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Original

Identification of canine parvovirus type 2C in puppies of Nicaragua

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ABSTRACT

Objetive. To identify genotypes of canine parvovirus circulating in puppies in two municipalities of Nicaragua. **Materials and methods.** Rectal swab samples from 45 puppies less under 6 months of age were collected and processed for presence of parvovirus bur conventional PCR technique. Puppies might or not have been vaccinated and with or without parvovirus infection symptoms. Two commercially available parvovirus vaccines in Nicaragua (vaccine nº1 and vaccine nº2) were also analyzed by conventional Polymerase Chain Reaction (PCR) resulting in a product of ≈630 bp of the VP2 gene. In addition, Sanger sequences of four randomly chosen field samples and both vaccine strains were obtained. **Results**. 28.9% (13/45) of the analyzed samples were positive by PCR, for CPV VP2 gene. No statistically significant differences (p≥0.05) were obtained in PCR detection between dogs with or without vaccination history. The four sequenced field samples were identified as CPV-2C genotype while both vaccine strains were identified as CPV-2A genotype. **Conclusions**. The aligned sequences showed high evolutionary divergence of filed strains with respect to vaccines strains, leading us to reconsider the efficacy of the analyzed vaccines commercially available in Nicaragua nowadays.

Keywords: Vaccine, PCR, genotype, parvoviruses, diarrhea (*Source: DeCS*).

RESUMEN

Objetivo. Identificar los genotipos de parvovirus canino-circulantes en cachorros en dos municipios de Nicaragua. **Materiales y métodos**. Se recolectaron muestras por hisopado rectal de 45 cachorros con y sin antecedentes de vacunación, menores de 6 meses de edad, con y sin sintomatología compatible con parvovirosis. Las muestras y dos de las vacunas que se comercializan en Nicaragua (vacuna nº1 y vacuna nº2) fueron analizadas por Reacción en Cadena de la Polimerasa (PCR) convencional para un producto de ≈630 pb del gen VP2. Además, el producto directo del PCR se secuenciaron en sentido reverso cuatro muestras de campo elegidas aleatoriamente y las dos cepas vacunales. **Resultados.** El 28.9% (13/45) de las muestras analizadas fueron positivas en PCR. No

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se encontraron diferencias significativas en la detección por PCR del fragmento de VP2, respecto al estado de vacunación de los animales (p≥0.05). Las cuatro muestras de campo secuenciadas fueron identificadas como genotipo CPV-2C y las dos cepas vacunales se identificaron como genotipo CPV-2A. **Conclusiones.** la inferencia evolutiva de las secuencias alineadas de cepas vacunales mostró alta divergencia evolutiva respecto a las cepas de campo, este hallazgo lleva a replantear el tema sobre la eficacia de las vacunas analizadas en este trabajo y que son aplicadas en Nicaragua.

Palabras clave: Vacuna, PCR, genotipo, parvovirosis, diarrea (*Fuente: DeCS*).

INTRODUCTION

Canine parvovirus type 2 (PVC-2), is the most important agent of deaths in puppies around the world, since its appearance in 1978 (1). This virus has been associated with a gastroenteric syndrome disease consisting of sudden onset, vomiting and severe hemorrhagic diarrhea (2).

CPV-2 appeared as a mutation of a virus similar to feline panleukopenia (FPLV), capable of infecting several canine species. Despite having commercially available vaccines, parvovirus is still the most important etiologic agent of viral gastroenteritis in puppies. The original strain called canine parvovirus type 2 (CPV-2) has undergone genetic alterations, leading to the appearance of new variants (3) since its emergence. In 1980, the CPV-2 mutation resulted in the CPV-2A variant. In 1984, another variant emerged designated as CPV-2B associated with a mutation, which resulted in a change of the amino acid asparagine for aspartate at codon position 426 of the VP-2 gene (4 ,5). In the year 2000 a third variant known as CPV-2C was detected in Italy for the first time, with another mutation in the same position, (glutamate). This new variant has spread rapidly in the canine population in several regions (5). In South America, more virulent CPV-2C strains have been diagnosed than their predecessors CPV-2A and CPV-2B (6–8). In Mexico, researchers reported that variant C is predominant (9,10). In Central America, molecular studies to identify CPV variants are inexistent. Therefore, it is necessary to know the canine-circulating parvovirus variants in Nicaragua and the phylogenetic relationship with the vaccine strains that are currently marketed in the region.

