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Typing of Canine Parvovirus using Tetra Primer Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra ARMS PCR)

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Abstract

Canine parvovirus 2 (CPV 2) causes haemorrhagic gastroenteritis in the dogs worldwide. The prevalence of CPV despite vaccination may be due to the presence of many variants that leads to disease. The emergence of antigenic variants is due to mutations in the gene VP2 that code for the viral capsid. The mutation in the VP2 is due to a single nucleotide polymorphism (SNPs) that leads to evolution of different variants. To identify a particular variant thus assumes great importance as it helps in adoption of the vaccine directed against the most prevalent variant or variants in a particular geographic area. In the present study, a Tetra Primer Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra ARMS PCR) was tested for typing antigenic variants of CPV. A total of 120 dogs that had parvovirus infection were further screened using Tetra ARMS PCR and it was observed that out of 120, 25 (20.83%) were CPV 2a, 38 (31.66%) were CPV 2b, 27 (22.5%) were both CPV 2a and 2b and 30 (25%) were variant other than 2a or 2b.

Keywords: Single Nucleotide Polymorphism (SNP); CPV 2; CPV 2a; CPV 2b; Diagnosis; VP2 Gene; Dogs; Validation

Introduction

Canine parvovirus 2 (CPV 2) is an important enteric pathogenic virus of dogs that was first recognized in the year 1978. CPV 2a and 2b emerged in 1979 due to antigenic drift and spread worldwide within a year replacing the CPV2 variant. It is responsible for causing acute haemorrhagic enteritis in dogs throughout the world with a high morbidity upto 100% and mortality up to 10% [1]. CPV 2 belongs to the family *Parvoviridae* which comprises of two sub-families *Parvovirinae* and *Densovirinae*. Among these *Parvovirinae* is very important from veterinary perspective as it has the genera *Protoparvovirus* having CPV 2 that infects vertebrates. CPV 2 has a single 5.3kb long linear DNA genome enclosed in an icosahedral structure of 18-28 nm in diameter having two open reading frames ORF1 and ORF 2. ORF 1 at the 3'end encodes two non-structural proteins that are essential for replication while ORF 2 encodes two structural proteins at the 5'end; VP1 and VP2 which mainly determine structural conformation. VP1 capsid proteins are responsible for the infectivity of the virus whereas VP2 protein interacts with cellular receptors and leads to internalisation of virus. VP2 protein is the major determinant of host range and host virus interactions [2] and mutations in VP 2 are mainly responsible for the emergence of newer antigenic variants [3]. Substitution at the amino acid (aa) position 426 of VP2 lead to development of CPV 2 variants (2a, 2b and 2c). At aa 426, CPV 2a has Asparagine, CPV 2b has Aspartic acid and CPV 2c has Glutamic acid leading to emergence of CPV 2 variants[1]. The CPV variants have exhibited a wider host range and higher morbidity in dogs of 2-4 months while low in <1and >4 month old puppies than CPV 2. Various diagnostic tests have been employed for the diagnosis of CPV like haemagglutination (HA), enzyme linked immunosorbent assay (ELISA) and dot ELISA, however using these we cannot type CPV variants. A gold standard method of identification of CPV 2 is using sequencing technique looking for single nucleotide polymorphism (SNPs) mainly at 426 aa. Since for sequencing the sample is to be send for some commercial vendor that takes time and in order to hasten the diagnosis various researches focus to identify simpler methods to type CPV2. Tetra-primer amplification refractory mutation system polymerase chain reaction (Tetra ARMS PCR) is one such method which is proving as simple, effective, rapid and economical SNP genotyping [4]. It is a type of nested PCR that work by combining PCR and ARMS for identifying specific allele and can be used to identify CPV2 variants without sequencing. It has been observed that the result of ARMS PCR is completely in agreement with the sequencing results [5]. However, using ARMS method typing was achieved in two steps, with the first reaction CPV 2a from the other two variants could be distinguished and the second step was used for identification of CPV 2c. These two steps could be combined using Tetra ARMS PCR to distinguish CPV2 variant targeting mutated aa at 426 of VP2. Partial success in typing was achieved in a study but they could not differentiate CPV 2 and CPV 2a [6]. Therefore, this study was aimed to develop a Tetra ARMS PCR for typing CPV 2 into CPV 2a and CPV 2b that are prevalent in Indian subcontinent.

Materials and Methods

Ethical Approval

The ethical approval to conduct this study was obtained from the institutional animal ethics committee (I-AEC) via GADVASU/2021/IAEC/60/07 dated 10.08.2021. All the samples collected from the dogs were collected after taking informing the owners about the use of sample in the research.

