Short communication

Prevalence and molecular epidemiology of Canine parvovirus 2 in diarrheic dogs in Colombia, South America: A possible new CPV-2a is emerging?

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A B S T R A C T

Since its identification in 1978, Canine parvovirus type 2 (CPV-2) has been considered a pathogen of great importance in the canine population because it causes severe enteritis with high mortality rates in pups. CPV-2 is a virus belonging to the family Paroviridae. Currently, there are three described antigenic variants (CPV-2a, CPV-2b, and CPV-2c). CPV-2c is an emerging virus that is seen as a global health hazard. The objective of this work was to confirm the presence of CPV-2 in dogs with acute gastroenteritis compatible with parvovirus and to molecularly characterize the antigenic variants circulating in two regions of Colombia. An analytical cross-sectional study was conducted with fecal samples collected from 71 dogs showing signs of acute diarrhea. The samples were processed and polymerase chain reaction (PCR), restriction fragment length polymorphism analysis (RFLP), sequencing and phylogenetic analysis was performed to detect and characterize CPV. A total of 70.42% of the individuals were confirmed positive for CPV-2. Statistically differences were found in the presentation of CPV-2 between the evaluated regions. Phylogenetic analyses confirmed the presence of the antigenic variants CPV-2a/2b. Moreover, we found the presence of two conserved substitutions Asn428Asp and Ala514Ser in the VP2 protein suggesting the presence of a possible new CPV-2a variant circulating in Colombia. This study demonstrates the importance of the CPV-2a/2b in the region and highlights the importance of performing molecular studies for the early detection of new antigenic variants of CPV-2.

1. Introduction

Canine parvovirus type 2 (CPV-2) is a globally distributed pathogen that is highly contagious in dogs. CPV-2 belongs to family Paroviridae, has an icosahedral morphology and its genetic material is single-stranded DNA. Evolutionary and genomic analyses have postulated that CPV originated from the feline panleukopenia virus and adapted to the canine species by various mutations after passing through wild animals, such as ferrets and foxes (Hueffer and Parrish, 2003).

CPV-2 features genomic substitution rates similar to those of RNA viruses (Shackelton et al., 2005), with values of approximately 10^{-4} substitutions per site per year (Truyen, 2006). Consequently, shortly after its appearance at the end of the 1970s, the original type of CPV-2 changed to antigenic variant CPV-2a through five/six amino acid mutations in the VP2 capsid protein (Parrish et al., 1991). Years later, a second variant (CPV-2b) was identified (Parrish et al., 1988), and in 2000, a third variant (CPV-2c) was reported in Italy (Buonavoglia et al., 2001). The three variants differ mainly in residue 426 of the VP2 protein of the viral capsid, for which CPV-2a, 2b and 2c have the amino acids Asn, Asp and Glu, respectively (Miranda and Thompson, 2016; Truyen, 2006).

Regarding the clinical picture in dogs caused by CPV-2c, the presence of more severe hemorrhagic enteritis has been observed.
in comparison to other antigenic variants, with higher mortality rates in pups and a greater presence of infections in adult dogs with complete and up-to-date vaccination schedules (Decaro et al., 2008).

In Colombia, studies of CPV-2 began in the 1980s with the only isolations reported to date (Galvis et al., 1984). However, currently there is a large gap in the knowledge about which antigenic variants are circulating in the country and their frequencies. Therefore, the objective of the present study was to perform a characterization of the presentation of CPV-2 in two regions of the country (Antioquia and Santander) and to molecularly confirm the antigenic variants circulating in those regions.

2. Materials and methods

2.1. Ethical considerations

The Ethics Committee for Animal Experimentation of the University of Antioquia and the Cooperative University of Colombia in Bucaramanga approved the realization of this study. Dog owners signed informed consent forms approved by the ethics committee.

2.2. Type of study, target population and sampling

An analytical cross-sectional study was conducted with convenience sampling in dogs brought to various hospitals and veterinary clinics in the Valley of Aburrá (Antioquia) and Bucaramanga (Santander), Colombia (Fig. 1), with clinical signs of hemorrhagic diarrhea and a presumptive diagnosis of parvovirus. Approximately one gram of feces were collected from those patients and stored at −80 °C until processing.

