



Global Scenario of Canine Parvovirus Mutants: Epidemiology, Diagnostics and Immunoprophylactic Agents

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Abstract

Canine parvovirus 2 (CPV-2) is one of the most important enteropathogen of dogs emerged in 1978 and manifested by two forms namely gastroenteritis and myocarditis. It is characterized by depression, loss of appetite, vomiting and leukopenia. CPV-2 is probably evolved from a very closely related virus in cats, feline panleukopenia virus (FPLV) or a closely related carnivore parvovirus. It caused high morbidity of 100% and low mortality of 10% in adult dogs and 91% in pups. Over the years a number of variants namely CPV-2a, CPV-2b, CPV-2c, New CPV-2a, New CPV-2b and Asp300 (2a/2b) have been reported from different countries in the world with varying degree of pathogenic potential. Although CPV-2 differ from FPV by 6 amino acids in the VP2 protein, subsequent variants differ from CPV-2 only in one or two places. Further, CPV-2 affects only dogs, new variants expanded their host range to cat as well. There are a number of different serological and molecular tests (PCR, nested PCR, SYBR Green based real time PCR, Taqman based real time PCR, Minor groove binding assay based PCR) available for prompt, specific and accurate diagnosis of the disease. Some molecular tests not only detect the CPV-2 but also identify the variant of CPV-2 involved in disease outbreak. Further, both live attenuated and inactivated vaccines are available to control the disease in animals. Besides, new generation vaccines namely recombinant vaccine, peptide vaccine and DNA vaccine have been developed for control of the disease in canines effectively and efficiently. However, new generation vaccines have not been issued license to be used in the field condition. Again, the presence of maternal antibodies often interferes with the active immunization with live attenuated vaccine and there always exists a window of susceptibility in spite of following proper immunization regimen. Lastly, judicious use of the vaccines in pet dogs, stray dogs and wild canids keeping in mind the new variants of the CPV-2 along with the proper sanitation and disinfection practices must be implemented for the successful control the disease.

Keywords: Canine parvovirus-2 (CPV-2); CPV-2a; CPV-2b; CPV-2c; New CPV-2a; New CPV-2b; Asp300 (2a/2b); Hemorrhagic enteritis; Myocarditis; Canine; Vaccination; Feline panleukopenia virus (FPV); Raccoon parvovirus (RPV); Mine enteritis virus (MEV); Blue fox parvovirus (BFPV)

Introduction

CPV-2 is the most important enteric virus affecting domestic and wild carnivores around the world [1]. In the late 1970s from feline panleukopenia virus or related carnivore parvovirus was found to be ancestor of the CPV-2 [2]. There are two forms of the disease : enteritis in dogs of all ages and myocarditis in pups of 6 weeks to 6 months old with 100% morbidity and mortality of 10% in adult dogs and 91% in pups. The virus is very sturdy and usually transmitted by faecal oral route. The virus has be propensity to replicate in the rapidly dividing cells such as lymphoid tissues, intestinal epithelium and bone marrow as well as myocardium in neonatal pups. Vomiting, haemorrhagic

diarrhoea, depression, loss of appetite, fever, dehydration are the prominent signs of the disease but myocarditis is seen mainly neonatal pups. Due to infection of bone marrow and lymphoid tissue there is sharp drop of WBC count. Although a galaxy of immunoprophylactic agents are available to prevent the CPV-2 infections in dogs, lot of outbreaks are still reported in many countries around the world due to presence of unvaccinated dogs, interference of active immunization by maternally derived antibodies and emergence of a number of different mutants over the years. Because of sudden and wide spread outbreaks of severe contagious enteritis in dogs, canine parvovirus (CPV) has become an issue of great concern to veterinarians. The review article is aimed to give an idea about the latest development in the field of newly emergent variants, diagnostics and immunoprophylactics to the scientific fraternities.

Etiology

The CPV-2 together with FPV, MEV, RPV is under the genus *Protoparvovirus* and family *Parvoviridae* according to the ICTV [3]. CPV-2 is a naked virus, 25 nm in diameter and having icosahedral symmetry. The virus has a linear, SS negative sense DNA genome of 5.2 Kb containing 2 ORFs. One of which encodes 2 nonstructural proteins NS1 and NS2 and other two structural proteins VP1 and VP2. The palindromic hairpin of about 150 bases present in the viral genomic DNA is used in the replication of the viral DNA [4]. The viral capsid contains 60 structural subunits of VP2 (54-55 copies) and VP1 (5-6 copies). The VP1 protein is 727 residues in

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length whereas VP1 is the 584 residues of the C' terminal of VP1 protein. The two structural proteins are produced by alternative splicing of viral mRNAs [5]. The VP2 protein is again cleaved near its N terminus by host proteases to produce another structural protein VP3. The highly conserved central core of capsid protein is composed of an eight stranded anti-parallel β barrel with flexible loops between β strands. There are depression (canyon) of 22 Å deep at 5 fold axes and depression (dimple) of 15 Å at the 2 fold axes. The spike proteins at the 3 fold axes contain the immunogenic epitopes and responsible for inducing neutralizing antibodies [6].

