Update on Canine Parvovirus: Molecular and Genomic Aspects, with Emphasis on Genetic Variants Affecting the Canine Host

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Abstract
Canine parvovirus (CPV), the etiology of hemorrhagic enteritis in dogs, was first isolated as CPV type 2 (CPV-2) almost 40 years ago, and was soon replaced by the emergence of new variant types. The major viral capsid proteins encoded by the VP2 gene are the sites where amino acids are often substituted, accounting for the unusual nature of this type of DNA virus. The alteration of specific residues has contributed to different antigenic variants which have affected the evolution of virus binding and host immunity to this virus. Sequence analysis of the VP2 gene and subsequent characterization have revealed three circulating CPV-2 strains, CPV-2a, CPV-2b, and CPV-2c, identified by mutations at amino acid residue 426. The latter strain displays increased pathogenicity in dogs and an extended host range. The present review article aimed at updating contemporary information on epidemiological studies and surveys from CPV field work. Moreover, we pointed out some sensitive and rapid diagnostic tools for detecting CPV in clinical samples, techniques which will be useful for health monitoring and management of CPV with currently available vaccines.

Keywords: Canine Parvovirus, CPV type 2, Genetic Variation, VP2 Gene, Mutation, Dog

INTRODUCTION
Canine parvovirus (CPV) is a contagious, life-threatening viral disease in young dogs, with a wide host range in many mammalian families: Mustelidae (ferrets, minks, and badgers), Canidae (dogs, foxes, and wolves), Procyonidae (raccoons), and Felidae (cats, lions, tigers, and cheetahs) [1]. This viral disease is very common in unvaccinated dogs living in densely populated areas. The transmission of CPV is mediated by persons, animals, and fomites that come in contact with infected secretions or materials, such as feces, blood, food bowls, clothing, or bedding. The most

Citation of This Article

Özet
Köpek parvovirusu (KPV), köpeklerde hemorajik enterit etkeni olan köpek parvovirus (CPV) neredeyse 40 yıl önce ilk olarak CPV türü 2 (CPV-2) olarak izole edildi ve daha sonra yeni türler olarak CPV-2'nin yerine geçti. CPV geni tarafından kodlanan büyük virüs kapsid proteinleri aynı zamanda en sık kullanılan amino asitlerin arasında bulunan ve virüsü bağlayabilen ve virüsü karşı konakçı bağışıklığını etkileyen en önemli alanlar olup bu tip DNA virüslerin olduğu reaksiyonun nedenini oluşturur. Specifik yapılardaki değişimler farklı antijenik varyantların oluşturmasına katkıda bulunarak virüsü birleştirmek ve virüs karışı konakçı bağışıklığının gelişimini etkileyebilir. CPV geninin sekans analizi ve takibi karakterizasyonu, 426. amino asitinde mutasyon ile şekillenen CPV-2a, CPV-2b ve CPV-2c olmak üzere toplamda 3 farklı CPV-2 suşunun olduğunu göstermiştir. CPV-2c köpeklerde artmış patojenite ve daha geniş konakçı yelpazesini göstermektedir. Bu derlemede güncel epidemiolojik çalışmalardan bilgi alalım ve CPV'nin kontrol edilebilmesi için genel bilgileri güncellememiz önemlidir. Ayrıca, klinik örneklerde CPV'nin tedavisinde kullanılmak suretiyle sağlık taramasında faydalı olabilecek ve mevcut aşılarla CPV'nin kontrol altına alınmasına faydali olabilecek bazı hassas ve hızlı yöntemleri de değerlendirilmiştir.

Anahtar sözcükler: Köpek Parvovirus, CPV türü 2, Genetik Varyasyon, VP2 Geni, Mutasyon, Köpek
climically significant forms induced by CPV are hemorrhagic enteritis, or bloody diarrhea. The general clinical signs may present as anorexia, depression, vomiting, fever, and mucoid or watery diarrhea. In severe cases, dehydration and hypovolemic shock may occur. The mortality rate in puppies can reach more than 70%, whereas the rate in the adults is less than 1% [2]. Canine parvovirus replication occurs in host cell nuclei and requires rapidly dividing cells of fetuses, newborns, lymphoid tissue, and intestinal epithelium of animals. The CPVs spread easily and are highly stable in the environment, able to survive in harsh conditions for about six weeks [1,3].

