Veterinaria Italiana, **46** (1), 69-81

Molecular characterisation and nucleotide sequence analysis of canine parvovirus strains in vaccines in India

Sukdeb Nandi[(1)], Rajendra Anbazhagan[(2)] & Manoj Kumar[(3)]

Summary

Canine parvovirus 2 (CPV-2) is one of the most important viruses that causes haemorrhagic gastroenteritis and myocarditis of dogs worldwide. The picture has been complicated further due to the emergence of new mutants of CPV, namely: CPV-2a, CPV-2b and CPV-2c. In this study, the molecular characterisation of strains present in the CPV vaccines available on the Indian market was performed using polymerase chain reaction and DNA sequencing. The VP1/VP2 genes of two vaccine strains and a field strain (Bhopal) were sequenced and the nucleotide and the deduced amino acid sequences were compared. The results indicated that the isolate belonged to CPV type 2b and the strains in the vaccines belonged to type CPV-2. From the study, it is inferred that the CPV strain used in commercially available vaccine preparation differed from the strains present in CPV infection in dogs in India.

Keywords

Canine parvovirus, DNA, Dog, Feline panleukopenia virus, Gastroenteritis, India, Parvovirus, PCR, Polymerase chain reaction, Sequencing, Vaccine, Virus.

Caratterizzazione molecolare e sequenze di nucleotidi in ceppi di parvovirus canino 2 in vaccini in India

Riassunto

Il parvovirus canino 2 (CPV-2) è uno dei più importanti virus responsabili di gastroenterite emorragica e miocardite nella popolazione canina mondiale. Il quadro epidemiologico è stato ulteriormente complicato dalla comparsa di nuovi mutanti del CPV ovvero CPV-2a, CPV-2b e CPV-2c. In questo studio la caratterizzazione molecolare dei ceppi presenti nei vaccini CPV disponibili sul mercato indiano è stata eseguita mediante sequenziamento del DNA e reazione a catena della polimerasi (PCR). Sono stati sequenziati i geni VP1/VP2 di due ceppi di vaccini e di un ceppo isolato sul campo (Bhopal) mettendo a confronto le sequenze nucleotidiche e aminoacidiche. Il ceppo isolato è risultato appartenere al tipo CPV-2b, i ceppi dei vaccini al tipo CPV-2. Questo studio ha permesso di concludere che il ceppo CPV utilizzato nei vaccini disponibili in commercio differisce dai ceppi presenti nell'infezione canina da CPV in India.

Parole chiave

Cane, DNA, Gastroenterite, India, Parvovirus, Parvovirus canino, PCR, Reazione a catena

(1) Senior scientist, Centre for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh 243122, India
snandi1901@yahoo.com
(2) MVSc scholar, Centre for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243122, India
(3) MVSc scholar, Centre for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243122, India

© IZS A&M 2010

della polimerasi, Sequenziamento, Vaccino, Virus, Virus della panleucopenia felina.

Introduction

'Parvo' means small (Latin), canine parvovirus (CPV) belongs to genus *Parvovirus*, family *Parvoviridae*. The genome is a single-stranded negative-sense DNA that is 5.2 kb in length with two promoters that result in the expression of three structural viral proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2) through alternate splicing of the viral mRNAs (19). VP2 (64 kDa) is an NH2-terminally truncated form of VP1 (84 kDa) and is the major component of the capsid. VP3 is derived from VP2 by post-translational proteolytic cleavage and is present only in complete (DNA-containing) virions. CPV has an icosahedral symmetry, is 25 nm in diameter and is non-enveloped (24). There is some evidence that the VP1 terminus is internal and may help stabilise the DNA. The main structural motif is an eight-stranded, antiparallel β-barrel, which has also been found in most other viral capsid structures. The β-barrel motif contains approximately one third of the amino acid composition of VP2, the major structural protein in most parvoviruses and which comprises approximately 90% of the capsid (19).

CPV causes the most dangerous and contagious disease that affects dogs. CPV infection is now considered to be most threatening to puppies between the time of weaning and six months of age. Adult dogs can also contract the virus, although this is relatively uncommon. The disease is clinically characterised by severe vomiting and diarrhoea that lead to fatal dehydration as well as myocarditis, particularly in young pups. Diarrhoea commences five to fourteen days after infection. This occurs in dogs of any age but has serious consequences in pups. Early symptoms are depression, loss of appetite, vomiting, high fever and severe diarrhoea (2, 9). There is no consistent characteristic of stools which may be watery, yellow in colour or tinged with blood in severe cases. Rapid dehydration is a danger and dogs may continue to vomit and suffer from diarrhoea until death. The morbidity and mortality vary according to the age of the animals, as do the severity of challenge and the presence of intercurrent disease problems. The second form of CPV is cardiac syndrome or myocarditis which can affect puppies under three months of age (2). Within an infected litter, 70% pups will die of heart failure by 8 weeks of age and the remaining 30% will have pathological changes which may result in death many months or even years later. The most dramatic manifestation of CPV type 2 (CPV-2) myocarditis is sudden death in young pups aged usually about four weeks (3).

CPV type 2 emerged in 1978 as the cause of a new disease in dogs throughout the world. It spread rapidly both in domestic populations, as well as in wild dogs (2). CPV-2 was soon shown to be a variant of the long recognised feline panleukopenia virus (FPV), from which it differed in less than 1% at the nucleotide sequence level (18, 29). Sequence analysis has also revealed that CPV strains have undergone a series of evolutionary selections in nature, which have resulted in the global distribution of new CPV variants. This was first seen in the global replacement between 1979 and 1981 of the original (1978) strain of the virus by a genetically and antigenically different strain termed CPV-2a (20). The two viruses differed in 5-6 amino acids, which constitute two different neutralising antigenic sites on the surface of the capsid. In 1984, an additional antigenically different virus strain was observed, which differed in only a single epitope and was designated CPV-2b (18). The genetic and antigenic variation in the virus strains was also correlated with changes in the host range of the virus both *in vivo* and *in vitro* (19). Currently, CPV-2a is predominant field strain in Italy and Germany, while CPV-2b is more common in the United States, Taiwan and Japan (3, 12, 30). More recently, strains have emerged in Italy in which the amino acid at position 426 (Asn in 2a and Asp in 2b) has been replaced by glutamic acid (Glu) residue (12). These Glu 426 variants, termed 'CPV-2c viruses', circulate and coexist with other CPV

© IZS A&M 2010

types in Italy (12), Spain (7), Vietnam (14), Germany, the United Kingdom and other European countries (8).