2.3. DNA extraction

The QIAamp DNA Stool kit (Qiagen®, Hilden, Germany) was used to extract viral DNA in the stool samples by following the manufacturer’s recommendations. All DNA samples were quantified using a NanoDrop 2000 (Thermo Fisher Scientific®, Waltham, MA, USA).

2.4. Amplification of the VP2 gene fragment and restriction enzyme analysis

To confirm the presence of CPV-2, a highly conserved portion of the parvovirus VP2 gene was amplified and enzymatically digested following the protocol described by Buonavoglia et al. (2001). The DreamTaq™ Green PCR Master Mix (2×) kit was used (Thermo Fisher Scientific, Waltham, MA, USA) with the primers 555for and 555rev, which amplified a 583 base pair (bp) fragment. As positive controls, DNA from commercial vaccines with modified live CPV-2 strains were used. Molecular biology grade water and DNA extracted from the feces of a negative dog were used as negative controls. The samples positive for CPV-2 were enzymatically digested with the restriction enzyme Mbol (Thermo Fisher Scientific®, Waltham, MA, USA). Plasmid DNA for CPV-2c was used as a positive control (kindly donated by Dr. Yanina Panzera of the Universidad de la República, Uruguay). Finally, after amplification of the VP2 gene fragment and enzyme restriction, the corresponding electrophoresis was performed in 1.5% agarose gels. For each sample, 5 μl of the final solution was used with a molecular weight marker (GeneRuler™ 100 bp DNA Plus Ladder, Thermo Scientific®). Gels were stained using EZ- VISION™ dye (Amresco® Solon, OH, USA) and viewed by trans-illumination with UV light on a transilluminator (Mini BIS Pro, DNR Bio Imaging

Fig. 1. Geographical location of sampling sites in Colombia. The pink area denotes the Antioquia and Santander provinces. The map was created using the DIVA – GIS software version 7.5.0 for Windows™. See the main text for References.
Systems, Jerusalem, Israel) using the image acquisition software ImageLab™ (Bio-Rad ™, Hercules, CA, USA).

2.5. Sequencing and sequence analysis

The amplicons of the VP2 gene fragment were sent to Macrogen Inc. (Macrogen Inc., Seoul, Korea) for purification and sequencing in duplicate using the primers 555for. This region covers critical residues of the capsid protein that are considered important for the antigenic properties of CPV-2. The chromatogram sequencing files were inspected using Chromas™ v2.6. The best-fit model of nucleotide substitution was selected (GTR + G + I) and Maximum-likelihood trees were inferred. All Phylogenetic analysis were performed by using MEGA™ 7.0 for Windows ™.

Selected CPV-2a and CPV-2b nucleotide fragment sequences amplified from Antioquia and Bucaramanga in this study were submitted to the GenBank accession numbers: KX89191-KX891212.

2.6. Data analysis

A descriptive analysis of the collected data was performed. The qualitative and quantitative variables were correlated and presented in tables and charts. A bivariate analysis was conducted to compare the status of positivity in the assessed regions using the Chi-square test or Fisher’s exact test. The tests were conducted using Prism 7.01™ for Windows ™ (GraphPad Software, San Diego, CA).

3. Results

A total of 71 individuals were sampled (39 belonging to the department of Antioquia and 32 belonging to Santander). All individuals were admitted to the clinics with acute gastroenteritis clinically compatible with parvovirus. In all cases, the patients were brought to the consultation with clinical signs of extensive and watery diarrhea, and 82% had bloody diarrhea.

By using PCR, it was possible to confirm a prevalence for CPV-2 in the study area of 70.42% (confidence interval, 95% CI: 59.81%–81.04%). The prevalence was higher in the department of Santander 84.85% (95% CI: 72.62%–97.08%) compared with the prevalence found in the department of Antioquia of 56.41% (95% CI: 40.85%–71.97%). Additionally, significant differences were found in the presentation of the infection between the two regions (p = 0.0047).

The gender distribution showed a population without significant differences in the total percentages (48% males and 52% females), and no difference were found in gender distribution between the two regions evaluated.