Origin

The phylogenetic analysis between CPV-2 isolates from dogs and viruses from cats (FPV), raccoon (RPV), raccoon dog (RDPV), mink (MEV) and blue fox (BFPV) showed that all have evolved from a common ancestor and that the strains were closely related to viruses from different wildlife including raccoons and foxes. CPV-2 has been derived as a host range mutant from a virus related to FPV in 1970s and acquire the capability to bind the canine transferrin receptor (TfR) type 1. There is >98% nucleotide sequence homology between CPV and FPV but have distinct host ranges, antigenic and haemagglutination properties [7]. There is 6 amino acid changes at 3' axis symmetry of VP2 protein between FPV and CPV namely 93 (Lys to Asn), 103 (Val to Ala) and 323 (Asp to Asn) responsible for successful transfer to canine host and 80 (Lys to Arg) 564 (Asn to Ser) and 568 (Ala to Gly) responsible for nonsusceptibility to cats. Although, residues 232 (Val to Ile) and 375 (Asp to Asn) also changes between FPV and CPV-2 sequences, the residue 375 variation was found only in some isolates of original stain of CPV-2. VP2 residue 373 along with 323 determines the pH dependence of HA [8]. Both the virus also differ from each other in respect to antigenicity, haemagglutination properties, TfR binding affinity and ability to replicate in cats [9]. Further, the evolution rate of CPV much higher than FPV [10] and mutation rate is comparable to RNA viruses of 10^{-4} substitutions per site per year [7]. In addition to that prolong active virus circulation in the host and host immunity pressure due to continual vaccination also contributed for the emergence of new variants of CPV-2.

Host Range, Susceptibility and Age

CPV-2 infects a range of different animals under family: *Mustelidae* (weasels, ferrets, minks, and badgers), *Canidae* (dogs, foxes, and wolves), *Procyonidae* (raccoons), and *Felidae* (cats, lions, tigers, and cheetahs) respectively. The high prevalence of anti-parvovirus antibodies has been reported in different wild animals probably due to interspecies transmission of canine and feline parvoviruses. Due to constant exposure of CPV-2, a large proportion of dogs in animal shelters, pet stores and breeding kennels also harbor antibodies to CPV-2. Although, CPV can affect dogs at any age, severe infection is most common in puppies between 6 weeks and 4 months old and often collapse due to myocarditis [11,12,13]. Crossbred dogs in comparison to pure bred like Doberman Pinchers, Rottweiler, Labrador, German Shepherd and English Springer Spaniels are less susceptible with the exception of Toy Poodles and Cocker Spaniels [14]. A

certain proportion of infected dogs may not necessarily exhibit clinical manifestations but they serve as source of virus to other susceptible animals [15]. Besides dogs, CPV-2 infection has been reported in other canidae including manged wolves, bush dogs, coyotes, crab eating fox and mink [16].

Emergence of CPV-2 variants

In early 1978, entire canine population in the world has been collapsed with hemorrhagic gastroenteritis and high mortality. It was found to be a viral agent and designated as CPV-2 to differentiate it from CPV-1, a relatively non pathogenic virus. In 1980 another mutant of CPV-2 was reported in USA and termed as CPV-2a. Subsequently it has been reported in Japan, Belgium, France, Denmark and Australia due to strong positive selection and strong epidemiological advantage over CPV-2 and became the most common and dominant virus in many other carnivores [17]. There are 6 amino acids difference between CPV-2 and CPV-2a mutants. These are 87 (Met to Leu), 300 (Ala to Gly) and 305 (Asp to Tyr) of VP-2 protein and responsible for replication in cats. Other changes in VP2 gene are 101 (Ile to Thr), 297 (Ser to Ala) and 555 (Val to Ile).

CPV-2b and CPV-2c

After the emergence of CPV-2a, 2 more antigenic variants CPV-2b and CPV-2c were detected in 1984 in USA and in 2000 in Italy respectively [18]. The differentiation of CPV-2a, CPV-2b and CPV-2c is based on the amino acid present at 426 residue of VP2 protein [CPV-2a (Asn), CPV-2b (Asp) and CPV-2c (Glu)] and affect the major antigenic region (epitope A) located at 3' fold spike in the VP2 protein. There is another change at 555 position (Ile to Val) of VP-2 protein between CPV-2a and CPV-2b. The substitution of VP2 residues 555 represented a reversion to or retention of the sequence of CPV-2. Thus CPV-2b and CPV-2c differ from CPV-2a only at 426 residue of VP2 protein. Presently CPV-2c is most dominant strain in Italy, Germany, Uruguay and Argentina [2]. Another mutant 300(Gly to Asp) of CPV-2a and CPV-2b has been recognized in leopard, cats in Vietnam in 1999 [19] and in a few dogs in Vietnam and Korea [20,21]. CPV-2c has become dominant strain throughout the world and reported from Europe, North & South America, Africa and part of Asia [22-28]. In Asia, CPV-2c has been reported in Vietnam, India and China [21,29,30] but not in Korea, Taiwan, Iraq, Turkey, Russia and Japan. Further a substitution at 324 Ile frequently confirmed in Korean and Chinese strains [31,32] has also been reported in the Japan [33]. The residues 324 and 323 located at 3 fold spike regulate the host range along with residue 101, 300 and 426 [12,34]. Another important feature of CPV-2c is that this mutant does not readily transmit in the dogs where CPV-2b prevails but causes severe clinical signs particularly in adult dogs. Further, in addition to this CPV-2a with substitution at residue 413 (Asp to Asn), 418 (Ile to Thr) 435 (Pro to Ser) and 440 (Thr to Ala) have been reported in Korea [20,31,32] Europe [35] and U.S. [26]. The residue 297 is located in the middle of epitope B and substitutions at this position influence the changes in antigenicity of CPV variants [36]. Although, the change T440A has been reported worldwide [20,26,37], but its implications are not clearly understood. Most of the changes in the VP-2 protein



lies between residues 267 and 498 and play an important role in the evolution of new variants due to its presence on the capsid surface and liable to undergo mutation.

