RESEARCH ARTICLE

High-Resolution Melting Analysis for Genotyping of Canine Parvovirus 2 Strains in Indian Context: Challenges and Insights

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Abstract

This study focuses on the detection and differentiation of Canine Parvovirus 2 (CPV-2) strains in India using High Resolution Melt (HRM) curve analysis. CPV-2 is a highly contagious virus causing acute haemorrhagic enteritis and myocarditis in dogs, with a high mortality rate. A total of 45 faecal swab samples were collected from suspected CPV cases, out of which 20 tested samples were positive for CPV using PCR targeting the VP2 gene. These positive samples were further analyzed using HRM, which predicted them to belong to the CPV2a strain based on their melting temperature (71.4° or less). Two CPV2a positive samples were sequenced, they were found to belong to CPV2b strains, indicating a discrepancy between HRM prediction and sequencing results. Single nucleotide polymorphic variations were observed at positions 4062 and 4064, confirming their classification as CPV2b strains. Phylogenetic analysis of VP2 capsid gene showed that these field samples were closely related to CPV2c strains, suggesting potential genetic diversity among circulating CPV strains in India. The study highlights the importance of developing geographically specific CPV strain primers for accurate detection and differentiation using HRM. It raises concerns about potential co-infections or the emergence of new strains, indicated by varying melting temperatures in field samples.

Key words: Canine faecal swabs, CPV-2, PCR-HRM, Sequencing, Strain differentiation.

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Introduction

Canine parvovirus (CPV) is a contagious, life-threatening viral disease in young dogs, one of the most significant canine enteric pathogens. This virus is extremely contagious, resulting in high morbidity and occurring more frequently in breeding kennels, pet stores, and shelters. In non-protected hosts, the disease has a rapid clinical course and death can occur within two to three days after signs appear (Miranda and Thompson, 2016). Any breed, age, or sex of dog can develop acute CPV-2 enteritis, however pups between the ages of 6 weeks and 24 weeks seem to be particularly vulnerable (Goddard and Leisewitz, 2010). All breeds are vulnerable to the illness, but the blended varieties are depicted to be less susceptible than numerous pure-breds. Purebreds such as the American Pit Bull Terrier, German Shepherd, English Springer Spaniel, Rottweiler, and Doberman Pinscher have been linked to an increased risk of CPV enteritis (Miranda and Thompson, 2016).

Parvoviruses are single-stranded, non-enveloped viruses with a diameter of approximately 25 nm and approximately 5.2 kb in size (Nandi and Kumar, 2010). The genome codes for two non-structural proteins (NS1 and NS2) and three structural proteins (VP1, VP2, VP3) through the same mRNAs being spliced alternatively (Vannamahaxay and Chuammitri, 2017). Ninety percent of the viral capsid is made up of the most prevalent structural protein, VP2, which may self-assemble to form virus-like particles (VLPs). In determining viral tissue tropism and host range, VP2 is a significant antigenic factor. Notably, simply a small number of amino acid changes in its sequence can change the relevant biological properties of a virus (Li *et al.,* 2017).

Furthermore, it is known that the antigenic variants of CPV have evolved as a result of mutations that alter the VP2 gene. Given that the prevalent CPV strains appear to be mutating away from the vaccine strains, rising CPV incidences in the population of vaccinated dogs are a cause for

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concern (Vimalan *et al.,* 2023). For CPV strain differentiation, conventional methods like virus isolation, haemagglutination inhibition (HI) test, molecular methods like PCR-RFLP, PCR followed by sequence analysis were used. However, these techniques are usually time consuming and labour intensive. A PCR-based high-resolution melting (HRM) curve analysis (PCR-HRM) was introduced to detect and differentiate CPV 2 genotypes (Bingga *et al.,* 2014). In the present study, this method was adopted for its applicability in the Indian context for differentiation of CPV strains currently in circulation.

Materials and Methods

A total of 45 number of faecal swab samples from CPV suspected dogs were collected from small animal outpatient ward of clinics, Madras Veterinary College, Chennai from November 2022 to March 2023. The swabs were immersed in 1 mL sterile 1X PBS and stored at -20°C until further use. DNA was isolated from all the field samples using QIAamp DNA Mini Kit as per the manufacturer's instructions and DHPPi vaccine was used as positive control.