Sample Collection

A total of 138 rectal swabs were collected in 2.0 ml phosphate buffered saline from dogs exhibiting signs of CPV (haemorrhagic gastroenteritis, fever) from Multi Speciality Veterinary Hospital, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana between August 2021 and March 2022. The samples were transported on ice to the laboratory and were subjected for extraction of DNA. The DNA was extracted from all the samples and a vaccine (positive control) (DHPPi Nobivac, Merck) using the phenol-chloroform extraction method [7]. The extracted DNA from all the samples was tested for its quality using Nanodrop (ThermoFisher, USA).

Primer Designing

The nucleotide sequences of CPV 2a (MT880781.1) and CPV 2b (MT353764.1) were download-

ed from National Centre for Biotechnology Information (NCBI; https://ncbi.nlm.nih.gov). The 426 aa site (nucleotide position 4062-4064) on the VP2 gene of the CPV was selected as site for allelic differentiation. The Accession No. MT880781.1 was only used to make primers using Primer 3[8]. Forward outer (Fo), reverse outer (Ro), forward inner (Fi) and reverse inner (Ri) primers having 20-22 base pairs were made and tested using primer BLAST (https://ncbi.nlm.nih.gov). Fo was made near to the mutated allele and Ro was made away from the mutated allele (Table 1 and Figure 1,2,3). The specific primers were selected to differentiate between the CPV types (CPV 2a and CPV 2b). VP2 gene is the region of the virus where the major mutations in the virus are encountered. Therefore, this specific region was selected for designing primers which could diffreentiate between the CPV types.

Table 1: Primers for Tetra Arms PCR along with their combination

Primers	**Sequence (5' to 3')**	**Position in VP2**	**Length (bp)**
Fo	GTGATCCAAGATATGCATTTGG	1121- 1142	22
Ro	TAATTTTCTAGGTGCTAGTTGAGA	1749- 1726	24
Fi	CTTTAACCTTCCTGTAACAA	1257-1276	20
Ri	GTTGGTAGCAATACATTATCATC	1298- 1278	23

Primer combinations	**Product size (bp)**	**CPV Variants Detection**
Fo + Ro (Set 1)	629	CPV
Fi + Ro (Set 2)	493	CPV 2a
Fo + Ri (Set 3)	178	CPV 2b

> MT880781.1
ATGAGTGATGGAGCAGTTCAACCAGACGGTGGTCAGCCTGCTGTCAGAAATGAAAGAGCTACAGGATCTG
GGAACGGGTCTGGAGGCGGGGGTGGTGGTGGTTCTGGGGGTGTGGGGATTTCTACGGGTACTTTCAATAA
TCAGACGGAATTTAAATTTTTGGAAAACGGATGGGTGGAAATCACAGCAAACTCAAGCAGACTTGTACAT
TTAAATATGCCAGAAAGTGAAAATTATAGAAGAGTGGTTGTAAATAATTTGGATAAAACTGCAGTTAACG
GAAACATGGCTTTAGATGATACCCATGCACAAATTGTAACACCTTGGTCATTGGTTGATGCAAATGCTTG
GGGAGTTTGGTTTAATCCAGGAGATTGGCAACTAATTGTTAATACTATGAGTGAGTTGCATTTAGTTAGT
TTTGAACAAGAAATTTTTAATGTTGTTTTAAAGACTGTTTCAGAATCTGCTACTCAGCCACCAACTAAAG
TTTATAATAATGATTTAACTGCATCATTGATGGTTGCATTACATAGTAATAATACTATGCCATTTACTCC
AGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGGAAACCATCCATACCAACTCCATGGAGATAT
TATTTTCAATGGGATAGAACATTAATACCATCTCATACTGGAACTAGTGGCACACCAACAAATATATACC
ATGGTACAGATCCAGATGATGTTCAATTTTACACTATTGAAAATTCTGTACCAGTACACTTACTAAGAAC
AGGTGATGAATTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACACATGGCAA
ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGTACTAACTTTG
GTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGAAATACAAACATTATTACTGA
AGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCACCATATTATTCTTTTGAGGCGTCTACACAA
GGGCCATTTAAAACACCTATTGCAGCAGGACGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATG
GTGATCCAAGATATGCATTTGGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGA
G
ATTTACATACATAGCACATCAAGATACAGGAAGATATCCAGAAGGAGATTGGATTCAAAATATTAACTTT
AACCTTCCTGTAACAAATGATAATGTATTGCTACCAACAGATCCAATTGGAGGTAAAGCAGGAATTAACT
ATACTAATATATTTAATACTTATGGTCCTTTAACTGCATTAAATAATGTACCACCAGTTTATCCAAATGG
TCAAATTTGGGATAAAGAATTTGATACTGACTTAAAACCAAGACTTCATGTAAATGCACCATTTGTTTGT
CAAAATAATTGTCCTGGTCAATTATTTGTAAAAGTTGCGCCTAATTTAACAAATGAATATGATCCTGATG
CATCTGCTAATATGTCAAGAATTGTAACTTACTCAGATTTTTGGTGGAAAGGTAAATTAGTATTTAAAGC
TAAACTAAGAGCCTCTCATACTTGGAATCCAATTCAACAAATGAGTATTAATGTAGATAACCAATTTAAC
TATGTACCAAGTAATATTGGAGGTATGAAAATTGTATATGAAAAATCTCAACTAGCACCTAGAAAATTAT
ATTAA

