Current Journal of Applied Science and Technology

40(19): 76-82, 2021; Article no.CJAST.72480
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

Identification and Typing of Canine Parvovirus Using Molecular Techniques

Koppu Vasavi[1], Gurpreet Kaur[1*], Mudit Chandra[1], P. N. Dwivedi[1] and Navjot Kaur[1]

[1]Department of Veterinary Microbiology, COVS, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141001, Punjab, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2021/v40i1931457
Editor(s):
(1) Dr. Yahya Elshimali, Charles Drew University of Medicine and Science, USA.
Reviewers:
(1) Qingliang Shen, USA.
(2) Asmarani Kusumawati, Gadjah Mada University, Indonesia.
Complete Peer review History: https://www.sdiarticle4.com/review-history/72480

Original Research Article

Received 11 June 2021
Accepted 15 August 2021
Published 20 August 2021

ABSTRACT

Aim: Canine parvovirus 2, the causative agent of acute hemorrhagic enteritis in dogs, is one of the most important pathogenic viruses. It causes a highly contagious and often fatal disease. The disease condition is complicated further due to emergence of a number of variants namely CPV-2a, CPV-2b, CPV-2c, new CPV 2a, new CPV 2b over the years and involvement of domestic and wild canines. The virus is shed in large numbers in the feces of infected dog and upto 7 to 10 days post-infection, therefore, the present study was designed to detect CPV and to identify the prevailing antigenic types of CPV using molecular techniques from rectal swabs of affected dogs.
Methods: The rectal swabs were collected from dogs suspected of Canine Parvovirus and subjected to PCR, Nested PCR and Realtime PCR for identification and typing of CPV in infected dogs.
Results: From the study it was found that the per cent positivity was high in dogs and was found to be 50% and 89% by PCR and nested polymerase reaction respectively when considered in suspected dogs. The most prevailing antigenic type as detected by Real time PCR was found to be CPV 2a.
Conclusions: The study indicated the animals vaccinated for CPV were also found positive for the disease. This study helps to detect percent positivity of CPV in dogs and also is important to identify the prevailing antigenic types of CPV in the region.

**Corresponding author: E-mail: gurpreet7502@rediffmail.com;*

Keywords: Dogs; canine parvovirus; antigenic types; PCR; nested PCR; real time PCR.

1. INTRODUCTION

Parvoviruses have small (~25 nm diameter), non-enveloped icosahedral capsids and among them Canine Parvovirus (CPV) has significance as is responsible for haemorrhagic enteritis causing high morbidity and mortality in dogs being more severe in pups. CPV have undergone a series of evolutionary selections in nature, resulting in global distribution of new variants that have replaced the original CPV-2. Currently, the three major antigenic variants of CPV-2 which are 2a, 2b and 2c are known to be distributed among the dog population worldwide [1]. Isolation of CPV-2 was done for the first time in India by Ramadass and Khader [2] since then several occurrences of disease have been reported from different parts of the country involving different variants of CPV (2, 2a, 2b and 2c) both in vaccinated and unvaccinated animals [3,4,5]. VP2 is the major capsid protein that plays an important role in the determination of antigenicity and host range of CPV. The virus has two major open reading frames (ORFs); one of which encodes the non-structural proteins NS1 and NS2, and the other encodes two structural proteins VP1 and VP2. At either end of the genome, palindromic hairpins of about 150 bases are present used in the replication of the viral DNA [6,7]. The capsid proteins have a highly conserved central core composed of an eight-stranded, anti-parallel β-barrel with flexible loops between the β-strands that interact to form most of the capsid surface. The surface features of the capsid include a 22 Å long raised region (spike) on the threefold axes, a 15 Å deep depression (canyon) surrounding cylindrical structures at the fivefold axes, and a 15 Å deep depression (dimple) at the twofold axes. In addition, the threefold axes are the most antigenic region of the capsid and serve as a target for neutralizing antibodies [8,9].

