Canine parvovirus-2: an insight into molecular diagnosis and characterization methods

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Abstract

Canine parvovirus is an extremely contagious and serious disease caused by Canine parvovirus type 2 (CPV-2). The disease was first reported in 1978 and since then it continues to pose a severe threat to world canine populations. The virus can infect dogs of all breeds and wild carnivores, leading to considerable morbidity and mortality in young canines. The mortality rate may approach up to 90% in untreated cases of CPV infection whereas with timely and appropriate therapy, survival rates can be 80-95%. Infected dogs during recovery from the disease and the subclinical shedder dogs are the major source of infection for contact animals and the environment contamination. To reduce the spread of the virus among the susceptible population and to help timely implementation of proper supportive treatment, rapid identification of the disease and isolation of CPV-2 positive animals at these stages of infection is vital. There are a number of methods developed for the laboratory confirmation of CPV infection viz., electron microscopy, virus isolation in cell culture, hemagglutination test, hemagglutination inhibition test, enzyme linked immunosorbent assay and many molecular assays, which are usually carried out on the feces of affected dogs and the blood samples at late stages of infection. Since the rapid and sensitive diagnosis of CPV-2 is important for the timely treatment and control of CPV-2, the present review is focused on various nucleic acid detection based tests for rapid, sensitive and optimal diagnosis and characterization methods to aid in formulation of effective control strategies.

Keywords: Canine parvovirus; Molecular diagnosis; Characterization

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Introduction

Canine parvovirus enteritis is a most common infectious disease of wild and domestic canines[(1-3)]. The disease has emerged due cross species jumping and adaptation of feline panleukopenia virus in wild canines[(1,2)]. This virus was classified as Canine parvovirus-2 (CPV-2) to differentiate it from the Canine parvovirus-1of mink. Since its emergence, different variants of canine parvovirus-2 (CPV-2a, CPV-2b, CPV-2c, new CPV-2a, and new CPV-2b) has evolved and were classified on the basis of positions of amino acid at 426Asp/Glu/Asn and 297Ser/Ala in the VP2 protein[(4-6)]. Currently, different variants of CPV-2 are circulating in different geographical areas of the world causing canine parvovirus enteritis or Parvo. The disease is characterized by gastroenteritis, anorexia, fever, vomiting and foul smelling diarrhoea leading to severe dehydration and death if not checked timely, leucopoenia and occasionally myocarditis in new born puppies from unvaccinated bitches[(3)]. To control the spread of virus to other susceptible hosts, prompt and accurate diagnostics are required. The clinical signs in this disease are merely not restricted to canine parvovirus-2 infection and many other canine pathogens manifest similar clinical picture making laboratory confirmation of the virus essential. The commonly employed methods for the confirmation of canine parvovirus-2 includes electron microscopy (EM), virus isolation, hemagglutination (HA) test, hemagglutination inhibition (HI) test, enzyme linked immunosorbent assay (ELISA), latex agglutination test (LAT) and nucleic acid detection. Among the available methods for CPV-2 detection, most methods are cumbersome to perform and comparatively less sensitive to the molecular assays for diagnosis of the canine parvovirus enteritis in dogs.

Molecular Diagnostics for CPV-2

Polymerase Chain Reaction

Polymerase chain reaction (PCR) analysis has been considered as the most reliable and widely applied diagnostic technique in detecting CPV nucleic acid from faecal samples[(7-9)]. Because of the high sensitivity and specificity, PCR can detect fewer number of virus particles than that of HA, EM and ELISA. When combined with the gel filtration, PCR can detect the CPV from faeces containing as little as 10^3 PFU/g of fresh faeces, whereas the ELISA test or the culture method can detected the virus only from faeces containing more than 10^6 PFU/g of fresh faeces[(10)]. Further, the sensitivity and specificity of the reaction can be increased by employing the nested PCR (n-PCR) methodology[(11,12)]. Hirasawa et al.[(13)] used PCR to assess the prevalence of CPV-2 in Japanese dogs which revealed 54.1% prevalence. Kim and Jang[(14)] used different diagnostic tests for the

detection of CPV from faecal samples and found that 45% were positive by HA test, 64% were positive in PCR whereas 87% were positive in nested PCR. Kumar et al.[15] develop n-PCR using published pCPV-2ab as external primer set and self-designed pCPV-2N as internal primer set and found that out of fifty two faecal samples tested, 27 and 31 were tested positive by one step PCR and n-PCR, respectively whereas when positive cell culture supernatants was tested, it was found that one step PCR had a detection limit of 10^{-5} dilution, whereas nPCR had detection limit up to 10^{-8} dilution. Schunck et al.[7] developed a touchdown PCR protocol for amplification of CPV DNA from faeces after a fast and simple boiling pre-treatment and found that its sensitivity was as high as ten infectious particles per reaction corresponding to a titre of about 10^3 infectious particles per gram of unprocessed faeces. Moreover, the PCR can be used to differentiate the different mutants of CPV-2 using the primers specific for particular mutants[12]. Multiplex PCR utilizes the power of PCR to check the presence of nucleic acids from several pathogens in one test reaction. It is a time as well as cost effective methods because it can detect several pathogens simultaneously. Ramadass and Latha[16] used multiplex PCR assay for simultaneously identification of canine *Leptospira* sp and CPV. The CPV-2a and CPV-2b strains were also differentiated using multiplex PCR assay[17].