After the isolation of CPV-2 for the first time in India by Ramadass and Khader (22) several occurrences of disease were reported from different parts of the country (4, 9, 21). In India, no detailed studies have been conducted on the characterisation of vaccine strain of CPV-2 or the prevalence of different variants of CPV (CPV-2, 2a and 2b) that have been implicated in outbreaks and serious cases of infection, both in vaccinated and non-vaccinated dogs (28). Vaccination against CPV infection with CPV-2 may not confer immunity against the disease and infection in dogs. This may be due to the mismatching of the vaccine strain and the outbreak strain of CPV. Moreover, the alarming populations of stray dogs, which harbour and shed the virus, act as a potential source of infection for other susceptible populations. Speculation about antigenic variation between the current vaccine strain and the field isolates of CPV called for the study of nucleotide sequencing of VP1/VP2 genes of vaccine strains and a comparison with the established nucleotide sequence of field strains.

Materials and methods

Processing of faecal samples

Rectal swabs were collected from 13 dogs suspected of suffering from CPV infection. These were suspended (ratio: 1:9) in Hanks balanced salt solution (HBSS) containing streptomycin (0.1 mg/ml) and penicillin (500 IU/ml) and were filtered through a disposable syringe filter (0.45 µ) (Millex, Millipore, Palm Springs, California) and then centrifuged at 10 000 rpm at 4°C for 15 min in a refrigerated centrifuge. The supernatant was carefully pipetted out and stored at –20°C until further use.

Virus isolation

Madin-Darby canine kidney (MDCK) cells were grown in 25 cm^2 cell culture plastic flasks containing Eagles minimum essential medium (EMEM) with 10% foetal calf serum (FCS). When the cell monolayer had grown to 70% of cell culture flask, it was washed with EMEM and 0.5 ml of processed faecal sample was used as innoculum which was incubated for 1 h at 37°C for adsorption. After incubation, the infected cell monolayer was washed three times with medium, followed by the addition of 5 ml of medium containing 2% FCS. The infected cells were incubated at 37°C for 4-5 days. The virus was then harvested when the cell monolayer started to show morphological changes; these samples were kept at –20°C for further use.

Canine parvovirus vaccines

The commercial vaccines against CPV available on the Indian market, namely: parvovirus vaccine (Bio-Core Inc. Company, Kissimmee, Florida) and 'Nobivac® Puppy DP' (DP: distemper parvo) (Intervet, Boxmeer), were used in the study. The viral DNA was extracted from the commercial vaccines (parvovirus vaccine and Nobivac® Puppy DP) and field isolates adapted in MDCK-infected cells using alkali lysis and the phenol-chloroform method (23).

Polymerase chain reaction for the amplification of entire VP1/VP2 gene of CPV-2

The primer set used to amplify the entire VP1/VP2 gene of the CPV was custom designed and synthesised to yield a polymerase chain reaction (PCR) product of 2 256 kbp (2 285 to 4 540 nucleotide position of CPV genomic DNA). The forward primer with the restriction site for *EcoR* I and the reverse primer with the restriction site for *Xba* I were designed to express the structural protein of CPV from an *Escherichia coli* based expression vector, as follows:

- pCPV-(F) GGG GAA TTC ATG GCA CCT CCG GCA AAG AGA (30mer)
- pCPV-(R) GGC TCT AGA TTA ATA TAA TTT TCT AGG TGC TAG (33mer)

The PCR reaction was performed in a final volume of 50 µl containing 1X *Taq* PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 5 µl of processed faecal sample or vaccines and 10 pmol of each

primer. A total of 0.5 µl of Taq DNA polymerase (5 U/ µl) was added and the mixture incubated at 94°C for 5 min in the thermocycler. The standardised PCR protocol for primer pair pCPV in a 50 µl reaction mix was incubated at 94°C for 5 min, followed by an amplification cycle consisting of denature-ation at 94°C for 30 sec, primer annealing at 57°C for 2 min and extension at 72°C for 3 min. The cycle was repeated 30 times in a thermocycler. This was followed by a final extension for 10 min at 72°C. After PCR, the amplified products were analysed on 1.0% agarose gel containing ethidium bromide to a final concentration of 0.5 µg/ml. A total of 10 µl of amplified product was mixed with 2 µl of bromophenol (6×) dye and loaded into the well and run along with 100 bp DNA ladder in 1X TAE electrophoresis buffer at 5 volts/cm^2; the progress of mobility was monitored by migration of dye. After electrophoresis, the gel was observed using the UV transilluminator (16).

Purification of polymerase chain reaction product from gel

The PCR products were purified from gel using the QIAquick gel extraction kit (QIAGEN Inc., Valentia, California) in accordance with the manufacturer's protocol. The DNA fragment was visualised with a UV trans-illuminator and excised from the agarose gel with a sterilised clean and sharp scalpel. The gel slice was weighed and collected in a sterilised 2 ml Eppendorf tube and three volume of buffer QG (guanidium chloride) was added to one volume of gel and was incubated at 50°C for 10 min to dissolve the gel. A yellow colour was noted and the product was transferred to a QIAquick spin column fitted to a 2 ml collection tube. The QIAquick column was centrifuged at 12 000 rpm for 1 min. The flow-through was discarded and the column placed back into the same tube. It was washed with 0.75 ml of buffer by centrifugation at 12 000 rpm for 1 min. The flow-through was discarded and the column was replaced into the same tube and centrifuged again at 12 000 rpm for 1 min. The QIAquick column was transferred to a clean sterile 1.5 ml microcentrifuge tube for elution. The DNA was eluted with 20 µl of elution buffer (EB) (Tris chloride) and stored at –20°C until further use. After purification, 1 µl of purified product was checked by agarose gel electrophoresis.

Cloning of the polymerase chain reaction product

The PCR product of 2.2 kbp was cloned in a TOPO® TA vector using a TOPO® TA cloning kit following the manufacturer's instructions (Life Technologies™, Carlsbad, California). The reaction mix included 4 µl of purified PCR product, 1 µl of NaCl solution and 1 µl TOPO® vector. The content was mixed gently, incubated at room temperature for 30 min and finally stored at –20°C until further use. *E. coli* DH5*α* competent cells were prepared using the calcium chloride method and heat shock was applied at 42°C for 90 sec (23). A total of 200 µl of the transformed competent cells were then plated on a lysogeny or Luria-Bertani broth (LB) agar plate containing 100 µg/ml ampicillin. The plates were incubated at 37°C for 16-20 h to enable the growth of the transformed bacteria. There was no growth in the negative control plates (without plasmid). The plasmid DNA from recombinant bacterial colonies was extracted using the alkaline lysis method described by Sambrook and Russell (23). The recombinant plasmid was checked for the presence of desired insert by PCR and restriction endonuclease analysis.