Further new CPV-2a and new CPV-2b have also been reported due to nonsynonymous substitution at 297 residues located close to epitope B (Ser to Ala) of the VP2 protein without affecting the antigenicity of those variants [38]. The mutation at 440 (Thr to Ala) located in the G-H loop and at 324 (Tyr to Ile) of VP2 protein have also been reported in the same isolates and may influence the antigenic structure and host range respectively [39]. The mutant 300 Asp (CPV2a/2b) detected in domestic or wild felids in Southern Asia as well as in raccoons indicate the adaptation of the mutant to replicate in these hosts [19,40].

Now-a-days 3 antigenic variants can infect a variety of different hosts and have a worldwide distribution. CPV-2 is considered to be a host range variant derived from FPV via wild carnivores and gained the ability to infect dogs. The phylogenetic analysis of VP2 protein of RPV and Bobcat parvovirus revealed the intermediate location between CPV-2 and CPV-2a strains. Hence, RPV might have played an important role in the evolution of CPV-2a and related strains which regained the ability to infect cats, a property that is absent in CPV-2. The CPV-2a specific residues at 87 and 101 might have acquired during evolution of the virus in raccoon while the changes in 300 and 305 were acquired in canine hosts. Other wild animals such as jackals, coyotes, foxes and wolves also played pivotal role in the evolution of CPV-2 and related strains. One of the most interesting facts about the CPV-2 evolution is the high rate of nucleotide substitutions associated with infection of canine population [41]. Two possible explanations for this high substitution rate are high intrinsic rate of mutation [2.957×10^{-4} substitution/site/year] in CPV and high persistent positive selection pressure [7]. This rate of substitution is higher for the DNA viruses and comparable to rapidly evolving RNA viruses, such as HIV and HCV [42].

Clinical Signs

Canine parvovirus causes haemorrhagic gastroenteritis in dogs of all ages and myocarditis in pups of 6 weeks to 6 months [43]. The incubation period varies from 3–7 days. The acute gastroenteritis form affects all age groups of dog but it is more severe in pups. The early symptoms include depression, loss of appetite, vomiting, high fever, severe diarrhoea and pain. Initially there is slight rise of temperature but gradually turn to subnormal level with advancement of vomiting and diarrhoea [44]. The consistency of the stool may be watery, yellowish or tinged with frank blood in severe cases. The affected dogs become dehydrated due to excessive loss of fluid through vomiting and diarrhoea and death occurs within 3-4 days after onset of clinical symptoms. Puppies may die suddenly due to shock as early as 2 days of illness [15]. Leukopenia is the constant feature with white blood cell (WBC) counts dropping below 2000–3000 cells/ml of blood. Subclinical and inapparent infections are frequently detected, mainly in pups with intermediate MDA titers and in adult dogs [22]. The mortality rates can be high (up to 91%) in pups, but are usually less than 10% in adult dogs [43].

The second form of CPV is cardiac form, or myocardial form, which can affect puppies under 3 months old [11]. In an infected litter, 70% pups will die in 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-2 myocarditis is the sudden death in young pups usually about 4 weeks of age [45]. The collapsed pup may have cold extremities, pale mucosae and show gasping respiration or terminal convulsions. Acute heart failure with respiratory distress occurs in pups between 4 and 8 weeks of age. Subacute heart failure occurs in older pups usually of 8 weeks or more. They are tachypnoeic or dyspnoeic especially on exercise. The abdomen is swollen with hepatomegaly and ascitic fluid is blood tinged [46]. There is tachycardia, sometimes with arrhythmias and a weak pulse. Most animals die due to cardiogenic shock. However, if the animal survives it will suffer from chronic myocardial and circulatory complications [47,48].

Epidemiology of CPV-2

CPV-2 was reported in May, 1978 after an epizootic of gastroenteritis and myocarditis occurring worldwide in pups [11,49,50] and in 1979, CPV-2a which subsequently becomes predominant subtype of CPV-2 worldwide was identified. In 1984 another variant, CPV-2b was emerged in canine population. Later, in 2000 an antigenic type CPV-2c was reported in Italy [18]. Presently original antigenic type CPV-2 has been completely replaced by its antigenic variants in the field. Molecular epidemiology of CPV revealed that different geographical locations of the world are facing canine parvovirus enteritis outbreaks caused by different CPV-2 variants in recent years. In Europe all strains with wide spread presence of CPV-2c, have been reported from Germany [51], Poland [52], and Portugal [53]. Unlike other European country, in Greece, Hungary, and Bulgaria, CPV-2a remains most common variant with sporadic presence of CPV-2b and CPV-2c [54-56]. Calderón et al., 2015 [57] have reported presence of all antigenic variants but CPV-2c is most dominant strain in South American countries like Ecuador [58], Brazil [59], and Argentina. North American continent like in USA and Canada all antigenic variants are circulating but CPV-2b is the dominant one [60], while the current dominant viral variant in Mexico is CPV-2c [61]. In Africa, both CPV-2a and 2b are present in South Africa but only CPV-2a in Nigeria [62] and in Tunisia all type of antigenic variants was reported [28]. New Zealand had reported only CPV-2a [63] where as in Australia CPV-2a was the most prevalent strain with sporadic presence of CPV-2b [64]. In Asia, China and Taiwan have reported all type of CPV-2 variants, while Korea (South) [65], Japan [33], Thailand [66], Iraq [67] and Turkey [68] reported both CPV-2a and CPV-2b strains.

In India canine parvovirus-2 was first reported by [69] Ramadass et al. (1982). Different canine parvovirus variants (CPV-2a, CPV-2b, new CPV-2a, new CPV-2b and CPV-2c) have been isolated from dogs of almost all the states of India [39,70,71,72,73]. Only single report was documented about the presence of CPV-2c strain [43]. Presently new CPV-2a has surpassed the earlier circulating strain of CPV-2b or new CPV-2b and established as a predominant strain of CPV-2 in the canine population of India [39,73].