Puppies without or inadequate titer of maternal-derived antibody (MDA) to this virus are prone to be infected [4]. In any circumstances, healthy dogs or infected dogs with hemagglutination inhibition (HI) titer of 320 or higher are suggested to be protected from virus replication [4]. With sufficient protective immunity, the feces of dogs challenged with CPV-2 remained undetectable of CPV DNA by real-time PCR if they had the HI titer level of 320 of MDA [4]. In case of a low HI titer, such as 160 and lower, active CPV can be demonstrated by utilizing a reliable, sensitive method such as real-time PCR to detect the presence of the viral genome [5-7].

**CANINE PARVOVIRUS AND ITS GENOMIC ASPECTS**

Canine parvovirus is a DNA virus and a member of the Parvoviridae family. This virus family consists of two subfamilies, Parvovirinae and Densovirinae. According to available information, Parvovirinae viruses are able to infect vertebrate hosts, while the latter subfamily can only infect insects. Currently, the Parvovirinae subfamily is comprised of eight genera, namely Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependonavirus, Erythroparvovirus, Protoparvovirus, and Tetraparvovirus [8]. The unique viruses in the genus Parvovirus are canine parvovirus (CPV) and feline panleukopenia virus (FPV), which are now well characterized [9].

Parvoviruses are non-enveloped viruses, single-stranded DNA approximately 25 nm in diameter. The parvovirus genome consists of approximately 5,323 nucleotides [9]. The full length of the viral genome contains two large open reading frames (ORFs). The first ORF is encoded for two nonstructural proteins (NS1 and NS2). The second ORF is built up of three structural proteins or capsid proteins (VP1, VP2, and VP3) through an alternative splicing of the same mRNAs [3]. The parvovirus capsid is icosahedral and consists mainly of 60 subunits of the polypeptides VP1 and VP2 [3,10]. VP3 is a product of VP2 from virus–host interactions when cleaved by proteolytic enzymes [9].

The global distribution of contemporary CPV is thought to be divergent from canine minute virus (CnMV) [9]. This virus, formerly known as canine parvovirus type 1 (CPV-1), has caused neonatal death in puppies [8]. It has been documented that CPV-1 emerged from feline parvovirus (FPV) and has been circulating worldwide since the 1970s [11]. A few years later, the first CPV-2 isolates were discovered [12]. CPV-2 causes severe hemorrhagic gastroenteritis in dogs, as well as myocarditis [11].

The evolution of the original CPV-2 was established in the mid-1980s [6]. Since that time, the original CPV-2 (simply called “CPV-2”) has been completely replaced by alternative variants, the first two of which are known as CPV-2a and CPV-2b [8]. This phenomenon suggests that CPV-2 has evolved a highly fit conformation [11]. In 2000, a new CPV subtype, CPV-2c, was detected, and it is now confirmed to be co-circulating with the other presenting subtypes [6].

At present, the antigens or subtypes of CPVs can be systematically identified using certain amino acid residues positioned within the VP2 protein. The antigenicity of CPVs, which determines the host range, is associated with VP2 capsid proteins. There is an antigenicity difference frequency of CPV-2a/2b detection [5,14,15]. The introduction of the CPV-2c strain was reported in 2001 [16]. CPV-2c is more widespread in South America [17,18], with the exception of Brazil where all circulating strains were characterized as CPV-2a or -2b [19,20]; few CPV-2c strains have been detected in India [21,22].

The VP2 protein is a favored location for mutations. This protein accounts for interactions with host transferrin receptor (TFR). Once alterations become permanent, the affinity to canine TFR could be significantly enhanced [11,23]. The favorability of mitotically active tissues, such as actively dividing intestinal cells and myocardiocytes in canine puppies, leads to the pathogenesis of CPV infection because the transferin receptors are highly expressed in those cells [20].