Real time Polymerase Chain Reaction

Real-time PCR, based on continuous optical monitoring of a fluorogenic reaction[18,19] is more sensitive, rapid and reproducible in comparison to conventional PCR[20,21]. Real-time PCR run in 96 well format and most of the steps are automated. The total time required for analysis of 20-30 faecal samples is almost half of the time required by the conventional PCR for CPV detection because of the preparation of DNA by simple boiling of faecal homogenates[22]. Decaro et al.[9] designed a simple TaqMan probe based real-time PCR assay for the detection and quantification of canine parvovirus from dog faeces on the basis of *VP2*gene sequence. It was found that the TaqMan CPV-2 real time assay is more sensitive than HA and conventional PCR and capable of detecting as few as 10^2 copies of CPV-2 DNA. This real-time assay for CPV-2 was highly reproducible and linear over eight orders of magnitude (10^2 to 10^9 copies), allowing a precise calculation of CPV-2 DNA loads in samples. The established TaqMan assay was successfully employed in pathogenesis studies carried out during natural[23] and experimental[24] infections and was also used to evaluate virus distribution in different tissues[25]. In 2006, Decaro et al.[26]developed a minor groove binder (MGB) probe assay to discriminate between vaccines and field strains of canine parvovirus. A TaqMan real-time RT-PCR assay was developed to detect CPV RNA transcripts for establishing the presence of replicating virus in CNS[27]. Li et al.[28] also used TaqMan probe in real-time PCR for diagnosis of CPV. Kumar and Nandi[29] proposed a SYBR Green based real-time PCR as an alternative to the TaqMan methodology with same detection limit. For simultaneously identification of CPV, FPV and PPV a multiplex real-time PCR based on *VP2* gene was validated. Multiplex real-time PCR has been used to detect and quantify CPV[30-32]. Further, Kaur et al.[33] reported that multiplex real-time PCR assay could be used for rapid detection of CPV as well as typing of its three antigenic types.

Nucleic Acid Hybridization/Dot Blot

In this process, the DNA extracted from the stool samples or inoculated cell culture supernatant is charged on nylon membrane or nitrocellulose paper which is then subjected to hybridization with CPV-specific probe, either radio-labelled or biotin labelled. In case of presence of CPV, there will be development of band with radio-labelled probe in the X-ray film after autoradiography or colour in the nitrocellulose paper in case of non-radio-labelled probe[34]. Nho et al.[35] developed digoxigenin labeled probe specific for VP1 and VP2 protein to detect CPV. Also, this probe can be used for *in-situ* hybridization and detection of CPV from formalin fixed paraffin embedded tissue samples. Further, Decaro et al.[22] develop two minor groove binders (MGB) fluorophores labelled probe for rapid quantification of CPV-2 variants in dog faecal samples. The MGB probe was able to detect single nucleotide polymorphisms (SNPs) in CPV 2a/2b and 2b/2c.Furthermore, peptide nucleic acid (PNA) probe are stable nucleic acid analogue and hybridize to cRNAs or cDNAs more efficiently than DNA. PNA based array was used to discriminate between the four CPV-2 antigenic types (CPV-2, 2a, 2b, and 2c) during ante-mortem diagnosis in dogs using newly developed PNA-DNA hybridization assay. The PNA array has high sensitivity and specificity compared with a real-time PCR using the TaqMan assay[36].

In-situ Hybridization Assay

In-situ Hybridization (ISH) assay is a rapid and sensitive method for detection and tracking the distribution of CPV specific nucleic acid in tissues using labelled probes. Since the techniques uses isotopic labelled probe it require longer incubation for development of CPV-specific signals and the digoxigenin-labelled probes provide a more rapid DNA detection, Nho et al.[35] designed a digoxigenin-labelled probe complementary to DNA sequences that code for the entire sequence of the VP1 capsid protein and the middle part of the sequence of the VP2 capsid protein to specifically detect the target cell populations for viral replication in tissue sections. The method involve a CPV-specific DNA probe produced by PCR amplification of *VP1* and *VP2* gene which was employed

to visualise the distribution of CPV specific nucleic acid in tissue specimens.