Noteworthy in this study, parvovirus infection was confirmed in animals over one year of age in the department of Antioquia. Fig. 2a presents the percentage distribution by age range of positive individuals in each of the evaluated regions. Regarding the breeds of animals confirmed as positive for CPV-2, the most affected were crossbreed, followed by the Pitbull, Schnauzer, Poodle and Pinscher breeds (Fig. 2b).

Following the protocol described by Buonavoglia et al. (2001) to differentiate CPV-2a/2b variants from emerging CPV-2c, all samples amplified in this study corresponded to CPV-2a/2b variants and the presence of CPV-2c was not identified in either of the two regions evaluated.

A total of 22 positive samples (9 from Antioquia and 13 from Santander) were sequenced. The results confirmed the presence of CPV-2a in both regions (66.6% in Antioquia and 100% in Santander), and the presence of CPV-2b only in 33.3 samples from the department of Antioquia. Analysis of the partial segment of VP2 showed a low level of variation between Colombian CPV-2 variants (<1%). The change Asp426Glu, which is unique to CPV-2c variants, was not observed in any of the sequences evaluated in this study. The presence of the Thr440Ala substitution, previously reported in CPV-2c or the Thr440Ser substitution reported in Ecuadorian CPV-2a strains were also not observed. We found the presence of two non-synonymous substitution (Table 1), the first one at position Asn428Asp in two CPV-2a samples belonging to the department of Antioquia and

![Table 1](image)

Amino acid variation in VP2 protein from Colombian CPV-2a and CV-2b. The amino acid changes observed in Colombian CPV-2 samples are indicated in bold.
the second, Ala514Ser in 79% of the CPV-2a sequenced samples from both evaluated regions.

A maximum-likelihood tree was constructed using Colombian (from Antioquia and Santander) and Genbank sequences. The CPV-2a from Colombia are located in two different positions in the phylogenetic tree. A major group with the change Ala514Ser formed a monophyletic group and a second one that clusters with the European and North American classic CPV-2a strains. The CPV-2b from Colombia (Only from Antioquia department) cluster with North American CPV-2b strains (Fig. 3).

4. Discussion

Despite long existence of CPV-2 strains in Colombia, few studies have described the disease in the country, and studies characterizing molecularly circulating variants, are lacking. This is the first study that molecularly characterizes antigenic variants of CPV-2 in the departments of Antioquia and Santander. The results demonstrate a high prevalence of CPV and confirm the presence of CPV-2a/2b causing disease even in dogs older than 6 months (Fig. 2a) and the presence of a possible new CPV-2a variant (Fig. 3).

Our results showed that the vast majority of acute gastroenteritis cases in the two evaluated regions (70.42%) were related to CPV-2a/2b infections. A small percentage of the cases may be associated with other infectious and non-infectious causes. Although CPV-2 infection has been described since the 1970s (Appel et al., 1979) and in our country since the 1980s (Galvis et al., 1984), our results show that CPV-2 remains a viral infection of great importance.

Regarding the general characteristics found in individuals positive for CPV-2a/2b, such as the breed, it is well known that breeds such as Rottweilers and German Shepherds and other large breeds exhibit more susceptible behavior and develop more severe clinical responses to CPV-2 infection (Sellon, 2005). Although our results showed a greater prevalence in mixed breed animals, these results should be interpreted carefully because the sampling did not allow us to demonstrate or suggest any association with breed but instead were a reflection of trends in the possession of pets.

It is clear that our results do not fully rule out the circulation of CPV-2c because its circulation could be low and it may not have been present in the collected samples. Similarly, the proximity and canine traffic with countries in which CPV-2c has been reported, such as Brazil (Streck et al., 2009), and Ecuador (Aldaz et al., 2013), suggest that the virus could be circulating in other regions of the country and could be detected in studies with a higher sample number.