**CANINE TRANSFERRIN RECEPTOR (TFR), AND CPV RECEPTOR RECOGNITION**

The adaptation of receptor binding to canine transferrin receptor (TFR) type-1 has resulted in the extension of the host range of this virus, which for the newer antigenic types now includes both dogs and cats [11,25,26]. *Canine parvovirus has evolved its ability to bind the TFR type-1 by naturally occurring mutation of capsid protein (VP2) which conferred small local changes* [27]. The binding of the canine TFR plays a critical role in the canine parvoviral infection [28]. The TFR-capsid interaction depicted asymmetrical docking conformation [29,30]. It is postulated in vitro study that binding of viral capsid to canine TFR, required only a small number of TFR (one to five TFRs per capsid) in initiation of infection [29-31].

The alteration of hydrogen bonds and amino acid sub-
The CPV-2a, which has descended from CPV-2, has a broad host range of both domestic and wild carnivores [27]. The certain substitution of AAs on the exterior surface of VP2 (G299K/A300K) has demonstrated the efficient binding to the receptor and eventually allowed virus entry into both feline and canine cells [29]. Within the virus-binding region of canine TfR and closely related canids (e.g. coyotes, and gray wolves), the glycan molecules at glycosylation site has been discovered to influence the binding of the virus thus promote the infection of canine cells [28,34,35]. The presence of glycosylation site prevents binding of the virus thus promote the infection of canine TfR [24,29,31,32,38].

TfR (A300D) causing reduced binding and infectivity of canine host, whereas there were some evident dictated substitutions at position 300 of VP2 are likely to cause a great susceptibility of the host receptor in binding of the viral particles [27,32]. Adjacent to residue 300, replacement of Gly299 (G299) increased hydrogen bonds with aiding in the flexibility of capsid surface loop [27]. The single point mutation between two AA residues is unlikely to cause the major change in protein structure, but this phenomenon can enhance the thermodynamic properties or entropy of surrounding AAs [27,33] and further influence the interaction between viruses and TfR.

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The study of glycosylation found at TfRs of some carnivorous animals demonstrated the variation in patterns in which highly suggesting that the presence of glycan of domestic dog TfR forces the susceptibility to CPV [32]. The binding of AA residues near the 3-fold spike of VP2, especially residues 299 to 301, with TfR required the change of residue 300 (A300G) of virus to gain access to dog host, whereas there were some evident dictated that the mutation of residue 299 (G299E) or residue 300 (A300D) causing reduced binding and infectivity of canine TfR [24,29,31,32,38,40].

The emergence of new CPV-2 subtypes, specifically CPV-2c, has drawn attention to how well they fit to the canine host. CPV-2c is thought to have a less severe clinical course and a lower mortality rate, as observed in dogs infected with the Glu-426 mutant (currently known as CPV-2c) compared with outbreaks caused by CPV-2a and CPV-2b [16,41]. The alterations of amino acids (AA) in the VP2 protein at specific residues - asparagine to glutamic acid (N426E) and aspartic acid to glutamic acid (D426E) - as determined by the antigenicity of antibodies has resulted in different antigenic detection of monoclonal antibodies, as shown in many studies [6,11,42] (Fig. 1). From the perspective of humoral immunity, the monoclonal antibodies (mAbs) A4E3 and C1D1 could recognize this novel antigenic determinant occurring within the major antigenic sites of VP2 in the CPV-2 virus [43]. The other site in VP2 where alterations are often detected is the amino acid residue at the 440 position. The threonine to alanine mutation (T440A) is of interest since it is located in close proximity to the Glu426 residue in the major antigenic site, or epitope A, found on the three-fold spike of the CPV capsid protein [44].