Prophylaxis of CPV infection relies mainly on extensive vaccination. Since inactivated vaccines are able to induce only short-term immunity, modified live virus vaccines are widely used. These vaccines, prepared by using either the original type CPV-2 or its variant are highly effective, being able to protect dogs against parvoviral disease as well as infection, and almost completely safe. A recent study showed that most dogs developing parvovirus-like diarrhea after vaccination were infected by the field virus alone or with the attenuated vaccine virus [10]. It is also known that the mutations which affect VP2 gene are mainly responsible for evolving different antigenic variants of CPV [11]. The early detection along with the knowledge of genetic variations of VP2 can be of immense help in identifying the emerging CPV strains. Thus, the present study was designed to detect CPV and identify the antigenic types of CPV using molecular techniques.

2. MATERIALS AND METHODS

The rectal swabs were collected from dogs suspected of Canine parvovirus infection during the period from October, 2020 to February, 2021 from Multispeciality Veterinary Hospital, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The rectal swabs were collected in 4ml of phosphate buffered saline (pH 7.2) and kept at 4□C till further processing. The vaccine Nobivac DHPPi (Intervet, Pvt. Ltd) was procured commercially from local market to be used as positive control. The DNA was extracted from all the samples and the vaccine using the phenol-chloroform extraction method as described by Sambrook and Russell [12].

2.1 Detection of CPV by PCR and Nested PCR

PCR reaction mixture was made for the confirmation of virus by adding 5µl of 10X PCR buffer (with 15mM $MgCl_2$), 1.0µl of forward and reverse primer each (20pm/µl each) as per Mizak and Rzezukta [13], 1.0µl of dNTPs (10mM), 0.2µl of Taq polymerase (5 units/µl), 15.0µl of the template DNA and 26.8µl of nuclease free water making a total of 50µl of the reaction mixture. The DNA from the vaccine was used as a positive control and the rectal swab from a healthy dog was used as negative control.

The PCR product was subjected to nested PCR by setting up a different PCR reaction mixture. To prepare the reaction mixture for nested PCR, 2.0µl of the PCR product obtained from the above procedure was added along with 2.5µl of 10X PCR buffer (with 15mM $MgCl_2$), 1.0µl each of forward primer and reverse primer as per Mizak and Rzezutka [13], 1.0µl of dNTPs (10mM), 0.2µl of Taq polymerase (5 units/µl) and 17.3µl of nuclease free water making a total of 25µl of nested PCR reaction mixture.

Similar thermocycling conditions were setup for amplification for both PCR and nested PCR. After preparing the PCR mix, the reaction was

put in a Thermocycler which was subjected to the following thermocycling conditions i.e. for the step of denaturation a temperature of 94°C was kept for 60 seconds, for the step of annealing a temperature of 55°C was kept for 60 seconds, the extension step was set up at 72°C for 150 seconds and these were carried out for a total of 35 cycles and final extension was done at 72□C for 10 minutes. PCR and Nested PCR products (10 µl) were run using 1.5% agarose at 5 volts/cm with Gene Ruler ladder plus 100bp (New England Biolabs, USA).

2.2 Typing of Canine Parvovirus types by Realtime PCR

The extracted DNA from the rectal swabs samples was subjected to Real Time PCR for antigenic typing using three assays for CPV 2, CPV 2a and CPV 2b. The fluorescence-probe based assays (Taqman assays) for the three antigenic types viz CPV-2, CPV-2a and CPV-2b (Table 1) were used.

