The Pharma Innovation Journal 2021; 10(7): 1165-1168

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(7): 1165-1168

www.thepharmajournal.com
Received: 23-04-2021
Accepted: 10-06-2021

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Identification of canine parvovirus variants by polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP)

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Abstract
The research study was conducted to identify the canine parvovirus variants (CPV 2a, CPV 2b, & CPV 2c) by using type specific primers based polymerase chain reaction (PCR) followed by Restriction fragment length polymorphism (RLFP). Totally 180 numbers of faecal samples were collected in the form of faecal swabs from the domesticated dogs. Type specific primer pair CPV 2ab and CPV 2b were used in PCR to identify the CPV type 2a and CPV type 2b followed by RFLP using the enzyme *Mbo II* to differentiate the CPV type 2c from CPV type 2b after recognize selectively the sequence GAGAA at 4062 – 4064. Out of 180 samples screened, 80 samples were found to be positive to CPV-2ab primers and 14 samples were positive to CPV-2b primers and indicated that 66 samples were positive to CPV – 2a. PCR-RFLP could not detect any CPV – 2c strain from this study.

Keywords: Canine parvovirus, type specific primers, PCR, RFLP

Introduction
Parvo viral infection is the highly infectious viral disease of domesticated dogs of great concern to pet owners, practicing veterinarians and scientists due to its high morbidity and mortality rates especially in young puppies (Bargujar *et al*., 2011) [1]. It is caused by the virus called canine parvovirus (CPV). Canine parvovirus genome made up of linear single stranded DNA and has two open reading frames (ORF) of which the first two capsid proteins (VP1 and VP2) which are translated from alternatively spliced mRNA (Reed *et al*., 1988) [14]. VP2 gene is the major component of the nonenveloped icosachedral capsid of CPV, and only a few amino acid substitutions in its sequence can alter relevant biological characteristics of the virus and generate new genotypes (CPV-2a, CPV-2b and CPV-2c) (Buonavoglia *et al*., 2001; Calderon *et al*., 2009) [2, 3].
Polymerase chain reaction is a technique for *in-vitro* amplifying a specific region of DNA by a thermos table DNA polymerase. It uses short oligonucleotide primer sequence, dNTPs, and DNA template and thermo stable DNA polymerase enzyme. Polymerase chain reaction (PCR) reaction involves three steps *viz*. denaturation, annealing and extension repeatedly for 30 - 40 cycles in the thermal cycler. It can be used for detection of virus specific nucleic acid. (Minakshi *et al*., 2014) [8]. Polymerase chain reaction is widely used as a tool for the diagnosis of canine parvoviral infection which provides rapid, sensitive and accurate diagnosis of the disease (Firoozjaii *et al*., 2011; Kumar *et al*., 2010) [5, 7]. Further, different antigenic variants of CPV can also be differentiated by employing PCR with different combination of primer sets (Nandi *et al*., 2010) [9] with some modification. PCR has increasingly been employed for detection of pathogens, especially when present at very low titers (Sakulwira *et al*., 2001) [15] of 0.002 pg/micro litre of CPV DNA (Sharma *et al*., 2012) [17].
The PCR can now be used to differentiate the different mutants of CPV-2 using the primer specific for particular mutants such as CPV-2a and CPV-2b. But, PCR typing approach was unable to identify the variation that occurred in Glu-426 (CPV – 2c) mutants of CPV-2b and this can be overcome by the development of RFLP by using *Mbo II* restriction enzyme. PCR-RFLP assay using enzyme *Mbo* II was developed which was proven to be useful for discrimination between types 2b and 2c. Similarly, Zhang *et al.* (2010) [18] found that restriction enzyme *Mbo* II was able to recognize selectively the sequence GAGAA at 4062 – 4064 for CPV-2c. Restriction enzyme digestion further substantiates the specificity of the PCR (Nandi *et al*., 2010) [9]. Keeping these in view, the present research study was conducted to identify the canine parvovirus variants by type specific primers based polymerase chain

reaction (PCR) followed by Restriction fragment length polymorphism (RFLP) in faecal samples of domesticated dogs.

Materials and Methods

In this research study, Totally 180 numbers of faecal samples were collected in the form of faecal swabs from the domesticated dogs which are brought to the teaching veterinary clinical complex, Veterinary College and Research Institute, Namakkal, Tamil Nadu. Among the one hundred and eighty faecal swabs, one hundred and twenty faecal swabs were collected from the clinically affected dogs with the signs of anorexia, diarrhoea, vomiting, and sixty faecal swabs were collected from apparently healthy dogs brought for vaccination and other purposes. The collected faecal swabs were emulsified in the phosphate buffer saline (PBS), centrifuged and supernatant was stored in -20 degree Celsius until further use.