Indeed, progressive changes inside the capsid protein (particularly VP2) have been occurring throughout the past three decades, and these changes are continuing; however, the transformation seems rather small [11]. Changes at the 426 amino acid residue may account for the spread of all CPV-2 subtypes. The new mutant VP2 structure may improve the biological properties of the virus, contributing to canine host adaptation, stabilization of the VP2 capsid structure, and enhanced antigenic escape from monoclonal antibodies [45].

Many studies have identified the changes in amino acid residues located within the full-length gene encoding the main capsid protein VP2. Here, we list some of these changes in amino acids (Table 1). It is currently unknown whether the various new mutants, such as S297A, D426E, or T265P, are also associated with altered receptor binding [11]. Analysis of the VP2 protein has shown that all CPV-2c strains and sequenced CPV-2a/2b strains retain the AA changes of the variants with respect to the original CPV-2 (M87L, I101T, A300G, D305Y, N375D) and display the S297A mutation typical of the recent CPV-2a/2b isolates [45]. The most relevant change was at T440A, which was encountered in one United States. type 2c strain (110/07-27), but also in two type 2a Italian strains (333/05 and 849

Table 1. Frequent amino acid mutation sites found in full-length VP2 genes of canine parvovirus compared with a reference strain (accession number M382465).

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This change was also present in some reference isolates, CPV-2a (northern India) and K022 (South Korea) and CPV-2b LCPV-V204 (Vietnam) [49]. In contrast, strain CPV-2b 311/04 (Italy) displayed a different change at the same position (T440N) and a further change in a nearby residue (D434V) [49].

The GH loop, situated between the βG and βH strands of the capsid surface (VP2) of the parvovirus, is formed by residues 267 to 498. This region contains sites with the most variability, influenced by its presentation on the capsid surface [10,45]. Aside from amino acids 297 and 440, changes detected in the GH loop of the VP2 protein of CPV-2c were R274K, F420L, N421Y, and V463I, of which the change at position 463 has been identified in a Korean CPV-2a isolate [49].

### PREVALENCE OF CANINE PARVOVIRUS TYPE 2 VARIANTS

A new antigenic variation, carrying the AA substitution Asp426Glu (D426E) in the major antigenic site of the viral capsid protein VP2, was first reported in 2001 by a group of Italian virologists [16,41]. This newest variant, designated CPV-2c, has already been detected in other European countries, as well as in Asia, Africa, and the Americas. Five AA changes are present in the VP2 capsid protein, while the antigenic differences observed in CPV-2b are the consequence of only one AA substitution (Asn426Asp; N426D) located in the major antigenic site of the capsid (epitope A) (Fig. 1). CPV-2, on the other hand, replicates poorly in feline cells in vitro; however, this finding was not in accordance with the results of an in vivo study of live cats [11]. CPV-2c displays a low genetic variability and shared amino acid changes already detected in recent CPV-2a/2b isolates [49].

The prevalence of CPV-2 subtypes has been intensively studied at only a few laboratories. The majority of reports were derived mainly from countries in Europe, America, and Asia, where there are suspected endemic areas. We have collected information from a public dataset, which is summarized in a phylogenetic tree (Fig. 2). The phylogenetic clusters accounting for the geographical distribution were created using selected full-length amino acid sequences of the VP2 capsid protein (full 584 AAs) from various types of viruses (FPV, CPV-2, CPV-2a, CPV-2b, and CPV-2c) with the corresponding GenBank accessions (Fig. 2).

CPV-2c has been identified by sequencing at the major antigenic variation within the VP2 capsid. CPV-2c is the dominant and most prevalent type of CPV-2 that has been spreading in Argentina [44,45,51], Ecuador [52], Uruguay [53], and Rio de Janeiro, Brazil [54]. In Colombia, the presence of the antigenic variants CPV-2a/2b with a possible new CPV-2a are currently circulating [55].