Pathogenesis

The incubation period may vary from 3-7 days following oral exposure to approximately 10^6 TCID₅₀ of CPV. After entering into the body, CPV replicates in the lymphoid tissues of the oropharynx and there is development of viraemia on 2-5 days P.I. with a peak viral titre of $10^{5.5}$ TCID₅₀/ml. Virus has been found in small intestinal crypt cells and lymphatic tissues (thymus, spleen, lymph node and bone marrow) by FAT or other serological tests [74]. The germinal cells at the base of the intestinal crypts constantly divide to replace the cells extruded out from the tips of the villi. It generally takes 3 days to reach to the tip of the villus from the base of the crypt. This phase is vital to the integrity of the gut epithelium. In the milk fed pups the rate of epithelium regeneration is relatively low, but as consumption of solids increases, the turnover of cells also increases and the pups become susceptible to CPV challenge [75].

One of the target sites of CPV is myocardial tissue of heart. CPV produces myocardial lesion in puppies if infections is received within the first few weeks of life when the heart muscle cells are in dividing state. However, a number of factors viz., immaturity of the animals, a concurrent disease, dietary changes and other form of stress etc play an important role to influence the severity of the disease [76]. With the advent of the effective immunization, many young pups are now protected during the first weeks of life due to maternally derived antibody and so myocardial infection is rarely noticed now-a-days [74,75].

Pathological Changes

The lesions are confined to jejunum and ileum but not the duodenum and colon. Affected segments may be somewhat flaccid with subserosal haemorrhage or congestion [16,77]. The lumen of the intestine is often empty, but may contain variable, watery ingesta. The mucosal surface is often congested but devoid of exudates. Mesenteric lymph nodes are frequently enlarged and oedematous. Multifocal petechial haemorrhages are often seen within the cortex of a cut section of affected lymph nodes during acute state of the disease. Thymic cortical necrosis and atrophy are common findings in young dogs [48,77].

In the enteric form of the disease, lesions consist of necrosis of the crypt epithelial cells. Crypt lumenae are often dilated with necrotic debris. There may be occasional I/N eosinophilic inclusion bodies in intact crypt epithelial cells. The villi and lamina propria may collapse completely as results of the loss of crypt epithelium and the failure to replace sloughed villous epithelial cells. Necrosis and depletion of small lymphocytes is seen in Peyer's patches, the germinal centers of mesenteric lymph nodes and splenic nodules [78].

In myocardial form, gross lesions include cardiac enlargement with prominent dilatation of the left atrium and ventricle. There is white, frothy fluid in the trachea and bronchi. Pulmonary oedema and passive congestion of the liver with the variable presence of ascites and pleural effusion are noticed. The ventricular myocardium frequently contains visible white streaks associated with the presence of a cellular infiltrate. Some pups may die from left sided heart failure weeks or months after.

Pulmonary hypertension and myocardial dilatation have also been noticed [47,79]. Evidence of pulmonary edema and passive congestion of the liver is often present, with the variable degree of ascites and pleural effusion. The ventricular myocardium frequently contains visible white streaks associated with the presence of a cellular infiltrate [80].

Diagnosis

The rapid and accurate diagnosis of CPV-2 infection in dogs is of paramount importance to isolate them from shelter or kennel, curtail the secondary infection and stop chances of further spread of the disease to other susceptible dogs. Merely, clinical signs are not decisive to diagnose CPV-2 infection as other viral infections such as coronaviruses, adenoviruses, morbilliviruses, rotaviruses, reoviruses and noroviruses show same types of clinical signs [81]. Therefore, a suspected case of CPV-2 infection must be confirmed through laboratory tests. There are several types of laboratory tests which mainly rely on the faecal samples collected from suspected dogs. In late stage of infection, whole blood have been proven more suitable for detection of CPV-2 as there is long lasting viremia in affected dog [22,82]. There are broadly two types of diagnostics approaches for CPV-2 and its variants infection; namely (1) conventional & serological and (2) molecular tests.

Conventional and Serological Methods

Conventional methods detect viral antigens or whole virus in clinical samples. These are electron microscopy [83], virus isolation [45], latex agglutination [84], haemagglutination [45,83], and enzyme-linked immunosorbent assay [83,85] and immunochromatographic test. Virus isolation (VI) requires cell culture facility with expertise and knowledge in cell culture. In addition to this, VI is cumbersome and requires long incubation time approximately 5-10 days. Moreover, cell lines (MDCK and CRFK) show negligible CPE following CPV infection and other techniques such as immunofluorescence or HA test are needed to confirm the presence of virus.

HA Test

HA test is a simple and rapid test for the detection of CPV-2 from faecal samples and this test can be performed by using erythrocytes of porcine, rhesus monkey and feline. The HA test can be carried out by incubating the plates at various temperature such as 4°C, 25 °C and 37°C and the best results were found at 4°C followed by at 25 °C and least titre at 37°C [86]. HA titre varies between 128 and 10,240, between days 4 and 7 after infection, or when the signs of enteritis commence and generally cease in between 7 and 9 days after infection. Apart from this, various buffer systems have been evaluated for HA test but optimum results were obtained with PBS in a pH range of 4-6 [86,87]. Due presence of nonspecific haemagglutinin factors, the nonspecific HA titer (1:32) is common that makes this test less reliable for CPV-2 diagnosis [45] but it may be reduced by brief treatment of samples with fluorocarbon (Genetron, Freon 113) or CHCl₃ (10% V/V).