The 20µl of Real Time PCR reaction mixture was prepared by adding 10µl of 2X Taqman® Universal Master Mix II with UNG (Applied Biosystems), 1µl of the 20X Taqman® assay (for the individual antigenic type), 2µl of the DNA template and 7µl of nuclease free water. The PCR reaction was carried out in Step One™ software version 2.1 Real-Time System (Applied Biosystems, USA) with the thermal condition of UNG incubation at 50°C for 2 minutes, polymerase activation at 95°C for 3 minutes and 40 cycles of denaturation at 95°C for 15 seconds and annealing at variable temperatures and time depending on the antigenic type detected. For detection of CPV-2 the annealing was done at 52°C for 30 seconds; for CPV-2a the annealing was done at 61°C for 45 seconds and for CPV-2b annealing was carried out at 57°C for 45 seconds. The samples were considered positive or negative in the Real-Time PCR depending upon the fluorescence of a particular wavelength emitted by the respective fluorophore attached to the particular probe for the three antigenic types (CPV-2, CPV-2a and CPV-2b) of CPV. Depending upon the highest and lowest relative fluorescence unit (RFU) value, the cut off value or end point was calculated by using StepOne™ software Version 2.1.

3. RESULTS AND DISCUSSION

CPV-2 is one of the most common viruses responsible for acute hemorrhagic enteritis in dogs. CPV-2 induced disease is observed mainly in 6-12 weeks old pups [14]. CPV-2 spreads from infected to susceptible dogs by the fecal-oral route and reached high titers in feces of infected dogs [15,16] and is shed in feces for approximately 8-12 days post-infection. A rapid diagnosis of CPV-2 infection is especially important in kennels and shelters in order to isolate infected dogs and prevent infections of susceptible contact animals. Since a clinical diagnosis is not definitive several laboratory methods have been developed to detect CPV-2. Routinely, feces from diarrhoeic dogs are screened using ELISA, immunochromatographic or haemagglutination tests but these techniques are affected by relatively low sensitivity [17]. Virus isolation is more sensitive but it is too labor-intensive and time consuming for routine diagnostic testing. On the other hand, methods based on detection of CPV-2 DNA by PCR have been shown to be highly sensitive [18]. A Real-Time PCR assays are also being used for the detection and quantitation of DNA in the feces of diarrhoeic dogs. The CPV-2 Real-Time PCR assay based on Taqman technology has been found to be highly specific, sensitive and reproducible; it also is less time consuming than conventional gel-based PCR [19].

3.1 Detection of Canine Parvovirus by PCR and Nested PCR

PCR is a robust and highly sensitive tool for the detection of CPV-2 in clinical samples. CPV is excreted in high numbers in faeces of dogs suffering from parvoviral infection. Therefore, feces or rectal swab are the best sample for the detection of CPV. Thus, in the present study, a total of sixty-eight (n= 68) rectal swabs were collected; 67 from Ludhiana (Punjab) and 1 sample from Rajasthan from the dogs exhibiting signs of diarrhea, gastroenteritis and hemorrhagic enteritis with pyrexia. The genomic DNA was extracted from these samples by using P:C:I method and were subjected to PCR, which revealed 34 samples positive for CPV yielding a product size of 1198 bp (Fig. 1). Thus, the percent positivity was found to be 50% (34/68) using PCR.

When the vaccination history of the dogs was evaluated, it was found that out of 34 PCR positive CPV samples, 15 (15/34, 44.11%) dogs were vaccinated and 19 (19/34, 55.88%) were not vaccinated. Thus, some of the dogs which were positive for CPV in PCR were vaccinated indicating that it might be possible that vaccination of pups against CPV is not conferring immunity against CPV. This might be due to the

mismatching of vaccine strain and the CPV strain causing infection in dogs.

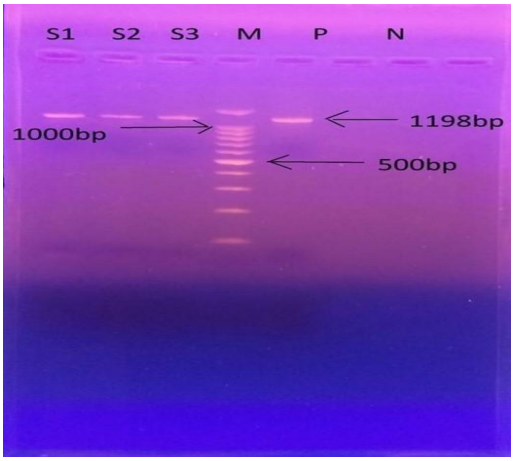

Fig. 1. PCR Product Visualization - Gel Run
M: 100 bp Plus DNA Ladder, P: Positive Control (DHPPi Vaccine), N:Negative Control (Rectal Swab from Healthy Dog), S1-S3:1198 bp PCR positive CPV Samples

