Ex Taq™ buffer] (Fermentas, South Africa), 0.25 µℓ of 20 µM of each primer [555 Forward and 555 Reverse primer (Table 1) (Buonavoglia *et al.,* 2001)], and 11 µℓ of water (PCR grade). The PCR conditions consisted of pre-incubation at 94 ℃ for 10 minutes and 35 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 50 ℃ for one minute and extension at 72 ℃ for one minute, with the final extension at 72 ℃ for 10 minutes and cooling at 4 ℃ in a Thermal cycler®, 9700 (AppliedBiosystems, South Africa).

After optimization, the PCR, as described by Buonavoglia *et al.,* (2001), was performed on all the controls and the South Africa and Nigeria field samples, using the optimised DNA and primer concentration as determined in the above sections 3.4.1 and 3.4.2.

3.5 Analysis of PCR products

PCR products were analysed on a 1.5% agarose gel (Celtic Molecular Diagnostic (Pty) Ltd, Bioline®, South Africa), stained with 3 µℓ ethidium bromide (EtBr) (stock concentration 10mg/ml) (Invitrogen,® South Africa). The gel was run at 115 V for 45 minutes. The bands were visualised under UV light and photographed with a Kodak DS electrophoresis documentation system using the Kodak digital science ID software programme. The CPV-2 amplicon size of 583 bp was determined with reference to a 100 bp DNA molecular weight marker (Gene Ruler™ 100 bp DNA ladder ready-to-use Fermentas, Life Sciences®, Southern Cross Biotechnology, South Africa).

3.6 Genotyping of CPV-2 strains by Real-Time PCR

Two real-time PCR assays for genotyping of CPV-2 strains were used in this study and were developed by Decaro *et al.,* (2006b). The first assay differentiates between CPV types 2a and 2b, using primers CPV-2a/b-Forward and CPV-2a/b-Reverse (Table 1) and MGB probes, CPVa-Pb-VIC and CPVb1-Pb-FAM (Applied Biosystem®, South Africa) (Table 1). The second assay differentiates between CPV types 2b and 2c, in which the primers CPV-2b/c-Forward and CPV-2b/c-Reverse and MGB probes, CPVb2-Pb-FAM and CPVc-Pb-VIC were used (Table 1).

The real-time PCR TaqMan ebox kit and LightCycler® 2.0 real-time PCR machine (Roche Mannheim Diagnostics,® Germany) were used rather than the IQ™ Supermix (Bio-Rad Laboratories, Italy) and i-Cycler iQ™ Real-Time Detection System (Bio-Rad Laboratories, Italy) used by Decaro *et al.,* (2006b).

Using specific primers and probes for each assay, a total reaction volume of 20 µℓ containing 4 µℓ of 5x concentration of Master Mix (Roche Mannheim Diagnostic®, Germany), 0.5 µℓ of 500 nM of each primer and 0.1 µℓ of 100 nM of each MGB probe (Applied Biosystem South Africa), 9.8 µℓ of water (PCR grade) and 5 µℓ DNA template

(5 ng/µℓ) were used for real-time PCR amplification. The thermal cycle conditions used were followed as described by Decaro *et al.* (2006b), including the pre-incubation of the Taq DNA polymerase at 95 ℃ for 10 minutes and 45 cycles consisting of denaturation at 95 ℃ for 30 seconds, primer annealing at 60 ℃ for one minute and extension at 72 ℃ for one second in the LightCycler® 2.0 machine.

A colour compensation reaction was performed using the probes (FAM at 530 nM and VIC at 560 nM) for accurate analysis of amplification curves to differentiate between CPV-2a, CPV-2b and CPV-2c variants/strains, the colour compensated data results were subsequently applied to all real-time PCR data.

Table 1 Sequence and position of oligonucleotide primers and MGB probes used for amplification and sequencing of the VP2 encoding gene of the CPV-2 virus as developed by Buonavoglia *et al*., (2001) and Decaro *et al.,* (2006b; 2008).

Assay	Primer/probe	Sequence 5' to 3	Polarity	Specificity	Position	Amplicon size (bp)
Conventional PCR[1]	555 Forward	CAGGAAGATATCCAGAAGGA	+	All types	4003-4022	583
	555 Reverse	GGTGCTAGTTGATATGTAATAAACA	-	All types	4561-4585	583
Conventional PCR[2]	CPV2655-F	CCAGATCATCCATCAACATCA	+	All types	2655-2675	837
	CPV3511-R	TGAACATCATCTGGATCTGTACC	-	All types	3489-3511	
Conventional PCR[3]	CPV3381-F	CCATGGAAACCAACCATACC	+	All types	3381-3400	717
	CPV4116-R	AGTTAATTCCTGTTTTACCTCCAA	-	All types	4093-4116	
Type 2a/2b MGB probe 1st assay[4] Real-time PCR	CPVa/b-Forward	AGGAAGATATCCAGAAGGAGATTGGA	+	All types	1719-1744	93
	CPVa/b-Reverse	CCAATTGGATCTGTTGGTAGCAATACA	-		1785-1811	
	CPVa-Pb	VIC–CTTCCTGTAACAAATGATA–MGB	+	Type 2a	1765-1783	
	CPVb1-Pb	FAM–CTTCCTGTAACAGATGATA–MGB	+	Type 2b	1765-1783	
Type2b/2c MGB probe 2nd assay[5] Real-time PCR	CPVb/c-Forward	GAAGATATCCAGAAGGAGATTGGATTCA	+	All types	1721-1748	150
	CPVb/c-Reverse	ATGCAGTTAAAGGACCATAAGTATTAAA–TATATTAGTATAGTTAATTC	-		1823-1870	
	CPVb2-Pb	FAM–CCTGTAACAGATGATAAT–MGB	+	Type 2b	1768-1785	
	CPVc-Pb	VIC–CCTGTAACAGAAGATAAT–MGB	+	Type 2c	1202-1219	

[1] Buonavoglia *et al.,* (2001)-Primers for amplification of CPV-2 partial gene region.
[1, 2 & 3] Decaro *et al.,* (2008)-Primers for CPV-2, full length VP2 gene used for amplification and sequencing.
[4 & 5] Decaro *et al*., (2006b)-Primers and MGB assays [4 & 5] for genotyping of CPV-2 Strains/variants.

3.7 Amplification of full length VP2 gene

For confirmation and screening of CPV-2 positive controls and samples, a partial region of the VP2 encoding gene of CPV-2 was amplified (583 bp) as described in Section 3.4. However, to genotype and compare field isolates of CPV-2 strains/variants from South Africa and Nigeria with other sequences of other parts of the world obtained from GenBank, the full length VP2 encoding gene of CPV-2 was sequenced.

Selected DNA samples (n=27) from South Africa (n=19) and Nigeria (n=6) and multivalent vaccines (n=2), already screened positive for CPV-2 as described in Section 3.4, were sent to Inqaba Biotechnology (South Africa) for amplification and sequencing of the full length VP2 encoding gene. Primers CPV2655-F and CPV3511-R; CPV3381-F and CPV4116-R (Decaro *et al.,* 2008) and 555 Forward and 555 Reverse (Buonavoglia *et al.,* 2001) (Table 1) were used for both amplification and sequencing. The positions of the primer pairs used for the amplification and sequencing of the VP2 gene is illustrated in Figure 1.

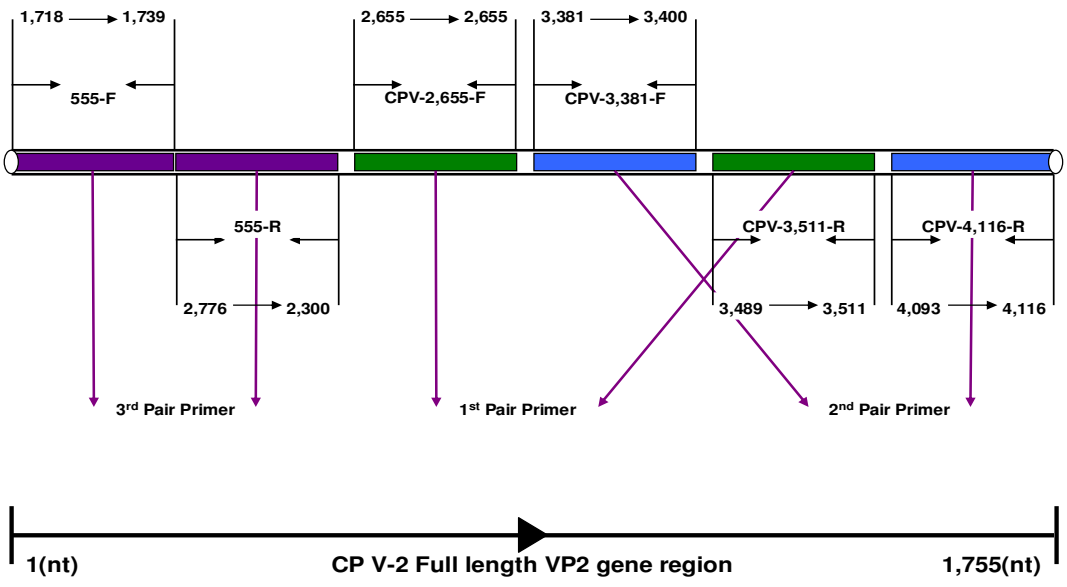


Figure 1 The full length of VP2 hypervariable gene region of the CPV-2 virus, indicating the positions of the tree pairs primers used in the amplification and sequencing of the complete VP2 gene region.

3.8 Editing and analysis of sequences

Sequences for the full-length VP2 encoding gene were assembled and edited using PREGAP and GAP 4 of the Staden package (Version 1.6.0 for Windows) (Bonfield *et al.*, 1995; Staden 1996; Staden *et al.*, 2000). The assembled sequences were aligned with sequences of related variants/strains, obtained from GenBank, using ClustalX (Version 1.81 for Windows). The alignment was manually truncated to the size of the shortest sequence (1,750 bp). Phylogenetic trees were constructed using neighbor-joining (Saitou and Nei, 1987) and the maximum parsimony methods using the Mega 4.0 software package (Kumar *et al.*, 2004). Bootstraping was performed in each analysis using 1000 replicates/tree for distance methods and 1000 replicates/tree for Maximum parsimony methods. Sequences of the full length VP2-encoding gene of CPV-2 field viruses from South Africa and Nigeria and as well as CPV-2 vaccines were deposited in GenBank and the accession numbers are shown in Table 3 below. The sequence accession numbers in the GenBank database of the CPV-2 reference strains used in this study are listed in Table 2 below.

Table 2 Shows the nucleotide accession numbers in GenBank used for the phylogeny

S/#	Isolate/strain name	Country	GenBank Accession no.	Author	Journal	Year
1	CPV-2c strain 56/00	Italy	FJ222821	Decaro, N	Virology 385 (1) 5-10	2009
2	CPV-2b strain 134/05	Italy	FJ005264	Decaro, N	Virology 385 (1) 5-10	2009
3	CPV-2b strain 29/97	Italy	FJ222823	Decaro, N	Virology 385 (1) 5-10	2009
4	CPV-2a isolate CPV-31	USA	M24000	Parrish, C R	Virology 166 (2), 293-307	1988
5	CPV-2a isolate CPV-15	USA	M24003	Parrish, C R	Virology 166 (2), 293-307	1988
6	CPV-2c strain GR51/08	Italy	GQ865518	Ntafis, V	J. Vet. Diagn. Invest. 22 (5) In press	2010
7	CPV-2 strain KU5_08	Thailand	FJ869126	Phromnoi, S	Virus Genes 41 (1), 23-29	2010
8	CPV-2b strain 42/05-49	Italy	FJ005263	Decaro, N	Virology 385 (1) 5-10	2009
9	CPV-2b strain G82/97	Germany	FJ005260	Decaro, N	Virology 385 (1) 5-10	2009
10	CPV-2b strain G162/97	Germany	FJ005261	Decaro, N	Virology 385 (1) 5-10	2009
11	CPV-2a strain CPV-ZD3	China	EU213085	Wei, W	Submitted to Prev. Vet. Med, Zhejiang Univ. China	2007
12	CPV-2a strain 54/08	Italy	FJ005257	Decaro, N	Virology 385 (1) 5-10	2009
13	CPV-2a strain 96/02	Italy	FJ005252	Decaro, N	Virology 385 (1) 5-10	2009
14	CPV-2a strain 67/05	Italy	FJ005253	Decaro, N	Virology 385 (1) 5-10	2009
15	CPV-2b strain SAH	Fort Dodge Vaccine	FJ222822	Decaro, N	Virology 385 (1) 5-10	2009
16	CPV-2c strain 128/08	Italy	FJ005246	Decaro, N	Virology 385 (1) 5-10	2009

CHAPTER 4 : RESULTS

4.1 Propagation of CPV-2

The replication of CPV-2 strain V01/02 was demonstrated by the degeneration of infected CrFK cells observed 6 days after infection. Negatively stained cell culture supernatant fluid (described in Section 3.2.1) confirmed the presence of parvovirus particles with the aid of electron microscopy (Figure 2).

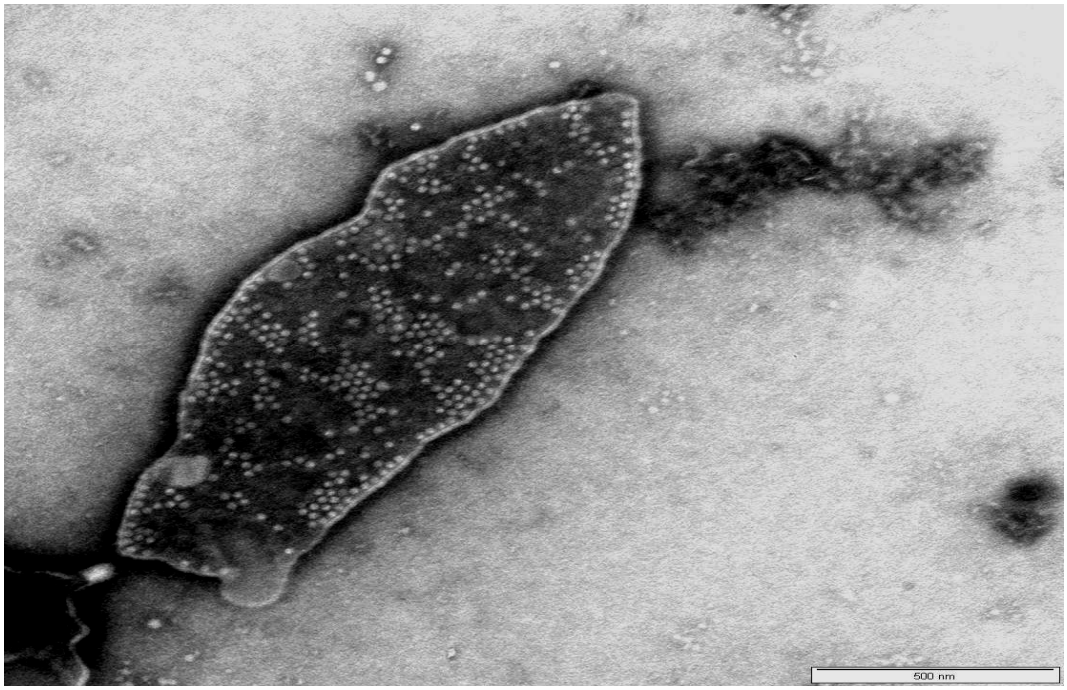


Figure 2 Electron micrograph of a group of negatively stained parvovirus particles cultured in CrFK cells

4.2 Optimization of conventional PCR

4.2.1 Positive controls

A volume of 1 µℓ of extracted DNA of the vaccine strains (Virbac, Fort Dodge and Vanguard vaccines) at a concentration of 10.31 ng/µℓ and cell culture material at a concentration of 15.12 ng/µℓ were used as positive controls. Twenty pM final concentration of the primer pair were used and amplified PCR products of the different vaccine strains and cell culture supernatant are shown in Figure 3.

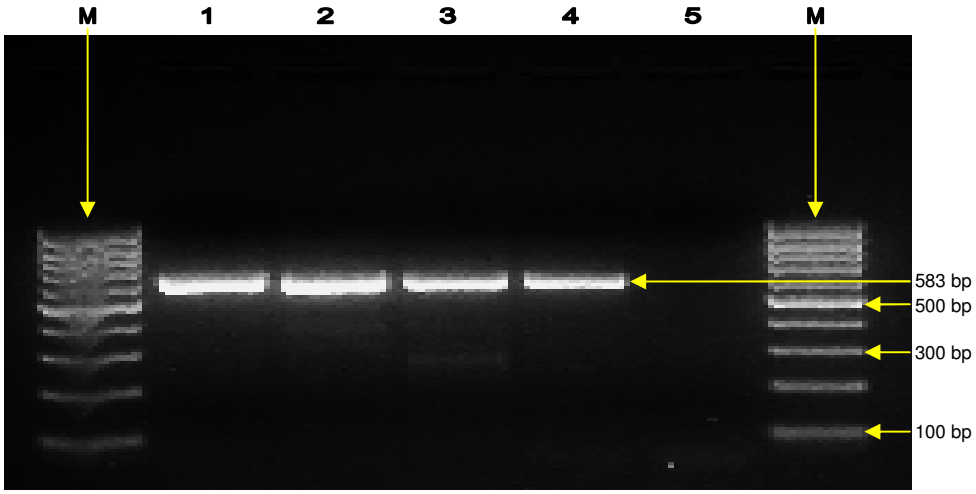


Figure 3 Conventional PCR results of the 583 bp amplicon of CPV-2. Lane 1: supernatant, cultured CrFK cells; lane 2: Virbac vaccine strain; Lane 3: Fort Dodge vaccine strain: Lane 4: Vanguard vaccine strain; Lane 5: (water) CPV-2 negative control, and M: 100 bp molecular marker.

4.2.2 Optimum concentration of DNA from field samples and primers used

In order to establish the optimum concentration of input DNA from field samples that should be used for screening for the presence of CPV-2, DNA from the Virbac vaccine at a concentration of 48.35 µg/µℓ was diluted ten-fold. The optimum DNA concentration where a strong PCR amplicon could be demonstrated with use of the primer concentration of 20 pM was regarded as the ideal DNA concentration for field samples. DNA was used at a final concentration of 10.31 ng/µℓ as seen in Figure 4, lane 6, using 20 pM of final primer concentration. The concentration of DNA in field samples was adapted accordingly.

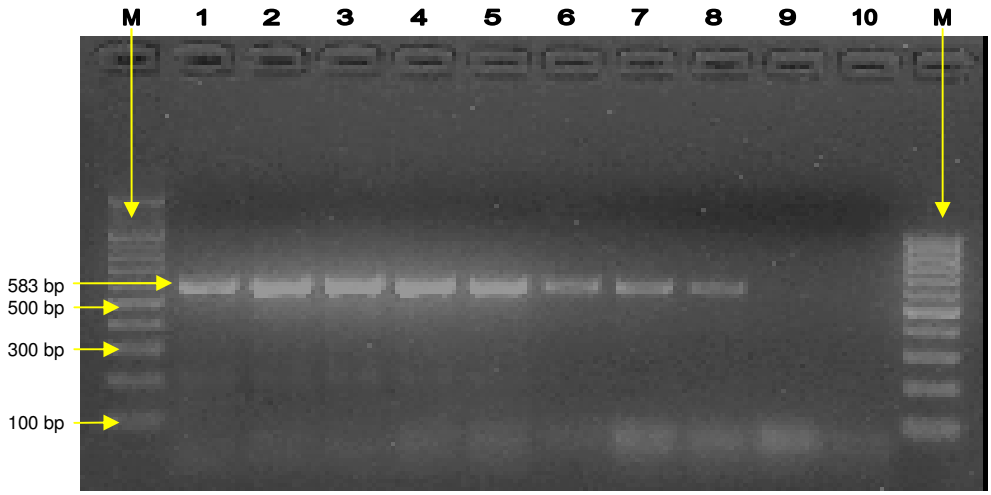


Figure 4 Agarose gel electrophoresis indicating PCR amplicons from a series of dilutions of the Virbac vaccine. Lane M: 100 bp molecular marker; Lane 1: concentration of 4.835 µg/µl DNA; Lane 2: concentration of 0.484 µg/µl DNA; Lane 3: concentration of 0.0484 µg/µl = 48.4 ng/ul DNA; Lane 4: concentration of 4.84 ng/ul DNA; Lane 5: concentration of 0.484 ng/ul DNA; Lane 6: concentration of 0.0484 ng/ul = 48.4 pg/ul DNA; Lane 7: concentration of 4.84 pg/ul DNA; Lane 8: concentration of 0.484 pg/ul DNA; Lane 9: concentration of 0.0484 pg/ul DNA; and Lane 10: negative control (water). The optimum DNA concentration chosen was lane 6 (10-6 = 0.0484 ng/ul = 48.4 pg/ul DNA).

4.3 Detection of CPV-2 in field samples by conventional PCR

PCR products of the expected size of 583 bp were obtained in 137 (97.85%) of the total of 140 samples collected. From the 108 faecal samples collected from South Africa, 106 (98.15%) tested positive and two (1.85%) were negative, as indicated in Figure 5, while 30 (96.77%) from 31 faecal samples from Nigeria were positive and one (2.23%) was negative, as indicated in Figure 6. The conventional PCR results of all the collected samples are shown in Appendix 1.

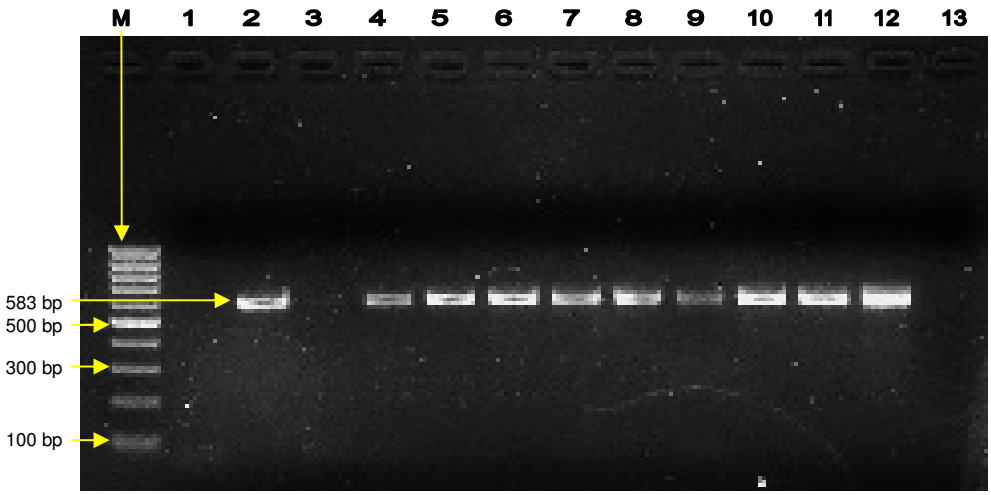


Figure 5 PCR amplicons of 583 bp obtained from faecal samples from South Africa. Lane 1 is a negative CPV-2 field sample; Lane 2 is a positive sample and lane 3 is a CPV-2 negative field sample. From lane 4 to lane 11 positive samples are present. Lane 12 is the CPV-2 Virbac vaccine positive control; Lane 13 is a negative control (water); and M is a 100 bp molecular marker.

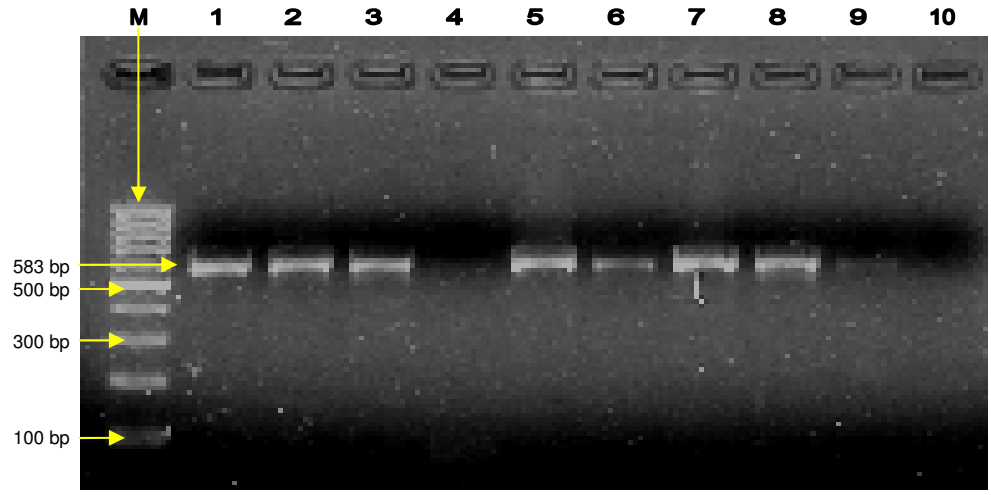


Figure 6 PCR results obtained from faecal samples from Nigerian dogs. Lane 1: CPV-2 Virbac vaccine positive control; lanes 2 and 3 are CPV-2 positive field samples. Lane 4 depicts a negative field sample and lanes 5 to 9 positive field samples. Lane 10 represents a CPV-2 negative control (water) and M represents a 100 bp molecular marker.

