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RESEARCH ARTICLE

Comparison of different diagnostic methods in detection of canine parvovirus infection

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Köpek parvovirus enfeksiyonunun tespitinde farklı teşhis yöntemlerinin karşılaştırılması

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Öz

Amaç: Bu araştırma ile klinik olarak gastroenterit semptomları gösteren köpeklerde CPV-2 enfeksiyonlarının teşhisi için kullanılan Enzyme Linked İmmunosorbent Assay (ELISA) ve Immunochromatographic (IC) testlerinin Polymerase Chain Reaction (PCR) yöntemi ile karşılaştırılarak sensitivite ve spesifitelerinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Araştırmada CPV-2'ye karşı aşılanmamış, 0-12 aylık gastroenterit semptomları gösteren 100 adet köpekten dışkı örnekleri toplandı. Daha sonra ELISA ve hızlı test kitleri ile CPV-2 teşhisi yapıldı. Testlerin sensivite ve spesifitelerinin belirlenmesi için örnekler CPV-2'nin VP-2 proteinine özgü primerler kullanılarak PCR testine tabi tutuldu. Testlerin sensitivite ve spesifiteleri hesaplandı.

Bulgular: IC testin sensitivitesi %36,6, ELISA'nın ise %24,24; her iki testin spesifiteleri ise %100 olarak tespit edildi. Ayrıca IC test sonuçları ve ELISA sonuçları, PCR sonuçlarına göre istatistiksel olarak farklı olduğu belirlendi ($p<0,05$).

Öneri: CPV-2'nin teşhisinde ELISA ve IC testin sensitivitelerinin oldukça düşük seviyede belirlenmesi gastroenterit semptomları bulunan köpeklerde CPV-2 yönünden yanlış negatif sonuçlar nedeniyle hastalığın yayılımı hızlanmaktadır IC ve ELISA yöntemleri ile negatif olarak belirlenen örneklerin PCR ile doğrulanması gerektiği ya da bu testlerin sensivitelerinin artırılması için gerekli çalışmaların yapılması önerilmektedir.

Anahtar kelimeler: CPV-2, ELISA, IC test, PCR, teşhis

Abstract

Aim: The study is to compare sensitivity and specificity rates of the enzyme-linked immunosorbent assay (ELISA), immunochromatographic assay (IC) and polymerase chain reaction (PCR) tests which are widely used to diagnose CPV-2 infections of dogs with severe gastroenteritis symptoms.

Materials and Methods: The stool samples were collected from 100 unvaccinated dogs with gastroenteritis symptoms. They were analyzed by ELISA and IC test kits for CPV-2. Also, the samples were investigated by PCR assay using the CPV-2 primer set amplify partial of VP2 gene to determine sensitivity and specificity of the tests.

Results: The sensitivity of IC was 36,6% and of ELISA was 24,2% compared to the PCR test. Also, both tests had 100% specificity. The IC test, and ELISA results were determined statistically different according to PCR ($p<0.05$).

Conclusion: The ELISA and IC assays had low sensitivity. Therefore, the tests can give false negative results in puppies with gastroenteritis symptoms and this situation can increase the spread of the disease. In conclusion, the negative determined samples by IC and ELISA methods should be verified by PCR and detailed studies should be carried out to increase the sensitivity of these tests.

Keywords: CPV-2, ELISA, IC test, PCR, diagnose

Introduction

Canine parvovirus type 2 (CPV-2) infection has been an important enteric pathogen for younger than 6 months domestic and wild carnivores across the world since the 1970s (Goddard and Leisewitz 2010, Ok et al 2015, Miranda and Thompson 2016). The causative factor, CPV-2, is a small, non-enveloped, 5.2 kb-long, single-helix DNA virus with negative polarity. This virus is classified as a virus of the *Parvoviridae* family, *Parvovirinae* subfamily, and the *Protoparvovirus* genus (Cotmore et al 2014). The CPV genome has two large open reading frames regions. One of these encodes non-structural proteins (NS1, NS2), whereas the other encodes capsid proteins (VP1, VP2) (Decaro and Buonavoglia 2012, Miranda and Thompson 2016). VP2 is the main capsid protein that plays an important role in determining host range and tissue tropism (Hueffer et al 2003, Vannamahaxay and Chuammitri 2017).

New antigenic variants of CPV-2 have emerged as a result of genetic and antigenic changes. The first variant was discovered in the early 1980s and was called CPV-2a. After this stage, the virus quickly mutated, and CPV-2b, which was a new strain, was identified in the UK in 1984 (Parrish et al 1985, Parrish et al 1991). In 2000, CPV-2c, a new strain of CPV-2, was identified in Italy as the third antigenic variant of CPV as a result of mutation of the amino acid Glu-426 (Asp-426→ Glu) (Buonavoglia et al 2001).