The present study also revealed that the sensitivity of NPCR was much more than conventional PCR for detecting CPV. Similar findings indicating increased sensitivity of NPCR has been reported by various earlier research works. The results of the present study are similar to work of other researchers [20,21,22,23] who have also stated that nested PCR is more sensitive than conventional PCR. The reason for this could be that the samples containing very few virus particles might be harboring inhibitory substances as reported by Kumar et al [24] leading to absence of visualization of the amplified product after a PCR, which could have been resolved using a NPCR leading to visualization of NPCR product in an agarose gel.

The PCR product from the 68 rectal swabs collected from the dogs exhibiting signs of CPV was subjected to nested PCR. Out of a total of 68 samples, 61 samples were positive with nested PCR yielding a product size of 548bp (Fig. 2) indicating that the per cent positivity of CPV with nested PCR was 89.70%. When age-wise status among positive was evaluated, it was found that 75.4% (46/61) were below 6 months of age. Thus, maximum animals affected by CPV were below 6 months of age followed by above 6 months substantiating the already established fact that the infection caused by CPV is more severe in young animals [3,4,22,23,25,26,27].

When breed-wise prevalence among positive by nested PCR was evaluated it was found that 68.85% (42/61) were pedigree dogs and 31.14% (19/61) were non-descript breed. Breed-wise comparison indicated that Pomeranian, German Shepherd and Labrador breeds of dogs were mostly affected by CPV as detected by nested PCR. These observations too were similar to the earlier reports in which researchers reported that in India German Shepherd, Labrador and Pomeranian breeds of dogs were most predisposed towards CPV [22-24,27].

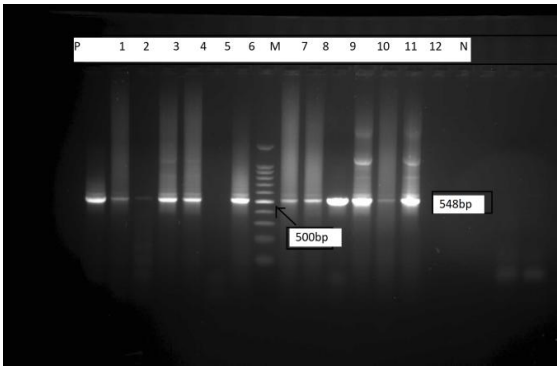

Fig. 2. Nested PCR Product Visualization - Gel Run
M: 100 bp Plus DNA Ladder, P: Positive Control (DHPPi Vaccine), N: Negative Control (Rectal Swab from Healthy Dog), Lane 1-12 except 5: 548 bp Nested PCR Positive CPV Samples

3.2 Real Time PCR typing for identifying CPV subtypes

RealTime PCR has been used by number of researchers for detection and quantitation of viral load in feces of infected dogs [10,19,28,29]. Real Time PCR has also been used for typing of CPV antigenic types [1,30,31]. Similarly in the present study the extracted DNA from a total of 68 samples collected were subjected to antigenic typing by Real Time PCR for detection of three antigenic types (CPV 2, CPV 2a and CPV 2b). Out of a total of 68 samples; 52 samples were found positive for Canine Parvovirus with a percent positivity of 76.47%. Out of 52 positive samples; a total of 7 cases were positive for CPV 2, 45 were positive for CPV 2a (Fig. 3) and none was positive for CPV 2b. Therefore CPV 2a antigenic type of Canine parvovirus was found to be most prevalent. The same observation was reported by Das et al. [32] of prevalence of CPV 2a from Punjab and Assam.