In the United States and Mexico, CPV-2 types have been documented as CPV-2, CPV-2b, as well as CPV-2c [1,6,53-55]. In Asia, CPV-2a and 2b are currently predominant in Japan [56,57], Taiwan [58,59], and South Korea [60,61]. In Vietnam, CPV-2c [42] is often used as a reference strain for the naturally occurring Vietnamese HNI-4-1 prototype [45]. In Thailand, dog populations are often crowded into urban and metropolitan areas such as Bangkok and Chiang Mai. It was previously reported that CPV-2, CPV-2a and CPV-2b were the pre-dominant types found in Bangkok and the vicinity [26].
More recently, we discovered that mixed types of CPV-2 - CPV-2a, CPV-2b, and CPV-2c - circulate in combination and cause mucoid or bloody diarrhea in dogs residing in the Chiang Mai municipality (unpublished data). There has also been a recent CPV-2c epidemic in Vientiane, Laos [64] and Taiwan [85]. In China’s capital city, Beijing, CPV-2a and 2b remain the dominant types of this virus [47,66-68], but more recently, the presence of CPV-2c has been confirmed in China [9,69-71]. In India and Iran, the dominant types in recent outbreaks were CPV-2a and -2b [21,72,73]. In 2016, CPV-2c was detected in northern and central India [74,75] and Iran [73].

In European countries, the co-circulation of CPV-2a, -2b, and -2c has been reported [76,77]. CPV-2c, causing gastroenteritis in dogs, has been detected in Spain [28], the United Kingdom (UK) [13], Italy [16,45], Germany, France [77], and Portugal [78,79]. In Albania [80], Hungary [81] and Turkey [82], CPV-2a and mutants are widely spread. In Australia, New Zealand, and Oceania, investigation of CPV in dogs has demonstrated that CPV-2a remains the predominant genetic variant, and has not been replaced by CPV-2b or CPV-2c, as in many other countries [83,84]. In Africa, infection of dogs with either CPV-2a or -2b was
reported in Nigeria, Mozambique, and South Africa \cite{85,86}, however, the CPV-2c strain was found to be present in Morocco \cite{87}. The numbers and percentages of detected CPV cases are summarized by region in Fig. 3.

**DIAGNOSTIC TOOLS FOR CPV ANTIGEN AND ANTIBODY DETECTION**

Canine parvovirus infection is the main viral etiology responsible for diarrhea in dogs. This disease may be differentially diagnosed by the time of clinical manifestation. Some viruses, such as morbillivirus, rotavirus, coronavirus, adenoviruses, reovirus, and norovirus, have contributed to causing diarrhea in dogs \cite{2}. The virus causing gastroenteritis should be confirmed by laboratory diagnosis in order to be distinguished from bacterial enteritis. CPV infection causing gastroenteritis is usually a major cause of illness in the early life of dogs. The feces, intestinal contents, or tissues from affected dogs, or even EDTA blood samples at the time of viremia, have proven to be useful in diagnosis \cite{25,43,88}. In recent years, many research groups have attempted to validate the use of various methods to detect the presence of CPV as a causative virus. Some are working on antibody-based tests for rapid detection of CPV antigens. Several studies have demonstrated that ELISA test kits are able to detect CPV antigens and have shown promising sensitivity and specificity toward new variants of CPV \cite{89,90}. A recent study compared commercial antibody-based tests for rapid detection of CPV antigens with other detection methods, i.e. PCR and immunoelectron microscopy (IEM). The results revealed the high specificity and low sensitivity of the antigen-detection kits \cite{91}. Molecular biology techniques, such as traditional polymerase chain reaction (PCR) and quantitative PCR (real-time PCR; qPCR) have been widely developed and used in the detection of CPV genetic materials from blood and fecal samples \cite{7,41,43,48}. Because of the sensitivity, specificity, and reproducibility of PCR and real-time PCR in detection of CPV DNA, this method might replace traditional methods such as virus isolation and antibody detection. Real-time PCR technology, using SYBR Green or minor groove binding (MGB) TaqMan probes for PCR assays, has many
advantages over conventional PCR [6,29]. The quantification of virus load is one example of the many applications for exploiting qPCR. Real-time PCR can also be performed with a large throughput to achieve an inexpensive and time-saving method [43].

