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CORELLATION BETWEEN THE REAL TIME PCR METHOD USED IN CANINE PARVOVIRUS DIAGNOSTIC AND CLINICAL MANIFESTATION

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Abstract

Canine parvovirus infection is one of the most common diseases of puppies. Dogs are affected by two viruses of the Parvoviridae family: CPV-1 (Bocavirus genus), thought to have minimal pathogenic potential, but having being associated with different disorder in all age category dogs, and CPV-2 (Parvovirus genus), known as Canine Parvovirus, the true parvovirus. Despite the extensive use of vaccination, the prevalence of infection registers an oscillating dynamic, the virus is evolving and the requirement for the confirmation diagnosis is being continuous. The aim of the research was to identify the possibly correlations of the clinical manifestations with the results of CPV-2 detection, using the real time PCR. Fourteen dogs with clinical manifestations associated with the suspicion of canine parvovirus, has been tested on feces samples by real time PCR: 13 were positive (92.85 %) to the real-time PCR test. The registered positiveness has been associated with different Ct values ranging from 6.82 to 35. One sample (7.14%) was negative. In this study, despite the variation range of Ct`s, the clinical pattern registered did not directly relate with the virus amount, rather with the age.

Key words*: canine parvovirus, real time PCR.*

INTRODUCTION

Canine parvovirus is one of the most common and yet underestimated diseases of canine youth. The diagnosis of canine parvovirus is a challenge for clinicians in terms of the ubiquity of the virus and rapid diagnostic methods with low sensitivity.

Dogs are affected by two viruses of the Genus Parvovirus: CPV-1, whose pathogenicity is not well known, being associated with "fading puppy" syndrome, causes lethargy, diarrhea, difficulty breathing and sudden death in puppies of 1-3 days. CPV-2 is known as Canine Parvovirus (Barr and Bowman, 2012).

CPV-2 first appeared in 1977 and is related to feline panleukopenia virus and mink viral enteritis virus. In 1980 and 1984, respectively, the variants CPV-2a and CPV-2b appeared, being characterized by different antigenic structures, increased pathogenicity and shorter incubation period compared to CPV-2 (Barr and Bowman, 2012; Carmichael, 2005).

In Italy, a third strain of canine parvovirus, strain CPV-2c, was isolated (Barr and Bowman, 2012).

The CPV-2c strain, known as Glu426, exhibits an amino acid substitution at position 426 from aspartic acid to glutamic acid, which altered the antigenic structure of the capsidal epitope. A (Greene, 2012). These changes in parvoviruses have been associated with genetics adaptation and changes in the B capsid epitope region, allowing the virus to replicate and spread effectively in susceptible dog populations, as well as the ability to infect cats (Greene, 2012).

CPV is a single-stranded, linear, icosahedral, non-enveloped DNA virus with dimensions between 18-26 nm in diameter (Greene, 2012; Gurpreet et al., 2015). The genome consists of 5323 base pairs (bp) encoding two structural proteins, VP-1 and VP-2, and two nonstructural proteins, NS-1 and NS-2. The gene for VP-1 is located between 2285-4537 (2253 bp) and for VP-2 between 2783-4537 (1755 bp) (Gurpreet et al., 2015). The parvoviral capsid contains approximately 10 copies of the VP1 protein and 60-70 copies of the VP2 protein. All the epitopes used to fix neutralizing antibodies are found in the VP2 protein. Mutations of this protein are responsible for the appearance of different antigenic strains (Gurpreet et al.,