The objective of the present study was to identify the genotypes of canine-circulating parvovirus in puppies in two municipalities of Nicaragua.

MATERIALS AND METHODS

Collection of samples and general information. A total of 45 Rectal swabs were collected individually from vaccinated or non-vaccinated puppies (n=30) of less than six months of age with symptoms compatible with parvovirus infection, and similarly from another group (n=15) with no disease symptoms. Data concerning general information and disease history of each puppy was collected at the sampling time.

Viral DNA extraction. For DNA extraction, swabs were placed in 1.5 ml vials with 200 µl of nuclease-free water. A 20 seconds vortex were applied before proceeding to the extraction of DNA using (QIAamp DNA Mini Kit QIAGEN) following the manufacturer protocol. In the last step, the DNA was eluted in 200 µl of nuclease-free water. DNA was also obtained from two commercial vaccines in Nicaragua, these were identified as vaccine No. 1 and vaccine No. 2. Nuclease free water was used as negative control For DNA extraction.

VP2 gene amplification. Conventional PCR technique was applied using the pair of primers Hfor: (5'-CAGGTGATGAATTTGCTACA-3' and Hrev: (5'-CATTTGGATAAACTGGTGGT-3'), as previously described (5). A 630 bp PCR product flanking a segment of the VP2 gene of the canine parvovirus was aimed. The final reaction volume was 50 µl having 25 µl of 2X Master Mix (Promega, USA), 12 µl of nuclease-free water, 4 µl of the Hfor primer (1x103 nM), 4 µl of Hrev primer (1x103 nM) and 5 µl of DNA sample. The PCR reaction was performed with the Applied Biosystem 2720 Thermocycler, raising the temperature to 94°C for 10 minutes, followed by 40 cycles (95°C for 50 seconds, 55°C for 1 minute, 72°C for 1 minute) and a final extension for 7 minutes at 72°C. DNA extracted from vaccines No. 1 and No. 2 were used as positive controls. The kit Elution

buffer was used as a negative control sample. Ten microliters of PCR Products were loaded per well in and run through a 1.3% agarose gel horizontal electrophoresis system and visualized through a UV light transilluminator after being stained with ethidium bromide.

VP2 Sequencing. Sanger sequencing was performed using the reverse primer (Hrev) for four randomly chosen field samples plus the 2 vaccines (vaccine No. 1 and No. 2) at the Research Center in Cellular and Molecular Biology (CIBCM), University of Costa Rica (UCR)

Phylogenetic Analysis. The sequence of each strain was aligned by ClustalW 1.6. The history of evolution was inferred using the Neighbor-Joining method, while the evolutionary divergences were calculated using the maximum likelihood method and were presented as base units of substitutions per site. The bootstrap test (500 repetitions) was performed as Statistic of Significance and is being shown as the percentage of occurrence of each taxon in the phylogenetic tree (11). The analysis was carried out with the help of the Molecular Evolutionary Genetics Analysis Version 6 (MEGA 6) software (12). Genotype identification was determined based on the amino acid corresponding to codon 426 of the VP2 gene (5). The sequences were sent to the National Center for Biotechnology Information (NCBI) using Banklt (13), obtaining access numbers MN517818, MN517819, MN541177, MN541178 for the samples, and numbers MN541179 and MN541180 were assigned to vaccine No. 1 and vaccine No. 2, respectively.

Statistical analysis. Results are presented in percentages and absolute numbers and the search for association from categorical variables, the exact Fisher and Student T test were applied for age in months.

Ethical considerations. Swabs were taken from the puppy without causing any significant alteration with the owner consent.

RESULTS

In the present study, 28.9% (13/45) of the puppies were PCR positive for canine parvovirus with a 66.7% (30/45) of them presenting diarrhea. Thirteen out of thirty puppies (43.3%) were positive for the VP2 gene with diarrhea and 100% (15/15) of dogs without diarrhea were negative for parvovirus, (p<0.01), (Table 1).