4.4 Genotyping of CPV-2 strains by means of real-time PCR

Results obtained from the genotyping of the CPV-2 strains using CPV-2a/b and CPV-2b/c assays as described in Section 3.7, revealed that from a total of 106 South African samples, 100 (94.34%) yielded CPV-2b and six (5.66%) CPV-2a strains. All the Nigerian samples [n=30 (100%)] contained only CPV-2a strain. There was no CPV-2c detected by the minor groove binder probes technology in any samples from South Africa or Nigeria previously screened positive for CPV-2 by conventional PCR.

Real-time PCR amplification curves demonstrating the presence of CPV-2a and b in samples obtained from South Africa (Figures 7 & 8) and Nigeria (Figures 9 & 10).

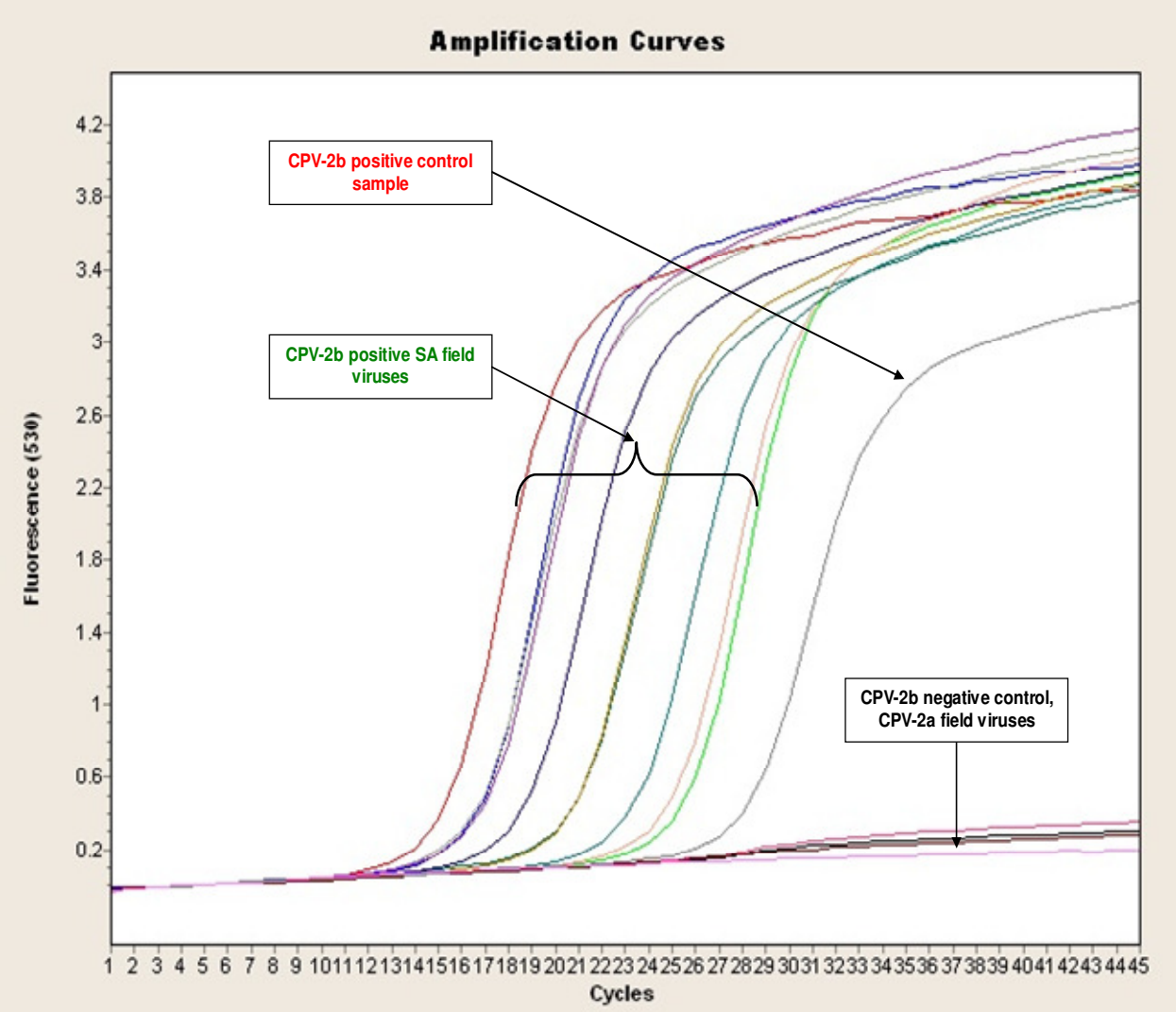


Figure 7 Results of the real-time PCR assay to differentiate CPV-2a/b in selected South African positive field samples using the fluorescence dye (FAM) at 530 nm. Field strains of CPV-2b (text in green) had a ***ct*** value of **12.5-26**. The positive CPV-2b control is indicted in red, and the negative control and other negative samples are indicated in black text.

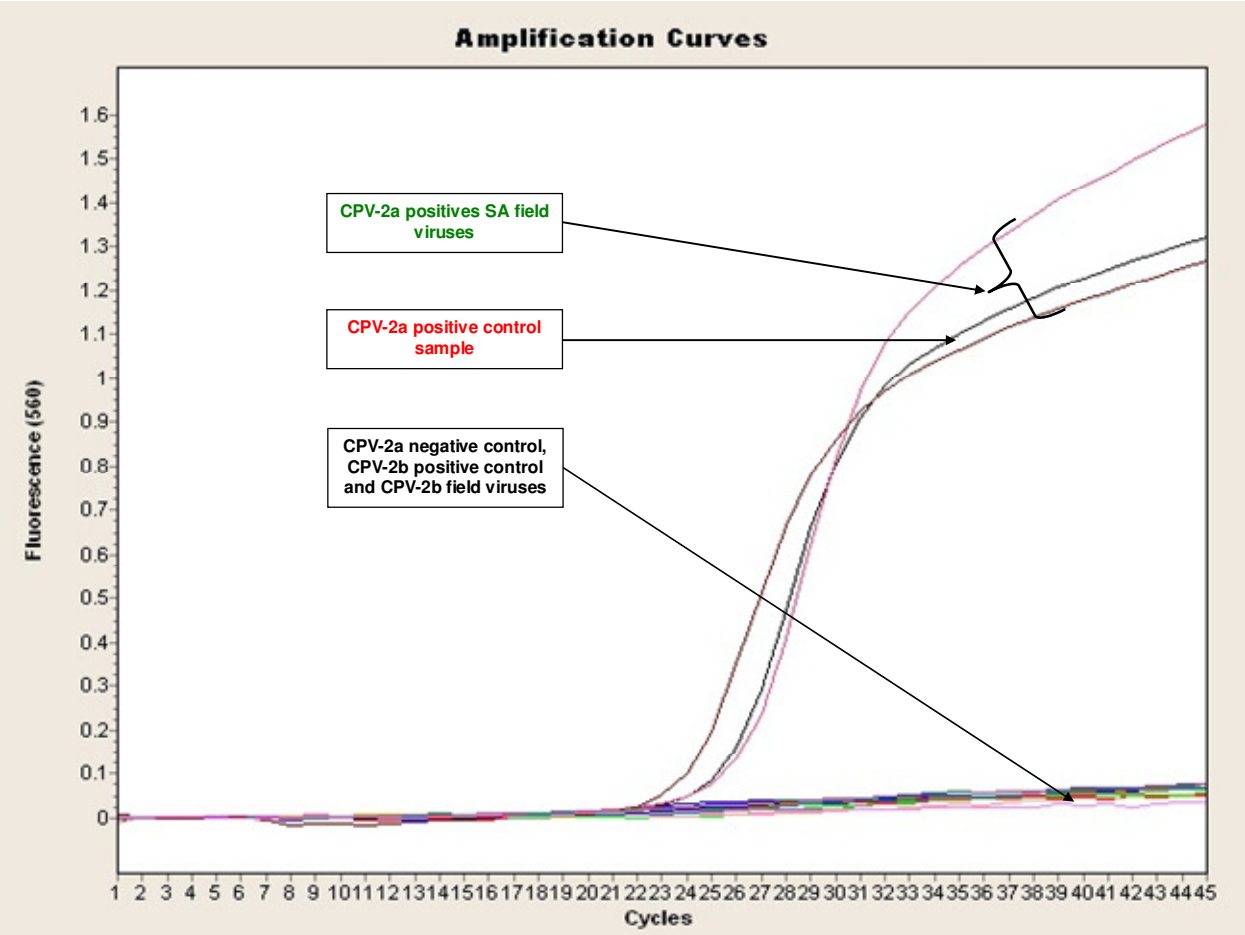


Figure 8 Results of the real-time PCR assay to differentiate CPV-2a/b in selected South African positive field samples using the fluorescence dye (VIC) at 560 nm. Field strains of CPV-2a (text in green) had a ***ct*** value of **24**. The positive CPV-2a control sample is indicated in red text. The CPV-2a negative control and other negative samples are indicated in text black.

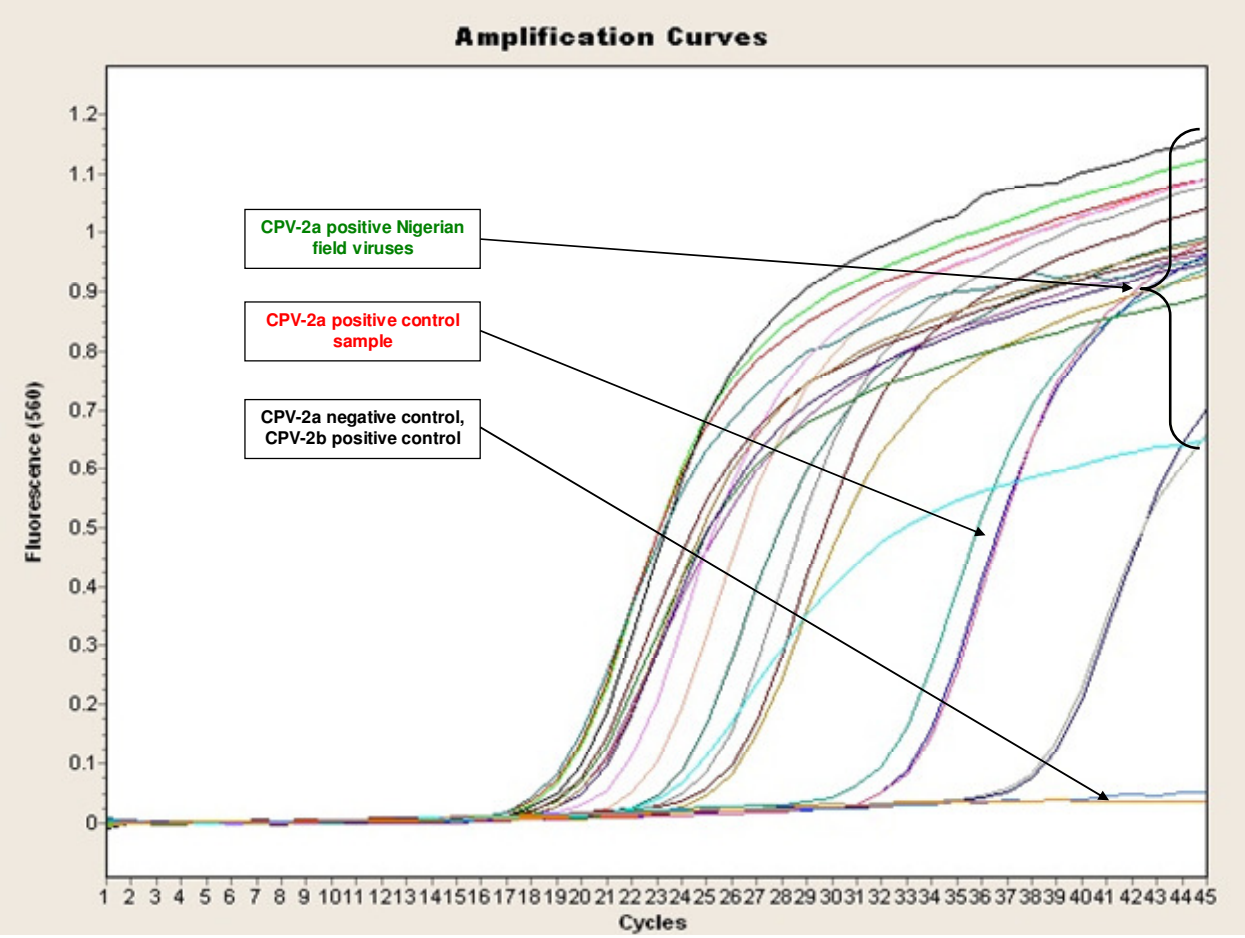


Figure 9 Results of the real-time PCR assay to differentiate CPV-2a/b in selected Nigerian positive field samples using the fluorescence dye (VIC) at 560 nm. Field strains of CPV-2a (text in green), had ***ct*** values from **18-38**. The positive CPV-2a control is indicated in red text. The negative control and CPV-2b positive control are indicated in black text.

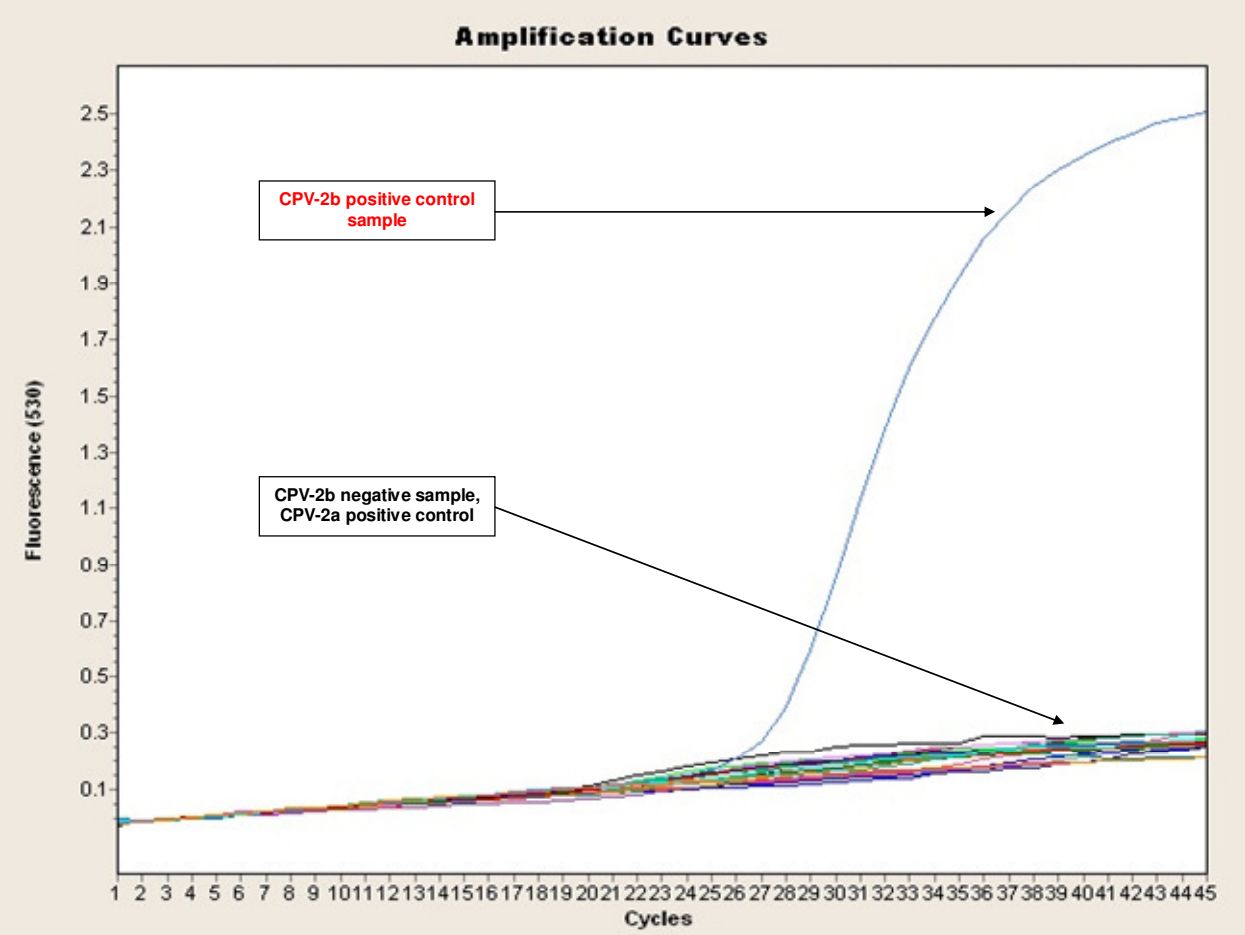


Figure 10 Results of the real-time PCR assay to differentiate CPV-2a/b in selected Nigerian positive field samples using the fluorescence dye (FAM) at 530 nm. No CPV-2b strain was detected in the field samples. The positive CPV-2b control (text in red) had a ***ct*** value of **26.5**. The negative control and other negative samples are indicated in black text.

4.5 Nucleotide sequence accession numbers

The accession numbers of the sequences retrieved from Genbank and used for the phylogeny as reference strains are listed in Table 2 on page 41.

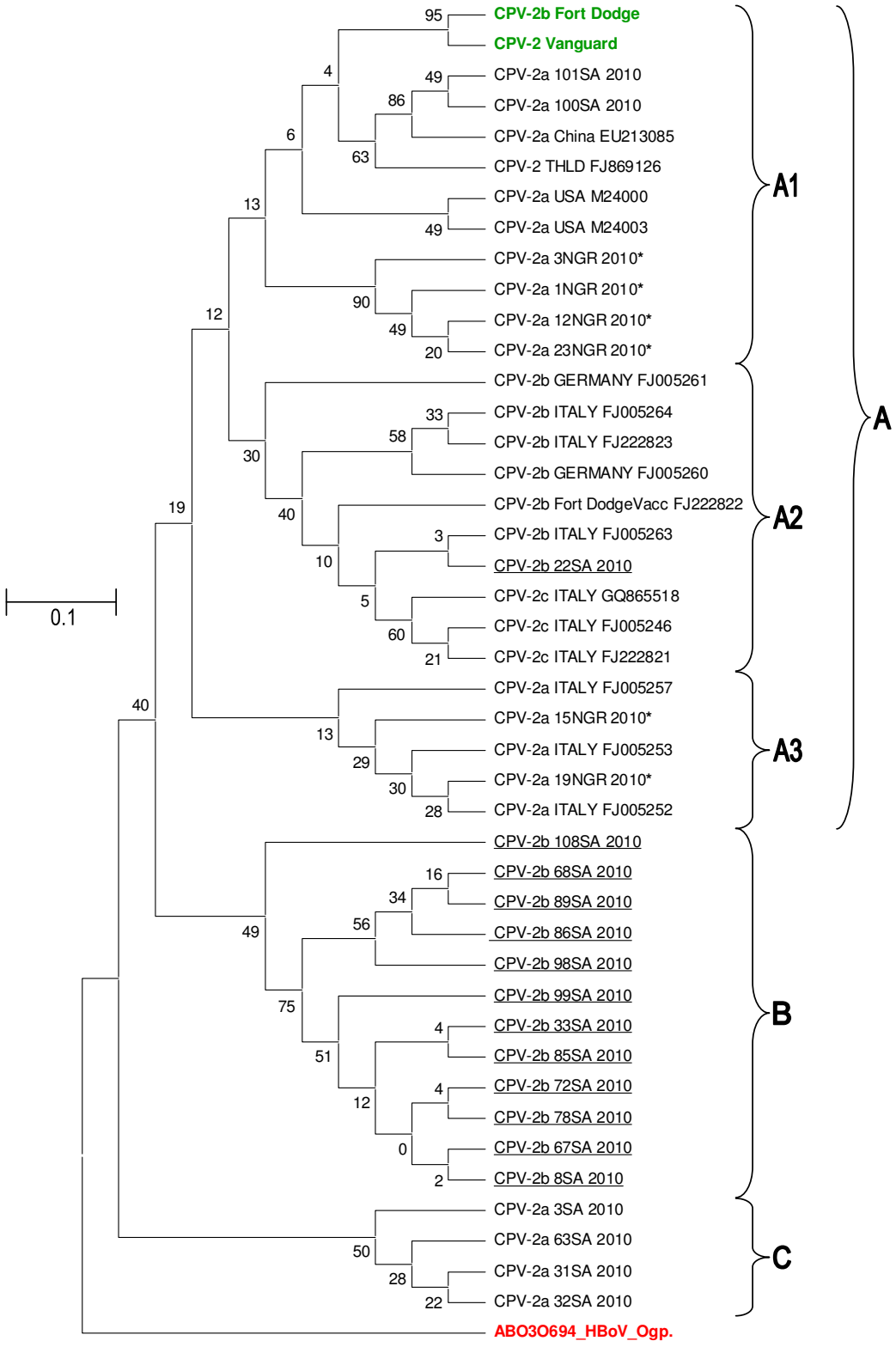


Figure 11 A phylogenetic tree of South African and Nigerian isolates compared with other sequences retrieved from GenBank using the neighbour joining (NJ) method. A, B and C are lineages and A1, A2 and A3 are clades.

The rooted NJ phylogenetic tree (Figure 11), was constructed using Mega 4, with a bootstrap value of 1000 replicates. The tree showed three major lineages group A, B and C that are separately grouped and different from the out-group human bocavirus (HBoV) indicated in red. The group that formed lineage A consists of three clades namely A1, A2 and A3. The South African commercially available vaccines (Fort Dodge and Vanguard) are indicated in green and grouped in clade A1. Two South African CPV-2a field viruses (101SA 2010 and 100SA 2010) also grouped in this clade, as well as four Nigerian field viruses (1NGR 2010, 23NGR 2010, NGR 2010, 12NGR 2010 and NGR 2010) indicated by an asterisk (*). Other CPV-2a strains in this clade include viruses from the USA, China and Thailand, emphasizing their close relationship. Clade A2 contains one South African field virus (CPV-2b 22SA 2010) that grouped together with other CPV-2b isolates from Italy and Germany. The CPV-2c viruses from Italy also grouped in clade A2. The last clade of lineage A namely A3, which contains two Nigerian field viruses (19NGR 2010 and 15NGR 2010) indicated with asterisks (*) grouped together with Italian field virus.

Lineage B interestingly, contains mainly 12 South African field viruses (CPV-2b 2010) (underlined), which grouped together separate from Lineage A. Lineage C contains four of the South African CPV-2a field viruses indicated by dots (.).