2015; Langeveld et al., 1993; Turiso et al., 1991). Different antigenic variants of CPV-2 are prevalent in varying proportions in different countries. CPV-2b has been reported predominantly in Brazil, the USA, Japan, Switzerland and South Africa (Nandi and Kumar, 2010). CPV-2a is considered to be the most common antigenic type in France, Taiwan and Italy. In Spain and the United Kingdom, strains CPV-2a and CPV-2b are eventy distributed (Nandi and Kumar, 2010). CPV-2c was first identified in Italy, and later was reported in Vietnam, Spain, the United Kingdom, South and North America (Nandi and Kumar, 2010). CPV is highly contagious, and most infections occur fallowing the contact with the environment elements, contaminated with the virus shed by feces. In addition, humans, tools (equipment in veterinary units or grooming operations), insects and rodents can serve as vectors. Dogs can keep the virus in the fur for long periods of time. The incubation period for the original CPV-2 strain, under natural conditions was 7-14 days, and the experimental infection was 4-5 days. In the case of new CPV-2 strains (-2a, -2b and -2c), the incubation period in natural infection can be up to 4-6 days (Greene, 2012). Intense elimination of CPV-2 virus begins 3-4 days after infection, usually before obvious clinical signs appear. Following ELISA tests and virus isolation, CPV-2 appears to be massively excreted in the faeces for a maximum of 7-10 days post-inoculation. However, using PCR tests, CPV-2 strains (a, b, c) were detected in faeces for several weeks after infection (Greene, 2012). The age and the immune status of the animal influence the form and severity of the disease. After a short incubation period, the animals with enteric form, suddenly show vomiting and anorexia. Apathy and fever can also be observed. Diarrhea, most often hemorrhagic, occurs after about 48 hours and in severe cases can be expressed as frank hemorrhage. The feces have a characteristic foul odor. The general condition deteriorates rapidly due to dehydration and weight loss (Quinn et al., 2011).

The cardiac form of the disease, whose frequency is quite rare, is found in puppies younger than 6 weeks and manifests itself as acute heart failure. Some animals develop congestive heart failure, weeks or even months after infection (Greene, 2012).

The neurological form, with cerebral damage, and cerebellar hypoplasia are common in cats infected with their specific parvovirus. In contrast, in the case of canines, neurological disorders are most often associated with bleeding in the central nervous system due to disseminated intravascular coagulation, hypoglycemia, sepsis and hydro-electrolyte imbalances. The skin shape is characterized by erythema multiform, ulcers and blisters.

The aim of the present research was to identify the possibly correlations of the clinical pattern with the result of CPV-2 detection, using the real time PCR.

MATERIALS AND METHODS

Fourteen dogs with clinical signs associated with the suspicion of canine parvovirus, has been tested on feces samples by real time PCR. The test samples used were rectal swabs from animals that have shown clinical signs of canine parvovirus infection. The samples were kept at a temperature of 3-4°C and analyzed in no more than 48 hours. A preliminary stage of nucleic acid extraction is required to perform Real-time PCR.

The extraction of the DNA was made with the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany), according with the insert kit (Table 1). Protein cleavage for the appropriate expression of the genetic material from the biological sample is done using proteinase K, in VXL buffer medium. After mixing by pipetting/vortexing and incubation for 15 minutes at room temperature, the mixture formed is spin-centrifuged to collect the liquid.

The CBA buffer is added to the liquid obtained and after centrifugation, the supernatant obtained is transferred to purification columns. Purification colonies are tubes that contain a single silica membrane that captures genetic material. They contain 2 inner chambers separated by the silica membrane: the upper one contains the liquid to be analyzed, and the lower one contains the solution that crossed the silica membrane. After centrifuge, the solution obtained from crossing the membrane is removed and the AW1 buffer is added to the upper chamber.

The colonies are centrifuged and the previous procedure is repeated with the addition of AW2 buffer. In order to eluate the genetic material, the AVE buffer is introduced in the purification colonies, which has the role of releasing the nucleic acids that were captured at the level of the silica membrane. The colonies are incubated for 1 minute at room temperature, after which there are centrifuged for 1 minute (Table 1). The eluate thus obtained will be used in the amplification step of the specific parvovirus genetic fragment within the Real-time PCR test.