CPV-2 infection often occurs in puppies. There are two clinical forms of the disease. These are acute forms of hemorrhagic enteritis and myocarditis (Goddard and Leisewitz 2010). A combination of symptoms such as vomiting, foul-smelling diarrhea which may range from mucoid to purely hemorrhagic, followed by depression, dehydration, fever, and leukopenia in unvaccinated offspring may be the indication of CPV diagnosis (Nandi and Kumar 2010, Mylonakis et al 2016). Isolation of infected dogs in kennels and shelters, prevention of secondary infections in susceptible animals, and rapid diagnosis of CPV are very important in order to reduce the risk of transmission. Another important aspect of rapid diagnosis is the prevention of its involvement with other diseases. Clinical diagnosis may not always yield an exact result. Diagnosis is attempted using enzyme-linked immunosorbent assay (ELISA), immunochromatographic (IC) test, or hemagglutination (HA) tests in the feces of suspected dogs; however, these techniques have relatively low sensitivity. Although virus isolation (VI) is a more sensitive method, it is not a preferred method in routine diagnostics, as it is a very labor-consuming and time-consuming diagnostic method. Detection of CPV-2 DNA by polymerase chain reaction (PCR) is considered a highly sensitive diagnostic method (Desario et al 2005). The most preferred IC test for diagnosis is a method based on binding of viral antigens excreted with feces to conjugated specific antibodies in the test kit, as this method allows rapid diagnosis (Sharma et al 2018). Another diagnostic method for canine parvovirus is ELISA, a diagnostic method based on antigen-antibody reaction usually using monoclonal antibodies (MAbs). ELISA, easily available to determine CPV-2 in virus-infected feces, is a fast, relatively cost-effective and easy to perform test (Prittie 2004). Molecular diagnostic methods such as PCR are often sensitive methods which are not affected by the host immune response but require intensive labor and expertise (Decaro and Buonavoglia 2012).

The purpose of this study is to compare ELISA and IC tests, which are important methods in the diagnosis of parvoviral enteritis infection, with by PCR test and to determine the sensitivity and specifications of these tests.

Material and Methods

For this study, stool samples were collected from 19 dogs with suspected parvovirus which were brought to the clinic of the Department of Internal Medicine of the Faculty of Veterinary Medicine of Selçuk University and which showed symptoms of gastroenteritis during clinical examination and from 81 dogs with clinical symptoms in the animal shelter of Konya Metropolitan Municipality. Overall, 100 samples were collected dogs who were 0-12 months and unvaccinated for CPV-2.

Preparation of stool samples

After the stool samples were brought to the laboratory, they were divided and transferred into sterile eppendorf tubes for use in IC test, ELISA and PCR test procedures. Stool samples were stored at −20oC until they were used in the tests.

IC test and ELISA

IC tests and ELISA/Antigen (Ag) (Biopronix – Agrolabo, İtaly) kits from the same company were used to detect the CPV antigen in stool samples. The tests were conducted in accordance with the procedure specified by the company.

DNA Extraction from stool samples and PCR

Viral DNA extraction was performed using "QIAamp DNA Stool Mini Kit" (QIAGEN, 51504, Germany) in the stool samples. Viral DNA products obtained from the extracted samples were examined using a commercial PCR kit (Taq PCR Master Mix — QIAGEN, Germany). Primers for CPV-2ab were used in the study. CPV-2ab (F) GAA GAG TGG TTG TAA ATA ATT, CPV-2ab (R) CCT ATA TAA CCA AAG TTA GTA (Nandi et al 2010). The CPV-2ab primary set amplify portion of VP2 gene of both CPV-2a and CPV-2b variants (3025—3706 nucleotide position of CPV genomic DNA) to yield a product size of 681 bp. For the amplification, the following conditions were applied: 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for