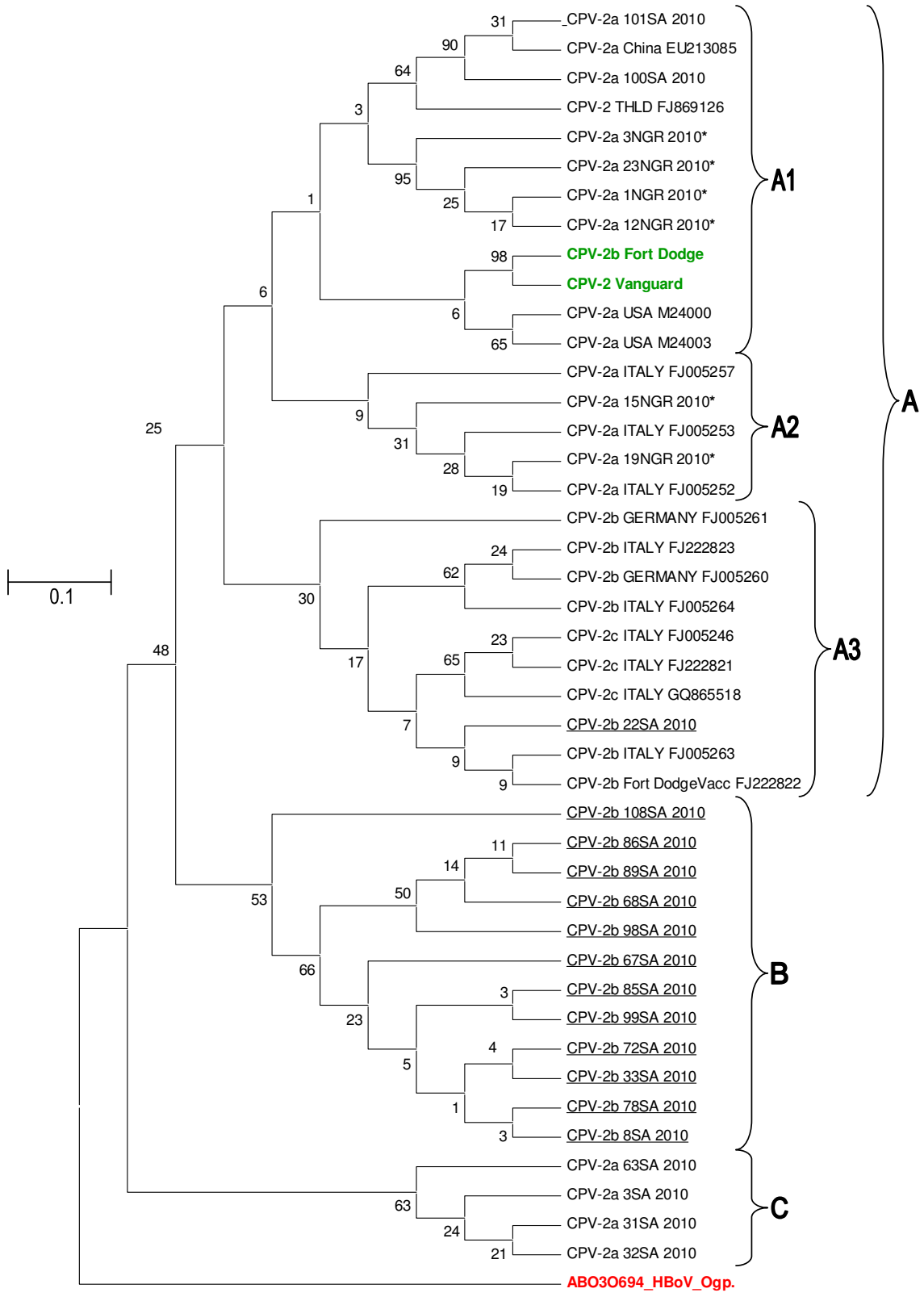


Figure 12 A rooted phylogenetic tree of South African and Nigerian Isolates compared with other sequences retrieved from GenBank using the Maximum parsimony (MP) method. A, B and C represent lineages and A1, A2 and A3 clades.

Maximum parsimony phylogenetic tree was also constructed using Mega 4, with a bootstrap value of 1000 replicates (Figure 12). It confirms the topology of the South African and Nigerian field viruses with other sequences retrieved from GenBank as in the case of the NJ phylogenetic tree with the same grouping for both South African and Nigerian field viruses and the Genbank sequences.

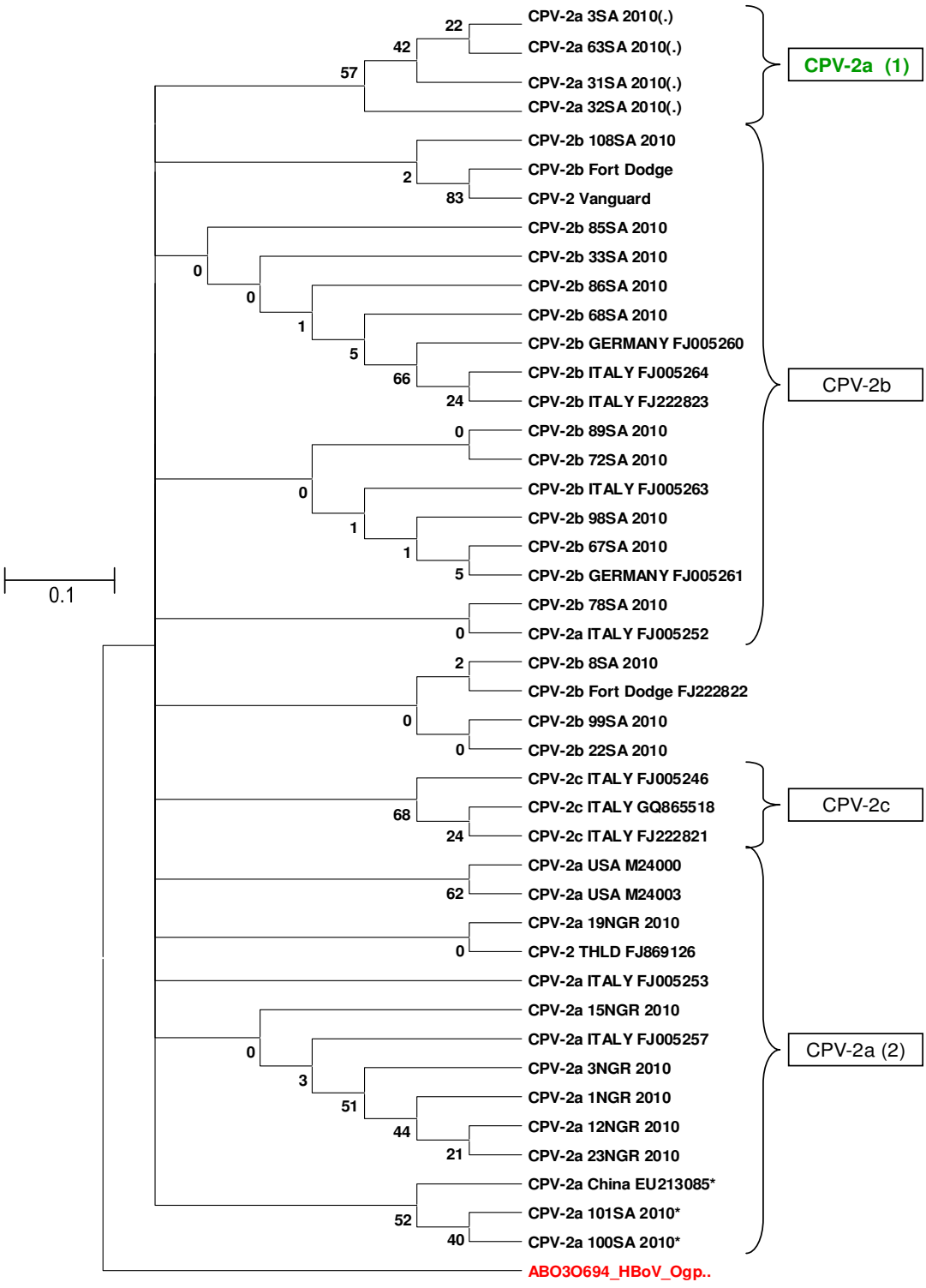


Figure 13 A rooted amino acid (protein) phylogenetic tree of South African and Nigerian viruses compared with other sequences retrieved from GenBank and contructed by using the NJ tree.

The amino acid or protein rooted phylogenetic tree was constructed using the NJ tree with the Mega 4 software and was subjected to 1000 replicates of bootstrap (Figure 13). The amino acid phylogenetic tree shows an interesting result for the first four CPV-2a viral strains namely CPV-2a (1) in green. This group of viruses was also grouped different from other South African field viruses and other viruses from other parts of the world. The CPV-2b represent the second group of viruses with four taxa including viruses from South Africa, Germany and Italy as well as the vaccine strains. The third group is the CPV-2c viruses mainly from Italy. The last group is the CPV-2a (2). This group contains CPV-2a viruses form USA, Nigeria, Thailand and China. It is noteworthy that only the CPV-2a (1) group and those viruses marked with an asterik in CPV-2a (2) as the fourth or last group also grouped together in both the nucleotide phylogenetic trees and the amino acid phylogenetic tree. Any other group that was contained in the nucleotide tree is not similarly presented the same in the amino acid tree. Human bocavirus (HBoV) is clearly seen as an outgroup.

4.6 Protein analysis

Nucleotide sequences were translated to amino acid sequences as shown in Figure 14 (Bonfield *et al*., 1995; Staden 1996; Staden *et al*., 2000). Amino acid sequence analysis showed seven amino acid changes at positions 265, 297, 324, 424, 426, 440 and 475. Residue 426 is involved in major antigenic mutations. All these changes observed were compared with other sequences including two vaccines strains. These changes in amino acid also are not major antigenic mutations of the CPV-2 strains, rather it can be referred to as minor antigenic mutations as it did not result in the generation of new CPV-2 strains.

Twelve SA CPV-2b strains {(72SA 2010); (33SA 2010); (67SA 2010); (86SA 2010); (89SA 2010); (68SA 2010); (98SA 2010); (99SA 2010); (78SA 2010); (8SA 2010); (85SA 2010); and (108SA 2010)}, as well as four CPV-2a {(3SA 2010); (32SA 2010); (63SA 2010) and (31SA 2010)} had an A→N (Ala to Asn) amino acid change at position 297 compared to the rest of the aligned amino sequences. Four South African CPV-2a strains (3SA 2010); (3SA 2010); (63SA 2010) and (31SA 2010)} had a T→K (Thr to Lys) amino acid change at position 265 when compared with other amino acid sequences in the aligment. Strains {CPV-2a (101SA 2010) and CPV-2a (100SA 2010)} had a Y→I (Tyr to Ile) amino acid change at position 324 when also compared to the rest of the

aligned amino acids sequences. Amino acid sequences from four SA strains {CPV-2a (3SA 2010); CPV-2a (32SA 2010); CPV-2a (63SA 2010) and CPV-2a (31SA 2010)} had a V→A (Val to Ala) amino acid change at position 424. Twelve strains {CPV-2b (72SA 2010); CPV-2b (33SA 2010); CPV-2b (67SA 2010); CPV-2b (86SA 2010); CPV-2b (89SA 2010); CPV-2b (68SA 2010); CPV-2b (98SA 2010); CPV-2b (99SA 2010); CPV-2b CPV-2b (78SA 2010); CPV-2b (8SA 2010); CPV-2b (85SA 2010) and CPV-2b (22SA 2010)} had an N→D (Asn to Asp) amino acid change at position 426 unlike other sequences in the alignment. Four SA strains {CPV-2b (3SA 2010); CPV-2b (32SA 2010); CPV-2b (63SA 2010) and CPV-2b (31SA 2010)} also had a T→A (Thr to Ala) amino acid change at position 440 and finally, strain CPV-2a (32SA 2010), had a D→N (Asp to Asn) amino acid change at position 475 when compared with other sequences in the alignment. Only one CPV-2a (3NGR 2010) virus of the six selected viruses from Nigeria had a T→A (Thr to Ala) amino acid change at position 440.

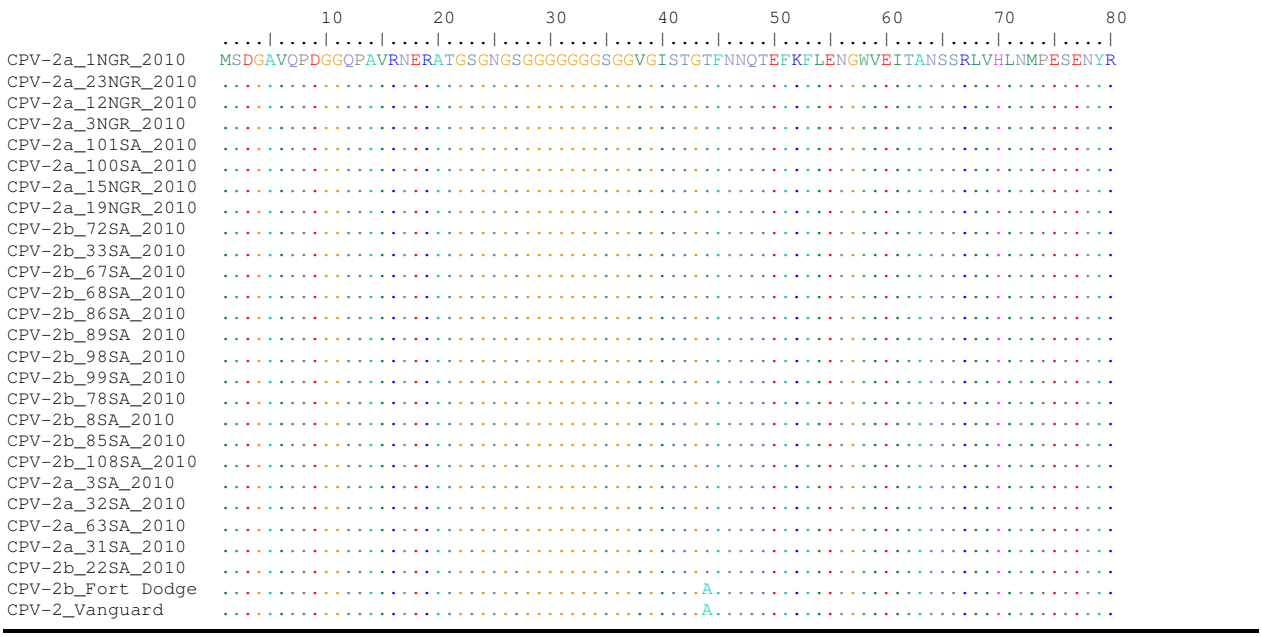
Some of the South African field isolates had more than two or four amino acid changes (Table 3). The CPV-2a of the SA strains (text in red with asteriks) had four different changes at positions 265, 297, 424 and 440 as seen in table 3, and one SA strain had five changes at positions 265, 297, 424, 440 and 475, while the CPV-2b (text in green) had two amino acid changes at position 297 and 426. One Nigerian CPV-2a had a shared amino acid change with SA field strains at position 440 (Figure 14).

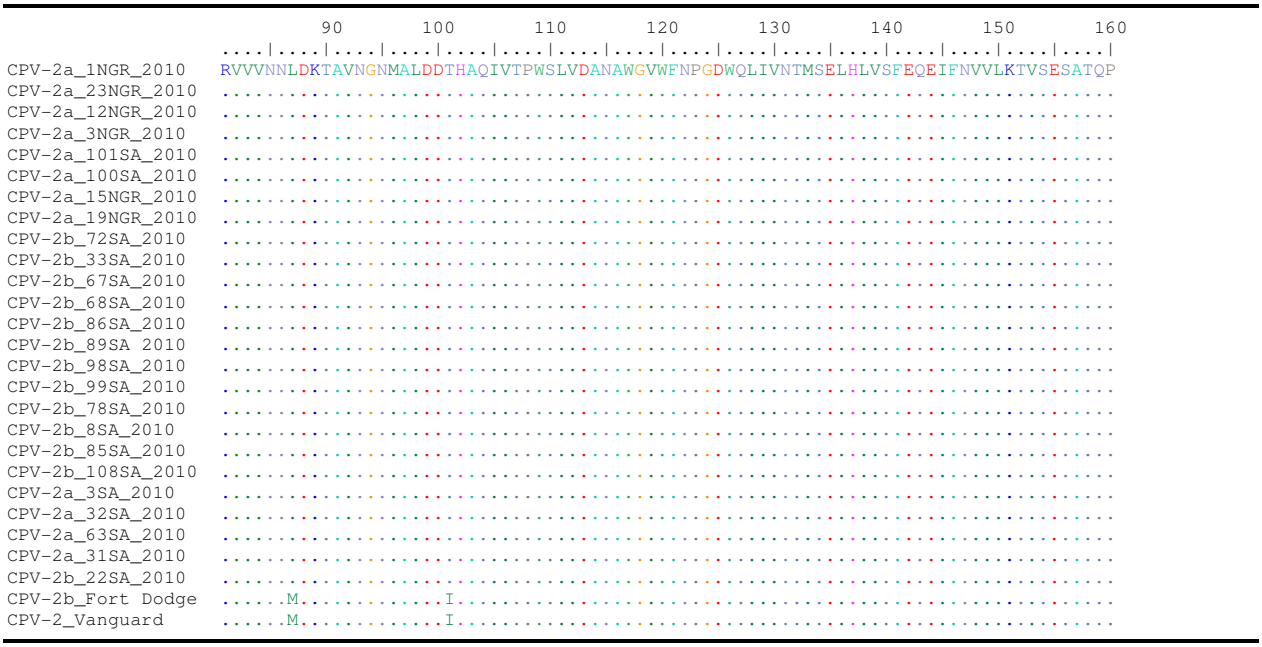
Table 3 Protein analysis showing the positions of changes of amino acids of South African and Nigerian CPV-2 field viruses

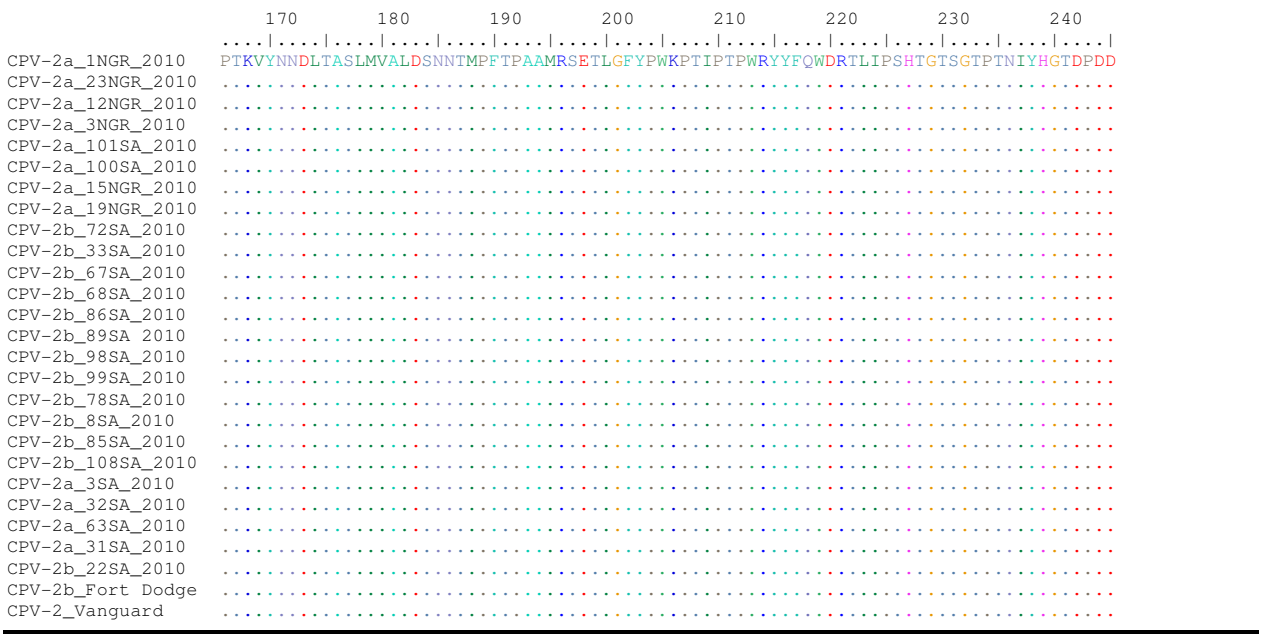
Position	265	297	342	424	426	440	475
Virus ID No.	3SA 2010*	72SA 2010	101SA 2010	3SA 2010*	72SA 2010	3SA 2010*	32SA 2010
	32SA 2010*	33SA 2010	100SA 2010	32SA 2010*	33SA 2010	32SA 2010*	
	63SA 2010*	67SA 2010		63SA 2010*	67SA 2010	63SA 2010*	
	31SA 2010*	86SA 2010		31SA 2010*	86SA 2010	31SA 2010*	
		89SA 2010			89SA 2010	3NGR 2010*	
		68SA 2010			68SA 2010		
		98SA 2010			98SA 2010		
		99SA 2010			99SA 2010		
		78SA 2010			78SA 2010		
		8SA 2010			8SA 2010		
		85SA 2010			85SA 2010		
		108SA 2010			22SA 2010		
		3SA 2010*					
		32SA 2010*					
		63SA 2010*					
		31SA 2010*					
Amino acid changes	T→K (Thr to Lys)	A→N (Ala to Asn)	Y→I (Tyr to Ile)	V→A (Val to Ala)	N→D (Asn to Asp)	T→A (Thr to Ala)	D→N (Asp to Asn)

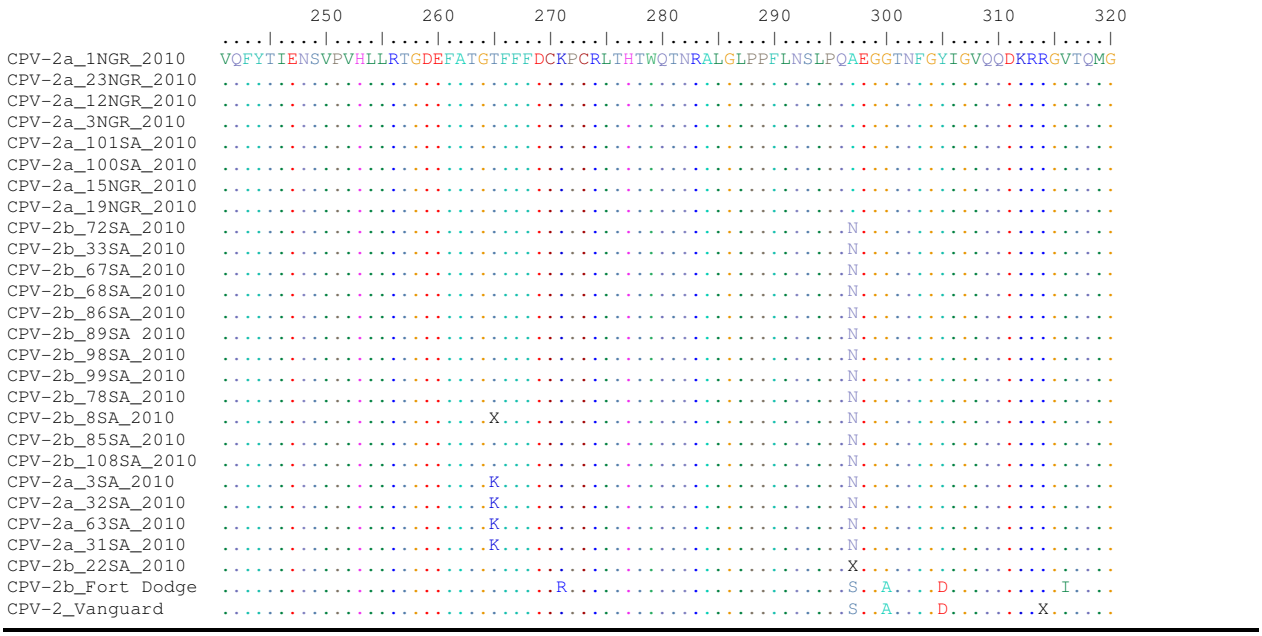
Figure 14 below indicates the amino acid sequence alignment of the VP2 encoding gene of CPV-2 South African field strains, Nigerian field strains and vaccines used in South Africa with the positions of the amino acid changes.