Various studies have reported that the clinical signs produced by CPV-2c are similar and equally as severe as the cases caused by new variants of CPV-2a (Aldaz et al., 2013; Decaro et al., 2005). Following its introduction into a new region, CPV-2c is capable of relatively quickly replace CPV-2a/2b, as has been demonstrated in different areas of Europe (Decaro et al., 2011). However, although initial studies of CPV-2c in South America showed a prevalence between 91 and 100% (Calderón et al., 2009; Pérez et al., 2012) most recent studies demonstrated a shift of the CPV-2c prevalence and the re-introduction of new CPV-2a variants (Calderón et al., 2015; Pérez et al., 2012). Because our study was cross-sectional,
Further studies of the incidence and phylogenetic characterization are required to understand whether there is a shift of variants or an established classical variant of CPV-2a/2b in our canine population. By now, our results have shown CPV-2a and CPV-2b cocirculation, in the Antioquia region.

It has been established that dogs under six months has higher risk of CPV-2 infection (Miranda et al., 2015) and that adult dogs are thought to be more resistant to CPV infection due to the age-reduced susceptibility and presence of specific immunity induced by vaccination or previous infections (Decaro and Buonavoglia, 2012). Our confirmation of CPV-2a and CPV-2b in patients over six months or even more than a year of age (Fig. 2a), could be due to the presence of new CPV variants in the field (Decaro and Buonavoglia, 2012; Decaro et al., 2008) and in some cases to failures in the protection of vaccination (Truyen, 2006).

This last context must be carefully taken into account because although vaccination against CPV-2a/2b using modified live vaccines was reported to protect dogs for up to 9 years (Lister et al., 2012), there is a strong disagreement between the studies addressing the protection of these traditional vaccines against antigenic variants of CPV-2c (Hernández-Blanco and Catala-López, 2015; Miranda and Thompson, 2016) and in some cases against emerging variants of CPV-2a/2b. (Maya et al., 2013; Pérez et al., 2012).

In our study, it was not possible to confirm the immunization status of the included individuals and to determine how many doses were administered to the vaccinated individuals (data not shown). Therefore, it is not possible to speculate regarding possible vaccine failures and cannot be rule out that the infections in older dogs may be related with an incomplete vaccination schedule. We assume that strengthening vaccination with modified live vaccines can reduce the incidence of CPV-2a/2b in the region.

Although CPV-2c was not detected in the analyzed samples, the present results have shown the circulation of different CPV-2 variants in the region. The amino acid changes Asn428Asp and Ala514Ser found in the CPV-2a positive samples have not been reported previously in dogs. Recently, a change Asn428His were reported in CPV-2c samples from Brazilian dogs (Fontana et al., 2013) and a change (Ala514Thr) was reported in CPV-2a viruses isolated from cats (Mukhopadhyay et al., 2016).

As has been reported recently for CPV from Ecuador (Aldaz et al., 2013), our findings suggest that the Colombian CPV-2a population includes strains from different origins and has marks of local differentiation through the acquisition of specific amino acid changes (Fig. 3). This substitutions caused by transition in the first position of the codon are located at important VP2 domains; although the functional impact of these mutations has yet to be determined. Further large-scale studies are needed to clarify the incidence and biologic function of those amino acid changes in the VP2 coat protein.

Similar to other members of family Paroviridae, CPV-2a has a high substitution rate that has already shown that it is capable of generating viral variants with changes that justify the designation of “new CPV-2a/2b” (Ohshima et al., 2008). The CPV-2a/2b capsid proteins can even adopt different antigenic structures to modify the host range and allow the virus to adapt to wildlife species (Allison et al., 2012). Taken together, our results encourage...
continuing evaluating the role of new substitutions in Colombian CPV-2a in order to know if our strains could be denoted as a possible "new CPV-2a".

5. Conclusion

The recent emergence and re-emergence of CPV-2 strains in different countries as well as in Colombia showed that CPV-2a/2b remains as an important agent for the canine population, and it is necessary to continue studying molecular epidemiology and evolution of CPV for the early detection of new antigenic variants in the region.

Conflict of interest

The authors of this work do not have any personal or financial conflicts or biases that can inappropriately influence the contents of the document.

Authors’ contributions

JRS conceived the study, and YDQ, MEZ, JTS and JRS were involved in all other aspects of the study, including data collection, data analysis, drafting and editing the paper. All authors read and approved the final manuscript.

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