All the samples were initially screened for CPV by PCR targeting the VP2 gene with the forward primer 5'-TCAACCTGCTGTCAGAAATGAAAGAGC-3' and reverse primer 5'-TTGAAAGTACCCGTAGAAATCCCCACA -3' for the product size of 100 bp. For each PCR reaction, a 20 µL reaction mixture was prepared containing 10 µL of Taq 2X master mix, 1 µL each of forward and reverse primers (10 pmol/ µL), 3 µL of DNA template and 5 µL of nuclease free water. The thermal cycling conditions for the amplification were 1 cycle for 5 min at 94°C, 30 cycles of 45 s at 94°C, 45 s at 67°C and 45 s at 72°C, with a final extension step of 5 min at 72°C. A non-template control (NTC) was maintained for every set of PCR reactions carried out and vaccine was used as positive control. Finally, 10 µL of the PCR amplified products were electrophoresed in a 2% agarose gel, stained using ethidium bromide and examined under gel documentation.

The VP2 gene from the faecal samples was pre-amplified to increase the reproducibility and reliability of the HRM curve analysis. This was to start the assay with similar amounts of the amplified PCR product. Pre-amplification was performed with specific primers and temperature combinations described by Bingga *et al.* (2014). HRM analysis was carried out for 20 µL volume reaction containing 10 µL of SYBR Premix ExTaq mixture (Tli RNaseH Plus), 1 µL each of forward and reverse primers (10 pmol/ µL), 7 µL of nuclease free water and 1 µL of template DNA from the pre-amplification step diluted 1:100. The amplification was performed on CFX Opus 96 real time PCR system (Bio Rad, USA) with the thermal cycling conditions as follows: 94°C for 5 min; 35 cycles of 94°C for 30 sec; 58°C for 30 sec; 72°C for 30 sec. After amplification, HRM analysis was carried out by increasing the temperature in 0.3°C increments from 65°C to 85°C. All specimens were tested in duplicates and their melting profiles were analysed using the Precision Melt Analysis Software Version 1.3.

Two CPV2a positive samples confirmed by HRM were sequenced using CPV-426F/426R and CPV-1270F/1270R (VP2 region) primers with the product size of 52 bp and 1270 bp, respectively, at M/S. Eurofins Genomics India Pvt. Ltd.., Bengaluru-560048. The nucleotide sequence data were subjected to BLAST analysis (www.ncbi.nlm.nih.gov), assembled and analyzed using Seqman and MegAlign programs of Lasergene package (version 7.1.0) (DNA Star Inc. Madison, WI). Nucleotide sequence alignment was performed by the Clustal W method and phylogenetic analysis of CPV isolate strain was performed using the Neighbor-joining method with 1000 bootstrap replication in the MEGA software version 11.

Results and Discussion

Among 45 suspected samples from dog, 20 samples were found to be positive for canine parvovirus (44.44%) by PCR (Fig. 1). Additionally, HRM analysis was conducted on all positive samples for CPV genotyping. Among the 20 positive samples, samples F21 and F25 exhibited a melting temperature of 71.40°C (Fig. 2), samples F4, F6, and F17 showed 70.95°C, samples F1, F2, F10, and F16 showed 70.80°C, samples F5, F7, F12, F15, F18, F19, F20, and F24 showed 70.65°C, sample F14 showed 70.50°C, and samples F11 and F23 showed 70.20°C.

Since the HRM assay is based on the detection of SNP on the target sequence, usually short amplicons are preferred (Bingga *et al.,* 2014). In the present study, two samples exhibited the melting temperature of 71.40°C, which is in agreement with Bingga *et al.* (2014) who reported melting temperature of the reference sample CPV-gd3 (CPV-2a) as 71.43 ± 0.03°C. The remaining 18 samples of our study revealed the melting temperatures below 71.40°C, and hence all of which could also be predicted as belonging to strain "2a". For the samples to be classified as "2b" or "2c" strains, their melt temperatures should have been greater than 72°C. Hence, all the field samples used in this study could be predicted to be belonging to strain "2a".