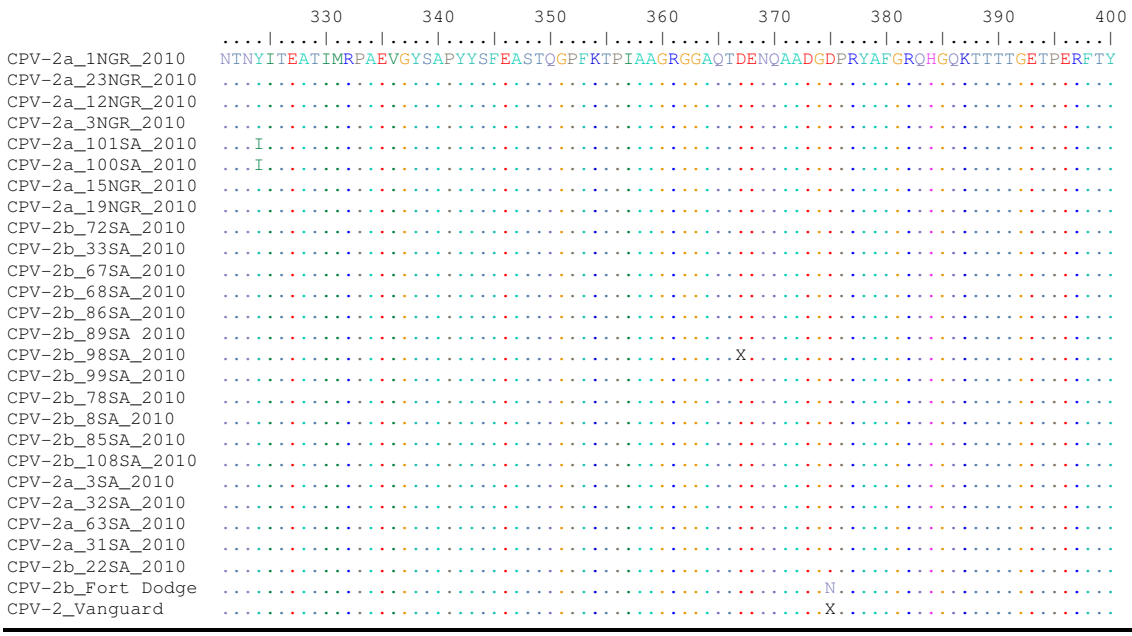
Key:
Dots (.) indicates similarities
Red=Nigerian field isolates
Orange=CPV-2a South African field isolates
Green=CPV-2b South African field isolates
Purple=Vaccines use in South Africa

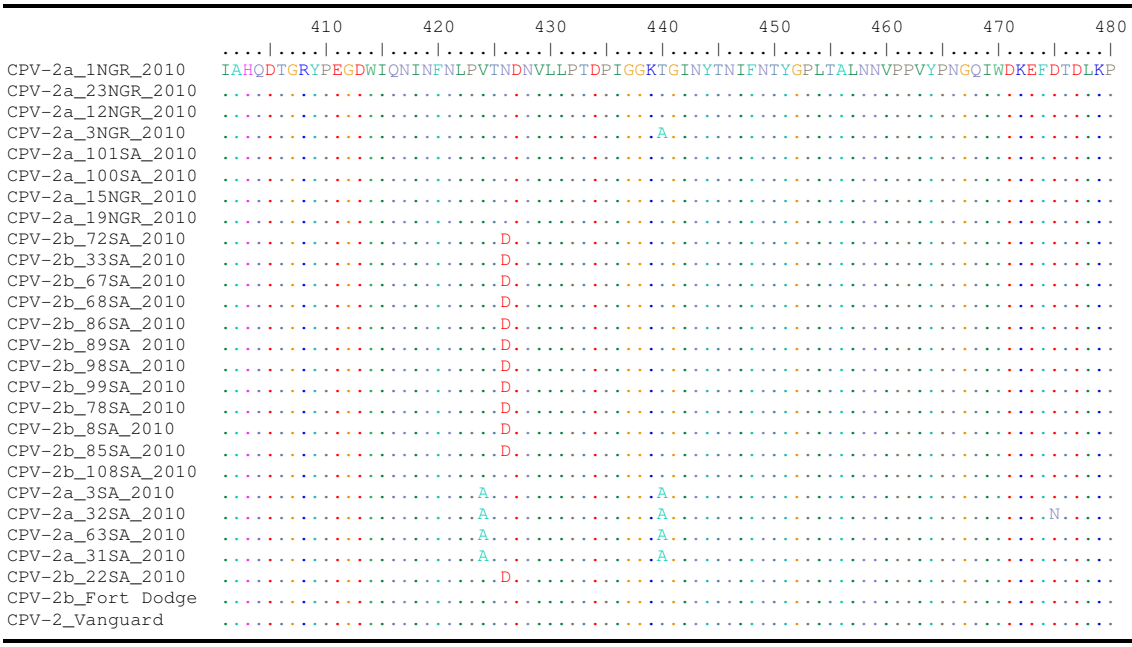





170 180 190 200 210 220 230 240
CPV-2a_1NGR_2010 PTKVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPWKPTIPTPWRYYFQWDRTLIPSHTGTSGTPTNIYHGTDPDD
CPV-2a_23NGR_2010
CPV-2a_12NGR_2010
CPV-2a_3NGR_2010
CPV-2a_101SA_2010
CPV-2a_100SA_2010
CPV-2a_15NGR_2010
CPV-2a_19NGR_2010
CPV-2b_72SA_2010
CPV-2b_33SA_2010
CPV-2b_67SA_2010
CPV-2b_68SA_2010
CPV-2b_86SA_2010
CPV-2b_89SA 2010
CPV-2b_98SA_2010
CPV-2b_99SA_2010
CPV-2b_78SA_2010
CPV-2b_8SA_2010
CPV-2b_85SA_2010
CPV-2b_108SA_2010
CPV-2a_3SA_2010
CPV-2a_32SA_2010
CPV-2a_63SA_2010
CPV-2a_31SA_2010
CPV-2b_22SA_2010
CPV-2b_Fort Dodge
CPV-2_Vanguard


250 260 270 280 290 300 310 320
CPV-2a_1NGR_2010 VQFYTIENSVPVHLLRTGDEFATGTFFFDCKPCRLTHTWQTNRALGLPPFLNSLPQAEGGTNFGYIGVQQDKRRGVTQMG
CPV-2a_23NGR_2010
CPV-2a_12NGR_2010
CPV-2a_3NGR_2010
CPV-2a_101SA_2010
CPV-2a_100SA_2010
CPV-2a_15NGR_2010
CPV-2a_19NGR_2010
CPV-2b_72SA_2010 N
CPV-2b_33SA_2010 N
CPV-2b_67SA_2010 N
CPV-2b_68SA_2010 N
CPV-2b_86SA_2010 N
CPV-2b_89SA 2010 N
CPV-2b_98SA_2010 N
CPV-2b_99SA_2010 N
CPV-2b_78SA_2010 N
CPV-2b_8SA_2010 X N
CPV-2b_85SA_2010 N
CPV-2b_108SA_2010 N
CPV-2a_3SA_2010 K N
CPV-2a_32SA_2010 K N
CPV-2a_63SA_2010 K N
CPV-2a_31SA_2010 K N
CPV-2b_22SA_2010 X
CPV-2b_Fort Dodge R S A D I
CPV-2_Vanguard S A D X


330 340 350 360 370 380 390 400
CPV-2a_1NGR_2010 NTNYITEATIMRPAEVGYSAPYYSFEASTQGPFKTPIAAGRGGAQTDENQAADGDPRYAFGRQHGQKTTTTGETPERFTY
CPV-2a_23NGR_2010
CPV-2a_12NGR_2010
CPV-2a_3NGR_2010
CPV-2a_101SA_2010
CPV-2a_100SA_2010
CPV-2a_15NGR_2010
CPV-2a_19NGR_2010
CPV-2b_72SA_2010
CPV-2b_33SA_2010
CPV-2b_67SA_2010
CPV-2b_68SA_2010
CPV-2b_86SA_2010
CPV-2b_89SA 2010
CPV-2b_98SA_2010
CPV-2b_99SA_2010
CPV-2b_78SA_2010
CPV-2b_8SA_2010
CPV-2b_85SA_2010
CPV-2b_108SA_2010
CPV-2a_3SA_2010
CPV-2a_32SA_2010
CPV-2a_63SA_2010
CPV-2a_31SA_2010
CPV-2b_22SA_2010
CPV-2b_Fort Dodge
CPV-2_Vanguard


410 420 430 440 450 460 470 480
CPV-2a_1NGR_2010 IAHQDTGRYPEGDWIQNINFNLPVTNDNVLLPTDPIGGKTGINYTNIFNTYGPLTALNNVPPVYPNGQIWDKEFDTDLKP
CPV-2a_23NGR_2010
CPV-2a_12NGR_2010
CPV-2a_3NGR_2010
CPV-2a_101SA_2010
CPV-2a_100SA_2010
CPV-2a_15NGR_2010
CPV-2a_19NGR_2010
CPV-2b_72SA_2010
CPV-2b_33SA_2010
CPV-2b_67SA_2010
CPV-2b_68SA_2010
CPV-2b_86SA_2010
CPV-2b_89SA 2010
CPV-2b_98SA_2010
CPV-2b_99SA_2010
CPV-2b_78SA_2010
CPV-2b_8SA_2010
CPV-2b_85SA_2010
CPV-2b_108SA_2010
CPV-2a_3SA_2010
CPV-2a_32SA_2010
CPV-2a_63SA_2010
CPV-2a_31SA_2010
CPV-2b_22SA_2010
CPV-2b_Fort Dodge
CPV-2_Vanguard

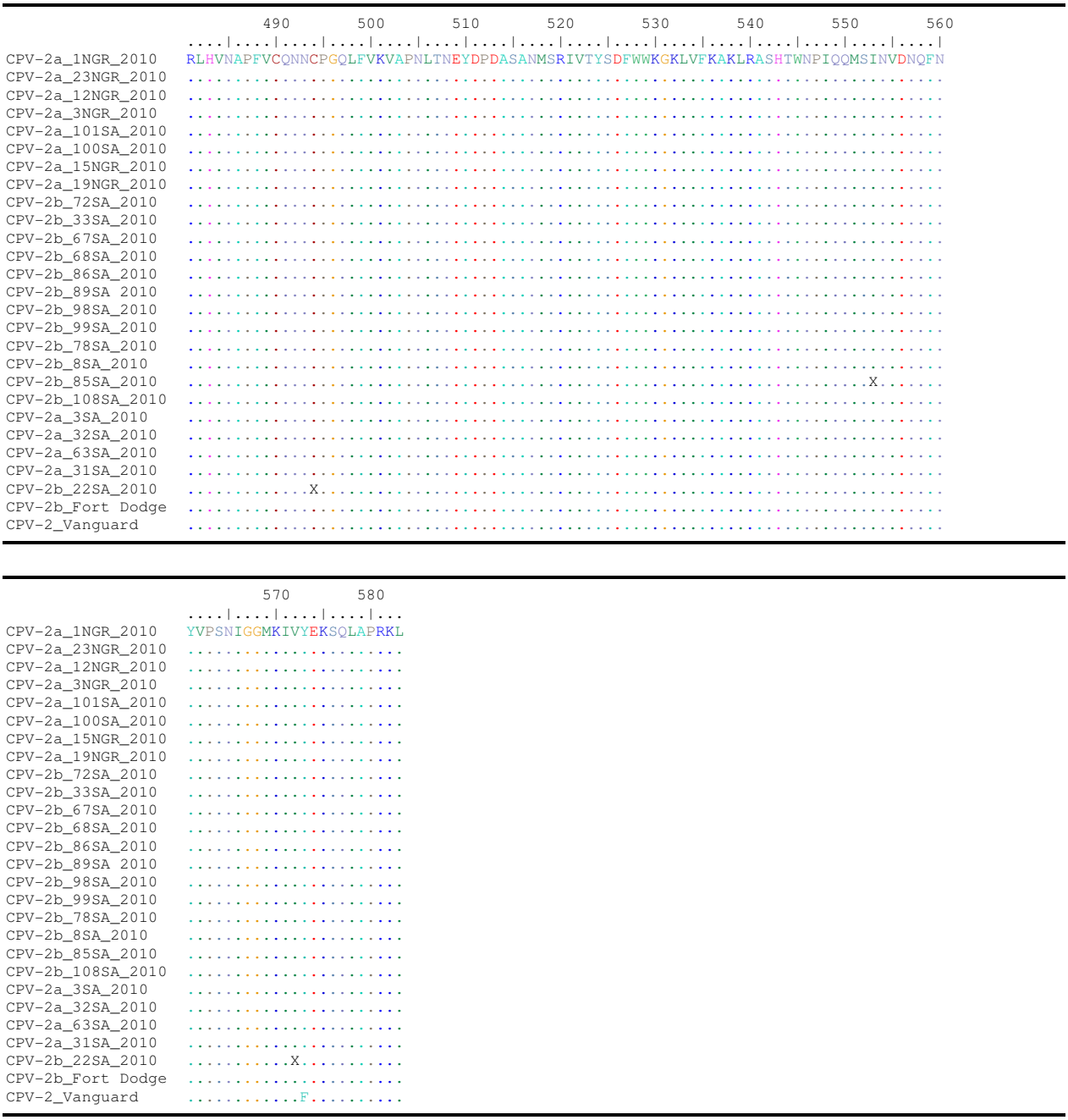


Figure 14 Amino acid sequence alignment of the VP2 encoding gene of CPV-2 South African field strains, Nigerian field strains and vaccines used in South Africa with the positions of the amino acid changes.

CHAPTER 5 : DISCUSSION

Canine parvovirus continues to be an important pathogen of dogs and is responsible for serious occurrences of morbidity and mortality, despite the availability of safe and effective vaccines (Decaro, *et al.,* 2006a, c). Since the replacement of the original CPV-2 virus by the CPV-2a/b variants and more recently the CPV-2c viruses (Parrish, *et al*., 1991; Martella, *et al.,* 2004), there have been concerns about the efficacy of canine parvovirus vaccines which are based on the original type 2 strains (Martella, *et al.,* 2005; Truyen, 2006). However, it has previously been demonstrated that the CPV-2 vaccines are able to provide protection against CPV-2a/2b field isolates (Greenwood *et al.,* 1995).

The molecular characterization of CPV-2 provides vital information about the strains circulating in a region at a particular time and also shows the relationship of these strains with CPV-2 strains from other parts of the world. Canine parvovirus type 2 has been studied and characterized in southern Africa. However, there is no documented record with regards to the genetic analysis of the full length VP2-encoding gene of the virus for the purpose of comparisons. This study represents the first documentation of this nature on the African continent. Similar studies have been completed outside Africa (Bo-Kyu Kang, *et al.,* 2008; Touihri, *et al.,* 2009).

The main objectives of this study were to detect CPV-2 viruses in faecal samples obtained from sick dogs in different areas of South Africa and Nigeria using conventional PCR, and to genetically characterize the strains using real-time PCR and sequencing. The use of the TaqMan assay and MGB probes technology could distinguish either between CPV-2a and 2b or and also CPV-2b and 2c. Finally sequences obtained from these isolates were compared and phylogenetically grouped with existing CPV-2 isolates from different parts of the world. Typing of parvoviruses from carnivores was done in 1998 in South Africa by means of the haemagglutination inhibition test (Steinel *et al.,* 1998), while parvoviruses from Nigeria have never been characterized.

The conventional PCR was used in this study for the detection of CPV-2 from field samples. A 583 bp section of the CPV-2 VP2-encoding gene was amplified according to the method of Buonavoglia *et al*. (2001). One hundred and six faecal samples from South Africa yielded positive results, while 30 of the 31 Nigerian samples were positive. The genotyping results revealed that 100 (94.34%) of the South African samples contained CPV-2b and 6 (5.66%) CPV-2a**,** while all the Nigerian samples [n=30 (100%)] contained only CPV-2a. There were no positive samples that contained CPV-2c. The result may imply that type 2c has not yet been introduced into South Africa or Nigeria by means of the movement of animals. Another explanation may be that the limited number of samples and the geographic areas that were targeted were not large enough to conclusively rule out the presence of type 2c strains in South Africa and Nigeria.

The finding that type 2b predominates in South Africa is in agreement with the study results of Steinel *et al*., (2001) in which CPV-2b was found to be the more predominant strain of CPV-2 in southern Africa. This is also in agreement with the prevalence of CPV-2b which was shown to be the predominant type in other parts of the world i.e. the United States of America (USA), Japan and Turkey (Parrish *et al.,* 1991; Sagazio *et al.,* 1998; Yilmaz *et al.,* 2005).

This is the first documented study to genetically characterize strains of CPV-2 using molecular methods in Nigeria. Canine parvovirus was first reported in Nigeria in 1985 by means of the haemagglutination inhibition test (Kamalu, 1985). In that study, CPV-2a [n=30 (100%)] was the only antigenic variant that was found circulating in Nigeria, A limiting factor in the present study was that all the samples from Nigeria were obtained from one geographical area and were mainly from local breeds and a few from German shepherd dogs. The CPV-2a variant has also been found to be the predominant one circulating in certain countries such as Italy and Germany (Buonavoglia *et al.,* 2000; 2001; Martin *et al.,* 2002; Mochizuki *et al.,* 2001).

The new antigenic variant, CPV-2c that was reported for the first time in Italy by Buonavoglia *et al.,* (2001) was not detected in any of the South African and Nigerian samples. It must be emphasized that most of the South African samples were collected in the Gauteng Province, with fewer samples collected in Mpumalanga, Eastern Cape and Western Cape Provinces, while the Nigerian samples were collected only in Jos, Plateau State located in Central Nigeria. These factors may have contributed to the

wide margin between the CPV-2b and CPV-2a strains in South Africa when compared with the results of Steinel *et al.,* (1998). The dominance of CPV-2a in Nigeria can most likely be ascribed to the small geographical area that was targeted.

The genotyping of the CPV-2 viruses was done targeting the same gene that was used for the detection of the virus in faeces by means of conventional PCR. Similar studies have also been done by Desario *et al.,* (2005); Decaro *et al.,* (2006b; 2008); Marina *et al., (*2009) and Renzhou *et al.,* (2010). It contributed to understanding the rapid mutation that canine parvoviruses underwent from the original CPV-2 to CPV-2a and CPV-2b strains that subsequently became the predominant types worldwide. A third mutant was recently described in Italy as CPV-2c by Buonavoglia *et al.,* (2001), and has since been reported in many European countries as well as the USA. The existence of CPV-2c can be attributed to the continuing mutation of the CPV-2 virus. It is important to know what strain of virus is circulating in a region at a particular point in time, so that vaccine producers can consider and review the antigens included in their products for the effective control of CPV-2-induced disease.

In this respect it should be mentioned that the canine parvovirus vaccine produced by Pfizer, namely Vanguard, still contains the original CPV-2 which is now extinct. Efficacy studies have however, confirmed that it provides partial cross-protection against CPV-2a and 2b. Other commercial vaccines include either type 2a or 2b or both, even though sequence differences of the strains may occur. The present generation of vaccines is yet to include the recently discovered CPV-2c.

Failure of vaccination as a result of the presence of maternally-derived antibody in puppies up to 16-weeks-of-age has been widely documented. It is the result of maternally-derived antibody falling below protective levels but which retains high enough levels to block an active immune response by the vaccinated puppies. In this study, some interesting cases of vaccinated dogs that yielded positive samples were encountered. One case involved a 9-week-old female German shepherd puppy vaccinated with Vanguard plus vaccine at six weeks of age. She became ill three-and-a-half weeks after vaccination. The most likely explanation is that the pup had not yet developed active immunity as a result of the blocking effect of maternally-derived antibody at the age of six weeks. It is reasonable to argue that most vaccinated

puppies that develop parvovirosis lacked active protective immunity as a result of colostral interference.

Another noticeable case was a vaccinated bitch which gave birth to two puppies. She was diagnosed with parvovirosis with the aid of conventional PCR and was found to be positive for CPV-2b by means of real-time PCR. One of the puppies also became sick and was found to be positive for CPV-2b following the first vaccination. The other puppy was not sick but was likewise found to be positive for CPV-2b. It is probable that the healthy pup became infected from the sick puppy shedding virus where they were kept by the breeder on the same premises.

The TaqMan and the MGB probe technology were used in this study for the real-time PCR assay because it is specific, more reproducible, allows the detection and quantification of CPV-2 nucleic acid within a few hours, and is less time consuming. The risk of carry-over contamination of the master mix or reactions is less than with conventional PCR (Desario, *et al.,* 2005). In addition, the real-time PCR minor groove binder probes™ technology was chosen because of it's utilization of the MGB which attaches to single-stranded DNA probes that enhances the stability of the duplex formed between the probes and the target region of the CPV-2 genome, allowing an increase in the melting temperature (Tm) of the DNA duplex, and enabling the use of smaller probes capable of detecting short conserved regions of the CPV-2 genome (Kutyavin, *et al.,* 2000). This technology has been used before by Decaro *et al.* (2005a; 2005c; 2006b) for diagnosis of parvovirosis with good results. Hong *et al.,* (2007) reported on the sensitivity of the real-time PCR compared to other techniques including the conventional PCR.

The two phylogenetic trees constructed using Mega 4 with a bootstrap value of 1000 replicates, yielded the same results. The same lineages and clades formed by the viruses and depicted in the NJ phylogenetic tree (Figure 11) were also observed in the case of the MP phylogenetic tree (Figure 12), confirming the similarity of the two phylogenetic trees. Therefore, only the NJ phylogenetic tree will be used for the purpose of discussion.

The rooted NJ phylogenetic tree revealed three major lineages, namely A, B and C that are separately grouped, and an out-group represented by canine distemper virus. The group that formed lineage A further consists of three clades namely A1, A2 and A3 (Figure 11). The commercial vaccines produced by Fort Dodge and Pfizer grouped as a sub-clade in clade A1. The sub-clade formed by these two vaccine viruses may have been due to their similarities in amino acids that made them group together but also made them different from other viruses from South Africa, Nigeria and other parts of the world. Two South African CPV-2a viruses also grouped in this clade as a sub-clade. They were obtained from the same geographical region (Cape Town), and likely represent the dominant type circulating in that region. Clade A1 also contains four Nigerian type 2a viruses (1NGR 2010, 3NGR 2010, 12NGR 2010, and 23NGR 2010). All these South African and Nigerian CPV-2a viruses grouped together with other CPV-2a strains from the USA, China and Thailand in clade A1 and this shows the relationship between them. In clade A2, one South African virus (22SA 2010) (Figure 12, underlined) grouped together with other CPV-2b viruses from Italy and Germany. The CPV-2c virus also grouped in clade A2. The last clade of lineage A is A3 which contains two Nigerian viruses (19NGR 2010 and 15NGR 2010) which grouped together with Italian isolates.

Lineage B represents mainly the South African field viruses and is likely the predominant type circulating at the time of sample collection. The last Lineage C includes four of the South African viruses that seem to be genetically different from CPV-2a field isolates from other parts of the world.

A rooted amino acid or protein phylogenetic tree was constructed using the NJ of the Mega 4 software and was subjected to 1 000 replicates of bootstrap (Figure 11). The amino acid phylogenetic tree shows an interesting result for the first four CPV-2a viruses indicated with dots (.). This group of viruses is different from other South African field viruses as well as other viruses from other parts of the world as indicated in the nucleotide phylogenetic trees presented in Figures 11 and 12. It indicates a unique SA CPV-2a virus group designated C in the nucleotide tree.

It must be emphasized that the resolution from the amino acid sequence tree is not as good as that from the nucleotide sequence tree because there are fewer variations at the amino acid level than those occurring at the nucleotide level. The CPV-2b viruses formed the second group of viruses that includes viruses from South Africa, Germany and Italy as well as the vaccine viral strains. The third group of viruses is the CPV-2c strains mainly from Italy which also grouped together. The last group is the CPV-2a viruses form the USA, Nigeria, Thailand and China which showed their relationship as a group. It is important to note that only CPV-2a (1) and CPV-2a (2) (Figure 11) grouped together in both the nucleotide phylogenetic trees and the amino acid phylogenetic tree but any other group that was contained in the nucleotide tree is not presented the same in the amino acid tree. Canine distemper virus (CDV) is clearly seen as an out-group indicated in red (Figure 14).

The protein analysis revealed a mutation site among the CPV-2a/2b isolates at position 324 that was first detected in China in 2004 and also reported in three isolates from Korea in the same year (Jeoung, *et al*., 2008). Other recent studies reported that mutations affecting important residues of the capsid proteins at positions 297, 300 and 426 suggest that canine parvovirus is still evolving (Martella, *et al*., 2006;Truyen, 2006).