Figure 1: Fo primer: 1121-1142 bp in VP2 gene (Red colour), Fi primer 1257-1276 bp in VP2 (underlined); 1276-1278 bp in VP2 gene is the mutated amino acid asparagine (Green colour)

Figure 2: Fo, Fi and Ri primer sequence in multiple alignment

Figure 3: Ro primer sequence in multiple alignment

Identification of CPV Positive Samples

The DNA extracted from all the samples and from a positive control was subjected to an initial PCR for the identification of CPV[9].The following components were used to set up the PCR reaction, 2.0µl of 10X PCR buffer, 1.0µl of dNTPs mix (2.5mM each), 0.8µl of forward and reverse primer (20 pmol/µleach), 1.0µl of the template DNA (50-100ng/µl), 0.5µl of 25mM $MgCl_2$, 0.2µl Taq polymerase 5 units/µl (Qiagen, Germany) and the reaction was made up-to 20 µl using nuclease free water. The bands obtained were observed in 1.5% agarose gel with ethidium bromide (2µg/ml concentration) in gel documentation system (Syngene, USA).

Evaluating the Efficacy of Tetra ARMS PCR on Positive CPV Samples

All the samples that were positive via PCR were retested using Tetra ARMS PCR. A PCR reaction was setup using 2.0µl of 10X PCR buffer (Qiagen, Germany), 1.0µl of dNTPs mix 2.5mM each (Qiagen, Germany), 0.5µl each of Fo, Ro,Fi and Ri (20 pmol/µl each) (IDT, USA), 2.0µl of the

template DNA (100ng/µl), 0.5µl of 25mM $MgCl_2$, 0.2µl Taq polymerase 5 units/µl (Qiagen, Germany) and the reaction was made upto 20 µl using nuclease free water. The PCR conditions used were initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 45 seconds, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. After the PCR, the products were subjected to gel electrophoresis (1.5% agarose with ethidium bromide (2µg/ml concentration) and bands were visualised and captured using the gel documentation system (Syngene, USA).

Results

In the present study a total of 138 dogs exhibiting signs of CPV were selected in the study. The DNA extracted from the 138 samples and a positive control was subjected to PCR. Out of a 138 samples tested, only 120 were found to be positive for CPV as they had a band of 611 bp (Figure 4).

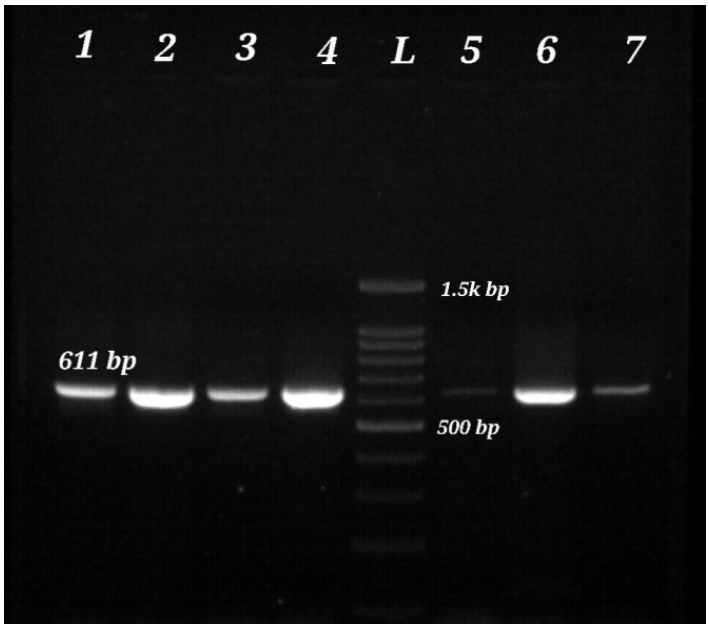

Figure 4: Conventional PCR for identification of CPV

Lane L: 100 bp DNA ladder; Lane 1: Positive control; Lane 2-7: positive samples with band at 611p

All the PCR positive CPV samples were then retested via Tetra ARMS PCR to type these CPV 2 into its variants. Interpretation of Tetra ARMS PCR include noting number of bands amplified in the Tetra ARMS PCR reaction and a total of 3 bands 629 bp, 493 bp and 178 bp can be obtained (Figure 5). Amplification of two bands of 629 bp and 493 bp corresponded to CPV 2a and a bands of 629 bp and 178 bp corresponded to CPV 2b. Amplification of all the bands 629bp, 493bp and 178bp corresponded to the presence of both the variants. A band of 629bp corresponded to CPV other than CPV 2a or 2b.