Polymerase chain Reaction

DNA extraction and primer pair selection

The viral DNA was extracted from all faecal samples by using QIAamp DNA Stool Mini Kit as per manufacturer protocol. The eluted DNA was collected from the final step of the protocol and stored in a 2 ml series tube. Primers were selected from variable regions in the VP1/VP2 capsid genes, according to published nucleotide sequences of CPV-2ab (Senda *et al*., 1995) [16] and CPV-2b (Pereira *et al*., 2000) [13] could detect new type-strains of CPV-2a and 2b and CPV-2b, respectively.

Canine parvovirus type 2a is recognized by the primer pair Pab only, but CPV type 2b is recognized by both primer pairs Pab and Pb (Firoozjaii *et al*., 2011) [5]. The primers were custom synthesized (Thermo Scientific, USA) and used in this study. The sequences of the primers are presented in the Table - 1

Table 1: CPV Type specific primer sequence for Amplification of VP2 gene

Sequence of primers used for VP2 gene amplification	Nucleotide position	Product size
CPV-2ab(F): 5' -GAAGAGTGGTTGTAAATAATA- 3' CPV-2ab(R):5' -CCTATATCACCAAAGTTAGTAG- 3'	3025-3045 to 3685-3706	681 bp
CPV-2b(F): 5' -CTTTAACCTTCCTGTAACAG- 3' CPV-2b(R): 5' -CATAGTTAAATTGGTTATCTAC- 3'	4043-4062 to 4449-4470	427 bp

Amplification of Canine parvovirus VP2 gene fragment

The resulting single stranded DNA was amplified in PCR, utilizing type specific CPV 2ab and 2b primers as described by Buonavoglia *et al*. (2001) [2] and Firoozjaii *et al*. (2011) [5] respectively. A 50 µl reaction mixture was prepared in 0.2 ml thin wall PCR tubes that contained following composition. Template DNA - 5µl, 2x concentration of master mix - 25µl, forward primer (10 Pico moles) - 1µl, reverse primer (10 Pico moles) - 1µl, Nuclease free molecular grade water - 18µl. The PCR tubes with all components were transferred to automated thermal cycler, (Eppendorf Master Cycler, Germany) for PCR amplification according to the program presented in Table – 2.

Table 2: Steps and conditions of thermal profile involved for PCR

Serial No	Steps	Temperature	Time
Step 1	Initial denaturation	95°C	5 min
Step 2	Denaturation	95°C	1 min
Step 3	Annealing	55°C	2 min
Step 4	Extension	72°C	30 sec
Step 2 to step 4 repeated for 30 cycles			
Step 5	Final extension	72°C	10 min
Step 6	Holding temperature	4°C	

Agarose gel electrophoresis

Agarose gel was prepared by clean dry gel tray in which the sides were sealed by using good quality adhesive tapes. The tray was placed on a levelled surface and a suitable comb was placed in the platform. Agarose (low EEO), 300 mg was taken in a conical flask and 15 ml of 1x Tris-acetate EDTA buffer was added to prepare two per cent agarose gel. The agarose (Low EEO) was melted for five minutes until it got dissolved completely and ethidium bromide (0.5µg/ml) was added to it. Agarose gel was carefully poured into the gel tray without air bubbles. Gel was allowed to solidify for 20 – 30 min at room temperature and then the comb was removed. After removing the tape, the electrophoresis buffer (1X TAE buffer) was poured off in the electrophoresis tank.

Submarine gel electrophoresis

The 1X TAE buffer was added over the gel to a depth of 1 mm. A volume of 10 µl of PCR product, 10 µl non-template samples as negative control, 10 µl of vaccine PCR product as positive control and 100 bp ladder were slowly loaded into the slots of the submerged gel using a micropipette. Electrophoresis tank was closed with a lid and attached to the electrical leads so that the DNA will migrate towards the positive anode (red lead). The submarine gel electrophoresis was carried out at 80 volt for 40 min.

Visualization of PCR products

The gel was visualized under UV transilluminator and bands of appropriate size were identified by comparison with the 100 bp ladder. The sample was scored as positive only if a 681 bp DNA fragment of expected size for CPV -2ab primers and 427 bp for CPV-2b primers were identified on the gel and no signal was obtained with negative control. The images were documented using gel documentation system (Vilber Lourmat, France).