In this study, sixteen (16) South African field isolates of CPV-2 virus comprising 12 CPV-2b and 4 CPV-2a nucleotide sequences were translated to proteins (Figure 14) for protein analysis (Bonfield *et al*., 1995; Staden 1996; Staden *et al*., 2000). The results of the protein analysis interestingly showed seven changes of amino acids at positions 265, 297, 324, 424, 426, 440 and 475 in the majority of the South African viruses. The following four South Africans strains (3SA 2010; 32SA 2010; 63SA 2010 and 31SA 2010) had a T→K (Threonine to Lysine) amino acid at position 265. All 16 strains: 72SA 2010; 33SA 2010; 67SA 2010; 86SA 2010; 89SA 2010; 68SA 2010; 98SA 2010; 99SA 2010; 78SA 2010; 8SA 2010; 85SA 2010; 108SA 2010; 3SA 2010; 32SA 2010; 63SA 2010 and 31SA 2010, had an interesting and unique A→N (Alanine to Asparagine) amino acid at position 297. This change was also recognised recently (Martella *et al.,* 2006; Truyen, 2006c). Other authors have also reported the replacement Serine297/Asparagine, which is unique for the local CPV-2 types (Marina *et al.,* 2009). It is not surprising that this number of South African viruses have this change because they were mostly collected in the same province (Gauteng). This substitution was not observed in any of the Nigerian field isolates.

It has been reported that mutation Ala297 does not change the viral antigenic type and so the Ala297 variant cannot be distinguished serologically. However, the emergence and spread of this variant indicates that the Ala297 mutation potentially has had a marked influence on the process of continuing host adaptation, and previous research has shown that site 297 is under strong positive selection pressure (Pereira, *et al.,* 2007). Strains 101SA 2010 and 100SA 2010 had a unique Y→I (Tyrosine to Isoleusine) amino acid at position 324. In the case of strains 3SA 2010; 32SA 2010; 63SA 2010 and 31SA 2010, there was a V→A (Valine to Alanine) amino acid change at position 424. Strains 72SA 2010; 33SA 2010; 67SA 2010; 86SA 2010; 89SA 2010; 68SA 2010; 98SA 2010; 99SA 2010; 78SA 2010; 8SA 2010; 85SA 2010 and 22SA 2010 had a unique N→D (Asparagine to Aspartic acid) amino acid change at position 426. This substitution of the CPV-2 VP2 protein has also been reported (Parrish *et al.,* 1991; Nakamara *et al.,* 2004; Ikeda *et al*., 2000 and Bo-Kyu *et al*., 2008).

There was a recent report of a mutant with a change at residue 426 (Aspartic acid→Glutamic acid) occurring in a strategic residue for the antigenicity of CPV-2 which has been detected in Italy by Buonavoglia *et al.,* (2001), and has been reported in other countries (Nakamara *et al.,* 2004; and Decaro *et al.,* 2006a). Moreover, while the mutations at residues 265, 297, 324, 424, 426, 440, and 475 are located at a minor antigenic site, the mutations at residue 426 affects the major antigenic region that has been taken into account for the classification of the strains of CPV-2a and CPV-2b (Parrish *et al.,* 1991). On the basis of the above considerations, Glutamic acid-426 mutant has been referred to as true antigenic strains of CPV-2c, whereas the mutations at T→K (Thr to Lys) position 265; A→N (Ala to Asn) position 297; Y→I (Tyr to Ile) position 324. V→A (Val to Ala) position 424. N→D (Asn to Asp) position 426, T→A (Thr to Ala) position 440; D→N (Asp to Asn) position 475 should be regarded as mutants of CPV-2a and CPV-2b.

Four strains, namely 3SA 2010; 32SA 2010; 63SA 2010 and 31SA 2010 also had a T→A (Thr to Ala) amino acid at position 440 and finally, strain 32SA 2010 revealed a D→N (Asp to Asn) amino acid at position 475. Some of the South African viruses had multiple or triple changes as observed from the translated nucleotide sequences.

One of the Nigerian parvoviruses (3NGR 2010) only revealed a T→A (Thr to Ala) amino acid at position 440 similar to the four South African field isolates as described above. This study is the first report of the full length VP2 encoding gene region of the CPV-2a and CPV-2b strains with mutations at the following residues 265, 297, 324,424, 426, 440, and 475 in South Africa. Furthermore, one Nigerian field virus also had a mutation at position 440 as the first of its kind. These changes were observed in the grouping of the nucleotide phylogenetic trees and was also confirmed or proven by the amino acid or protein tree analyses and is seen in the amino acid alignment in Figure 13.

CONCLUSIONS AND RECOMMENDATION

Canine parvovirus remains an important pathogen of dogs and is responsible for serious occurrences of morbidity and mortality, despite the availability of safe and effective vaccines (Decaro, *et al.,* 2006a, c). Since the replacement of the original CPV-2 virus by the CPV-2a/b variants and more recently the CPV-2c viruses (Parrish, *et al*., 1991; Martella, *et al.,* 2004), there have been concerns about the efficacy of canine parvovirus vaccines which are based on the original type 2 strains (Martella, *et al.,* 2005; Truyen, 2006). However, it has previously been demonstrated that the CPV-2 vaccines are able to provide protection against CPV-2a/2b field isolates (Greenwood *et al.,* 1995).

In this study, it was found that in South Africa, CPV-2b is the most predominant strain. Among the Nigerian field viruses only the CPV-2a type was recorded. The CPV-2c was not reported in this study. The relationship among the CPV-2 strains in South Africa and Nigeria and other parts of the world was determined by phylogenetic methods in which some of the South African field viruses CPV-2a/b grouped separately. The protein analysis also showed the CPV-2a/b mutants in some residues of these field viruses that caused them to cluster separately. However, sampling was partially biased, since not all Provinces/States of the two countries were covered. Ideally, molecular epidemiological studies should be carried out country-wide in order to have an in-depth knowledge of the CPV-2 strains that are predominantly circulating within countries and to enable proper planning towards developing efficacious vaccines that will contain the various CPV-2 strains circulating in those countries for proper control of CPV-2.

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APPENDIX 1 :
Experimental data form

An example of an experimental data form for samples collection

Name: **Dogonyaro, B.B.** Date: **04/01/2009**

Type: **Faeces** Time: **8:30 am**

Name/ID No.									History of Vaccination		Sheltered	
S/no.	**Patient**	**Age**	**Sex**	**Breed**	**Area**	**Clinical signs**	**Pos.**	**Neg.**	**Yes**	**No**	**Yes**	**No**
1N	Jack	4 Months	M	C. breed	Gada biyu	Vomiting, diarrheoa, Anorexia & 37.1 ℃	+			√	√	
2N	General	6 Momths	M	C. breed	Rukuba road	Vomiting, diarrheoa, Anorexia & 38 ℃	+			√	√	
3N	Rambo	5 Months	M	Mongrel	Bauchi road	Vomiting, diarrheoa, Anorexia & 38.7 ℃	+			√		√
4N	Black Jnr.	6 Months	F	Mongrel	Rukuba road	diarrheoa, Anorexia & 37.9 ℃	+			√	√	
5N	S.M.	1½Months	F	C. breed	Janta Adamu	Foul odour stool, vomiting, 37.9 ℃	+			√	√	
6N	Osama	1 Months	M	Mongrel	Rukuba road	diarrheoa, 39.9 ℃ vomiting,& anorexia	+			√	√	
7N	Chelsea	1½Months	M	C. breed	Janta Adamu	Vomiting, diarrhea, Mucoid foul odour stool & 38.7 ℃	+			√	√	
8N	Sauki-1	5 weeks	M	Mongrel	Rikkos	Vomiting, diarrheoa, foul odour stool, anorexia & 37.9 ℃	+			√		√

APPENDIX 2 :
Samples screened

The 140 field samples screened by conventional PCR from both South Africa and Nigeria
(Bonavoglia *et al.,* 2001), Y=vaccinated, N=not vaccinated, NA=vaccination history not available, Y=positive, N=negative and CPV-2 positive controls (100/09/CPV-2a and 243/07/CPV-2c) from Italy and Virbac vaccine obtained in South Africa.

S/No.	Name	Origin	Sample Type	Vaccinated	Conv. PCR
1	1N	Nigeria	Faeces	N	P
2	2N	Nigeria	Faeces	N	P
3	3N	Nigeria	Faeces	NA	P
4	4N	Nigeria	Faeces	N	P
5	5N	Nigeria	Faeces	N	P
6	6N	Nigeria	Faeces	N	P
7	7N	Nigeria	Faeces	N	P
8	8N	Nigeria	Faeces	N	P
9	9N	Nigeria	Faeces	N	P
10	10N	Nigeria	Faeces	NA	P
11	11N	Nigeria	Faeces	N	P
12	12N	Nigeria	Faeces	N	P
13	13N	Nigeria	Faeces	N	P
14	14N	Nigeria	Faeces	N	P
15	15N	Nigeria	Faeces	N	P
16	16N	Nigeria	Faeces	N	P
17	17N	Nigeria	Faeces	N	P
18	18N	Nigeria	Faeces	N	P
19	19N	Nigeria	Faeces	N	P
20	20N	Nigeria	Faeces	N	P
21	21N	Nigeria	Faeces	N	P
22	22N	Nigeria	Faeces	N	P
23	23N	Nigeria	Faeces	N	P
24	24N	Nigeria	Faeces	N	P
25	25N	Nigeria	Faeces	N	P
26	26N	Nigeria	Faeces	Y	N
27	27N	Nigeria	Faeces	N	P
28	28N	Nigeria	Faeces	N	P
29	29N	Nigeria	Faeces	N	P
30	30N	Nigeria	Faeces	N	P

S/No.	Name	Origin	Sample Type	Vaccinated	Conv. PCR
31	31N	Nigeria	Faeces	N	P
32	SA1	South Africa	Faeces	NA	P
33	SA2	South Africa	Faeces	NA	P
34	SA3	South Africa	Faeces	NA	P
35	SA4	South Africa	Faeces	NA	P
36	SA5	South Africa	Faeces	NA	P
37	SA6	South Africa	Faeces	N	P
38	SA7	South Africa	Faeces	N	P
39	SA8	South Africa	Faeces	N	P
40	SA9	South Africa	Faeces	N	P
41	SA10	South Africa	Faeces	N	P
42	SA11	South Africa	Faeces	Y	P
43	SA12	South Africa	Faeces	Y	P
44	SA13	South Africa	Faeces	N	P
45	SA14	South Africa	Faeces	N	P
46	SA15	South Africa	Faeces	Y	P
47	SA16	South Africa	Faeces	NA	P
48	SA17	South Africa	Faeces	NA	P
49	SA18	South Africa	Faeces	N	P
50	SA19	South Africa	Faeces	NA	P
51	SA20	South Africa	Faeces	N	P
52	SA21	South Africa	Faeces	Y	P
53	SA22	South Africa	Faeces	Y	P
54	SA23	South Africa	Faeces	NA	P
55	SA24	South Africa	Faeces	NA	P
56	SA25	South Africa	Faeces	Y	P
57	SA26	South Africa	Faeces	NA	N
58	SA27	South Africa	Faeces	NA	P
59	SA28	South Africa	Faeces	Y	N
60	SA29	South Africa	Faeces	Y	P
61	SA30	South Africa	Faeces	Y	P
62	SA31	South Africa	Faeces	NA	P
63	SA32	South Africa	Faeces	Y	P
64	SA33	South Africa	Faeces	N	P
65	SA34	South Africa	Faeces	N	P
66	SA35	South Africa	Faeces	N	P
67	SA36	South Africa	Faeces	Y	P
68	SA37	South Africa	Faeces	Y	P
69	SA38	South Africa	Faeces	N	P
70	SA39	South Africa	Faeces	NA	P

S/No.	Name	Origin	Sample Type	Vaccinated	Conv. PCR
71	SA40	South Africa	Faeces	N	P
72	SA41	South Africa	Faeces	Y	P
73	SA42	South Africa	Faeces	Y	P
74	SA43	South Africa	Faeces	Y	P
75	SA44	South Africa	Faeces	Y	P
76	SA45	South Africa	Faeces	Y	P
77	SA46	South Africa	Faeces	N	P
78	SA47	South Africa	Faeces	Y	P
79	SA48	South Africa	Faeces	Y	P
80	SA49	South Africa	Faeces	NA	P
81	SA50	South Africa	Faeces	NA	P
82	SA51	South Africa	Faeces	NA	P
83	SA52	South Africa	Faeces	NA	P
84	SA53	South Africa	Faeces	NA	P
85	SA54	South Africa	Faeces	NA	P
86	SA55	South Africa	Faeces	NA	P
87	SA56	South Africa	Faeces	NA	P
88	SA57	South Africa	Faeces	Y	P
89	SA58	South Africa	Faeces	Y	P
90	SA59	South Africa	Faeces	N	P
91	SA60	South Africa	Faeces	N	P
92	SA61	South Africa	Faeces	N	P
93	SA62	South Africa	Faeces	Y	P
94	SA63	South Africa	Faeces	Y	P
95	SA64	South Africa	Faeces	N	P
96	SA65	South Africa	Faeces	Y	P
97	SA66	South Africa	Faeces	N	P
98	SA67	South Africa	Faeces	N	P
99	SA68	South Africa	Faeces	Y	P
100	SA69	South Africa	Faeces	NA	P
101	SA70	South Africa	Faeces	NA	P
102	SA71	South Africa	Faeces	NA	P
103	SA72	South Africa	Faeces	NA	P
104	SA73	South Africa	Faeces	NA	P
105	SA74	South Africa	Faeces	NA	P
106	SA75	South Africa	Faeces	NA	P
107	SA76	South Africa	Faeces	N	P
108	SA77	South Africa	Faeces	Y	P
109	SA78	South Africa	Faeces	Y	P
110	SA79	South Africa	Faeces	NA	P

S/No.	Name	Origin	Sample Type	Vaccinated	Conv. PCR
111	SA80	South Africa	Faeces	NA	P
112	SA81	South Africa	Faeces	NA	P
113	SA82	South Africa	Faeces	NA	P
114	SA83	South Africa	Faeces	NA	P
115	SA84	South Africa	Faeces	NA	P
116	SA85	South Africa	Faeces	Y	P
117	SA86	South Africa	Faeces	Y	P
118	SA87	South Africa	Faeces	Y	P
119	SA88	South Africa	Faeces	Y	P
120	SA89	South Africa	Faeces	Y	P
121	SA90	South Africa	Faeces	Y	P
122	SA91	South Africa	Faeces	NA	P
123	SA92	South Africa	Faeces	N	P
124	SA93	South Africa	Faeces	NA	P
125	SA94	South Africa	Faeces	NA	P
126	SA95	South Africa	Faeces	NA	P
127	SA96	South Africa	Faeces	N	P
128	SA97	South Africa	Faeces	N	P
129	SA98	South Africa	Faeces	Y	P
130	SA99	South Africa	Faeces	Y	P
131	SA100	South Africa	Faeces	Y	P
132	SA101	South Africa	Faeces	Y	P
133	SA102	South Africa	Faeces	Y	P
134	SA103	South Africa	Faeces	Y	P
135	SA104	South Africa	Faeces	Y	P
136	SA105	South Africa	Faeces	NA	P
137	SA106	South Africa	Faeces	NA	P
138	SA107	South Africa	Faeces	Y	P
139	SA108	South Africa	Faeces	Y	P
140	111	Italy	100/09/CPV-2a		P
141	112	USA	Virbac vaccine		P
142	113	Italy	243/07/CPV-2c		P

APPENDIX 3 :
MGB results

The 136 genotyped using the MGB results as revealed by a real-time PCR assay for CPV-2a/b and CPV-2b/c (Decaro *et al.,* 2006b) from SA and Nigeria. Y=vaccinated, N=not vaccinated, NA=vaccination history not available, and CPV-2a/c positive controls from Italy=green, CPV-2b positive control (Fort Dodge vaccine=blue, SA CPV-2a=red.

S/No.	Name	Origin	Sample Type	MGB genotyped	Vaccinated	Sheltered
1	1N	Nigeria	Faeces	2a	N	Y
2	2N	Nigeria	Faeces	2a	N	Y
3	3N	Nigeria	Faeces	2a	NA	N
4	4N	Nigeria	Faeces	2a	N	N
5	5N	Nigeria	Faeces	2a	N	N
6	6N	Nigeria	Faeces	2a	N	N
7	7N	Nigeria	Faeces	2a	N	Y
8	8N	Nigeria	Faeces	2a	N	N
9	9N	Nigeria	Faeces	2a	NA	Y
10	10N	Nigeria	Faeces	2a	N	N
11	11N	Nigeria	Faeces	2a	N	N
12	12N	Nigeria	Faeces	2a	N	Y
13	13N	Nigeria	Faeces	2a	N	N
14	14N	Nigeria	Faeces	2a	N	N
15	15N	Nigeria	Faeces	2a	N	Y
16	16N	Nigeria	Faeces	2a	N	Y
17	17N	Nigeria	Faeces	2a	N	N
18	18N	Nigeria	Faeces	2a	N	Y
19	19N	Nigeria	Faeces	2a	N	N
20	20N	Nigeria	Faeces	2a	N	N
21	21N	Nigeria	Faeces	2a	N	N
22	22N	Nigeria	Faeces	2a	N	N
23	23N	Nigeria	Faeces	2a	N	N
24	24N	Nigeria	Faeces	2a	N	Y
25	25N	Nigeria	Faeces	2a	N	N
26	27N	Nigeria	Faeces	2a	N	Y
27	28N	Nigeria	Faeces	2a	N	N
28	29N	Nigeria	Faeces	2a	N	N
29	30N	Nigeria	Faeces	2a	N	N

S/No.	Name	Origin	Sample Type	MGB genotyped	Vaccinated	Sheltered
30	31N	Nigeria	Faeces	2a	N	Y
31	SA1	South Africa	Faeces	2b	NA	Y
32	SA2	South Africa	Faeces	2b	NA	Y
33	SA3	South Africa	Faeces	2a	NA	Y
34	SA4	South Africa	Faeces	2b	NA	Y
35	SA5	South Africa	Faeces	2b	NA	Y
36	SA6	South Africa	Faeces	2b	N	Y
37	SA7	South Africa	Faeces	2b	N	Y
38	SA8	South Africa	Faeces	2b	N	Y
39	SA9	South Africa	Faeces	2b	N	Y
40	SA10	South Africa	Faeces	2b	N	Y
41	SA11	South Africa	Faeces	2b	Y	Y
42	SA12	South Africa	Faeces	2b	Y	Y
43	SA13	South Africa	Faeces	2b	N	Y
44	SA14	South Africa	Faeces	2b	N	Y
45	SA15	South Africa	Faeces	2b	Y	Y
46	SA16	South Africa	Faeces	2b	NA	Y
47	SA17	South Africa	Faeces	2b	NA	Y
48	SA18	South Africa	Faeces	2b	N	Y
49	SA19	South Africa	Faeces	2b	Y	Y
50	SA20	South Africa	Faeces	2b	N	Y
51	SA21	South Africa	Faeces	2b	Y	Y
52	SA22	South Africa	Faeces	2b	Y	Y
53	SA23	South Africa	Faeces	2b	N	Y
54	SA24	South Africa	Faeces	2b	N	Y
55	SA25	South Africa	Faeces	2b	Y	Y
56	SA27	South Africa	Faeces	2b	N	Y
57	SA29	South Africa	Faeces	2b	Y	Y
58	SA30	South Africa	Faeces	2b	Y	Y
59	SA31	South Africa	Faeces	2b	NA	Y
60	SA32	South Africa	Faeces	2a	Y	Y
61	SA33	South Africa	Faeces	2b	N	Y
62	SA34	South Africa	Faeces	2b	N	Y
63	SA35	South Africa	Faeces	2b	N	Y
64	SA36	South Africa	Faeces	2b	Y	Y
65	SA37	South Africa	Faeces	2b	Y	Y
66	SA38	South Africa	Faeces	2b	N	Y
67	SA39	South Africa	Faeces	2b	NA	Y
68	SA40	South Africa	Faeces	2b	N	Y

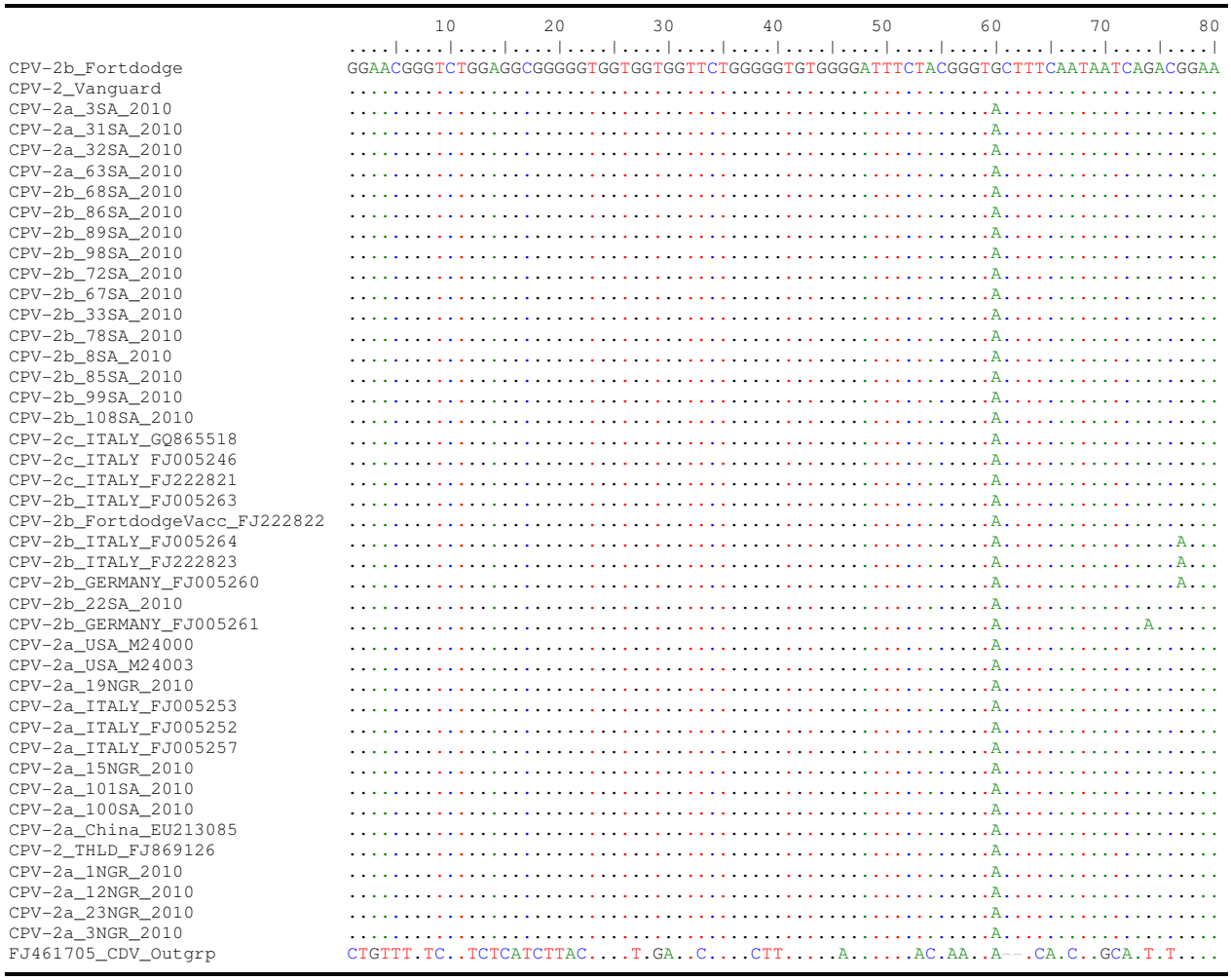
S/No.	Name	Origin	Sample Type	MGB genotyped	Vaccinated	Sheltered
69	SA41	South Africa	Faeces	2b	Y	Y
70	SA42	South Africa	Faeces	2b	Y	Y
71	SA43	South Africa	Faeces	2b	Y	Y
72	SA44	South Africa	Faeces	2b	Y	Y
73	SA45	South Africa	Faeces	2b	Y	Y
74	SA46	South Africa	Faeces	2b	N	Y
75	SA47	South Africa	Faeces	2b	Y	Y
76	SA48	South Africa	Faeces	2b	Y	Y
77	SA49	South Africa	Faeces	2b	NA	Y
78	SA50	South Africa	Faeces	2b	NA	Y
79	SA51	South Africa	Faeces	2b	NA	Y
80	SA52	South Africa	Faeces	2b	NA	Y
81	SA53	South Africa	Faeces	2b	NA	Y
82	SA54	South Africa	Faeces	2b	NA	Y
83	SA55	South Africa	Faeces	2b	NA	Y
84	SA56	South Africa	Faeces	2b	NA	Y
85	SA57	South Africa	Faeces	2b	Y	Y
86	SA58	South Africa	Faeces	2b	Y	Y
87	SA59	South Africa	Faeces	2b	N	Y
88	SA60	South Africa	Faeces	2b	N	Y
89	SA61	South Africa	Faeces	2b	N	Y
90	SA62	South Africa	Faeces	2b	Y	Y
91	SA63	South Africa	Faeces	2a	Y	Y
92	SA64	South Africa	Faeces	2b	N	Y
93	SA65	South Africa	Faeces	2b	Y	Y
94	SA66	South Africa	Faeces	2b	N	Y
95	SA67	South Africa	Faeces	2b	N	Y
96	SA68	South Africa	Faeces	2b	Y	Y
97	SA69	South Africa	Faeces	2b	NA	Y
98	SA70	South Africa	Faeces	2b	NA	Y
99	SA71	South Africa	Faeces	2b	NA	Y
100	SA72	South Africa	Faeces	2b	NA	Y
101	SA73	South Africa	Faeces	2b	NA	Y
102	SA74	South Africa	Faeces	2b	NA	Y
103	SA75	South Africa	Faeces	2b	NA	Y
104	SA76	South Africa	Faeces	2b	Y	Y
105	SA77	South Africa	Faeces	2b	Y	Y
106	SA78	South Africa	Faeces	2b	NA	Y
107	SA79	South Africa	Faeces	2b	NA	Y