To corroborate the HRM findings, the two samples (F21 and F25) initially designated as CPV2a were subjected to sequencing for genotype confirmation. Contrary to the HRM analysis, the sequencing results revealed that these samples actually belonged to CPV2b, as detailed in Table 1. The HRM primer set used in this study, CPV-426F/426-R, amplified a short 52 bp amplicon to detect the SNPs A4062G and T4064A in the CPV VP2 capsid gene that distinguish between CPV-2a and CPV-2b (A4062G) and between CPV-2b and CPV-2c (T4064A).

CPV2 genotyping based on the SNP at nucleotide positions 4062 and 4064 could be used to differentiate between the different strains of CPV2. Strain CPV 2a showed nucleotide sequences A and T, at positions 4062 and 4064, respectively. For CPV2b, it is G and T, respectively and CPV 2c exhibited the nucleotide sequence G and A, respectively (Bingga *et al.,* 2014). However, in our current investigation,

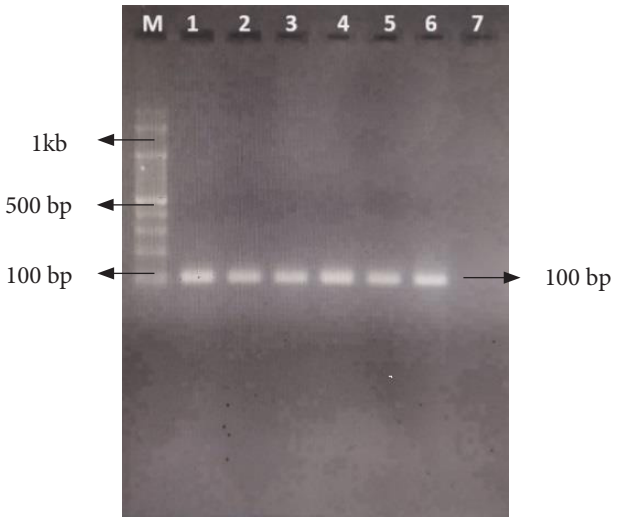

Fig. 1: PCR amplification of VP2 gene of Canine Parvovirus from suspected clinical cases; Lane M-100 bp ladder, Lanes 1to 5- CPV positive samples, Lane 6 – Positive control, Lane 7- NTC

Table 1: Comparison of SNP variation at nucleotide position 4062 and 4064 at CPV 2a, 2b, 2c and samples from present study

Position/Genotypes	4062	4064
HRM Ref (CPV 2) M38245	A	T
CPV 2a KJ754511	A	T
CPV 2b KJ754509	G	T
CPV 2c KJ754515	G	A
Present study 1	G	T
Present study 2	G	T