Nucleotide sequencing of the cloned insert

The cloned insert was sequenced at the DNA Sequencing Facility, Department of Biochem-istry, University of Delhi, South Campus, using a M13 reverse primer with the help of an automated DNA sequencer (ABI PRISM310, Perkin Elmer, Waltham, Massachusetts). The entire VP1/VP2 gene of CPV cloned in the TOPO® TA vector was sequenced by Sanger's dideoxy chain termination method using primer walking.

Sequence analysis

The sequences were aligned against the other published CPV VP1/VP2 gene sequences (Table I) using software from DNASTAR® Inc. (Madison, Wisconsin). The amino acid

© IZS A&M 2010

sequence, phylogenetic maps and percentage homology were deduced and analysed from the sequences using the same software.

Results

The 2 256 kbp amplicon denoting the entire length of the structural gene coding for the structural protein of the CPV-2 was amplified using the primer set pCPV. Of 13 samples screened for presence of CPV, the specific PCR amplicons (2 256 kbp) was obtained in five samples (Fig. 1). These samples were used as inoculum to infect the 70% confluent MDCK cell monolayers. Of the five samples, only two produced cytopathic changes including rounding of cells, granulation and aggregation of cells in MDCK cells after 72 h post infection; this increased subsequently and was widely distributed across the entire monolayer. The infected cells subsequently detached from the plastic culture bottle 4 days post infection.

PCR was also performed with DNA extracted from the parvovirus vaccine, Nobivac® Puppy DP vaccines and cell culture supernatant and specific amplicons of 2 256 kbp were obtained. The PCR amplicons of 2 256 kbp from cell

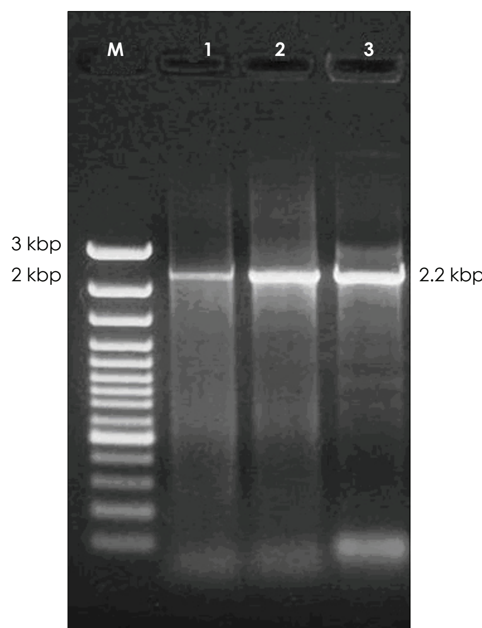

Figure 1
Agarose gel showing amplification of the entire VP-1/VP-2 gene (2.2 kbp) by using primer set canine parvovirus (pCPV) (forward) and pCPV (reverse)
Lane M DNA marker 100 bp 3 kbp
Lane 1: 2 256 bp (2.2) kbp PCR product of Bhopal isolate
Lane 2: 2.2 kbp PCR product of vaccine strain (NOBI)
Lane 3: 2.2 kbp PCR product of parvovirus vaccine strain (PVV)

Table I
Isolates of canine parvovirus variants used to compare nucleotide and amino acid sequences

CPV isolate	Genbank accession No.	CPV-type strain	Reference
CPV	NC_001539	CPV-2a	24
CPV-India	AJ698134	CPV-2b	10
CPV-JapanV217	AB054220	CPV-2a	11
CPV-Africa3	AJ007498	CPV-2b	26
CPV-Africa9	AJ007500	CPV-2a	26
CPV-ChowChow	AJ002927	CPV-2	30
CPV-Quinn	AJ002929	CPV-2a	30
CPV-Taiwan4 (strain T4)	U72695	CPV-2b	5
CPV-Thailand30	AY262281	CPV-2a	25
CPV-Polish	Z46651	CPV-2b	13
CPV-Italy695	AF401519	CPV-2b	3
CPV-Italy699	AF393506	CPV-2b	3
FPV	M38246		20
FPV-JapanV208	AB054226		11
FPV-Dohyvac	AJ002931		30
FPV-Panocell	AJ002932		30

CPV canine parvovirus
FPV feline panleukopenia virus

culture supernatant, parvovirus vaccine and Nobivac® Puppy DP vaccines were purified from agarose gel.

The purified PCR products were used for cloning into TOPO® TA vectors and confirmation for the presence of desired insert in recombinant plasmid DNA was performed by double digestion with restriction enzymes (*Eco R*I & *Xba* I) which showed the release of inserts from the vector as shown in Figure 2.

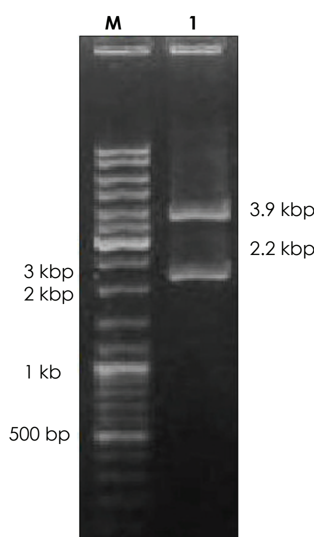

Figure 2
Agarose gel showing release of 2.2 kbp insert from recombinant plasmid after double digestion of recombinant with *Eco*RI and *Xba* I enzymes

The recombinant plasmids containing the correct inserts of three different clones (Bhopal field strain), parvovirus vaccine strain (PVV) and Nobivac® Puppy DP strain (NOBI) were sequenced. The sequences of entire VP1/VP2 structural genes (2 256 kbp) of all three clones were obtained. These sequence data also confirmed that our desired inserts were cloned in the correct orientation in the TOPO® TA vector.

The CPV VP1/VP2 gene sequence of the Bhopal field strain, PVV and NOBI were compared with the published VP1/VP2 gene sequence of CPV-2 variants (Table I). Both nucleotide and deduced amino acid sequences of different strains of CPV-2 variants were aligned and compared to the CPV-2 sequence using the 'Editseq and Megalign' program in the DNASTAR® software. The comparative analysis was made of the nucleotide sequence of VP1/VP2 gene of CPV-2 of the Bhopal field sample, two different vaccine strains (PVV and NOBI) and different CPV-2 variants from various world locations.

There was no appreciable variation between the nucleotide sequences of VP1/VP2 gene of PVV and NOBI vaccine strains and CPV type 2. The percent homology of PVV and NOBI at the nucleotide level was 99.3 and 99.4, respectively, when compared with CPV type 2; the percent divergence was 0.6 for both strains. The percent similarity between both vaccine strains was 99.1 and the percent divergence was 0.9. Both vaccine strains (PVV and NOBI) showed 98.7% and 98.4% homology with FPV-Dohyvac and FPV-Panocell, respectively (Fig. 3).