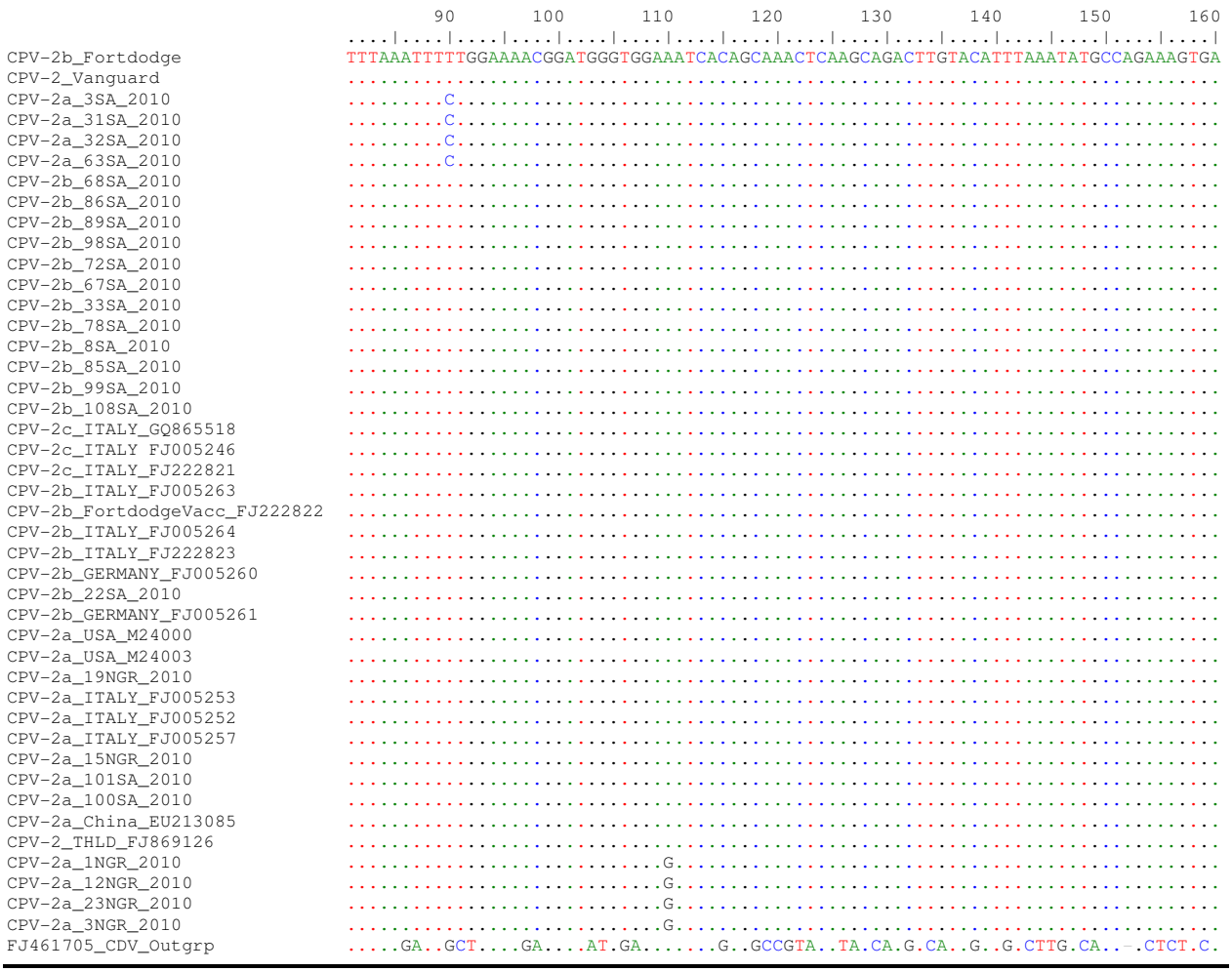
S/No.	Name	Origin	Sample Type	MGB genotyped	Vaccinated	Sheltered
108	SA80	South Africa	Faeces	2b	NA	Y
109	SA81	South Africa	Faeces	2b	NA	Y
110	SA82	South Africa	Faeces	2b	NA	Y
111	SA83	South Africa	Faeces	2b	NA	Y
112	SA84	South Africa	Faeces	2b	NA	Y
113	SA85	South Africa	Faeces	2b	Y	Y
114	SA86	South Africa	Faeces	2b	Y	Y
115	SA87	South Africa	Faeces	2b	Y	Y
116	SA88	South Africa	Faeces	2b	Y	Y
117	SA89	South Africa	Faeces	2b	Y	Y
118	SA90	South Africa	Faeces	2b	Y	Y
119	SA91	South Africa	Faeces	2b	NA	Y
120	SA92	South Africa	Faeces	2b	N	Y
121	SA93	South Africa	Faeces	2b	NA	Y
122	SA94	South Africa	Faeces	2b	NA	Y
123	SA95	South Africa	Faeces	2b	NA	Y
124	SA96	South Africa	Faeces	2b	N	Y
125	SA97	South Africa	Faeces	2b	N	Y
126	SA98	South Africa	Faeces	2b	Y	Y
127	SA99	South Africa	Faeces	2b	Y	Y
128	SA100	South Africa	Faeces	2a	Y	Y
129	SA101	South Africa	Faeces	2a	Y	Y
130	SA102	South Africa	Faeces	2b	Y	Y
131	SA103	South Africa	Faeces	2b	Y	Y
132	SA104	South Africa	Faeces	2b	Y	Y
133	SA105	South Africa	Faeces	2b	NA	Y
134	SA106	South Africa	Faeces	2b	NA	Y
135	SA107	South Africa	Faeces	2b	Y	Y
136	SA108	South Africa	Faeces	2b	Y	Y
137	111	Italy	100/09/CPV-2a	2a		
138	112	USA	Fort Dodge vac.	2b		
139	113	Italy	243/07/CPV-2c	2c		

APPENDIX 4 :
Phylogenetic tree

Nucleotide Sequence alignment of the VP2 Gene of CPV-2 South African field strains, Nigerian field strains and commercial vaccines used in South Africa

Key:
Dots (.) indicates similarities
Red colour=Nigerian field isolates
Orange colour=CPV-2a South African field isolates
Green colour=CPV-2b South African field isolates
Purple colour=Vaccines use in South Africa