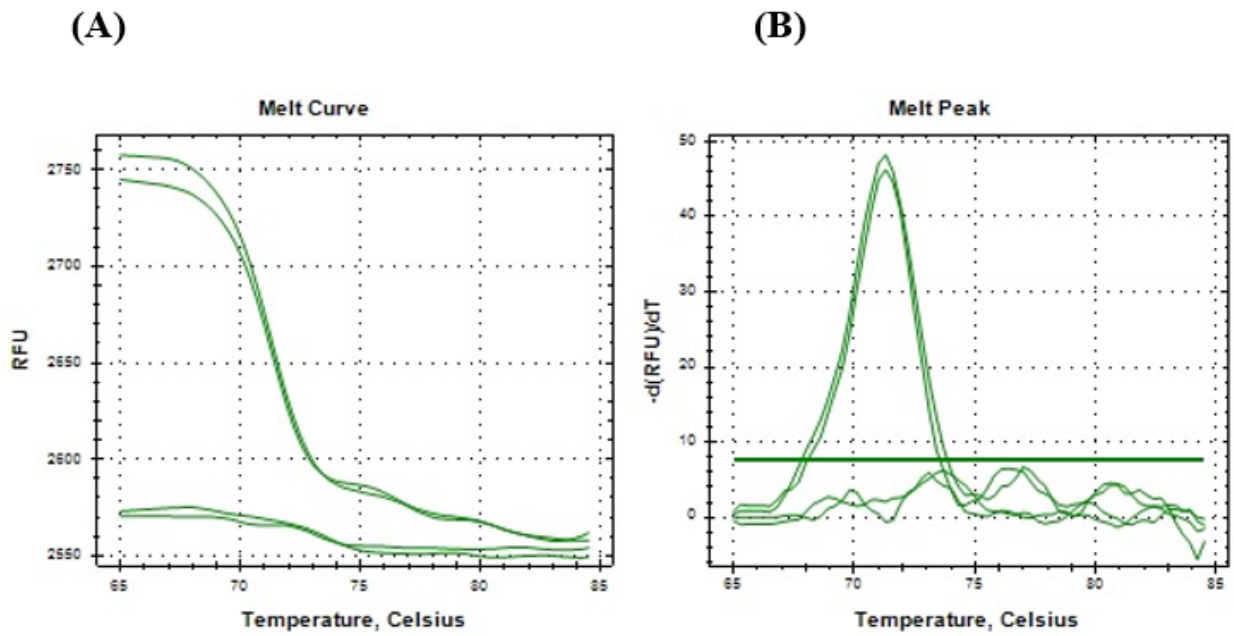

Fig. 2: (A) Normalized and (B) conventional melting curves analysis of nested PCR products from field specimen using the primer set CPV-426F/426R showing melt temperatures as 71.40°C (duplicates) along with NTC.

at nucleotide position 4062, the SNP was identified as G and at position 4064, it was T (Table 1). These findings suggest that the samples under study were associated with CPV2b.

In order to authenticate the HRM primer sequence data, the same two field samples were sequenced for genotype confirmation, employing the primer set CPV-1270F/1270R. Subsequent analysis, including sequencing results and phylogenetic examination, demonstrated that both field samples of CPV clustered with the CPV-2c strain of Canine parvovirus (Fig. 3).

The contrary result in the present study was mainly due to limitations of the HRM assay wherein the quality and quantity of the DNA matters to a great extent. Purity of the DNA affects the melting profile. Moreover, a change in base from A/T to C/G can easily affect the assay. It is because, when there is a change in the base from purine to pyrimidine or vice versa, there is a change in the number of hydrogen bonds, which in turn changes the melting temperature (around 1°C change in small PCR products)(Liew *et al.,* 2004).

Similarly, on the other hand, a base change from A/T to T/A or C/G to G/C is very difficult to identify based on the melting temperature alone as there is no change in the base pair and thus the number of hydrogen bonds remain the same. Hence, there is only a minute difference in the melting

temperature (Tm), which is predicted to be around 0-0.4°C (Li *et al.,* 2014). Due to the above-mentioned limitations, it is easy to identify CPV-2a strains from CPV-2b and CPV-2c strains as there is approximately a 0.6°C difference in Tm between 2a and the other two strains. But the Tm difference between the 2b and 2c strains is around 0.2°C, a difference too little to detect (Bingga *et al.,* 2014). Thus, there arises a need for heteroduplex formation to differentiate 2b strains from 2c strains when performing HRM assay.

Bingga *et al.* (2014) introduced a 1:1 mixture of the CPV-gd1 reference sample, representing the 2b strain, with PCR products to create heteroduplexes. Should the unknown field sample also pertain to the 2b strain, the melting profile would remain unaltered. Conversely, if disparate strains constituted the heteroduplex, such as the unknown field sample belonging to the 2c strain, the melting profile would undergo a change. These findings suggest that the HRM assay can effectively distinguish between 2b and 2c strains based on the observed melting profiles. In this study, the HRM assay was conducted directly on field samples, introducing the possibility of multiple strains/viruses coexisting in these samples. This co-presence could potentially influence the melt temperature outcomes of the HRM assay. The observed deviations in melt temperatures among the field samples, in comparison to the reference strains, suggest the potential presence of multiple strains or even the emergence of new strains. Further investigations are warranted to delve into the intricacies of potential co-infections or the evolution of novel strains, representing avenues for future research.

Conclusion

To conclude, this research underscores the significance of region-specific primers in devising swift and economical methods, such as high-resolution melting analysis, for discerning the prevalent CPV strain in a given geographical area. The application of such techniques is crucial for tailoring vaccines to the circulating strains and mitigating the incidence of CPV infections in the canine population. In contrast to conventional and sequence-based genotyping approaches, the HRM assay offers a cost-effective and streamlined alternative. PCR-HRM analysis can be accomplished within approximately 2 h, and it is amenable to the use of a generic DNA-binding dye, obviating the necessity for specific probes.