Electron microscopy

The canine parvovirus can be readily identified in faeces by negative staining in electron microscopy [50, 76]. Compared to other techniques it is less sensitive and expensive in nature. The electron microscope facility is not available in routine diagnostic laboratory. However, it is possible to concentrate viral particles by means of specific antibodies in the immunoelectron microscopy technique for specific detection of virion in faecal samples [50].

Virus isolation (VI)

A number of primary cell cultures (canine and feline kidney cells) and cell lines like MDCK (Madin-Darby Canine Kidney) or CRFK (Crandall Resse Feline Kidney) can support the replication of canine parvovirus-2 and used for virus isolation from suspected clinical samples. A new cell line named A-72 established from canine fibroma, can be used for isolation of virus particularly from field samples with characteristic CPE after 3-4 blind passages. Due to absence of CPE in MDCK and CRFK the presence of virus need to be confirmed by HA and FAT [88]. The virus isolation in cell culture is time consuming, less sensitive and there is a need of cell culture laboratory with expert personnel. CPV-2 can be detected by HA and/or VI only for few days post-infection and these techniques may give false-negative results despite high amounts of viral DNA detected by PCR or real-time PCR.

Antigen Detection

Viral antigens can be also detected by means of immunohistochemistry carried out on intestinal, brain or tongue sections. The diagnosis of CPV infection by immunochromatographic test (IC) is simple and adopted by the veterinarians in the clinics [89]. Although, it is simple, rapid and easy to use but require large amount of virus antigen to produce appreciable band. ELISA technique utilizes specific monoclonal antibodies coated on the plastic, nitrocellulose membranes for the detection of viral antigen [90]. The SNAP Parvo test from IDEXX Laboratories is a faecal CPV antigen ELISA, which is popular and used worldwide as a rapid in-house test. Although it is highly specific, but very variable data on its sensitivity of 81.8% [91], 56.2% [92], and 18.4% [93] has been reported. False negative test results are common and can lead to under diagnosis of CPV infection that can result in inadequate treatment and spread of the virus. Low faecal CPV-2 load and antibodies binding to CPV-2 antigen in faeces are likely to be important reasons for false negative faecal antigen results in ELISA [94].

Slide agglutination test (SAT) and slide inhibition test (SIT)

SAT and SIT are the test used for detection of CPV antigen in faeces and antibody in clinical serum samples respectively [95]. In SAT presence of antigen is demonstrated with haemagglutination activity of RBCs, while in SIT presence of antibody in serum is detected by haemagglutination inhibition of hyper-immune sera of CPV-2b. Results revealed that SAT is comparatively less sensitive than standard HA test while in SIT, titers of hyper-immune serum is much higher for homologous CPV-2b isolate than for heterologous CPV-2c isolates. The results of these assays

are available in 1 min without need of any special instrument except requirement of fresh porcine RBCs and SAT is not sensitive for detection of CPV having titre of less than 1:40. The test is useful for rapid detection of CPV antigen and determination of immune status before vaccination in pups.

CPV-2 detection by quartz crystal microbalance biosensor (QCM)

It is a novel, label free technology based on measurement of mass per unit area by measuring the change in the frequency of a quartz crystal resonator. It is a convenient method for detecting antigen-antibody interactions. Kim et al. (2015) [96] used QCM biosensor, ProLinker™ B, and antibody for rapid diagnosis CPV infection. A QCM biosensor with a cut-off value of -205 Hz showed 95.4% (104/109) sensitivity and 98.0% (149/152) specificity when used to test 261 canine fecal samples compared to PCR.

Molecular Techniques

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has been considered as the most reliable and widely applied diagnostic technique because of high degree of sensitivity and specificity in detecting CPV from faecal samples and vaccines [22,93,97,98]. PCR has been able to detect fewer number of virion particles than that of HA, EM and ELISA from faecal samples. The PCR assay combined with the gel filtration was able to detect the virus from feces containing as little as 10^3 PFU/g fresh faeces, whereas the virus was detected by either the ELISA test or the culture method only from faeces containing more than 10^6 PFU/g fresh faeces [99]. The nested PCR has been employed for the detection of canine parvoviral DNA and to improve the specificity and sensitivity of PCR [47]. The nested PCR is 100 times more sensitive compared to conventional PCR. Thus, the nested PCR seems to be a sensitive, specific and practical method for the detection of CPV in faecal samples [41,81]. Schunck et al. (1995) [48]. 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 10 infectious particles per reaction which corresponds to a titer of about 10^3 infectious particles per gram of unprocessed faeces. This renders the PCR about 10 to 100-fold more sensitive than electron microscopy, the standard method for parvovirus diagnosis. Moreover, the PCR can now be used to differentiate the different mutants of CPV-2 using the primers specific for particular mutants [41].

Real time PCR

Real-time quantitative PCR is based on continuous optical monitoring of a fluorogenic PCR reaction [100,101]. It is more sensitive, rapid and reproducible compared to conventional PCR assays and allowing a precise DNA quantitation even when a high sample throughput is required [102,103]. Real time PCR can be run both in 96 well or 8/12 strips formats and most of the steps are automated. Due to use of inexpensive procedure for preparation of DNA by simple boiling of fecal homogenates, the total time required for analysis of 20–30 samples was about 6 hours and 3 hours for conventional and real-time PCR, respectively [104].