There were appreciable changes in the nucleotide sequence of the Bhopal field strain compared to both vaccine strains and published of CPV type 2 sequences. The Bhopal field sample showed 99.2% homology and 0.8% divergence when compared with CPV type 2. While comparing the two vaccine strains with the field strain, the percent similarity at both nucleotide and deduced amino acid levels was 98.8 and the percent divergence was 1.2. The Bhopal field isolate showed 98.8% homology and 1.2% divergent with CPV-India. The percent similarities and divergence of the nucleotide and deduced amino acid sequence by comparison of different CPV isolates based on VP1/VP2 gene is given in Figures 3 and 4, respectively.

The difference at nucleotide positions 760, 803, 1400, 1414, 1600, 1642 and 1777 of VP1/VP2 gene and at amino acid residues 87, 101, 300, 305, 367, 375 and 426 in the capsid protein VP1/VP2 nucleotide and deduced amino acid sequences, of different CPV isolates is given in Table II. The analysis of the VP1/VP2 gene sequence of the Bhopal field strain (nucleotide position at 760, 803, 1400, 1414, 1600, 1642 and 1777 and amino acid residues at 87, 101, 300, 305, 367, 375 and 426) showed that it belongs to CPV type 2b. This was further confirmed as the presence of aspartate at position 426 in the deduced amino acid sequence of the Bhopal.

© IZS A&M 2010

Percent identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		99.4	98.4	98.8	98.6	98.6	98.8	98.4	98.4	98.6	98.5	98.5	98.8	98.8	99.1	98.6	98.8	99.5	99.3	1	FPV-JAPAN208.SEQ
2	0.6		98.3	98.8	98.5	98.5	98.8	98.4	98.3	98.5	98.4	98.5	98.7	98.8	99.2	98.5	98.8	99.8	99.2	2	FPV-PANOCELL.SEQ
3	1.6	1.7		99.2	99.3	99.3	99.2	98.8	100.0	99.4	99.3	98.8	99.5	99.2	98.5	99.4	99.3	98.4	98.6	3	CBPP-BHO.SEQ
4	1.2	1.2	0.8		99.6	99.6	99.8	99.4	99.2	99.6	99.5	99.4	99.7	100.0	99.0	99.6	99.9	98.9	99.1	4	CPV.SEQ
5	1.4	1.5	0.7	0.4		99.6	99.6	99.1	99.3	99.7	99.6	99.2	99.9	99.6	98.8	99.6	99.6	98.6	98.9	5	CPV-AFRICA3.SEQ
6	1.4	1.5	0.7	0.4	0.4		99.6	99.1	99.3	99.6	99.6	99.2	99.8	99.6	98.8	99.6	99.6	98.6	98.9	6	CPV-AFRICA9.SEQ
7	1.2	1.2	0.8	0.2	0.4	0.4		99.3	99.2	99.6	99.5	99.5	99.7	99.8	99.0	99.6	99.8	98.9	99.1	7	CPV-CHOWCHOW.SEQ
8	1.6	1.6	1.3	0.6	0.9	0.9	0.7		98.8	99.1	99.0	99.1	99.2	99.4	98.6	99.1	99.3	98.5	98.7	8	CPV-INDIA.SEQ
9	1.6	1.7	0.0	0.8	0.7	0.7	0.8	1.3		99.4	99.3	98.8	99.5	99.2	98.5	99.4	99.3	98.4	98.6	9	CPV-ITALY695.SEQ
10	1.4	1.5	0.6	0.4	0.3	0.4	0.4	0.9	0.6		99.7	99.2	99.9	99.6	98.8	99.7	99.6	98.6	98.9	10	CPV- ITALY699.SEQ
11	1.5	1.6	0.7	0.5	0.4	0.4	0.5	1.0	0.7	0.3		99.1	99.8	99.5	98.8	99.7	99.6	98.5	98.8	11	CPV-JAPAN217.SEQ
12	1.5	1.5	1.2	0.6	0.8	0.8	0.5	0.9	1.2	0.8	0.9		99.3	99.4	98.6	99.2	99.4	98.6	98.8	12	CPV-NOBI.SEQ
13	1.3	1.3	0.5	0.3	0.1	0.2	0.3	0.8	0.5	0.1	0.2	0.7		99.7	99.0	99.8	99.7	98.8	99.0	13	CPV-POLISH.SEQ
14	1.2	1.2	0.8	0.0	0.4	0.4	0.2	0.6	0.8	0.4	0.5	0.6	0.3		99.0	99.6	99.9	98.9	99.1	14	CPV-PW.SEQ
15	0.9	0.8	1.5	1.0	1.2	1.2	1.0	1.4	1.5	1.2	1.3	1.4	1.0	1.0		98.8	99.0	99.2	99.3	15	CPV-QUINN.SEQ
16	1.4	1.5	0.6	0.4	0.4	0.4	0.4	0.9	0.6	0.3	0.3	0.8	0.2	0.4	1.2		99.6	98.6	98.9	16	CPV-TAIWAN4.SEQ
17	1.2	1.2	0.7	0.1	0.4	0.4	0.2	0.7	0.7	0.4	0.4	0.6	0.3	0.1	1.0	0.4		98.9	99.1	17	CPV-THAILAND30.SEQ
18	0.5	0.2	1.6	1.1	1.4	1.4	1.1	1.5	1.6	1.4	1.5	1.4	1.3	1.1	0.8	1.4	1.1		99.3	18	CPV.SEQ
19	0.7	0.8	1.4	0.9	1.1	1.1	0.9	1.3	1.4	1.1	1.2	1.3	1.0	0.9	0.7	1.1	0.9	0.7		19	CPV-DOHYVAC.SEQ
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Figure 3
Percent similarity and divergence of the nucleotide sequence of VP1/VP2 gene of two vaccine strains and the Bhopal field isolate and their comparison with several published nucleotide sequences of canine parvovirus isolates