Table 1. Association of the PCR with variables of the sampled puppies.

Variables		PCR Result		Total	Sig
		Positive	Negative		
Diarrhea	Yes	13	17	30	
	No	0	15	15	p<0.01[a]
	Total	13	32	45	
Hematochezia	Yes	11	7	18	
	No	2	25	17	p<0.01 [a]
	Total	13	32	45	
Previous vaccination*	Yes	7	11	18	
	No	4	15	19	p≥0.05 [a]
	Total	11	26	37	
Age in months		2.70	5.05		p<0.01 [b]

Sig=Significance; *Data from only 37 puppies;
a: Fisher test; b: T Student test

The samples that were PCR positive for the Vp2 gene, were from animals with an average age of 2.7 months, whereas PCR parvovirus negative animals, had an average age of 5.05 months (p<0.01) (Table 1).

Of the 45 dogs sampled, only 37 obtained some information related to vaccination against parvovirus. Of these, 18 owners claimed to have vaccinated their puppies, and 19 received no vaccination. However, 38.9% (7/18) of the vaccinated dogs were positive, and 21.0% (4/19) of the unvaccinated were infected with parvovirus (p≥0.05) (Table 1).

Based on the nucleotide sequence of the codon at position 426 of the VP2 gene, the four field samples sequenced were identified as CPV-2C genotype and the two vaccine strains were identified as CPV-2A genotype (Figure 1).

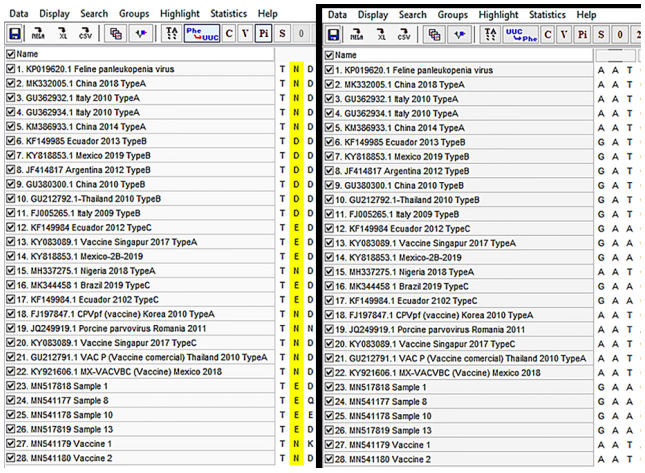

Figure 1. CPV genotypes according to codon 426 of VP2. On the left are the amino acids Asparagine (N) for genotype A, Aspartate (D) for genotype B and Glutamate (E) for genotype C, on the right the nucleotide sequence.

The evolutionary inference of the nucleotide sequences of aligned field strains showed divergence between 7.7% and 8.9% with respect to vaccine No. 1 and between 18.0% and 19.0% with respect to vaccine No. 2. The divergence between the two vaccines was 17.7% and between the four samples ranged between 0.5% and 5.4%. Among the field samples with respect to the CPV sequences found in other countries (GenBank) it was from 3.2% to 6.3%, while with respect to the FPLV a divergence between 5.7% and 6.5% was observed (Figure 2).