Decaro et al. [104] designed a simple TaqMan probe based real time PCR assay for the detection and quantification of canine parvovirus on the basis of VP-2 DNA from dog faeces. It was found that the TaqMan CPV-2 real time assay is more sensitive than both HA and conventional PCR and being able to detect as few as 10^2 copies of CPV-2 DNA. The established CPV-2 real-time assay was highly reproducible and linear over a range of eight orders of magnitude, from 10^2 to 10^9 copies, allowing a precise calculation of CPV-2 DNA loads in samples containing a wide range of viral DNA amounts. The established TaqMan assay was successfully employed in pathogenesis studies carried out during natural [82] and experimental [22] infections and was also used to evaluate virus distribution in different tissues. A TaqMan real-time RT-PCR assay developed for detection of CPV RNA transcripts demonstrated the presence of replicating virus in CNS [105]. A SYBR Green based real-time PCR assay was proposed as an alternative method to the TaqMan technology, displaying the same detection limit (10 copies of viral DNA) [72]. Due to high sensitivity and reproducibility of real time PCR, it may be more useful for detection of dogs shedding CPV at low titers in their feces, and to take preventive measures during the outbreak of CPV-2 especially dog shelter and kennels.

Nucleic Acid Hybridization/Dot Blot

The extracted DNA from the stool samples or cell culture supernatant inoculated with the stool sample suspected for canine parvovirus is charged on the nitrocellulose paper or nylon membrane. The DNA is then subjected to hybridization with CPV-specific probe either radio-labelled or biotin labeled. In the positive case there will be development of band in the X-ray film after autoradiography in case of radio-labelled probe or colour in the nitrocellulose paper in case of non-radio-labelled probe [106].

Detection of Canine Parvovirus by in situ Hybridization

This technique was developed to detect viral antigen in tissue sections obtained from CPV-infected animals. In this method a CPV-specific DNA probe was produced by PCR amplification of a genome segment encoding capsid proteins VP-1 and VP-2 and was used to know the distribution of CPV specific nucleic acid in tissue specimens obtained from infected dogs [107].

Nucleic Acid Sequencing

The PCR product as it is or cloned in the suitable cloning vector can be sequenced with the help of automated DNA sequencer for typing of CPV strains. The sequence is analysed using the appropriate software. This is an important technique to know with certainty the particular variant of the CPV present in the field sample. Both the nucleotide and amino acid sequence data can also be used to know the percent homology and for phylogenetic analysis of CPV-2 isolates from different geographical regions. Based on sequence analysis CPV-2a and CPV-2b type could be differentiated and none of the isolates were belonging to original CPV-2 type in India [70]. In a further study, in India field isolates as well as vaccine strains of CPV were sequenced and it was

found to be CPV-2b type and CPV-2 respectively [29]. CPV-2c variant has been reported from various countries based on the nucleotide sequence analysis [26,97].

Insulated isothermal PCR method

An insulated isothermal PCR (iiPCR) method was developed for on-site detection of all circulating CPV-2 strains and FPV [108]. Originally the iiPCR technology was developed by Tsai et al. (2012) [109]. It utilizes a hydrolysis probe (similar to TaqMan based qPCR) and runs in a commercially available device, POCKITTM Nucleic Acid Analyzer (POCKITTM; GeneReach USA, Lexington, MA, USA). Limit of detection, reaction sensitivity and performance of CPV-2 iiPCR test was determined and compared with in house real time PCR by testing of serial dilution of CPV-2b strain and 100 canine fecal samples. The 95% limit of detection of the iiPCR method was 13 copies of standard DNA and detection limits for CPV-2b DNA were equivalent to real-time PCR. The iiPCR has 98.41% sensitivity and 100% specificity to detect CPV-2 in faeces compared to real time PCR.

Loop mediated isothermal amplification assay (LAMP)

Loop mediated isothermal amplification assay is a novel, highly sensitive, rapid and reliable technique for the detection of genomic material of different microorganisms. The assay was reported by Notomi et al. (2000) [110]. The whole procedure generally requires 6 to 8 primers, simple boiling on water bath at 60-65°C and approximately 1 h of incubation. The end-product can be visualized as a white precipitate of magnesium pyrophosphate, colour change of different dye (SYBR green-1, Calcein) under UV-ray or visible light and specific ladder pattern in gel electrophoresis. The established LAMP assay targeting VP-2 gene of CPV-2 has 100% relative sensitivity and 76.9% relative specificity compared to conventional PCR [106]. The detection limit of the LAMP method was 10^{-1} median tissue culture infective doses (TCID₅₀)/ml, compared with 10 TCID₅₀/ml for PCR analysis [106]. A total of 104 (74.28%) out of 140 fecal sample, were found positive by LAMP, whereas 81 samples (57.85%) were found positive by conventional PCR and the detection limit of the LAMP and PCR were 0.0001 TCID₅₀ ml⁻¹ and 1000 TCID₅₀ ml⁻¹ respectively [111]. Another, LAMP based assay demonstrated fg level of CPV-2 DNA, whereas single-step PCR and nested PCR detected 10 ng of DNA and 1 pg of DNA, respectively [112]. Again LAMP assay in conjunction with lateral flow dipstick (LFD) and LAMP-ELISA with objective of visual detection of canine parvovirus was reported. It was found that CPV detection limits by PCR, PCR-ELISA, LAMP, LAMP-ELISA and LAMP-LFD were 10^2 , 10^2 , 10^{-1} , 10^{-1} and 10^{-1} TCID₅₀/ml, respectively [113]. 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 canine parvovirus-2 in 1978, different antigenic variants of canine parvovirus-2 have been reported and they have replaced completely the original canine parvovirus-2 (old type) [22]. These antigenic variants namely (CPV-2a, CPV-