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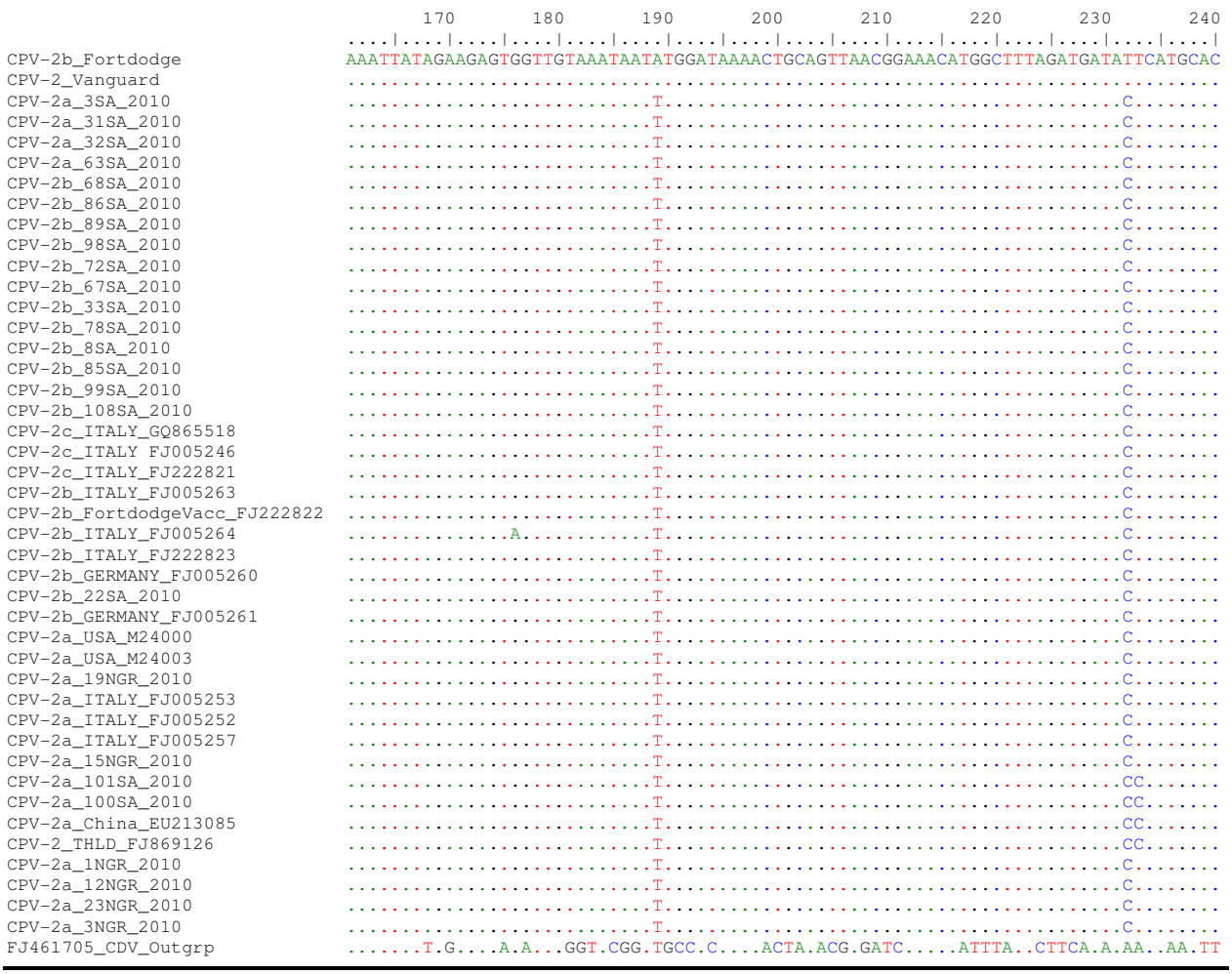
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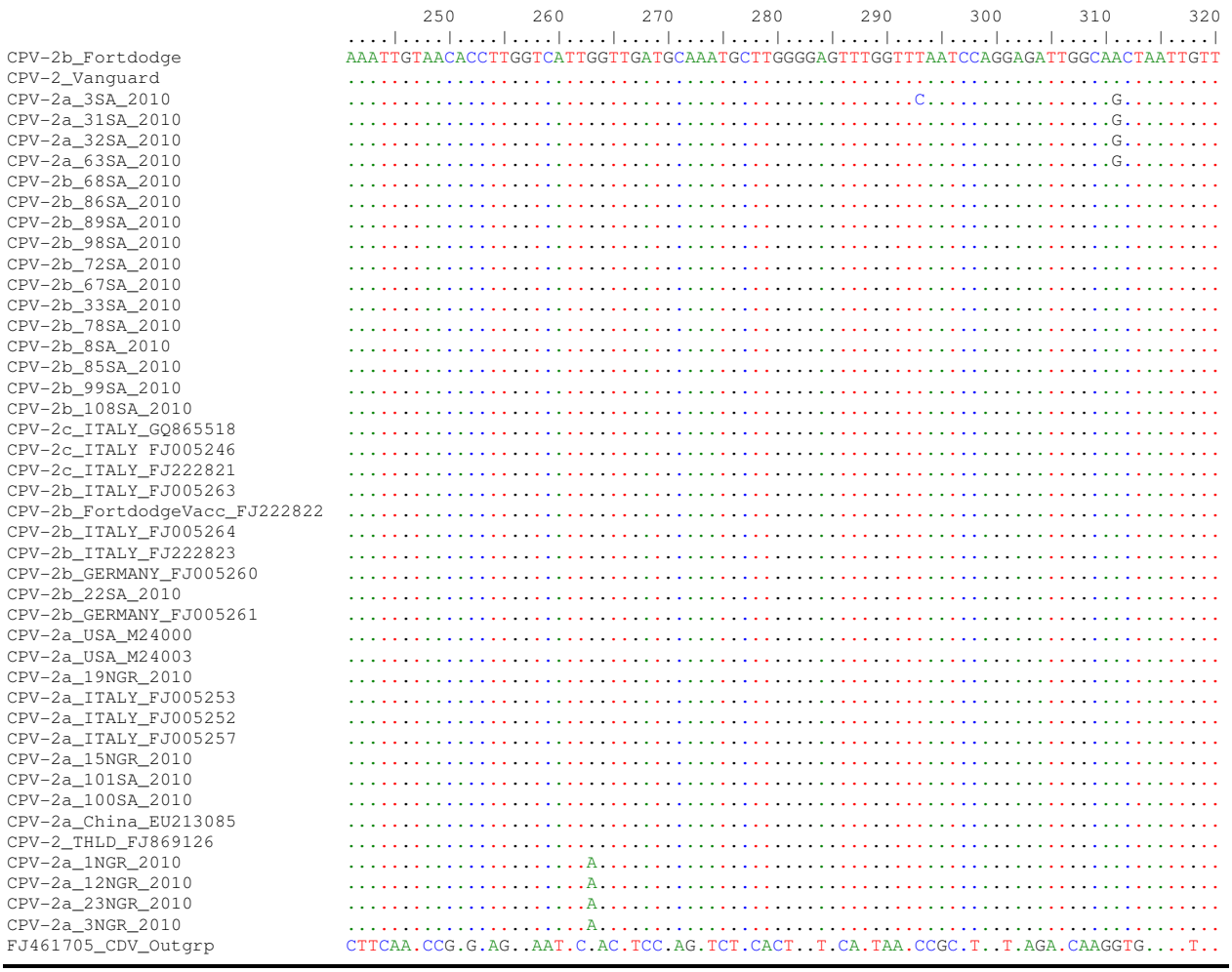
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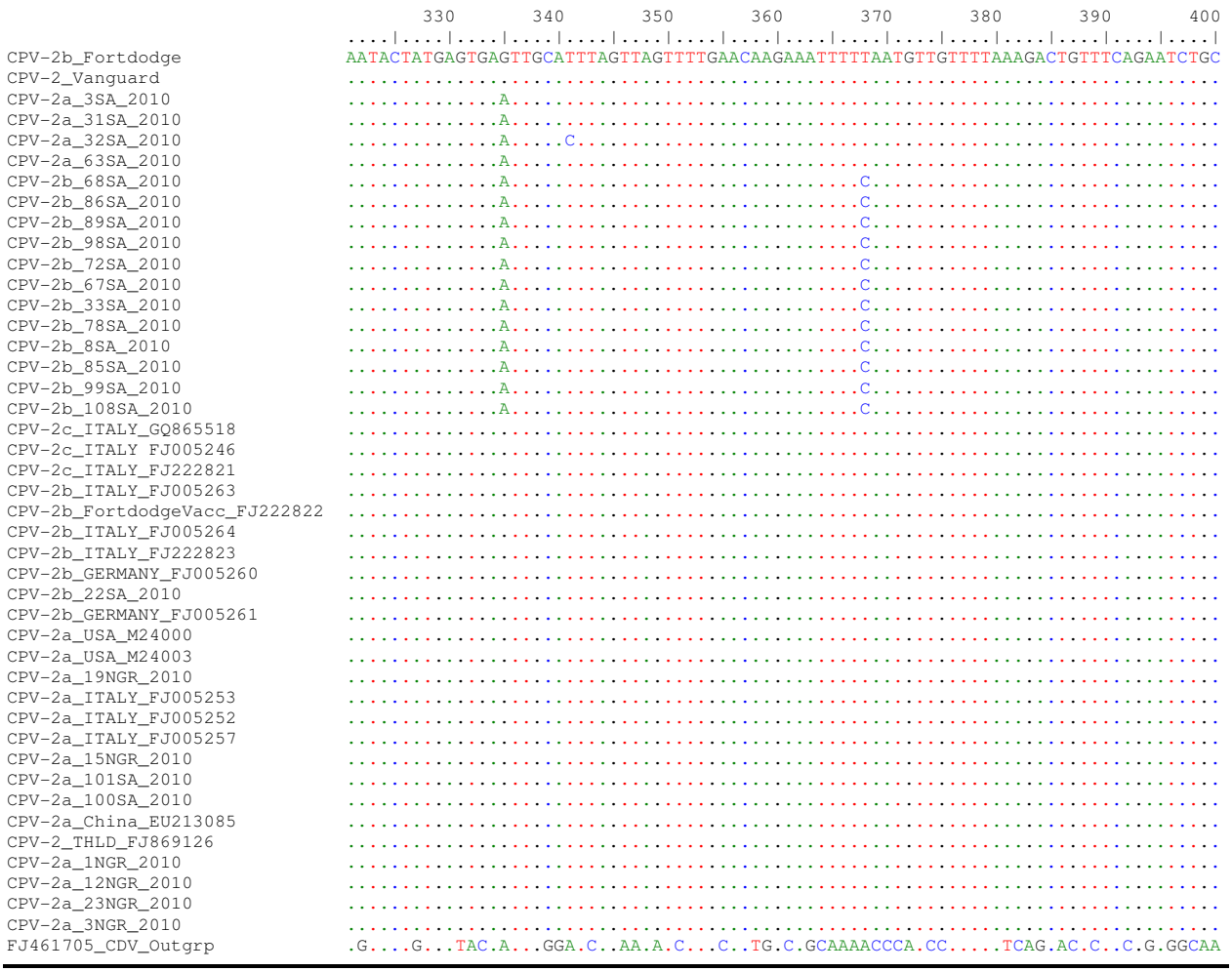
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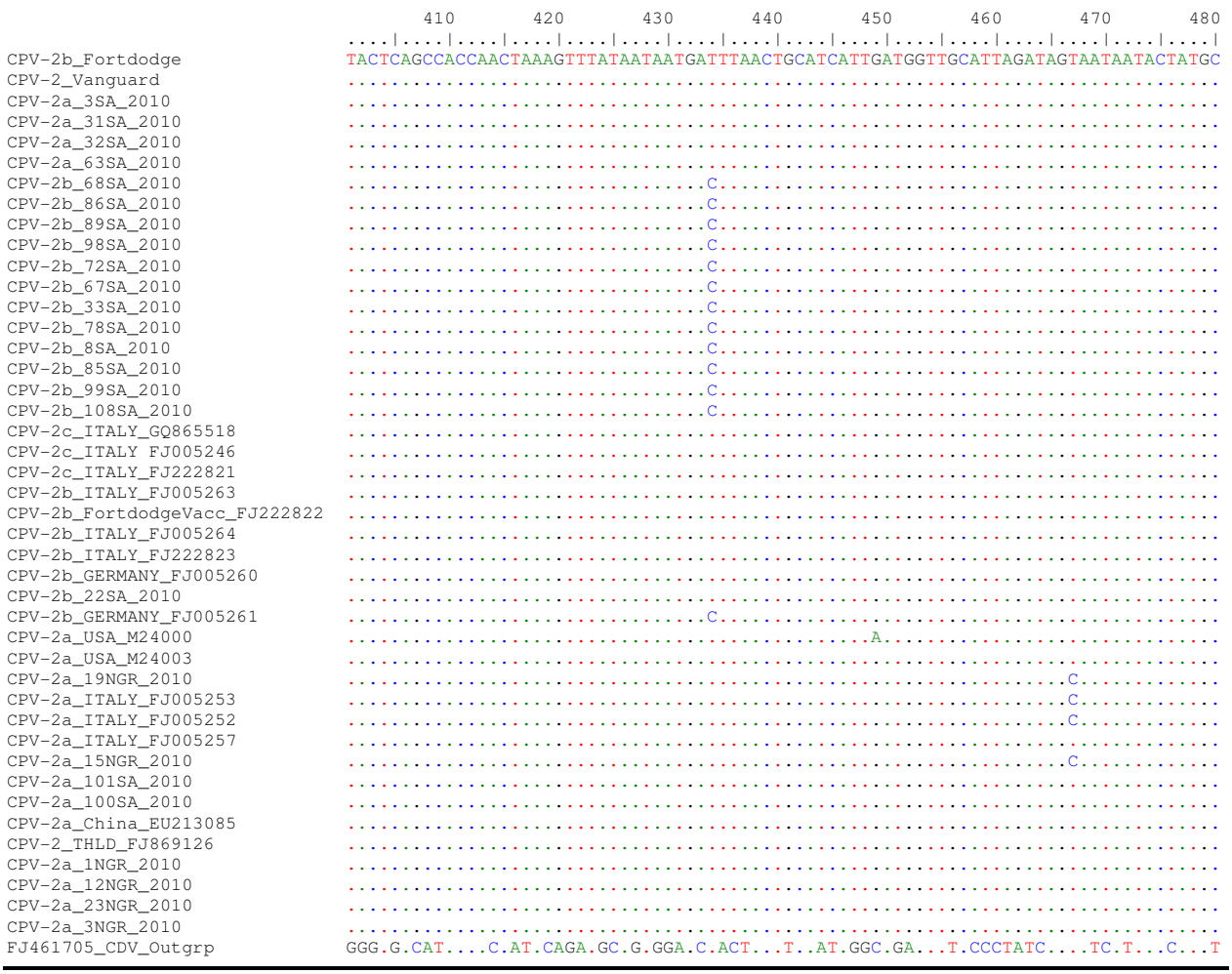
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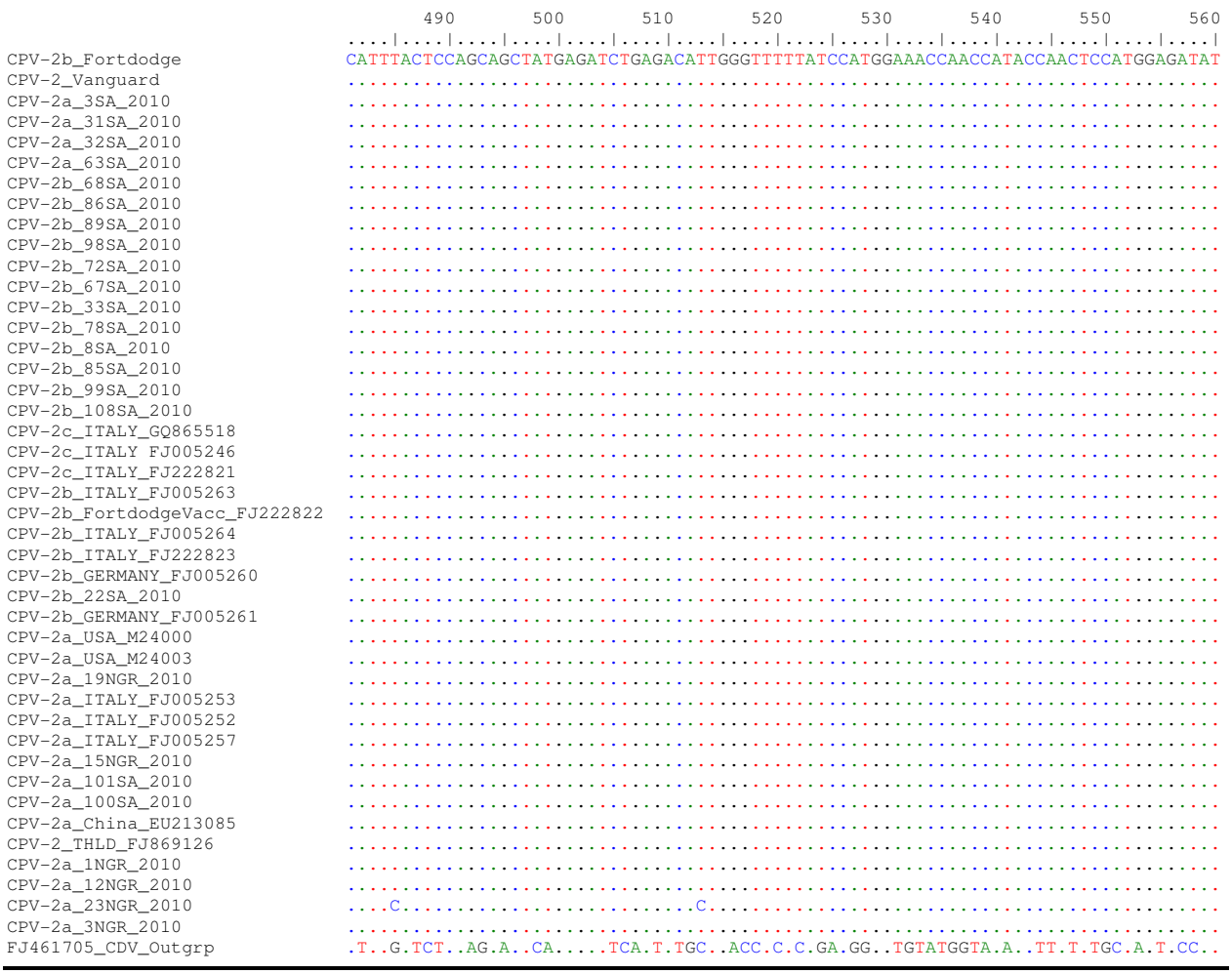
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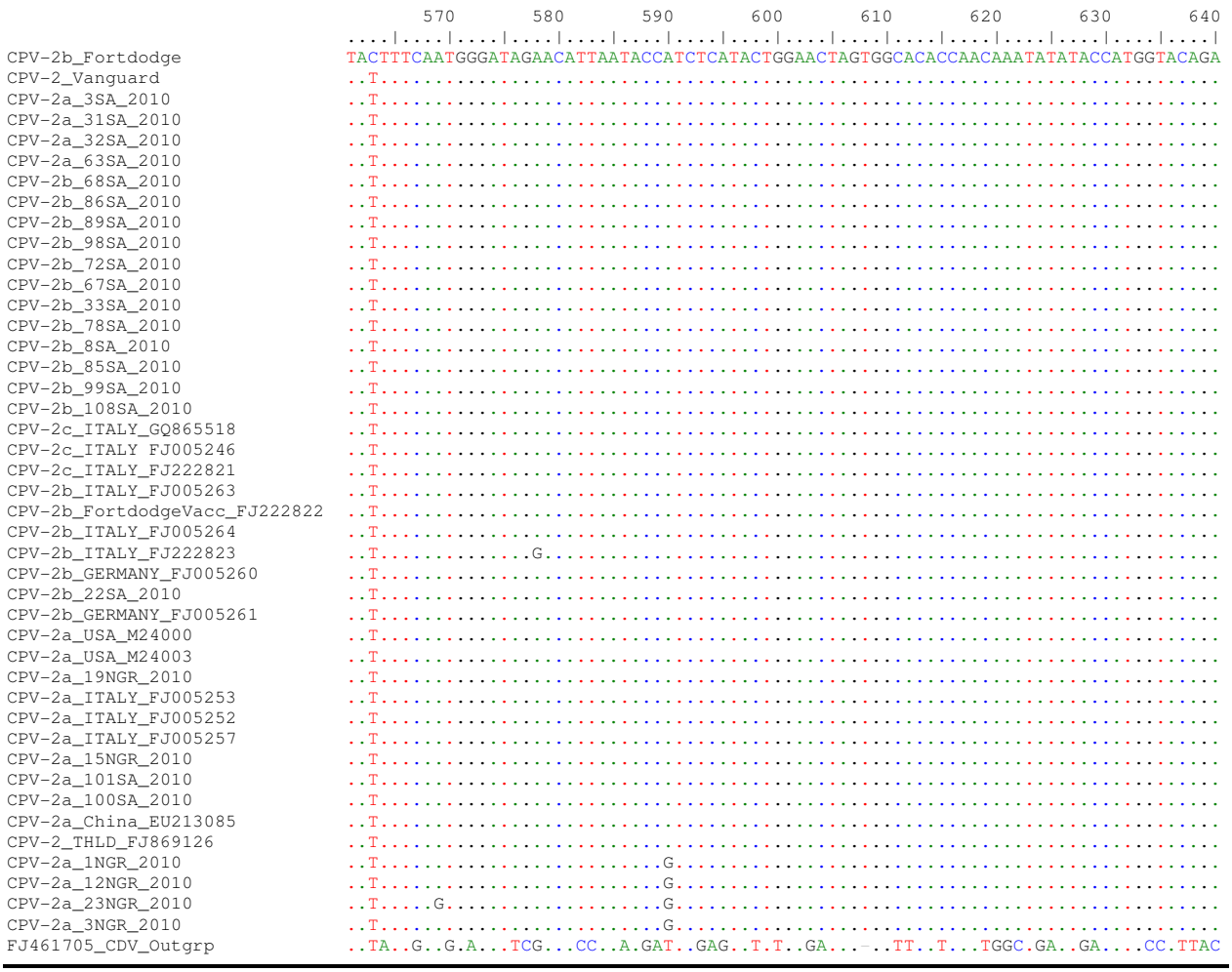
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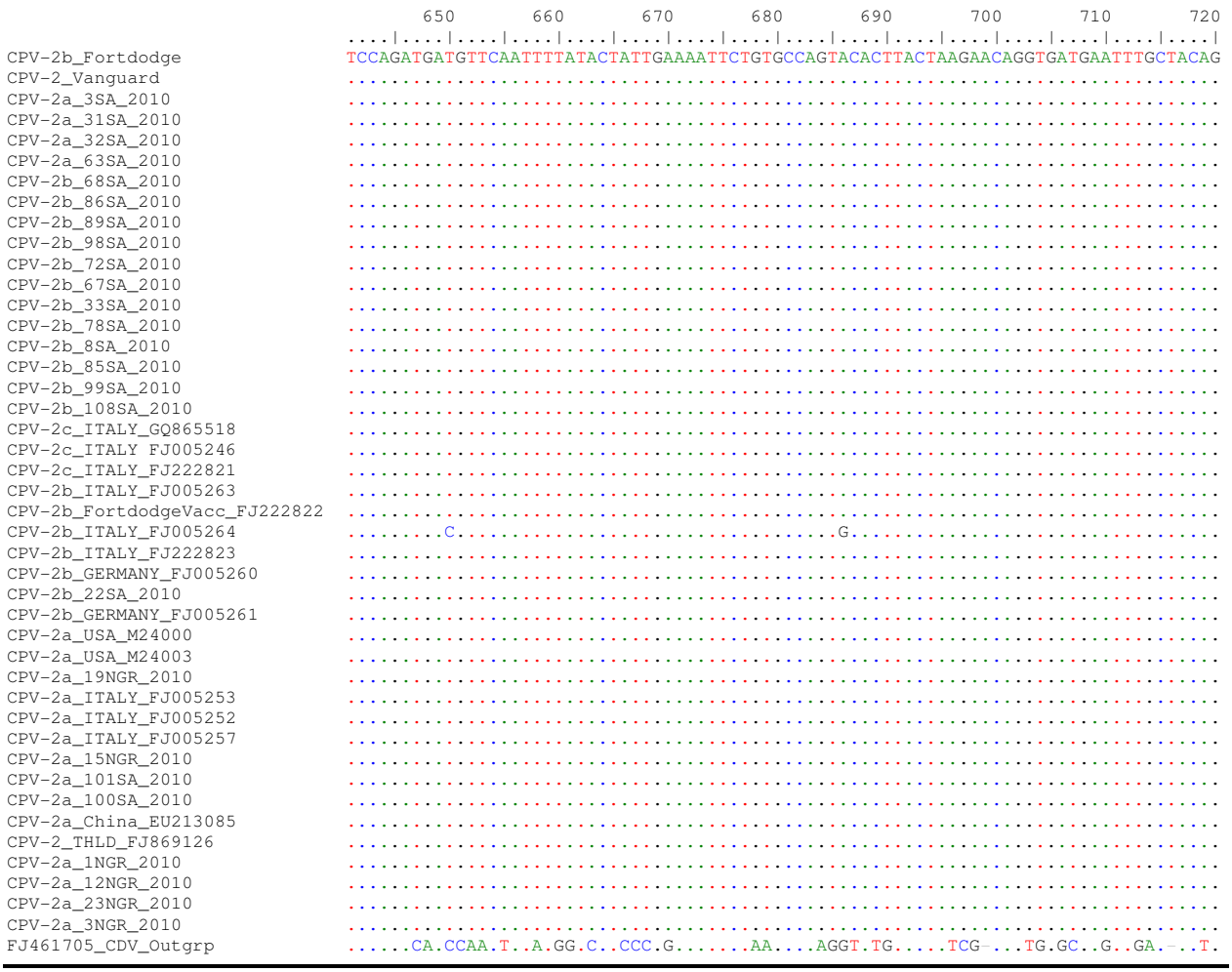
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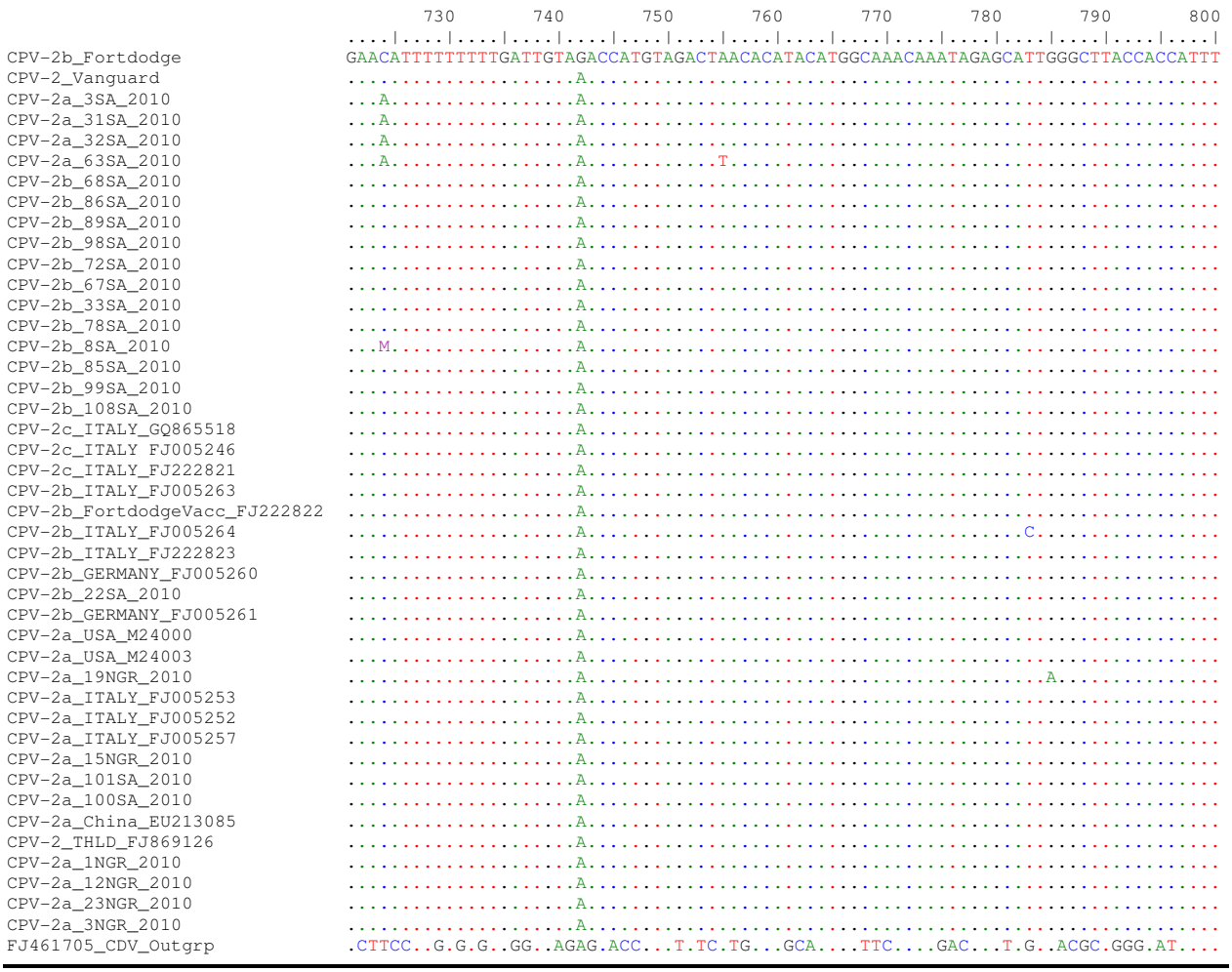
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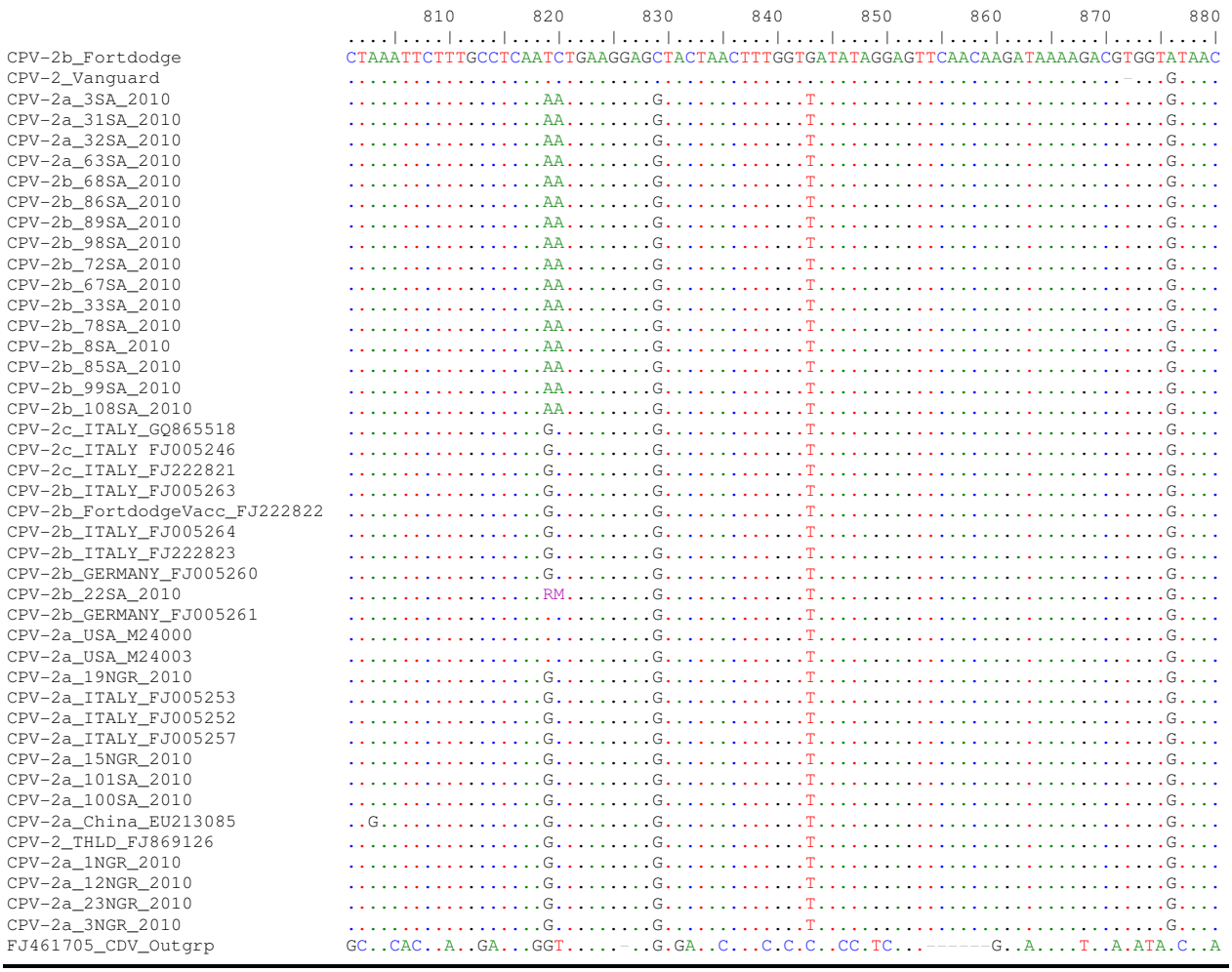
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FJ461705_CDV_Outgrp