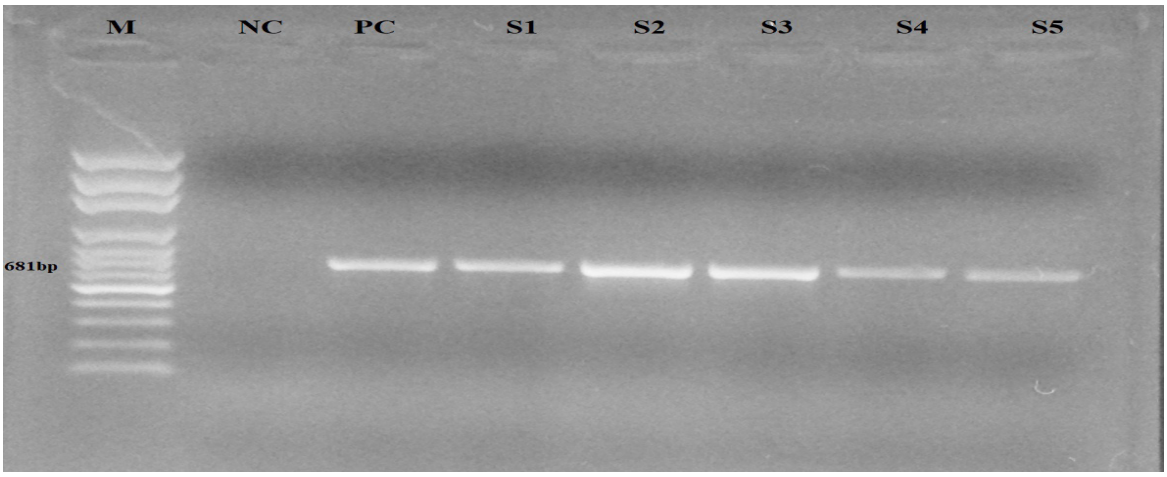

Figure 1. Electrophoresis image of the CPV (681 bp) positive samples.

M: molecular weight marker (100 bp DNA ladder, Solis Biodyne), PC (Positive Control), NC (Negative Control), S1-5: CPV positive samples.

45 s, 72°C for 60 s and a final extension of 72°C for 10 min followed by electrophoresis on a 1.5% agarose gel (Figure 1.). All steps included a positive control and RNA free water as negative control.

Statistical analysis

Sensitivity and specificity values for the data obtained from the tests in the study were calculated by the method specified by Martin et al (1987). Mc Nemar Test (SPSS statistical software, version 22.0) was used for the comparison of diagnostic methods used in the study.

Results

The results of the three tests used for CPV detection in the feces of dogs with gastroenteritis are summarized in Table 1. The rapid test was able to detect CPV antigen in 24/100 (24%) of analyzed samples. Approximately 16/100 (16%) positivity was achieved as a result of the ELISA test, while the PCR result demonstrated 66/100 (66%) positivity.

Discussion

Canine parvovirus is the most common cause of viral enteritis in dogs and leads to significant morbidity and mortality (Qi et al 2020). Accurate and rapid diagnosis of the virus in the very early stages of infection is very important in controlling the disease. Numerous studies conducted in different countries of the world (Miranda and Thompson 2016) have demonstrated the importance and prevalence of CPV-2 infection.

Large quantities of viral antigens are needed to obtain visible bands from IC tests. The sensitivity of the test decreases in the presence of small quantities of antigen. Therefore, methods such as virus isolation (VI) and PCR are important for accurate diagnosis (Desario et al 2005). Although the commercial IC test kit manufacturer information indicates a very high percentage of the sensitivity of 90-96%, in a study conducted by Schmitz et al (2009) which compared PCR testing of three different commercial IC kits (i.e. Snap Test, FASTtest and Witnesscard), the sensitivity was reported to be very low: 18.4%, 15.8% and 26.3%, respectively. In the study by Kantere et al (2015), it was reported that the IC test was less sensitive in the comparison of IC test and PCR for CPV-2 because CPV-2 amount decreased in stool as the sampling time was delayed, viral antigens decreased due to the intes-

Table 1. Statistical comparison of positive results obtained as a result of the tests used in the study

Tests	Results
PCR	66/100
IC Test	24/100*
ELISA	16/100**

*: Based on the Mc Nemar Test, IC test results are statistically different from those of PCR ($p < 0.05$).

**: Based on the Mc Nemar Test, ELISA results are statistically different from those of PCR ($p < 0.05$).

Table 2. Specificity and sensitivity results of comparison of PCR with immunochromatography (IC) and Enzyme Linked Immunosorbent Assay (ELISA)

		PCR +	PCR -
IC TEST	+	24[a]	0[b]
	-	42[c]	34[d]

Sensitivity (%) = 36.36

Specificity (%)=100

		PCR +	PCR -
ELISA	+	16[a]	0[b]
	-	50[c]	34[d]

Sensitivity (%) = 24.24

Specificity (%) = 100

a = True Positive (TP), b = False Positive (FP), c = False Negative (FN), d = True Negative (TN).

Sensitivity (%) = TP/(TP+FN) × 100, Specificity (%) = TN/(TN+FP) × 100.

tinal antibodies, depending on the freezing and thawing of the samples at -20oC. In the study conducted by Tinky et al (2015) to compare the diagnostic potential of PCR with rapid testing used in CPV diagnosis in diarrheal dog stools, the sensitivity of the rapid test compared to PCR was 72.73% and specificity was 92.86%. However, there was no statistically significant difference between the two tests (According to McNemar statistics test) ($p > 0.05$).