Percent identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		99.5	99.1	98.9	98.0	99.1	99.6	99.7	98.4	99.9	99.1	97.7	99.1	99.6	99.1	98.0	98.0	98.0	98.0	1	CPV-AFRICA3.SEQ
2	0.5		99.1	98.9	98.0	99.1	99.5	99.5	98.4	99.6	99.1	97.7	99.1	99.6	99.1	98.0	98.0	98.0	98.0	2	CPV-AFRICA9.SEQ
3	0.9	0.9		98.5	97.6	100.0	99.3	99.3	98.0	99.2	98.7	97.3	98.7	99.5	98.9	97.9	97.6	97.9	97.9	3	CPV-BHO.SEQ
4	1.1	1.1	1.5		98.5	98.5	98.9	98.9	99.2	99.1	99.6	98.0	99.6	99.1	99.6	98.5	98.3	98.3	98.5	4	CPV-CHOWCHOW.SEQ
5	2.0	2.0	2.4	1.5		97.6	98.0	98.0	98.3	98.1	98.9	97.3	98.9	98.1	98.7	97.9	97.6	97.6	97.9	5	CPV-INDIA.SEQ
6	0.9	0.9	0.0	1.5	2.4		99.3	99.3	98.0	99.2	98.7	97.3	98.7	99.5	98.9	97.9	97.6	97.9	97.9	6	CPV-ITALY695.SEQ
7	0.4	0.5	0.7	1.1	2.0	0.7		99.9	98.4	99.7	99.1	97.7	99.1	99.9	99.3	98.0	98.0	98.0	98.0	7	CPV- ITALY699.SEQ
8	0.3	0.5	0.7	1.1	2.0	0.7	0.1		98.4	99.9	99.1	97.7	99.1	99.9	99.3	98.0	98.0	98.0	98.0	8	CPV-JAPAN217.SEQ
9	1.6	1.6	2.0	0.8	1.7	2.0	1.6	1.6		98.5	99.1	97.5	99.1	98.5	99.1	98.0	97.7	97.7	98.0	9	CPV-NOBI.SEQ
10	0.1	0.4	0.8	0.9	1.9	0.8	0.3	0.1	1.5		99.2	97.9	99.2	99.7	99.2	98.1	98.1	98.1	98.1	10	CPV-POLISH.SEQ
11	0.9	0.9	1.3	0.4	1.1	1.3	0.9	0.9	0.9	0.8		98.1	100.0	99.2	99.7	98.7	98.4	98.4	98.7	11	CPV-PW.SEQ
12	2.3	2.3	2.7	2.0	2.7	2.7	2.3	2.3	2.6	2.2	1.9		98.1	97.9	98.1	98.7	98.7	98.7	98.7	12	CPV-QUINN.SEQ
13	0.9	0.9	1.3	0.4	1.1	1.3	0.9	0.9	0.9	0.8	0.0	1.9		99.2	99.7	98.7	98.4	98.4	98.7	13	CPV.SEQ
14	0.4	0.4	0.5	0.9	1.9	0.5	0.1	0.1	1.5	0.3	0.8	2.2	0.8		99.5	98.1	98.1	98.1	98.1	14	CPV-TAIWAN4.SEQ
15	0.9	0.9	1.1	0.4	1.3	1.1	0.7	0.7	0.9	0.8	0.3	1.9	0.3	0.5		98.7	98.4	98.4	98.7	15	CPV-THAILAND30.SEQ
16	2.0	2.0	2.2	1.5	2.2	2.2	2.0	2.0	2.0	1.9	1.3	1.3	1.3	1.9	1.3		98.9	99.7	100.0	16	FPV-SEQ
17	2.0	2.0	2.4	1.7	2.4	2.4	2.0	2.0	2.3	1.9	1.6	1.3	1.6	1.9	1.6	1.1		98.9	98.9	17	FPV-DOHYVAC.SEQ
18	2.0	2.0	2.2	1.7	2.4	2.2	2.0	2.0	2.3	1.9	1.6	1.3	1.6	1.9	1.6	0.3	1.1		99.7	18	FPV-JAPAN208.SEQ
19	2.0	2.0	2.2	1.5	2.2	2.2	2.0	2.0	2.0	1.9	1.3	1.3	1.3	1.9	1.3	0.0	1.1	0.3		19	FPV-PANOCELL.SEQ
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Figure 4
Percent similarity and divergence of the deduced amino acid sequence of VP1/VP2 gene of two vaccine strains and the Bhopal field isolate and their comparison with several published amino acid sequences of canine parvovirus isolates

Table II
Nucleotide sequence differences in the capsid protein gene of different isolates of canine parvovirus variants

Virus strain	Nucleotide in VP1/VP2 gene of CPV-2						
	760	803	1400	1414	1600	1624	1777
CPV	A Met	T Ile	C Ala	G Asp	T Tyr	G Asp	A Asn
CPV-India	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	G Asp
CPV-JapanV217	A Met	T Ile	C Ala	G Asp	T Tyr	G Asp	A Asn
CPV-Africa3	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	G Asp
CPV-Africa9	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	A Asn
CPV-ChowChow	A Met	T Ile	C Ala	G Asp	G Asp	A Asn	A Asn
CPV-Quinn	A Met	C Thr	C Ala	G Asp	G Asp	G Asp	A Asn
CPV-Taiwan4	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	A Asn
CPV-Thailand30	A Met	T Ile	C Ala	G Asp	G Asp	G Asp	A Asn
CPV-Polish	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	G Asp
CPV-Italy695	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	G Asp
CPV-Italy699	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	G Asp
FPV	A Met	T Ile	C Ala	G Asp	G Asp	G Asp	A Asn
FPV-JapanV208	A Met	C Thr	C Ala	G Asp	G Asp	G Asp	A Asn
FPV-Dohyvac	A Met	C Thr	C Ala	G Asp	G Asp	G Asp	A Asn
FPV-Panocell	A Met	C Thr	C Ala	G Asp	G Asp	G Asp	A Asn
CPV-Bho	T Leu	C Thr	G Gly	T Tyr	G Asp	G Asp	G Asp
CPV-PVV	A Met	T Ile	C Ala	G Asp	G Asp	A Asn	A Asn
CPV-NOBI	A Met	T Ile	C Ala	G Asp	G Asp	A Asn	A Asn
Residue in VP2	87	101	300	305	367	375	426

CPV canine parvovirus
FPV feline panleukopenia virus
Bho Bhopal
PVV parvovirus vaccine strain
NOBI Nobivac® Puppy DP strain

field strain was found to be a CPV-2b mutant The sequence analysis and the presence of methionine at 87, isoleucine at 101, alanine at 300 and asparagine at 367 and 426 in the deduced amino acid sequence of PVV and NOBI vaccine strain further confirmed that these strains belong to CPV-2 variant.