As documented in Desario et al. [43], monoclonal anti-bodies (mAbs) can be raised against CPV-2 types in order to determine the hemagglutination inhibition (HI) titers in different viral variants. Abs clones A4E3, B4A2, C1D1, and B4E1 were unequally recognized major epitopes in original CPV type 2, CPV-2a, CPV-2b, and CPV-2c (formerly known as Glu-426 mutant) [43]. The clones A4E3 and C1D1 demonstrated superior reactivity with nearly all CPV variants [43]. The change in CPV-2c at amino acid residue 426 has shown differences in antigenic determination by the monoclonal antibodies 21C3 and 19D7 [6,29].

CANINE PARVOVIRUS VACCINES: FUTURE PERSPECTIVES

The original CPV-2-based vaccines have been proven to provide secure immunization against CPV-2c in Italian isolates [1]. The antibodies produced in dogs vaccinated with the latest CPV-2b field strain have shown more promising reactivity than the traditional CPV-2 strain vaccines [59]. The observed antigenic contrasts may drive the selection of CPV strains by producing differential immunogenic pressures among canine populations, which raises concerns about immunization efficiency [44]. The CPV-2c variation displayed a one-of-a-kind antigenic example, since it was inadequately recognized by specific antibodies of dogs inoculated with CPV-2, CPV-2a, and CPV-2b strains [44]. Several studies have demonstrated that CPV-2 vaccines can be used to promote CPV-2 antibodies against CPV variants [13-96]. A new modified live CPV vaccine (CPV-MLV) recently launched in the marketplace is designed from the CPV-2b variant, or can be genetically engineered to simulate the new CPV-2c variant [44,93]. In the serum neutralization (SN) test, titers to the antigenic variants CPV-2a, CPV-2b, and CPV-2c in immunized dogs were significantly lower than the homologous titers (raised to the original type) [11,44,95]. As previously observed by Pratelli et al. [95], the greatest antigenic differences were found in comparison with the original CPV-2, which is still largely utilized in vaccine manufacturing [44]. The SN immunologic method has been found to provide greater clarity and contrast than HI in cross-antigenic assessment of CPV-2 variability. The heterologous SN titers (versus CPV-2a and -2c) were significantly lower than the homologous SN titer (versus CPV-2b) [44]. After inoculation with the CPV-2c variant, lower SN titer was found in the sera of dogs and rabbits immunized with heterologous (CPV-2, -2a, and -2b) viruses. Moreover, these discoveries suggest the opportunity to develop modified live virus (MLV) vaccines from the CPV-2c strain [44]. Another study has also confirmed the notion that the current vaccine regimen, made from nucleotide sequences of CPV-2b, can provide antigenic cross-protection of dogs from the CPV-2c variant [97]. In the case of maternal antibodies, a specific titer might provide adequate resistance to disease caused by homologous CPV infection, but it may not fully protect puppies if they encounter a heterologous virus strain [11].

CONCLUSIONS

The continuous antigenic evolution of CPV-2 has caused the rapid displacement of older strains by a new antigenic variant strain, CPV-2c, which emerged in Italy in early 2000 [78] is spreading with high morbidity and mortality in the dog populations of Italy and neighboring countries. This progressive mutant is now replacing the antigenic variants CPV-2a and -2b [15]. Studies by authors from many countries, i.e., Italy, Portugal, Spain, France, United Kingdom, Belgium, Germany, Greece, Bulgaria, Tunisia, United States, Uruguay, Argentina, China, Taiwan, Vietnam, Thailand, Laos, and India, have demonstrated that the new variant 2c is a global threat for puppies. The substitution of CPV-2 by strains -2a and -2b, and then -2c, has been connected with expanded receptor-binding capacity to canine transferrin receptors. The mutation at Glu-426 confers the benefit of infectivity and, even more, influences clinical disease status. Continuing progress in vaccine development will determine whether the CPV vaccines currently in use will still provide full protection against the new variant or whether we should be prepared to replace those homologous vaccines with a novel technology, heterologous vaccines.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this paper.

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