Nucleic acid Sequencing

This is most precise methodology to know the particular variant of the CPV present in the field sample. The purified PCR product as it is or cloned in suitable vector can be sequenced using the suitable primers with the help of automated DNA sequencing machine for typing of CPV strains. The sequence is then analysed using the appropriate bioinformatics software. Both, nucleotide and amino acid sequence data could be used to determine the percent homology and evolutionary analysis of CPV-2 isolates of different geographical origins[37]. CPV-2a and CPV-2b type could be differentiated with certainty based on the sequence analysis[38]. In a study in India, field isolates as well as vaccine strains of CPV were sequenced and found to be CPV-2b and CPV-2 type, respectively[39]. CPV-2c variants have been reported from various countries based on the nucleotide sequence analysis[39,40]. To explore the co-infection and recombination events and to assess the genetic divergence in the CPV-2a and CPV-2c variants, Perez et al.[41] generated complete sequences of coding region of CPV-2a and CPV-2c strains from fecal sample of 40 puppies and analyzed two samples by deep sequencing. The sequence analysis of one of the samples revealed the presence of CPV-2c and CPV-2a strains that differed at twenty nine nucleotides while the other sample included a minor CPV-2a strain (13.3% of the viral population) and a major recombinant strain (86.7%).

Insulated isothermal PCR method

Nested PCR and real-time PCR are highly sensitive but their application is limited to professional laboratories. Realising the need of an on-site sensitive molecular test for CPV-2 diagnosis for early and efficient management of the disease, Tsai et al.[42] developed a new convection PCR method, termed as insulated isothermal PCR (iiPCR). It utilizes a hydrolysis probe and runs in a commercially available device, POCKITTM Nucleic Acid Analyzer. The reaction mixture is passed automatically in sequence through different temperature zones in a capillary tube which is placed within the device to complete the three stages required for PCR. Optical signals produced by probe hydrolysis during the reaction are processed and reported automatically on the display screen as positive or negative result. Wilkes et al.[43] developed an iiPCR method for on-site detection of all circulating CPV-2 strains and FPV. The 95% limit of detection of the iiPCR method was 13 copies of standard DNA. The DNA detection limits for CPV-2b was equivalent for real-time PCR and iiPCR. The iiPCR have 98.41% sensitivity and 100% specificity to detect CPV-2 in faeces compared to real time PCR. Excellent sensitivity and specificity of iiPCR in detecting target pathogens from clinical samples have also been reported for different DNA and RNA viruses[44-47].

Loop mediated isothermal amplification

Loop mediated isothermal amplification (LAMP) assay is a rapid and highly sensitive technique for amplification of DNA for the detection of pathogens. The assay was reported for the first time by Notomi et al.[48]. It involves auto-cycling strand displacement DNA synthesis carried out by use of a DNA polymerase with high strand displacement activity. It generally require two sets of primers for amplification of target gene by simple boiling on water bath at 60-65˚C for about 1 hr. The end-product of the technique is a white precipitate of magnesium pyrophosphate that can be visualized as a white precipitate, colour change of different dye under UV-rays or visible light and specific ladder pattern in gel electrophoresis. The established LAMP assay targeting *VP2* gene of CPV-2 has 100% relative sensitivity and 76.9% relative specificity than conventional PCR[49]. The detection limit of LAMP assay was 10^{-1} median tissue culture infective doses ($TCID_{50}$)/ml in comparison to PCR with detection limit of 10 $TCID_{50}$/ml[49]. Parthiban et al.[50] demonstrated fg level of CPV-2 DNA detection by LAMP assay whereas single-step PCR and nested PCR detected 10 ng and 1pg of DNA, respectively. Mukhopadhyay et al.[51] standardized a highly sensitive and specific LAMP assay for detection of CPV DNA from faecal samples. In addition to this, LAMP assay in conjunction with lateral flow dipstick (LFD) and LAMP-ELISA with objective of visual detection of canine parvovirus were reported[52].Thus LAMP, LAMP-ELISA and LAMP-LFD methods hold promise for use as a diagnostic assay for rapid, sensitive and efficient CPV detection in a clinical setting.

Characterization and Typing of canine parvovirus

Since the first emergence of CPV-2 in 1978, different antigenic variants of CPV-2 have been reported and in course of time they completely replaced the original CPV-2[9]. These antigenic variants namely, CPV-2a, CPV-2b, CPV-2c, new CPV 2a and new CPV2b differ for the presence of one or two SNPs in the sequence of the capsid protein gene. SNPs at positions 4062 and 4064 of genome determine the presence of amino acids Asn, Asp and Glu at residue 426 of the capsid protein in types 2a, 2b and 2c, respectively[53,54]. At the beginning of typing of virus strains, HI test using monoclonal antibodies (MAbs) was the most frequent method. Isolated strains of CPV-2 were subjected to antigenic characterization in a HI assay using panel of MAbs[54-57] and could be typed as original CPV-2, CPV-2a, CPV-2b or CPV 2c on the basis of MAb reactivity[26].