In the present study, 100 stool samples collected from dogs with diarrhea were examined virologically for CPV-2 using IC test and PCR diagnostic methods, and the results of these two methods were compared in terms of sensitivity and specificity. The sensitivity of the IC test was 36.36%, and the specificity was 100% (Table 2). In addition, the data obtained were statistically significantly different compared to the McNemar statistical test ($p < 0.05$) (Table 1). In the current study, the specificity of the IC test was 100% consistent with the results of many studies (Schmitz et al 2009, Decaro et al 2013, Silva et al 2013). However, the sensitivity of the IC test was low according to the PCR test. The IC test may have caused false negative results and statistical difference according to the PCR test due to reasons such as freezing and thawing samples, low amount of viral particles excreted with feces because of high antibody level in the intestines of the dogs, and delayed sampling time. Molecular techniques such as PCR may yield positive results even when there is a small amount of viral antigen in the stool. However, it is necessary to have a fairly large amount of viral antigen in order to form a clearly visible band in IC tests, which may affect the interpretation of the results by the test user. The false negative results of the IC test are very common at the initial stage of infection, in the late stages of acute infection, and subclinical infections in vaccinated animals due to amount of the virus is very low. Proksch et al (2015) reported that they obtained a false negative result of 51.3% in their study to determine the cause of frequently reported false negative results in the rapid test commonly used in the detection of CPV-2. In the comparison of dogs with negative rapid test results and dogs with positive rapid test results, less defecation, decreased viral particulates in the stool and high serum antibody levels were observed. Investigators (Decaro et al 2005) emphasized that in natural and experimental infection studies using techniques such as HA and virus isolation, CPV-2 could only be detected within a limited time, such as a few days after infection, and even in samples with high amounts of viral DNA based on real-time PCR, cases with false negative results based on these techniques were quite common. This contradiction was explained by the appearance of CPV-2-specific antibodies in dog stools shortly after infection, which may affect the results of the tests (Decaro et al 2005, Goddard and Leisewitz 2010).

Since ELISA method is based on antigen-antibody binding, amount of viruses excreted with feces may affect the results of the test. Based on the results of the present study, it had lower specificity compared to molecular diagnostic methods such as ELISA, PCR and real-time PCR (Richards et al 2003, Izzo et al 2012). This can be explained by the fact that the results may vary depending on virus quantity in the stool. It is also reported that the detectable limit indicated in the leaflets of ELISA kits generally does not reflect reality (Izzo et al 2012). However, the presence of antigen-antibody complexes in feces, excessive fecal contaminations in samples, low affinity of coated antibodies on the surface of ELISA or the presence of proteases may adversely affect the results (Richards et al 2003). In the present study, 100 stool samples collected from dogs with diarrhea were examined virologically for CPV-2 using ELISA and PCR diagnostic methods. The results of these two methods were compared in terms of sensitivity and specificity. The sensitivity of ELISA compared to PCR was 24.24%, and the specificity was 100% (Table 2). In addition, the data obtained were statistically significantly different compared to the McNemar test ($p < 0.05$) (Table 1). While sample preparation was made in accordance with the kit procedure in this study, ELISA sensitivity was low. This may be partly caused by fecal contamination, as well as low affinity of the antibodies on the ELISA kit surface.

In this study, ELISA and IC test results showed a very low positivity rate, which supports the hypothesis that fecal antibodies that bind to CPV-2 virions prevent the detection of the presence of viral antigens by these tests. Contrary to all this,

it is known that PCR-based methods (classical or real-time) are much more sensitive in the detection of viral antigens compared to conventional methods, even in the presence of antibodies. Therefore, PCR testing may be considered as a factor that explains the higher number of positive results compared to other tests. It is suggested that clinically suspected dogs should be re-tested for the presence of CPV-2 with PCR using stool samples, even if the results are negative with other test methods.

Conclusion

This study used primers which could show both CPV-2a and CPV-2b variants in the PCR method used in molecular diagnostics of the presence of CPV-2 in stool samples. Therefore, ELISA and IC test sensitivities may have been low due to the fact that ELISA or IC test antibodies are sensitive to only one of these antigenic variants, virus titer is decreased due to freezing and thawing of samples, antibody titer is decreased due to the presence of antibodies in the gastrointestinal tract, and the acute period of infection is missed. It is necessary to carry out new studies in the future by standardizing each of these reasons.

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Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Writing the Article: Irmak Dik
Critical Review: Atilla Şimşek, Irmak Dik

Ethical Approval

All procedures and animal care were complied with the guidelines of the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2017/22 on 09/03/2017).

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