2b, CPV-2c, new CPV 2a and new CPV2b) differ for the presence of one or two single nucleotide polymorphisms (SNPs) in the sequence of the VP-2 gene. SNPs at positions 4062 and 4064 of the viral genome determines the presence of a specific amino acid; Asn, Asp, and Glu at residue 426 of the VP2 protein in types 2a, 2b and 2c, respectively [18,114]. In the beginning for determination of virus strain type, Haemagglutinin inhibition test using monoclonal antibodies were the most frequent test. Pereira et al. (2000) [115] had developed PCR based genotyping method to detect point mutation of different CPV-2 strains. As 2a and 2b strains differ by single nucleotide polymorphism at A4062 T and G4449A that determine the replacement of asparagine by aspartic acid at position 426 and of isoleucine by valine at position 555, in types 2a and 2b, respectively. A 2b specific primers were designed such that one such mutation at the very 3' end, as nucleotide mismatches that occur at the 3' end of a primer are highly detrimental to primer extension and strongly decrease PCR amplification. However, these mismatches, albeit present at the very 3' end of the primers, were not sufficient to completely prevent the amplification of the other CPV types [116]. Moreover, currently circulating type 2a strains have the mutation I555V, due to the nucleotide change G4449A [116]. Therefore, the PCR based genotyping system developed by Pereira et al. (2000) [115] is no longer able to discriminate between CPV 2a and CPV 2b strains, as almost all the novel type 2a strains (555-Val) would be mischaracterized as type 2b. Finally, by the type-specific PCR assays, type 2c CPVs are not detectable, 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 [18]. PCR-RE analysis based characterization of different strains employed amplification of VP1/VP2 gene (2.2 kb) and its RE digestion with *Hpa* I and *Rsa* I, can differentiate between original CPV-2 and CPV-2a/2b. Kumar et al., (2004) [86] used *Alu*I to differentiate CPV-2a and CPV-2b. The typing of field samples using PCR followed by *Rsa* I based PCR-RE mapping showed that the vaccine strain used in India are CPV-2 type while field isolates are either of CPV-2a/2b [117]. *Mbo* II restriction enzyme was used efficiently to discrimination between CPV2b and CPV2c [18].

Further for easier discrimination and quantification of these strains two minor binding grooves assays (MBG assay) were developed by using two type-specific probes labelled with different fluorophores (FAM and VIC) [118]. The MBG assay is specific, sensitive, reproducible, and unambiguously detect the single nucleotide polymorphisms existing between types 2a/2b and 2b/2c. The only limitation of this assay is 2a-specific probe is not able to discriminate type 2a CPVs from the original type and create problem in differentiation of vaccinal strain and wild type CPV-2a. In addition to this some of the CPV-2c strains uncharacterized by MGB assay has been reported [35]. Such strains display noncoding mutation in probe binding region that prevented the correct hybridization of the type-specific probe. These findings highlight the need to update the CPV-typing methods based on single nucleotide polymorphisms as additional mutations may hinder the correct strain characterization, reinforcing previous suggestions to keep using diagnostic

molecular tools that target more conserved regions to avoid false-negative results [23]. Recently a novel technique, high melting resolution (HRM) curve method was developed to identify the CPV-2 strains by using nested PCR [119]. Two sets of primer were used. The region amplified by CPV-426F/ 426R(primer) included the A4062G and T4064A mutations in CPV-2a, CPV-2b and CPV-2c, while the region amplified by CPV-87F/87R included the A3045T mutation in the vaccine strains of CPV-2 and CPV-2a, CPV-2b and CPV-2c. The PCR-HRM assay is able to distinguish single nucleotide polymorphisms between CPV-2a, CPV-2b and CPV-2c using CPV-426F/426R. CPV-2a is distinguished from CPV-2b and CPV-2c by differences in the melting temperature. CPV-2b and CPV-2c could be distinguished based on the shape of the melting curve after generating heteroduplexes using a CPV-2b reference sample. The vaccine strains of CPV-2 were identified using CPV-87F/87R. Limitation of HRM-PCR assay is that the quality and quantity of DNA used in the assay may affect the melting pattern [120] and using amplicons that have not been pre-amplified had poor reproducibility in terms of T_m on different days and/or using different templates made from individual samples [119]. So, to obtain reproducible results with HRM, the reference DNA templates, unknown samples collected in the field or DNA isolated using different extraction methods must be pre-amplified by PCR for subsequent HRM analysis. Amplification refractory mutation system PCR (ARMS-PCR), a sequencing independent method was developed for simultaneous detection and typing of the CPV-2 variants [121]. The method involves two steps, where first reaction is used for differentiation of CPV-2a from CPV-2b/CPV-2c by using two common detection primers, CPV-2a specific primer and CPV-2b/CPV-2c primer while second reaction is carried out for the presence of CPV-2c by using CPV-2c specific primer.

Prevention and Control

The canine parvovirus is naked without envelope and relatively hardy in the environment. Although most disinfectants cannot kill it, chlorine bleach is quite effective in the ratio of 1 part bleach and 30 parts of water. There is no way to completely disinfect contaminated dirt and grass, although sunlight and drying has some effect [14]. Mechanical decontamination through irrigation may also be helpful, but the area must be allowed to dry thoroughly between applications. Potassium peroxymonosulfate is very much effective on organic faecal matter, and can be sprayed on contaminated areas using a pesticide sprayer or other applicator [122]. But, vaccination program is the most effective and economical way to control the disease in dogs and the best way to protect the pups is by vaccinating the pregnant bitch.

Vaccination

Immunization of pups against CPV is common but not reliably effective because of interference by maternally derived antibodies to active immunization and present of intercurrent disease. Pups receive immunoglobulins while *in-utero* and via colostrums. Pups are protected by passively acquired antibody against CPV infection, but make them refractory to active immunization until the level of maternally derived antibody has reached to below 1:20. All pups born to CPV immune bitches experience a period



known as window of susceptibility when they are susceptible to infection but refractory to vaccination [123].