Pereira et al.[12] developed PCR based genotyping method to detect point mutation of different CPV-2 strains. CPV-2a and CPV-2b strains differ by SNP at A4062T and G4449A which results in the replacement

of asparagine by aspartic acid and isoleucine by valine at position 426 and 555, respectively. These mismatches, even if present at the very 3' end of CPV-2b primers, were not sufficient to completely prevent the amplification of the other CPV types. Addition to this, currently circulating type 2a strains have the mutation I555V, due to the nucleotide change G4449A[57]. Therefore, the PCR based genotyping system developed by Pereira et al.[12] is no longer able to discriminate between CPV 2a and CPV 2b strains. Also, the CPV-2c are not detectable by the type-specific PCR assays since the substitution D426E is due to a change (T-A) at nucleotide 4064 in the third codon position, so that this mutant is recognised as type 2b by this PCR strategy[54]. To address this, a PCR-RFLP assay using *Mbo*II enzyme was developed to discriminate between types 2b and 2c[54]. However, RFLP analysis was unable to differentiate between CPV-2b and CPV-2a as both types remained undigested after *Mbo*II digestion. The typing of field samples using PCR followed by *Rsa*I based PCR-RE analysis showed that the vaccine strain used in India are CPV-2 type while field isolates are either of CPV-2a or CPV-2b[58]. Parthiban et al.[17] carried out a typing study using PCR based assays to assess the CPV types circulating in Pondicherry and found the CPV-2a and CPV-2b in the region. Kumar and Nandi[59]analyzed 129 fecal samples and found seventy eight samples positive by PCR and out of these, twenty seven were CPV-2a, thirty nine were CPV-2b and twelve were CPV-2c type, respectively.

Further, to speed up the detection of CPV types, two real-time PCR assays based on the MGB probe technology were developed to recognize SNPs existing between types 2a/2b (A4062T) and between types 2b/2c (T4064A)[22,60]. Both assays were found to be highly sensitive, reproducible, and unambiguously detect the SNPs existing between types 2a/2b and 2b/2c. The only limitation of this assay was that the 2a-specific probe cannot differentiate CPV-2a from the original type and thus unable to discriminating the vaccine strain from the wild type CPV-2a. In addition to this, some of the CPV-2c strains uncharacterized by MGB assay have been reported[61]. Such strains display non coding mutation in probe binding region that prevented the correct hybridization of the type-specific probe. Recently, high melting resolution (HRM) curve method was developed to identify the CPV-2 strains by using nested PCR[62].The PCR-HRM assay is able to distinguish SNPs between CPV-2a, CPV-2b and CPV-2c using two sets of primer. CPV-2a can be distinguished from CPV-2b and CPV-2c on the basis of differences in the melting temperature while CPV-2b and CPV-2c could be differentiated on the basis of the shape of the melting curve using a CPV-2b reference sample. Limitation of HRM-PCR assay is that the quality and quantity of DNA used in the assay may affect the melting pattern[63].

Conclusion

CPV-2 is highly contagious disease causing high mortality. Rapid and sensitive diagnostics are very important in order to provide timely treatment for better survival rate and isolation of infected dogs to prevent transmission to susceptible contact animals especially in case of kennels and shelters. The diagnosis of the disease on the basis of clinical signs is not definitive and should always be confirmed with laboratory tests. Definitive diagnostic tests include demonstration of CPV in the feces, sero-daignosis and necropsy findings with histopathology. Methods available to detect CPV antigen in feces include EM, viral isolation, ELISA, LAT, fecal hemagglutination, immune-chromatography, counter immune-electrophoresis and polymerase chain reaction. The PCR based methods, specifically nested PCR and real-time PCR are more sensitive than traditional techniques. Though there are various methods for the diagnosis of CPV but the molecular techniques are quick, easy and reliable. The characterization of CPV-2 provides vital information about the strains circulating in a region and also shows the relationship of these strains with other CPV-2 strains in the region or different parts of the world. Emergence of new antigenic variants with different antigenic and biological properties may demand an extensive review of the vaccination policy and update of the viral strains contained in the commercially available products. Also, the sophisticate molecular methods to detect and characterize the CPV strains based on perfect matching between viral DNA and assay oligonucleotides will be affected by the onset of point mutations and should therefore be updated as well. Country-wide molecular epidemiological studies should be carried out to gather in-depth knowledge of the CPV-2 strains circulating to aid in planning towards developing efficacious vaccines and effective control strategies.

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