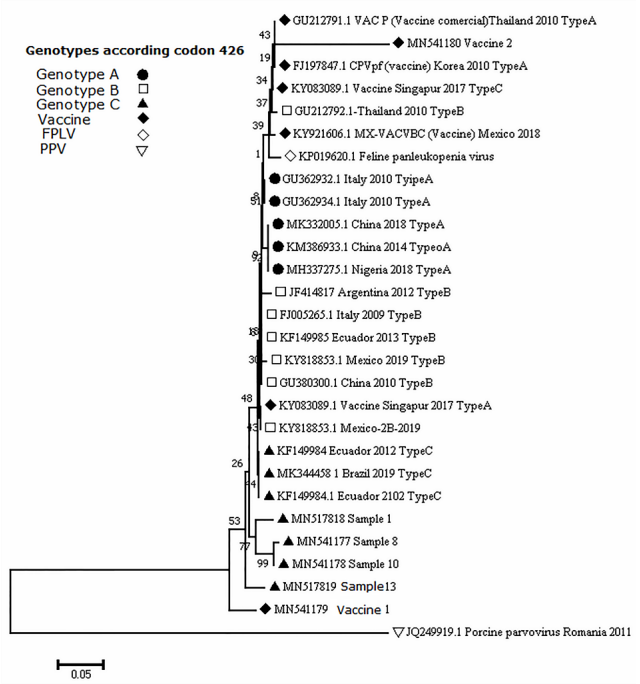

Figure 2. Phylogenetic tree based on the nucleotide sequence of the VP2 gene of canine parvovirus, the four field samples from Nicaragua (MN517818, MN541177, MN541178, MN517819) and the two vaccines (MN541179, MN541180) are presented and compared with sequences obtained from the GenBank for canine parvovirus, Feline Panleucopenia Virus (FPLV) and Porcine Parvovirus (PPV).

The evolutionary history was inferred using the Neighbor-Joining method, the percentage of replicated trees in which the associated taxa grouped in the bootstrap test (1000 repetitions) are shown next to the branches. The evolutionary distancing factors were calculated using the Maximum Composite Likelihood method. The analysis involved 28 nucleotide sequences with a total of 383 positions in the final data set.

DISCUSSION

The genotypic variants present in the four sequenced samples were identified as CPV-2C genotype according to codon 426 of the VP2 gene. This result is similar to reported from other countries in which CPV-2C is the most frequent virus variant. A study carried out in Argentina by Calderón et al (7), describes 91% CPV-2C variant between 2003 and 2010, being the most prevalent genotype since 2008. CPV2A and CPV2B accounted for only 3.6% and 5.4% of the population, respectively. It is worth mentioning that having found CPV-2C in this study does not exclude the presence of the other two genotypic variants.

A clear association was observed between the diarrhea onset and parvovirus PCR positive samples, given that in all dogs with CPV had diarrhea but none without diarrhea had. This result clearly indicates that infected animals would present diarrhea which is in accordance to what is reported by Zhao et al. (14), who found that all experimentally infected dogs had symptoms of diarrhea four days post infection.

In the analysis of the association parvovirosis with the presence of hematochezia, it was observed that 61.1% (11/18) of dogs with hematochezia showed amplification of the portion of the VP2 gene, and only 7.2% (2/27) did not. These results coincide with what Mauro et. al. (15), who reported 7 out of 10 cases positive for the virus are symptomatic, but also indicates that other etiologies should be considered as causative agents in our study.

Regarding the age as a risk factor, it was observed that the average age of positive dogs was less than the age of the negative ones. In the study conducted by Nandi et al (1) the highest infection rates were observed in puppies older than 1.5 months. As with other canine infectious diseases, puppies that are born from immunized bitches are protected during the first week due to colostrum consumption rich in antibodies, but then with time, titers decrease would reach a critical period in which the maternal antibodies become insufficient to protect the descendant (16).

The evolutionary divergence observed in the vaccines with respect to the field strains, raises doubts over the efficacy of the two vaccines from this study. Our data clearly indicates that

vaccination is not protecting the puppies, given that the diarrhea symptoms and hematochezia with CPV PCR positive dogs were observed despite vaccination. As mentioned in the study conducted by Ohshima et al (17), antibodies produced by dogs vaccinated with a different vaccine strain CPV-2B induced better protection against recent parvovirus field isolates compared to those vaccinated with conventional CPV- 2. The mutations in the VP2 protein can lead to vaccination failures. Vaccine sequence No. 2 showed a high homology with that described for vaccines analyzed in Korea by Yoon et al (18), as well as in Mexico (ID KY921606). while vaccine No.1 showed greater homology with the analyzed field samples. However, to determine the effectiveness of a given vaccine, a specific serologic evaluation for neutralizing antibodies against newly emerging variants of the virus must be conducted.

Conflict of interests

All authors declare that there is no conflict of interest for the publication of this manuscript.

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