Restriction fragment length polymorphism

For RFLP analysis, *Mbo II* enzyme was purchased from Thermo Scientific, USA and used in this study. Positive CPV 2b PCR reaction mixture was used in RFLP technique after polymerase chain reaction amplification procedure to differentiate the CPV type 2c from CPV type 2b. The protocol for digestion of PCR products directly after amplification was followed as manufacturer instruction. RFLP reaction contains, PCR reaction mixture - 10 µl, Nuclease – free water -18µl, 10X Buffer- 2µl, *MboII* -1-2µl. Reaction Mixture was mixed gently and spin down for few seconds and Incubated at 37°C for 1 – 16 hours. The cleavage pattern of the amplicon was observed on 1.5% agarose gels stained with ethidium bromide.

Results and Discussion

All the one hundred and eighty faecal samples were subjected

to PCR using 'both CPV-2ab and CPV-2b primer pairs and revealed a product size of 681 bp and 427 bp respectively. Out of 180 samples screened, 80 samples were found to be positive for CPV-2ab primers (figure -1) and 14 samples were positive to CPV-2b primers and indicated that 66 samples were positive to CPV-2a. Observed rate of type specific prevalence CPV 2a and CPV 2b were 82.5 and 17.5 per cent respectively. This is in agreement with Narayanan *et al*. (2001) [10], Chinchikar *et al*. (2006)[4] and Panneer *et al*. (2008) [11] who reported that prevalence of CPV 2a is more when compared to other mutants in South India.

Fourteen CPV-2b positive samples were subjected to RFLP using *Mbo II* enzyme for detection of CPV-2c circulating in the canine population. The absence of bands sizes of 426 bp and 1 bp indicated the absence of CPV-2c in Namakkal, Tamil Nadu. Parthiban *et al*. (2010) [12] also reported that molecular typing of clinical samples by PCR and PCR- RFLP based assays did not reveal any involvement of CPV-2c type in the field cases of canine parvovirus infections in Pondicherry.

As far as health status concerned, all the 80 positive faecal samples are belongs to clinically affected animals with the clinical signs. Polymerase chain reaction could not detect any canine parvoviral DNA from the faecal samples of apparently healthy animals. Higher positivity was observed in clinically affected dogs, when compared with apparently healthy dogs. This is in agreement with Islam *et al*., (2014) [6] who reported that significantly higher prevalence of CPV was recorded in diarrhoeic dogs compared with those having no diarrhoea. He also stated that dogs with poor health condition were more vulnerable to canine parvovirus infection compared to those with normal health status.

The PCR based genotyping system developed by Pereira and colleagues (Pereira *et al*., 2000) [13] is no longer able to discriminate between type 2a and type 2b strains, as almost all the novel type 2a strains (555-Val) will go mischaracterized as type 2b. Type specific PCR assays could not detect CPV variant type 2c, since the substitution D426E is due to a change (T - A) in the third codon position, at nucleotide 4064, so that this mutant is erroneously recognised as type 2b by this PCR strategy. Applying PCR and subsequent RFLP analysis with the restriction endonuclease MboII enabled us to differentiate CPV-2c from CPV-2b

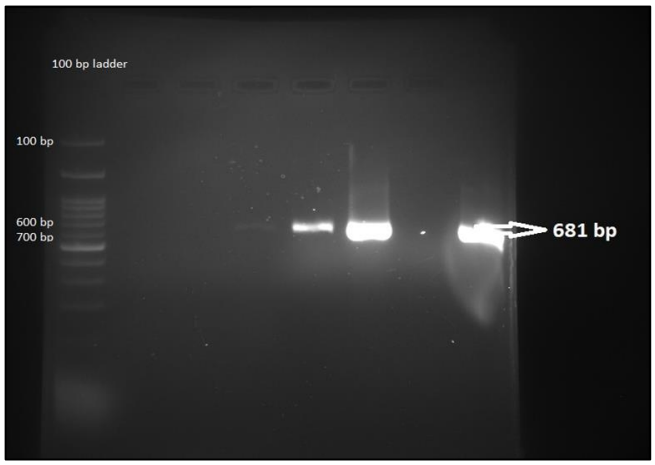

Fig 1: PCR products of VP2 gene of canine parvovirus yielding 681bp product.

Conclusion

Polymerase chain reaction followed by DNA sequencing is the very expensive method to differentiate the various strains of canine parvovirus. To overcome the economical disadvantage in diagnosis in above method, type specific primers based polymerase chain reaction followed by Restriction fragment length polymorphism (RFLP) is the cost effective and accurate detection of canine parvo strains especially CPV type 2c.

Acknowledgements

The authors are grateful to the Dean, Veterinary College and Research Institute, Namakkal, Tamil Nadu for providing necessary facilities and fund to conduct this research.

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