Thus, using Tetra ARMS PCR it was observed that out of the 120 positive samples, 25 (20.83%) were CPV 2a, 38 (31.66%) were CPV 2b, 27 (22.5%) were positive for both CPV 2a and CPV 2b and 30 (25%) were belonged to a variant other than 2a or 2b. Thus, Tetra ARMS PCR designed in the study was able to differentiate between CPV 2a and CPV 2b.

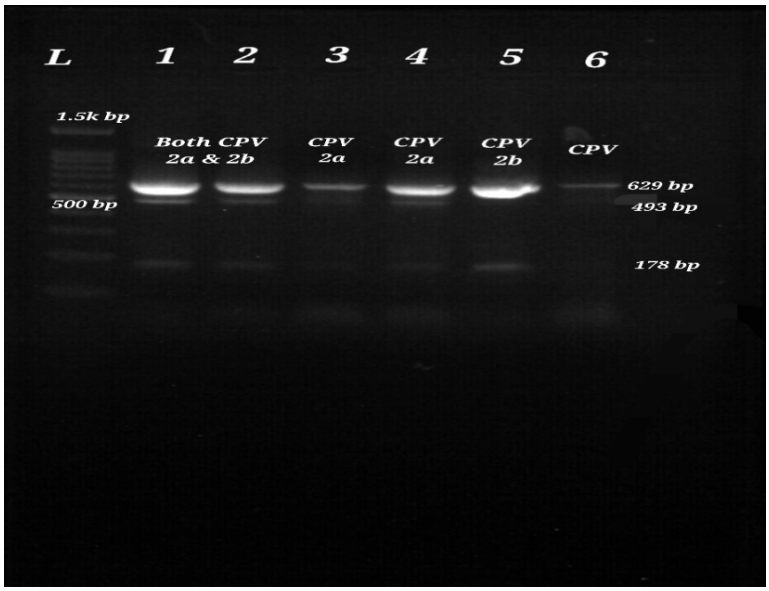

Figure 5: Typing of CPV in TETRA ARMS PCR

Lane L: 100bp ladder; Lane 1 and 2: positive for both CPV 2a and 2b; Lane 3 and 4: positive for CPV 2a; Lane 5: positive for CPV 2b; Lane 6: positive for CPV but neither for CPV 2a or 2b

Discussion

Canine Parvovirus is a very important disease in dogs particularly puppies as it leads to high morbidity and mortality [1]. In order to control CPV infection in dog's vaccination is advocated but many a times the requisite success is not achieved despite proper administration of vaccine raising suspicion in both owners and clinicians. One reason attributed for vaccine ineffectiveness is due to multiple CPV variants. Several studies have been conducted to determine the epidemiological distribution of the CPV 2 variants in order to improve the immunisation strategies and thereby reducing the incidence of the infections [10]. It has also been observed that distinguishing CPV 2a from 2b using PCR fails to differentiate thereby making differentiation by PCR difficult [11-15]. Since the current methods available fail to distinguish CPV 2 into its variants thus, most researches have resorted to sequencing for identifying antigenic variants of CPV 2. Sequencing invariably is the gold standard but is time consuming and requires either proper knowledge of sequencing or a commercial vendor. Hence, newer methods to type CPV at a faster pace is required.

Tetra primer ARMS PCR approach was proposed as having a 100% agreement with sequencing results [16]. Further, Tetra Primer ARMS PCR has been reported as reliable, sensitive and accurate as conventional ARMS PCR method, and it is suggested that it can be utilised as a complementary method for identification of CPV variants[4]. In the present study with the help of Tetra ARMS PCR, 120 CPV positive samples could be typed into its variants CPV 2, CPV2a and CPV 2b successfully. Therefore, Tetra ARMS PCR is an easy and quick way to differentiate or type among the virus types which differ by single nucleotide polymorphism.

Conclusion

It can be concluded that the Tetra-ARMS PCR developed in the present study was successful in differentiating between CPV 2, CPV 2a and 2b in the single reaction. It is a simple, cost effective and time saving method for typing field isolates.

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Conflict of Interests

The authors declare that there are no conflicts of interests with respect to the research, authorship and/or publication of this article.

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