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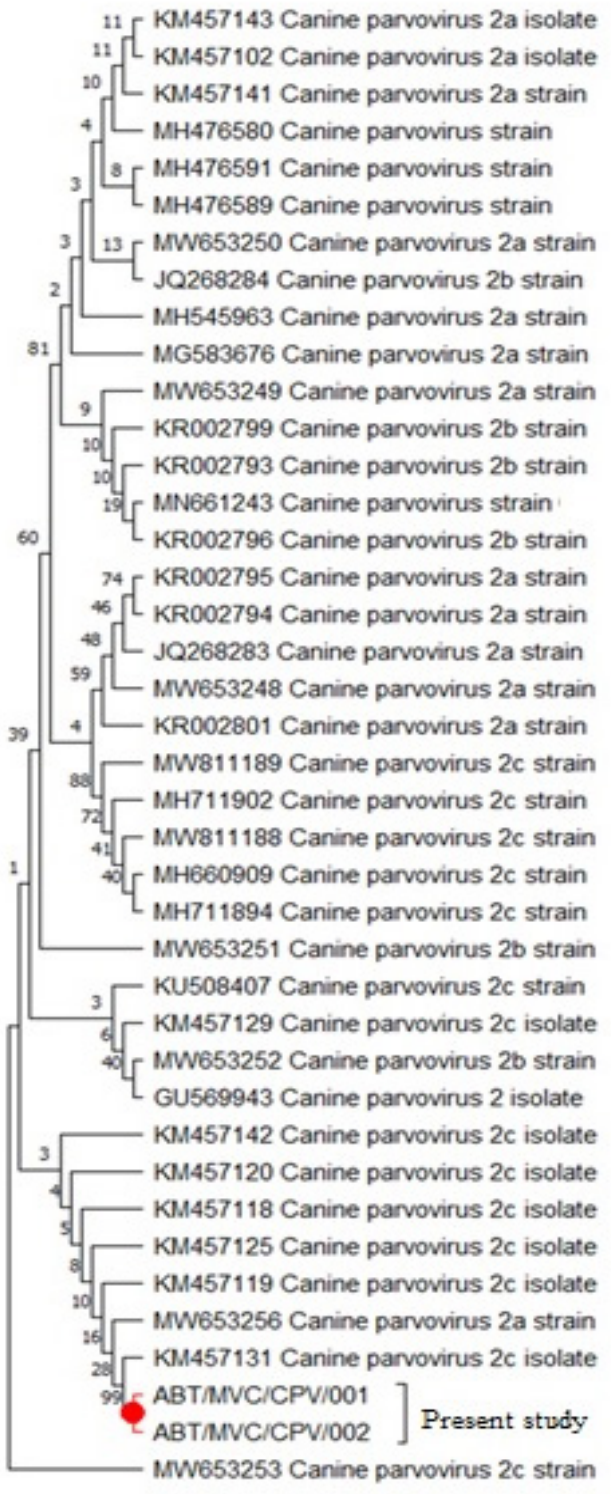

Fig. 3: Phylogenetic analysis of VP2 partial CPV sequences. The phylogenetic analysis revealed that both the samples of CPV were clustered with CPV-2c strain.

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SECOND ANNOUNCEMENT

XI Annual Convention and National Conference of SVSBT-2024

XI Annual Convention of the Society for Veterinary Science & Biotechnology ***(SVSBT) and National Conference on "Biotechnological Innovations to Augment Health and Productivity of Livestock and Poultry for Sustainable Livelihood"*** will be **organized** by College of Veterinary Science, **Proddatur-516 360, YSR District, Andhra Pradesh,** under Sri Vekateswara Veterinary University (SVVU), Tirupati, **during 23rd to 25th October, 2024**. The detailed Brochure cum First Announcement showing Theme Areas/Sessions, Registration Fee, Bank Details for online payment and deadlines, etc. has been floated on the Whatsapp group and e-mails of all life members. The organizing committee ***invites abstracts*** of original and quality research work on theme areas of seminar limited to 250-300 words for oral and poster sessions by **e-mail on or before 10th October, 2024 to: svsbt2024@gmail.com** OR **rajakishorekonka9@gmail.com** for inclusion in the Souvenir cum Compendium to be published on the occasion.

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