The phylogenetic relationship based on nucleotide and deduced amino acid sequences of the different CPV isolates is given in Figures 5 and 6, respectively. The phylogenetic tree based on the nucleotide sequence of VP1/VP2 gene revealed that CPV-2 and both CPV vaccine strains were in separate

© IZS A&M 2010

monophyletic groups, whereas the field sample formed another group (Fig. 6). The FPV belongs to a separate monophyletic group when analysed with nucleotide sequences of the VP1/VP2 gene of the Bhopal field strain, PVV and NOBI. The FPV-Panocell, FPV-Dohyvac, FPV-JapanV208 and CPV-Quinn isolates are grouped under a different monophyletic group of the phylogenetic tree (11, 18, 30). The Bhopal field strain was grouped in same monophyletic group that includes CPV-Italy699, CPV-Taiwan4 and CPV-Japanv217 isolates. The nucleotide sequence analysis revealed that CPV-Italy699, CPV-Italy695, CPV-India, CPV-Polish, CPV-Taiwan4 and CPV-Africa 3 are CPV type 2b. Both the PVV and NOBI vaccine strains have been placed in the same monophyletic group formed by CPV-ChowChow, CPV-India and CPV-Thailand. Among the strains included in this study, only CPV-ChowChow was found to be CPV type 2 and the original CPV-2 strain was completely replaced by the newer antigenic types CPV-2a and CPV-2b (18), currently prevalent in different parts of the world.

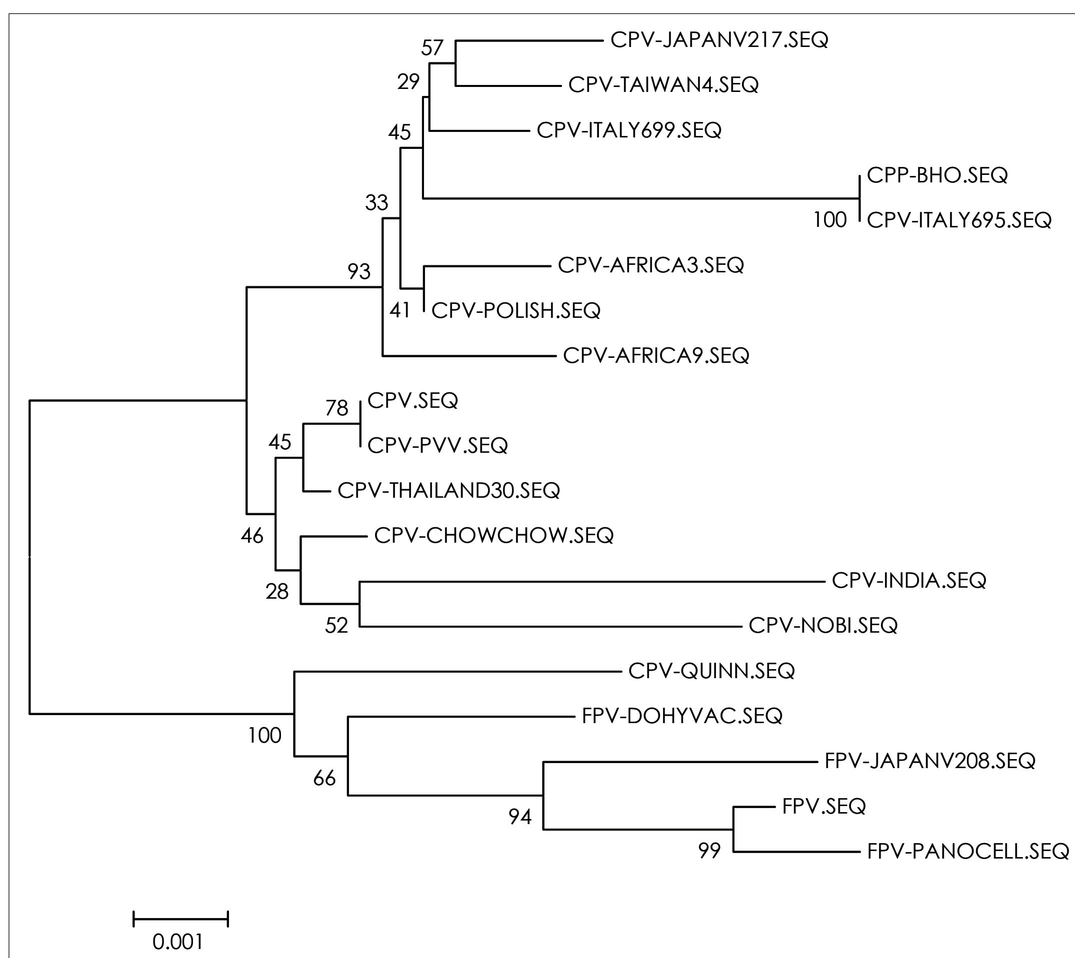

Figure 5
Phylogenetic analysis based on nucleotide sequence of VP1/VP2 gene of two vaccine strains and the Bhopal field isolate and their comparison with several published nucleotide sequences of canine parvovirus isolates