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CPV-2a_31SA_2010                  ................................................................................
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CPV-2a_63SA_2010                  ..........................C.....................................................
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CPV-2a_USA_M24003                 ................................................................................
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CPV-2a_23NGR_2010                 ................................................................................
CPV-2a_3NGR_2010                  ................................................................................
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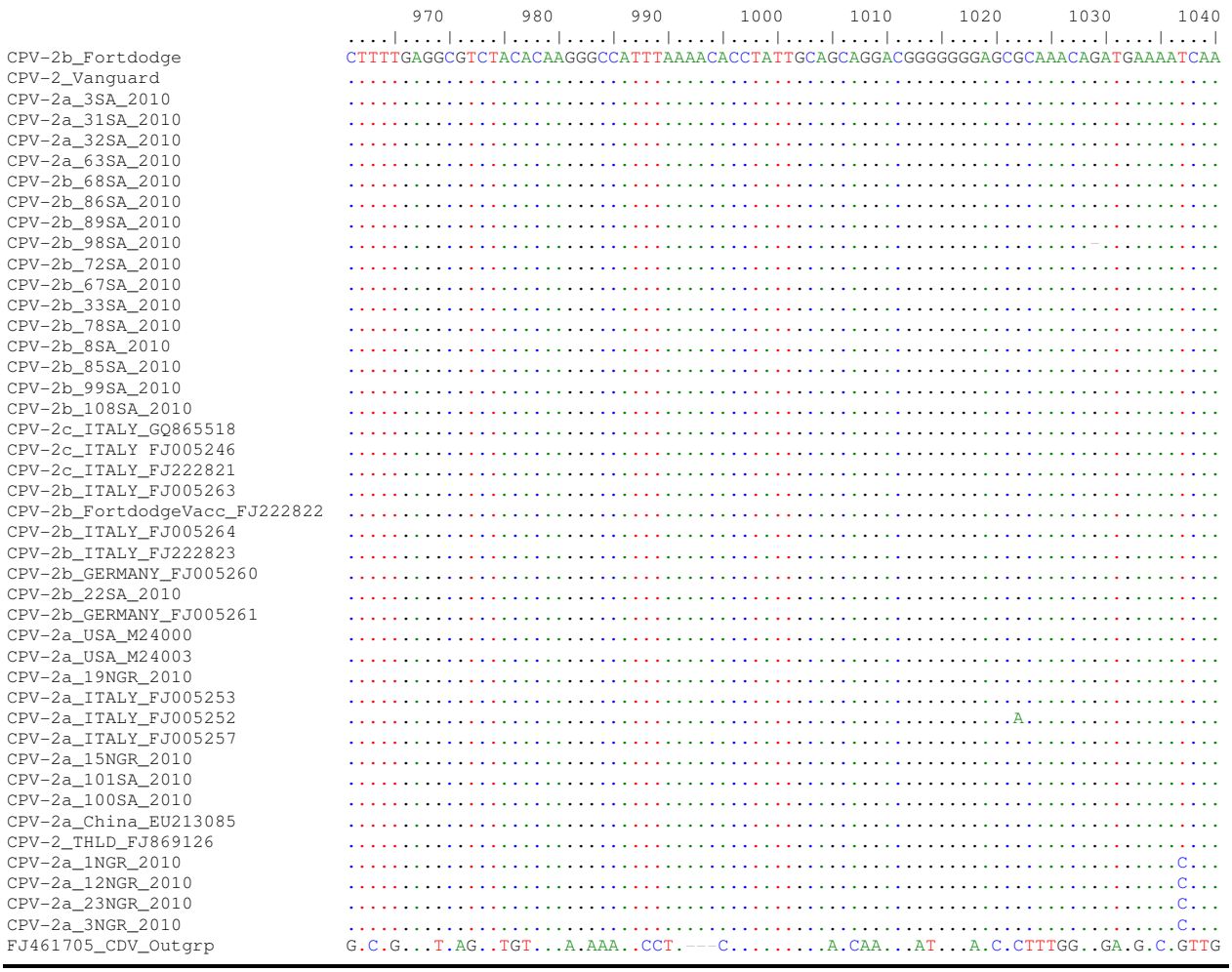
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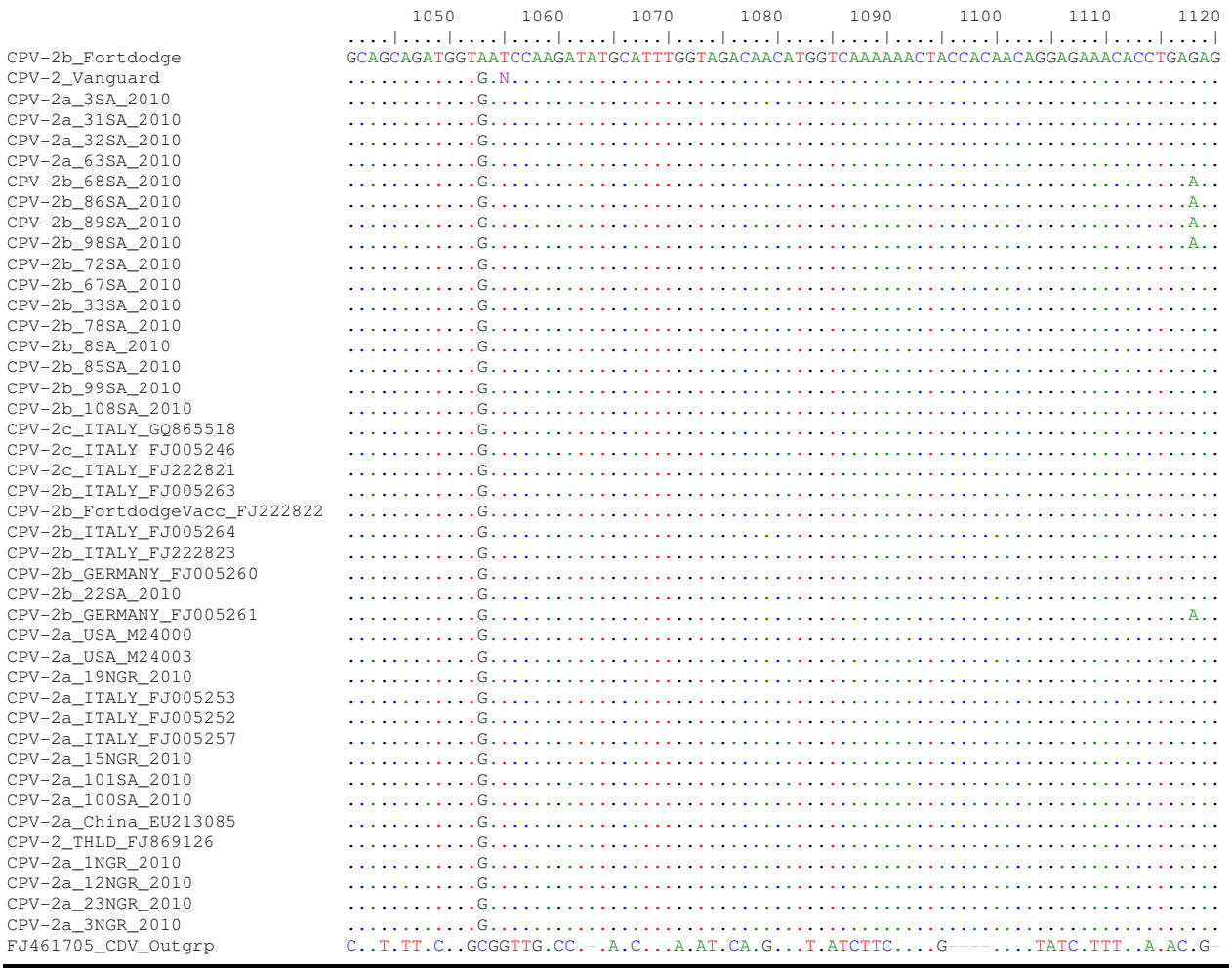
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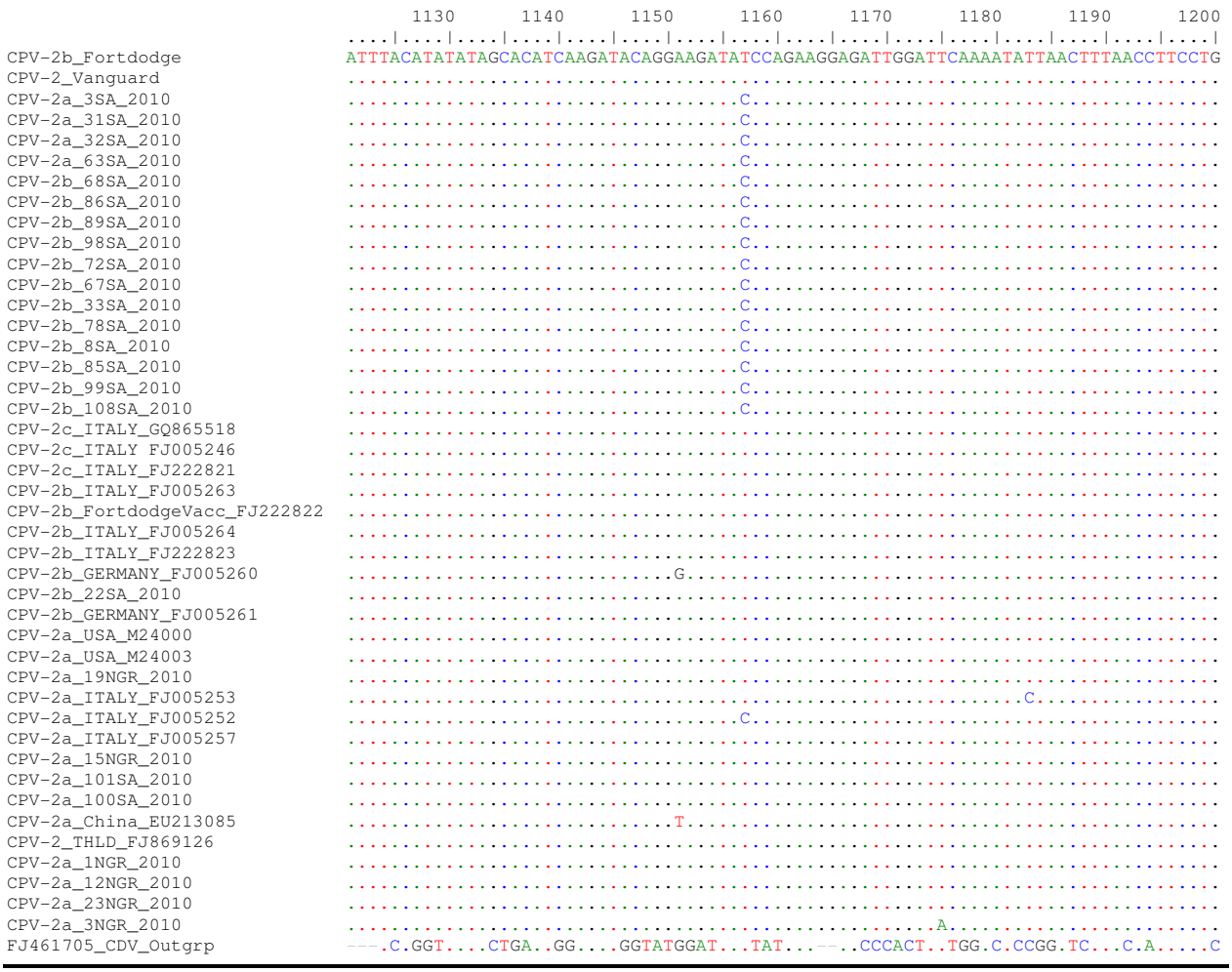
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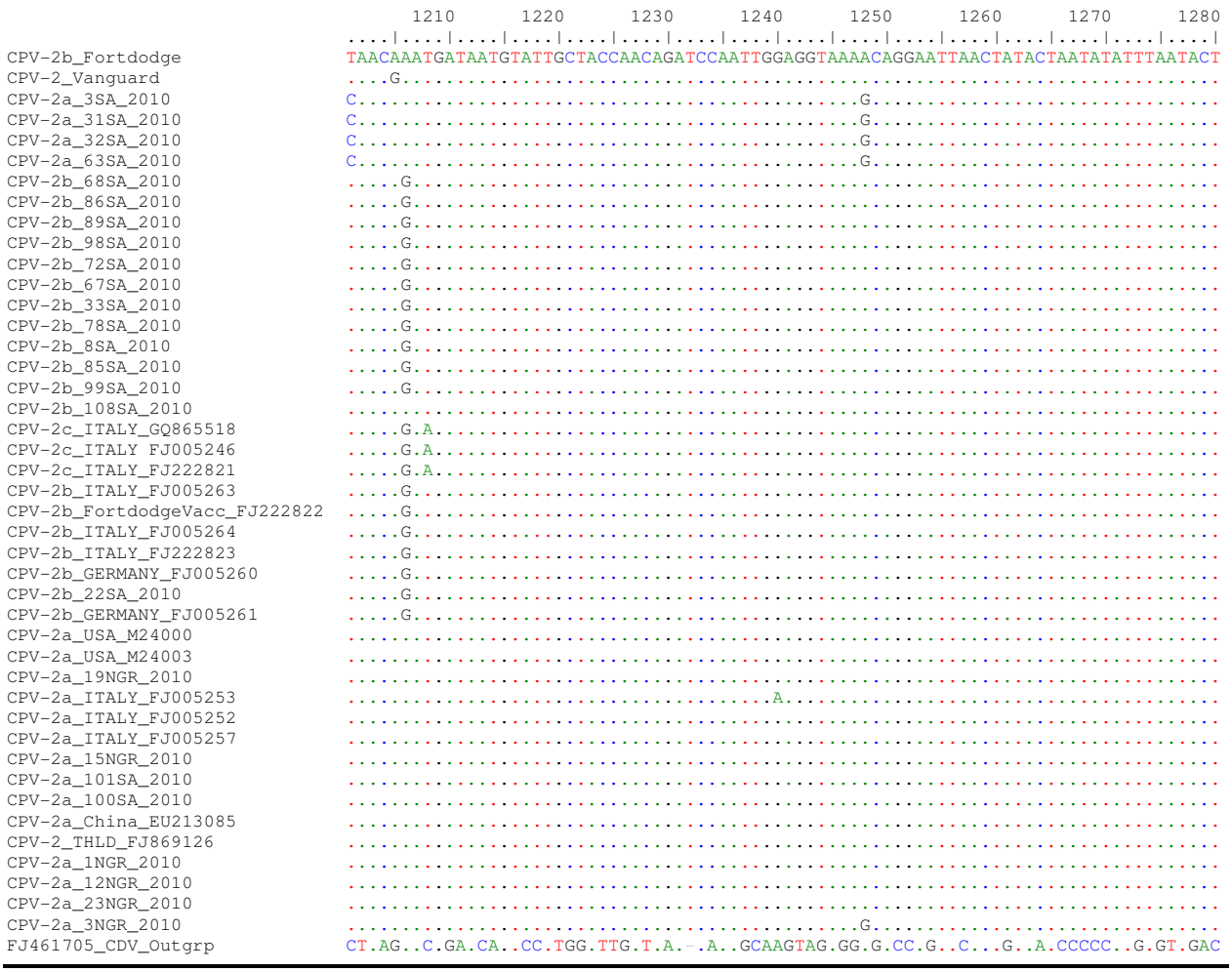
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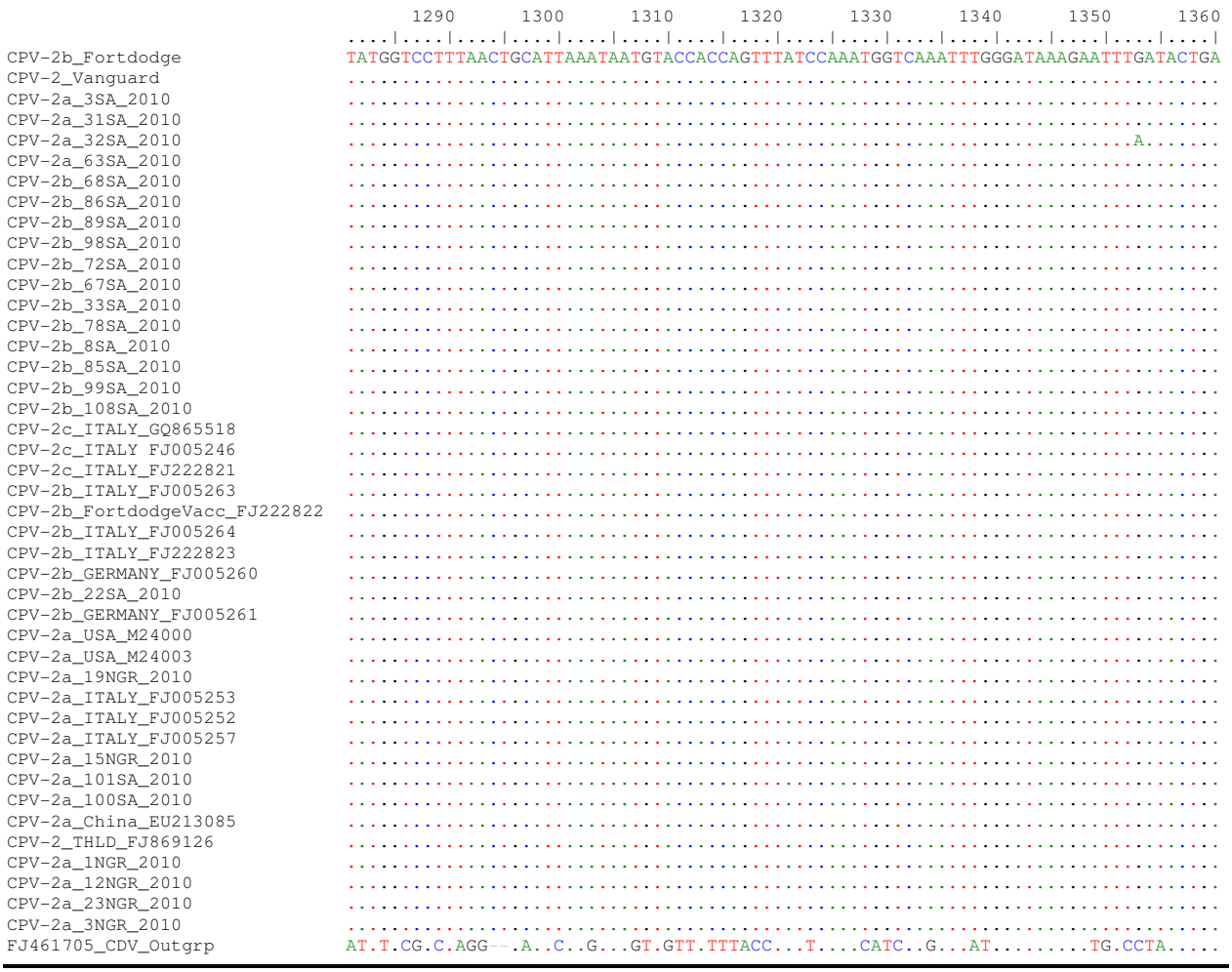
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CPV-2c_ITALY_FJ222821
CPV-2b_ITALY_FJ005263
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CPV-2b_ITALY_FJ222823
CPV-2b_GERMANY_FJ005260
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CPV-2a_USA_M24003
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CPV-2a_100SA_2010
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CPV-2a_12NGR_2010
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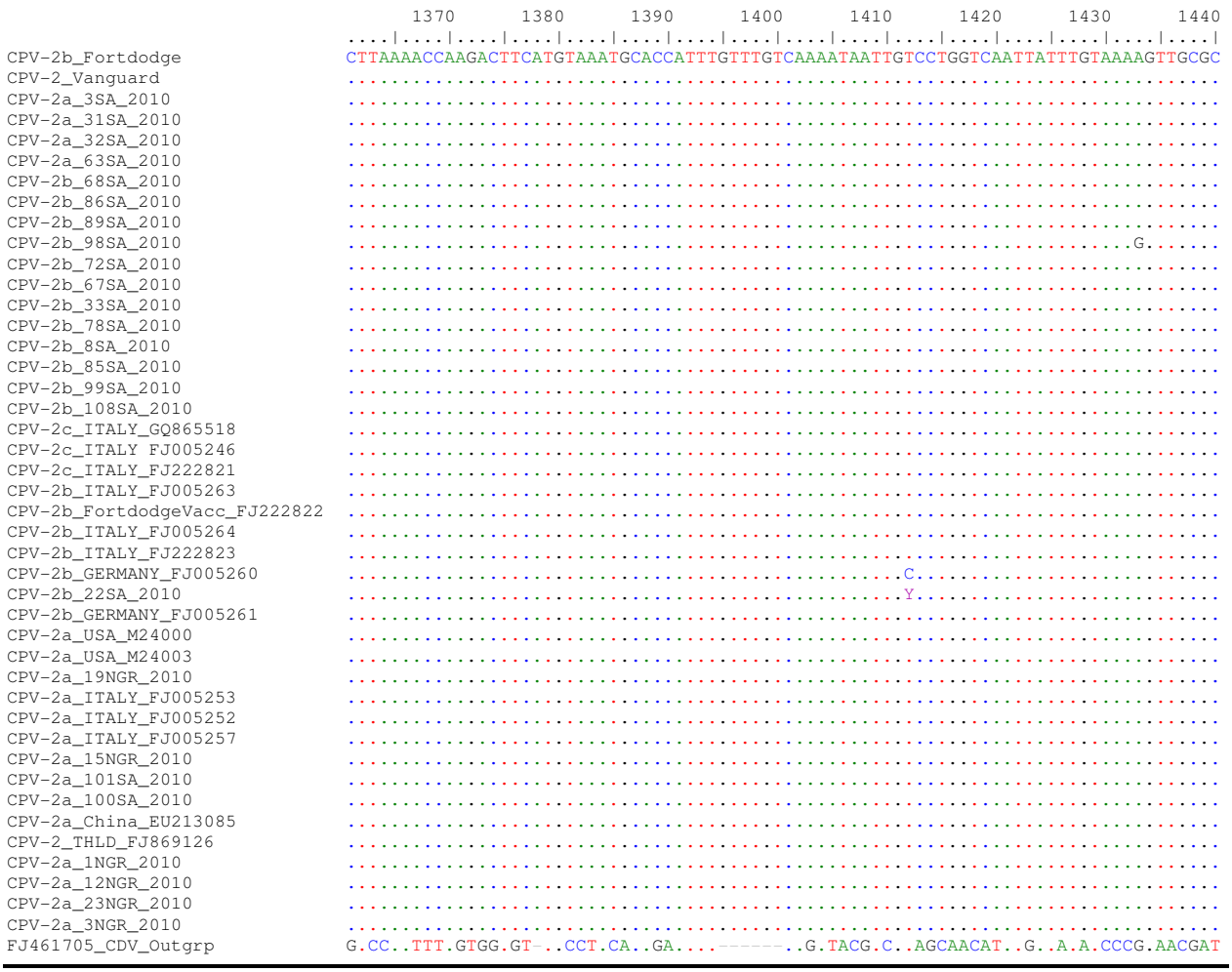
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CPV-2a_100SA_2010
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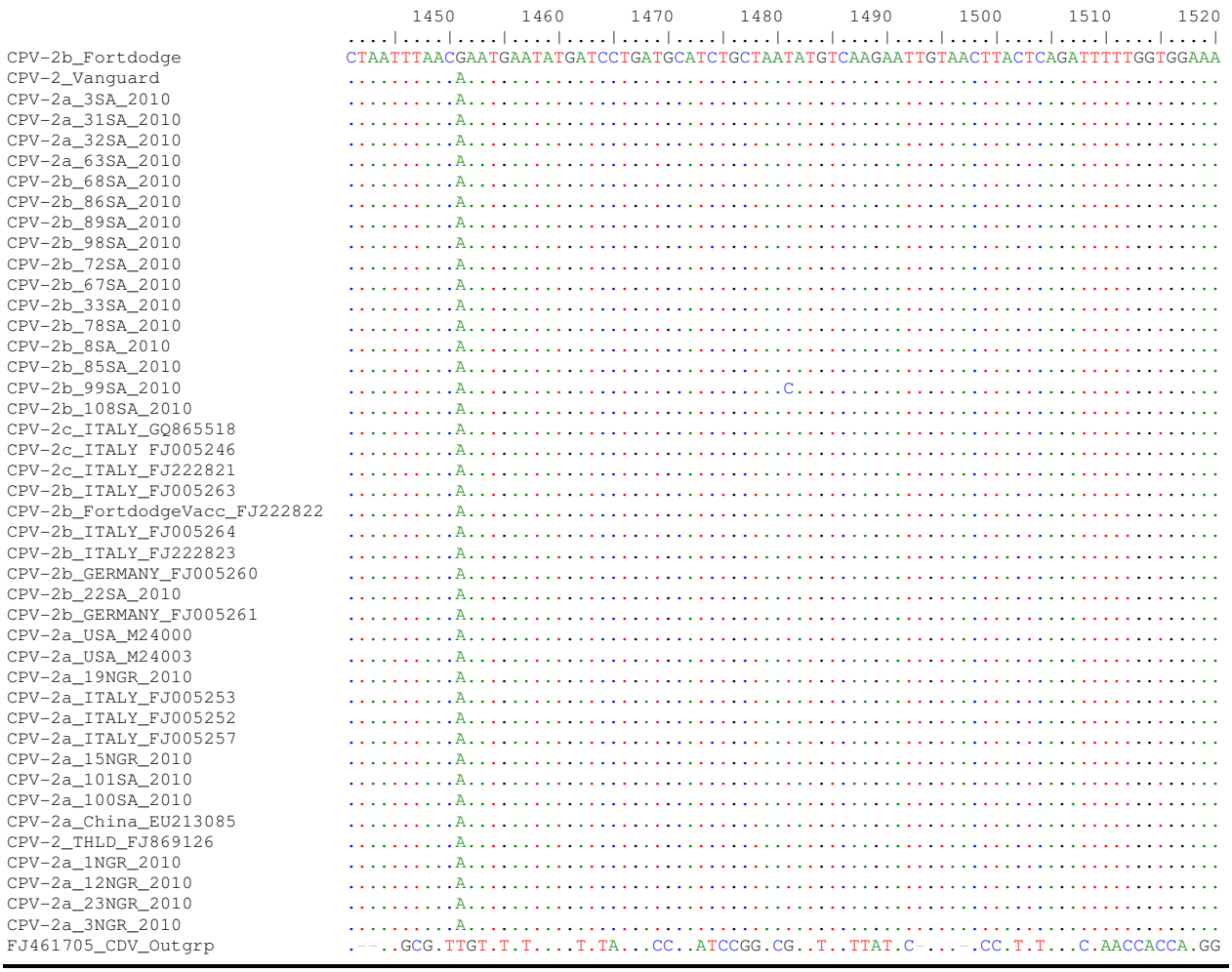
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CPV-2_Vanguard
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CPV-2a_63SA_2010
CPV-2b_68SA_2010
CPV-2b_86SA_2010
CPV-2b_89SA_2010
CPV-2b_98SA_2010
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FJ461705_CDV_Outgrp


1530 1540 1550 1560 1570 1580 1590 1600
CPV-2b_Fortdodge GGTAAATTAGTATTTAAAGCTAAACTAAGAGCCTCTCATAGGAATCCAATTCAACAAATGAGTATTAATGTAGATAACCA
CPV-2_Vanguard
CPV-2a_3SA_2010
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CPV-2_THLD_FJ869126
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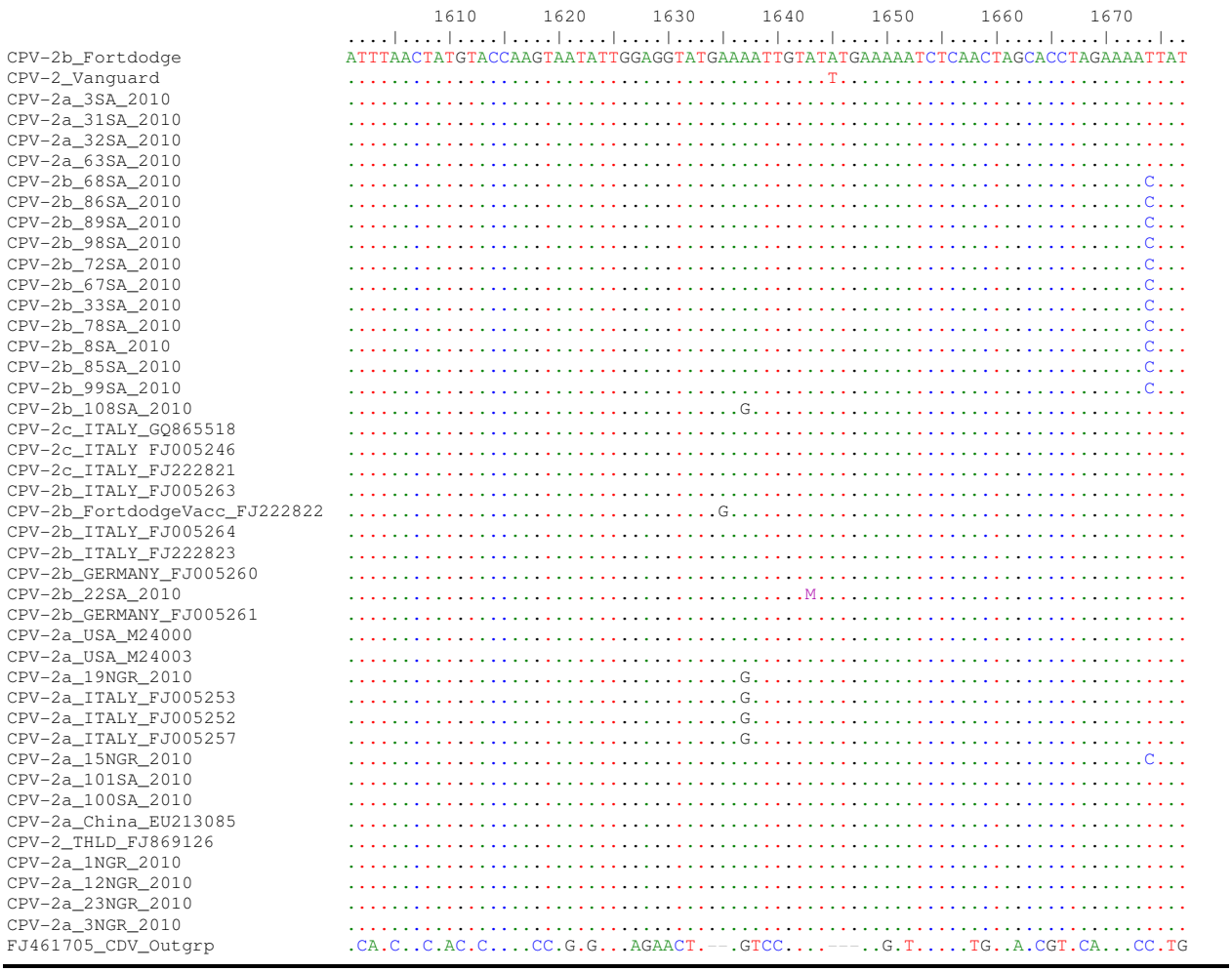
CPV-2a_12NGR_2010
CPV-2a_23NGR_2010
CPV-2a_3NGR_2010
FJ461705_CDV_Outgrp .TA..CC.GA.T.CCT...---G.T.G.AT.TT.TGTG.G...TGA.G...TGTGGTG.C.CC.A.TT.AC....GG.TG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FJ461705_CDV_Outgrp