Table 1. Nucleic acid extraction protocol (QIAamp cador Pathogen Mini Kit)

REAGENT	µl/sample	No. samples	Total
Proteinase K	20 µl		
Sample	200 µl		
Buffer VXL	100 µl		
Pipetting/vortex mixing			
Incubate for 15 minutes at room temperature			
Spin centrifuge for liquid collection			
Buffer ACB	350 µl		
Pipetting/vortex mixing			
Spin centrifuge for liquid collection			
Transfer of samples to purification colonitis			
Centrifuge at 8000-10.000 rpm for 1 minute. Replacement manifold tube.			
Buffer AW 1	600 µl		
Centrifuge at 8000-10.000 rpm for 1 minute.			
Eluted remove			
Buffer AW2	600 µl		
Centrifuge at 8000-10.000 rpm for 1 minute. Replacement manifold tube.			
Eluted remove			
Centrifuge at maximum speed for 2 minutes. Introduction of colonitis into the collection tube.			
Buffer AVE	50 µl		
Incubation for 1 minute at room temperature.			
Centrifuge at maximum speed for 1 minute.			
Storage the elute at 1-2°C until the amplification step.			

The PCR amplification temperature protocol for the detection of the canine parvovirus was: 95°C 5 minutes, 40 cycles with 96°C for 5 seconds, 60°C for 5 seconds and 68°C for 3 seconds. The final elongation: 72°C for 1 minute.

The mix was made in a total volume of 25 µl from which 8 µl DNA template, 4.5 µl primers and probes specific for canine Parvovirus, 12.5 µl enzyme mix (Table 2).

Table 2. The reagents and the quantities of the reaction mix for detection of the canine Parvovirus genome

Reaction mix	
Reagents	**µl/sample**
Primeres and probes	4.5 µl
Enzyme mix	12.5µl
DNA template	8 µl
Total	25 µl

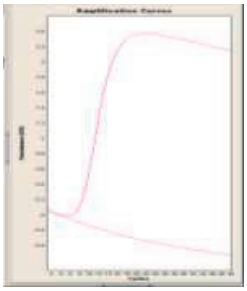
RESULTS AND DISCUSSIONS

From the fourteen tested samples, 13 were positive (92.85%) to the real-time PCR test. The registered positiveness has been associated with different Ct values ranging from 6.82 to 35. One sample (7.14%) was negative. In this study, over 50% of the animals examined were 16 weeks age or younger. Positive Ct value has been registered in samples belonging to dogs aged from 7 to 12 months old (28%), thus proving that canines over 6 months old may develop clinically manifest infection (Table 3).

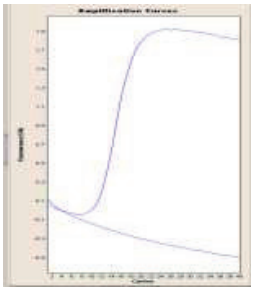
Table 3. The results of the tested samples

Sample No.	Age	Sex	Clinical features	RT-PCR result	Ct value
1	4 m	F	apathy, loss of appetite, diarrhea and vomiting	P	27.88
2	6 m	F	apathy, diarrhea and vomiting	P	32.31
3	2 m	F	apathy, loss of appetite, diarrhea and vomiting	N	-
4	3 m	M	lethargy, loss of appetite	P	18.22
5	3 m	M	apathy, loss of appetite, diarrhea	P	10.22
6	3 m	F	apathy,vomiting,diarrhea, exitus	P	6.82
7	1 y	F	apathy, loss of appetite,vomiting, hypersalivation.	P	32.60
8	8 m	M	apathy, anorexia, vomiting, bloody feces	P	13.09
9	3 m	F	apathy, anorexia, bloody feces	P	22.96
10	4 m	M	apathy, anorexia, vomiting, bloody feces	P	30.93
11	4 m	F	apathy, anorexia, fecal diarrhea	P	33.51
12	9 m	M	apathy, anorexia, vomiting, diarrhea	P	35
13	8 m	M	lethargy, anorexia, repeated vomiting, hemorrhagic diarrhea	P	35
14	4 m	F	apathy, vomiting, abdominal pain	P	30.28