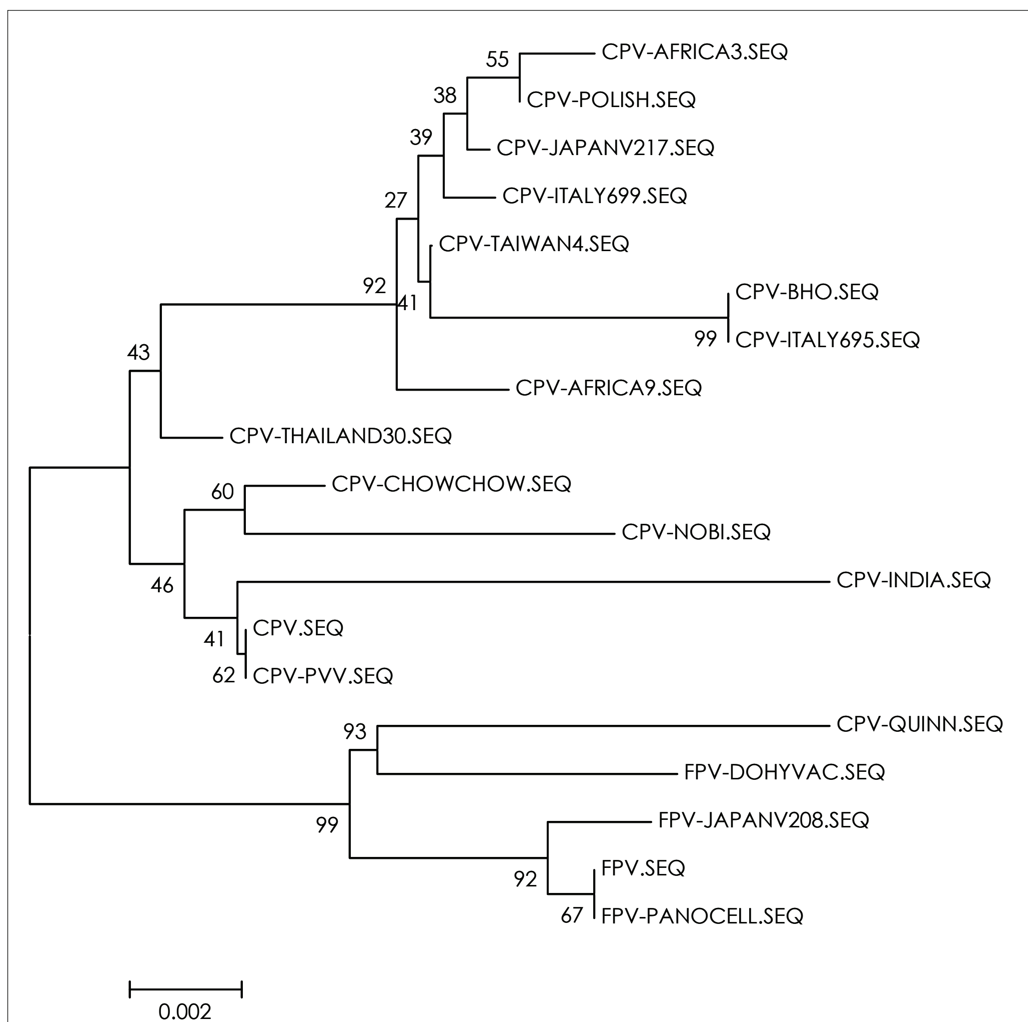

Figure 6
Phylogenetic analysis based on deduced amino acid sequence of VP1/VP2 gene of two vaccine strains and the Bhopal field isolate and their comparison with several published amino acid sequences of canine parvovirus isolates

Discussion

From the nucleotide sequence data analysis of CPV, vaccine strains were found to be CPV-2 whereas the Bhopal field strain proved to be a CPV-2b antigenic variant. CPV-India was also a CPV-2b variant similar to the Bhopal field strain which indicated that CPV-2b prevailed in the fields of northern India (15). However, to obtain a comprehensive picture, a greater number of samples needs to be collected and tested from various part of India to generate information regarding the molecular epidemiology of CPV. Our results clearly revealed that CPV variants can be differentiated by sequence analysis of the VP1/VP2 gene. The comparison of VP1/VP2 gene sequences among different CPV variants revealed that the major part of the gene is conserved. However, changes in specific nucleotide position affect the major antigenic site of the viral capsid that determines the unique variant of CPV. Consequently, to obtain better protection against the field strains of CPV, the incorporation of a specific new variant of CPV-2 (CPV-2a and CPV-2b) in the vaccine is recommended based on the prevalence in the country.

Numerous CPV outbreaks in dogs have been reported over the last few years from different parts of India (4, 15, 21). Nandi *et al.* studied the occurrence of CPV infections in dogs and CPV-2b was found to be the predominant strain in northern India (15). Furthermore,

© IZS A&M 2010

Chinchkar *et al.* studied the epidemiology of CPV infections in dogs in India and found that CPV type 2a isolates were predominant over the CPV-2b variant in central and southern India (6). Upon comparison of the VP2 gene sequences, the Indian isolates formed separate lineages distinct from the South-East Asian isolates. The Indian CPV isolates appear to evolve independently and distinct geographic patterns of evolution could not be identified in the isolates (6). However, there is no detailed information available on the genetic makeup of any of the CPV strains used in commercial vaccines. This is of paramount importance in the context of the Indian subcontinent as massive numbers of both pet and stray dogs are involved. The CPV vaccine used in dog populations in India is mostly imported by vaccine companies from abroad. It is always advisable to use an indigenous CPV vaccine strain as the immuno-prophylactic agent against prevailing field strains. The VP1/VP2 gene sequence is important in the determination of antigenic types based on the epitopes located on the VP2 capsid protein. The characterisation of the VP1/VP2 gene is most important due to the presence of neutralising epitopes, amino acids responsible for important biological properties of the virus and for designing an effective and efficacious DNA vaccine for the control of CPV infection (5, 15, 18). Therefore the study was performed to characterise the VP1/VP2 gene of vaccine strains as well as Indian field strains so that it would generate useful information on the genetic composition of the CPV which, in turn, would provide the baseline data for epidemiological surveillance of different CPV antigenic variants that prevail in India. The recombinant VP1/VP2 capsid protein can be used to screen serum samples during serological surveillance of the disease and to determine the efficacy of the vaccine.

The antigenic variants (types 2a and 2b) differ from CPV type 2 in five or six amino acids and variant type 2b differs from type 2a in only two positions, Asn-426 to Asp and Ile-555 to Val (17, 18). Residue 426 is placed at a major antigenic site (epitope A) over the threefold spike of the capsid and the mutation Asn-426 to Asp differentiates from CPV-2b not only from CPV types 2 and 2a, but also from FPV and mink enteritis virus (MEV) (19). Conversely, residue 555 lies at a minor antigenic site and the mutation of Ile-555 to Val represents a reversion to or a retention of the sequence of the original type 2 (1, 18, 27). The nucleotide sequence analysis of the VP1/VP2 gene of CPV revealed that both vaccine strains belonged to CPV type 2 and differed from the Bhopal field strain that belonged to CPV type 2b. The original CPV type 2 strain has been replaced by the newer antigenic types (CPV-2a, CPV-2b and CPV-2c) around the world. Based on the prevalence of strains in field outbreaks and to achieve greater protection against CPV infection, it is now envisaged that the more recent variants of CPV will be incorporated in the vaccine. This is especially important in regard to the control emerging novel strains of CPV that cause a disease that has devastating consequences in dog populations.

Conclusions

Since the emergence of CPV-2 in 1978, a number of outbreaks have been reported due to the development of new variants, namely: CPV-2a, CPV-2b and CPV-2c. In India, there is an extremely high dog population and, in particular stray dogs that not protected by the immunisation umbrella against CPV infections and remain a source of infection to other susceptible populations. Furthermore, most vaccines used in India are based on the strain that was isolated about 25 to 30 years ago. The problem has been amplified manifold due to disease outbreaks, not only in unvaccinated but also in vaccinated animals. Keeping this in mind, it is therefore necessary to analyse not only CPV field strains but also the vaccine strains.

This study revealed that vaccines used in India are based on CPV-2 strain and are different from outbreak strains which belong to type CPV-2b. It is thus prime time to debate whether use of the CPV-2 strain should be discontinued in the face of CPV-2b outbreaks or whether a new indigenous strain might

offer greater and more effective protection against CPV infections.

Acknowledgement

Sincere thanks are extended to the Director of the Indian Veterinary Research Institute in Izatnagar for providing the facilities in which this study was conducted.