Modified live Vaccine

For the control of canine parvovirus enteritis in canine population, live attenuated vaccine based on original canine parvovirus-2 (CPV-2) and its variants CPV-2b were being used for the prophylaxis of the young puppies. The vaccine was found protective against infection of canine parvovirus infection. Emergence of new variants of canine parvovirus-2 raises the concern about the efficacy of vaccine having original CPV-2 [26, 35, 118]. Recently CPV-2b based vaccine has also been developed to deal with infection caused by CPV-2 variants. Different groups have variable opinion about the CPV-2 based vaccines: some says it is still effective against the newly emerging strains of CPV-2 but others in the opinion of using region specific CPV-2 strain prevalent in a particular area. Although a particular strain of CPV-2 confer better protection against the homologous strain, it has been reported that CPV-2b provide better protection against all the mutants compared to other mutants of CPV-2 [124]. Dogs that recover from the infection have standard HI titres ranging from 1: 2560 to 1: 20480 which persist for at least one year. Such animals are solidly immune. Dogs vaccinated with MLV-FPV have HI titers in the range of 10 to 1600 (usually 160-640) [88] lasting for one year. On the other hand, dogs vaccinated with killed FPV vaccine developed a HI titre of 20-160 (usually 40-80) for a period of 12-14 weeks [90].

Recombinant Protein Vaccine

Recombinant vaccine containing the baculovirus expressed VP2 protein was found to be structurally and immunologically indistinguishable from authentic VP2. The recombinant VP2 also shows the capability to self assemble, forming virus like particles similar in size and appearance to CPV virions. The VP2 protein at the rate of 10 µg was able to elicit good protective response as measured by ELISA and shown to be better than commercially available inactivated CPV vaccine in terms of immune response [125].

DNA vaccines: Both the prokaryotic and eukaryotic vectors harboring the genes coding for the structural proteins of the canine parvovirus has shown the encouraging results. The dogs immunized with the DNA vaccines withstood the challenge with virulent canine parvovirus. However, the DNA vaccines still is in the experimental stage and not yet licensed to be used in the field condition [126].

Peptide Vaccine

The N-terminal domain of the major capsid protein VP2 of canine parvovirus was shown to be an excellent target for development of a synthetic peptide vaccine. The shortest sequence sufficient for neutralization induction was nine residues. Peptides longer than 13 residues consistently induced neutralization, provided that their N termini were located between positions 1 and 11 of VP2 [127].

Treatment

The principle of treating CPV infection in dogs is restoration

of fluid therapy. The fluid therapy with bicarbonate may be recommended as soon as the dogs come to hospitals. Metabolic acidosis develops if the diarrhoea is severe. All oral intake must be withheld in case of severe vomiting [128] and should be given parenterally. It is generally suggested to give antibiotic therapy in case of parvoviral enteritis. Any animal with parvoviral infection should certainly receive a broad spectrum bactericidal antibiotic such as ampicillin, polymyxin, cephalosporin or gentamicin parenterally [128]. The antiemetics are also suggested by I/V route. The coagulants are recommended by I/V route as long as haemorrhagic faeces were passed by the animal. During the early phase of the disease, the application of hyperimmune serum may help to reduce the virus load and render the infection less dramatic. Such treatment has been shown to reduce the mortality and shorten the length of the disease however hyperimmune serum is difficult to obtain. In case of vomiting, chlorpromazine or metoclopramide (Reglan), out of which Reglan can be given @ 0.5 mg /kg body weight parenterally may be given at 8 hours interval. To correct the gastric problem cimetidine, ranitidine, famotidine and to check diarrhoea, loperamide or bismuth subnitrate or other astringent preparations may be given. A dog with persistent vomiting should not be given any food until the diarrhoea and vomiting subsides. Deaths resulting from myocarditis usually occur so rapidly that therapy is unsuccessful [80]. Dog that survives the initial episode may die of heart failure six months later [46].

Conclusion

CPV-2 is one of the most significant viral pathogen of canines causing high morbidity and mortality and manifested by haemorrhagic gastroenteritis and myocarditis. Over the years a number of highly sensitive and specific diagnostic both serological and molecular has been developed to provide prompt and accurate diagnosis of the disease conditions in dogs. Further, a galaxy of potent and efficacious live attenuated and inactivated vaccines is available in India to control the disease in dogs. In spite of presence of excellent diagnostics and vaccines, large numbers of cases are reported both in vaccinated and unvaccinated dogs and other wild animals. Although pet dogs are vaccinated against CPV-2 infections, unvaccinated stray dogs without any conspicuous clinical signs remain carrier of the virus and source of infection to other susceptible dogs. Extensive studies must be undertaken to know the molecular epidemiology of the canine parvovirus infections in different canine species and the variants of the CPV involved in the outbreak of the disease. The necessary preventive measures must be undertaken to immunize the susceptible dogs including the stray dogs with the potent and efficacious vaccines against the disease to check the spread of the disease. Prompt symptomatic treatment, restoration of fluid and antibiotics to prevent bacterial infection will increase survivability in infected puppies but vaccination program should be considered the best way to control the disease in dogs. The pups are usually best protected through vaccination of the pregnant bitch. In addition, the newer CPV-2c strain presents new challenges as it causes severe disease in adult dogs and it is not easily detectable in laboratory tests and current vaccines may not be as effective in providing protection against it. However, if



the new virus gains wider host ranges, deadly outbreaks could be observed in dogs. In that case, recent isolates of CPV need to be investigated to anticipate and assess the risk imposed by them. Further the homologous vaccine should be based on current or newer variant to tackle the disease more efficiently. Also, zoo sanitary measures must be employed to prevent the disease both in pet and wild animals.

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