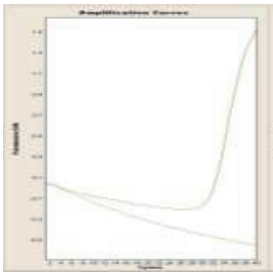
m = month; y = year; P = positive; N = negative; M = male; F = female



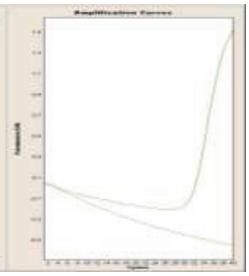
Sample 6-Ct. 6.82



Sample 5-Ct. 10.22



Sample 10-Ct. 30.93



Sample 14-Ct. 30.28

Figure 1. Images with different Ct values from Real-Time PCR; in each image are selected the tested sample with the melting curve register and the negative control with no melting curve

Real-Time PCR analysis of the tested samples revealed the following results: 28.57% of cases had a large amount of parvoviral DNA (Ct range 6.82-18.22) in feces, reflecting severe infection of the digestive tract with massive elimination of viral particles; 14.28% of the cases had an average amount of parvoviral DNA (Ct = 22.96-27.88) in feces reflecting the active infection of the digestive tract; 50% of cases had a small amount of parvoviral DNA (Ct = 30.28-35) in feces reflecting the reduced infection of the digestive tract with low elimination of viral particles; in one case (7.14%) the result was negative, there was no genetic material of parvovirus in the examined fecal swab. The 3-4 months old dogs register the highest share, their samples containing large and medium amounts of virus. However, high concentrations of canine parvoviral DNA can also be seen in individuals older than 6 months in the present study. The increased prevalence of parvovirus in dogs, under 6 months old, described in the literature is in accordance with the results of this study, which shows that over 50% of the tested dogs had canine parvovirus infection at 3-4 months of age. Clinically expressed infection in individuals older than 6 months is increasingly common, especially in the case of CPV-2c infection (Decaro et al., 2009; Decaro and Buonavoglia, 2012). Females are represented in greater numbers compared to males. This is in contradiction with studies from the literature that state either that there is no predisposition to sex, or that males are represented in a higher percentage compared to females (Behera et al., 2015; Khare et al., 2019; Mokhtari et al., 2017). Real-time PCR is a method of diagnosing canine parvovirus that has high specificity and sensitivity compared to other laboratory tests due to the fact that detect a small quantities of virus and has 100% of specificity because of the primers (Decaro et al., 2005; Desario et al., 2005). Low Ct values are mainly registered in the subjects of young age and, so, with an unfavorable survival prognosis. Clinical manifestations cannot be attributed to a category dictated by Ct, being quite uniform regardless of the amount of CPV eliminated in the feces.

CONCLUSIONS

In the studied group, 50% of the positive subjects by Real-Time PCR were less than 6 months old. Positive results by Real-Time PCR were also recorded in 2.28% of subjects over 7 months of age: this demonstrates that the calendar age it self is not a criterion for excluding/including suspicion. Also, regarding the sources of infection, this result supports the observation according to which the parvovirus is carried and eliminated by the subjects that were previously contaminated.

In the studied group, the ratio of females is 14% higher than that of males. The characteristic symptoms of canine parvovirus infection - vomiting/diarrhea, has been manifested in only 4.92% of dogs.

Using the Real-time PCR, we found the highest amount of viral particles (DNA material) in the subjects of 3-4 months aged. Dogs older than 6 months may suffer severe infection of the digestive tract and clinically expressed disease.

Despite the variation range of Ct`s, the clinical pattern registered did not directly relate with the virus amount, rather with the age.

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