References

1. Agbandje M., Parrish C.R. & Rossmann M.G. 1995. The recognition of parvovirus capsids by antibodies. *Sem Virol*, **6**, 219-231.
2. Appel M.J.G., Scott F.W. & Carmichael L.E. 1979. Isolation and immunization studies of a canine parvo-like virus from dogs with hemorrhagic enteritis. *Vet Rec*, **105**, 156-159.
3. Battilani M., Scagliarini A., Tisato E., Turilli C., Jacoboni I., Casadio R. & Prosperi S. 2001. Analysis of canine parvovirus sequences from wolves and dogs isolated in Italy. *J Gen Virol*, **82**, 1555-1560.
4. Biswas S., Das P J., Ghosh S.K. & Pradhan N.R. 2006. Detection of canine parvovirus (CPV) DNA by polymerase chain reaction and its prevalence in dogs in and around Kolkata, West Bengal. *Indian J Anim Sci*, **76** (4), 324-325.
5. Chang W.L., Chang A.C.H. & Pan M.J. 1996. Antigenic types of canine parvoviruses prevailing in Taiwan. *Vet Rec*, **138**, 447.
6. Chinchkar S.R., Mohan S.B., Hanumantha R.N., Rangarajan P.N., Thiagarajan D. & Srinivasan V.A. 2006. Analysis of VP2 gene sequences of canine parvovirus isolates in India. *Arch Virol*, **151**, 1881-1887.
7. Decaro N., Martella V., Desario C., Bellaciccio A. L., Camero M., Manna L., d'Aloja D. & Buonavoglia C. 2006. First detection of canine parvovirus type 2c in pups with haemorrhagic enteritis in Spain. *J Vet Med B*, **53**, 468-472.
8. Decaro N., Desario C., Addie D.D., Martella V., Vieira M.J., Elia G., Davis C., Thompson G., Truyen U. & Buonavoglia C. 2007. The study of molecular epidemiology of canine parvovirus, Europe. *Emerg Infect Dis*, **13**, 1222-1224.
9. Deepa P.M. & Saseendrannath M.R. 2000. Serological studies on canine parvoviral infection. *Indian Vet J*, **79**, 643-644.
10. Gupta P.K., Rai A., Rai N., Raut A.A. & Chauhan S. 2004. Cloning of canine parvovirus VP2 gene and its use as DNA vaccine in dogs. *Curr Sci*, **88** (5), 778-782 (www.ias.ac.in/currsci/mar102005/778.pdf accessed on 4 November 2009).
11. Ikeda Y., Mochizuki Naito, R., Nakamura K., Miyazawa T., Mikami T. & Takahashi E. 2000. Predominance of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. *J Virol*, **278** (1), 13-19.
12. Martella V., Cavalli A., Pratelli A., Bozzo G., Camaro M., Buonavoglia D., Narcisi D., Tempesta M. & Buonavoglia C. 2004. A canine parvovirus mutant is spreading in Italy. *J Clin Microbiol*, **42**, 1333-1336.
13. Mizak B. & Plucienniczak A. 1995. Antigenic typing polish isolates of canine parvovirus. *Bull Vet Inst Pulawy*, **39**, 71-76.
14. Nakamura M., Tohya Y., Miyazawa T., Mochizuki M., Phung H.T., Huynh L.M., Nguyen L.T., Nguyen N.P. & Akashi H. 2004. A novel antigenic variant of canine parvovirus from a Vietnamese dog. *Arch Virol*, **149**, 2261-2269.
15. Nandi S., Kumar M., Anbazhagan R., Chidri S. & Chauhan R.S. 2007. A sensitive method to detect canine parvoviral DNA in the stool samples by polymerase chain reaction. *Indian J Comp Microbiol Immunol Infect Dis*, **27** (1&2), 56-57.
16. Nandi S., Pandey A.B., Sharma K. & Chauhan R.S. 2008. Polymerase chain reaction for detection of canine parvoviral DNA in vaccines. *Indian J Virol*, **19** (1), 9-11.
17. Parker J.S.L. & Parrish C.R. 1997. Canine parvovirus host range is determined by the specific conformation of an additional region of the capsid. *J Virol*, **71**, 9214-9222.

© IZS A&M 2010

18. Parrish C.R. 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. *Virology*, **183** (1), 195-205.
19. Parrish C.R. 1999. Host range relationship and the evolution of canine parvovirus. *Vet Microbiol*, **69**, 29-40.
20. Parrish C.R., O'Connell P.H., Evermann J.F. & Carmichael L.E. 1985. Natural variation of canine parvovirus. *Science*, **230**, 1046-1048.
21. Phukan A., Deka D. & Boro P.K. 2004. Occurrence of canine parvovirus infection in and around Guwahati. *Indian J Anim Sci*, **74** (4), 930-931.
22. Ramadass P. & Khader T.G.A. 1982. Diagnosis of canine parvovirus infection by agar gel precipitation test and fluorescent antibody techniques. *Cheiron*, **11**, 323-325.
23. Sambrook J. & Russell D.W. 2001. Molecular cloning; A laboratory manual, 3rd Ed. Cold Spring Harbor Laboratory Press, New York, 1.31-1.38, 1.116-1.118.
24. Reed A.P., Jones E.V. & Miller T.J. 1988. Nucleotide sequence and genome organization of canine parvovirus. *J Virol*, **62**, 266-276.
25. Sakulwira K., Vanapongtipagorn P., Theamboonlers A., Oraveerakul K. & Poovorawan Y. 2003. Prevalence of canine coronavirus and parvovirus infection in dogs with gastroenteritis in Thailand. *Vet Med Czech*, **48** (6), 163-167 (www.cazv.cz/2003/VM6_03/3-Sakulwira.pdf accessed on 30 November 2009).
26. Steinel A., Venter E.H., Van Vuuren M., Parrish C.R. & Truyen U. 1998. Antigenic and genetic analysis of canine parvoviruses in southern Africa. *Onderstepoort J Vet Res*, **65**, 239-242.
27. Strassheim M.L., Gruenberg A., Veijalainen P., Sgro J.Y. & Parrish C.R. 1994. Two dominant neutralizing antigen determinants of canine parvovirus are found on the threshold of the three fold spike of the virus capsid. *Virology*, **198**, 175-184.
28. Truyen U.A. 2006. Evolution of canine parvovirus – a need for new vaccines. *Vet Microbiol*, **117**, 9-13.
29. Truyen U.A., Gruneberg S.F., Chang B., Obermaier P. & Parrish C.R. 1995. Evolution of the feline-subgroup of parvoviruses and the control of canine host range *in vivo*. *J Virol*, **69**, 4702-4710.
30. Truyen U., Geissler K., Parrish C.R., Hermanns W. & Siegl G. 1998. No evidence for a role of modified live virus vaccines in the emergence of canine parvovirus. *J Gen Virol*, **79**, 1153-1158 (vir.sgmjournals.org/cgi/reprint/79/5/1153.pdf accessed on 28 December 2009).