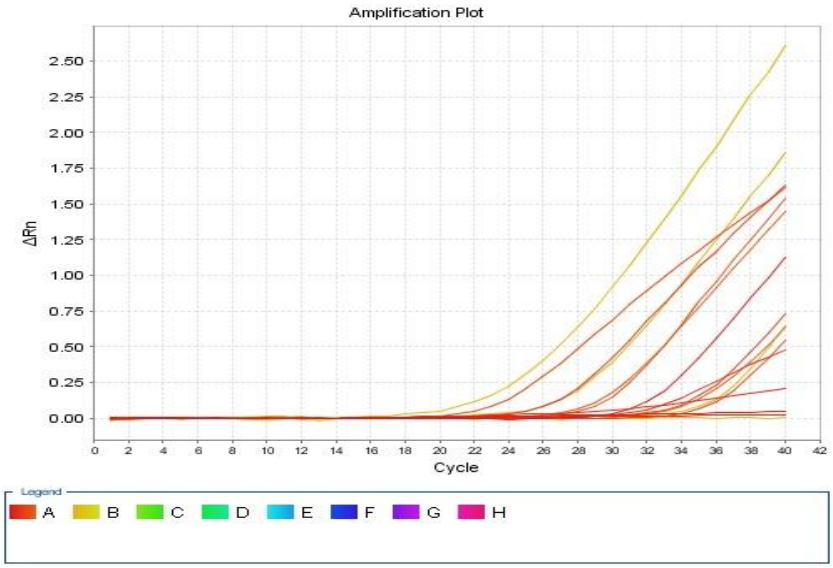

Fig. 3. Real-time PCR graph for positive samples for CPV 2a

Table 1. Typing of CPV types using three Taqman Assays

Antigenic Type	Taqman Assay	Sequence	References	Position in genome
CPV 2	F	5‘- AAACAGGAATTAACTATACTAATATATTTA-3‘	[9]	-
	R	5‘-AAATTTGACCATTTGGATAAACT-3‘		-
	Probe	5‘-/6-FAM/TGGTCCTTT/ZEN/AACTGCATTAAATAATGTACC/IowaBlack/3‘		-
CPV 2a	F	5‘-TGACCAAGGAGAACCAACTAAC-3‘	[8]	847-866
	R	5‘-TGATCTGCTGGCGAGAAATATAA-3‘		1013-993
	Probe	5‘/6-FAM/ACGCTGCTT/ZEN/ATCTTCGCTCTGGT/IowaBlack/-3‘		867-896
CPV 2b	F	5‘-ACAGGAAGATATCCAGAAGGAGA-3‘	[13]	1216-1238
	R	5‘-TGACCATTTGGATAAACTGGTGG-3‘		1403-1381
	Probe	5‘-/HEX/TATTAACTT/ZEN/TAACCTTCCTGTAACAGATGA-/IowaBlack/-3‘		1251-1280

However, when the results were thoroughly observed, some cases were found to be positive for more than a single subtype simultaneously. Therefore the number of dogs positive for CPV 2 alone were 1, for CPV 2a there were 39 cases and number of positive cases for both CPV 2 and 2a were 6.

4. CONCLUSION

It can be concluded from the study that the percent positivity of CPV was found to be 50% and 89% by PCR and nested PCR respectively indicating nested PCR more sensitive. Majority of CPV positive samples belonged to dogs below 6 months of age and were among Labrador, Pomeranians and German Shepherd breeds of dogs. Dogs vaccinated for CPV were also found to be positive for the CPV with percent positivity of 44.11%. CPV 2a was found to be most prevalent in the study as indicated by Real-Time PCR.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely

no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Decaro N, Elia G, Desario C, Roperto S, Martella V, Campolo M, Buonavoglia C. A minor groove binder probe real-time PCR assay for discrimination between type 2-based vaccines and field strains of canine parvovirus. Journal of Virological Methods. 2006;136(1-2): 65-70.
2. Ramadass P, Khader TGA. Diagnosis of Canine Parvovirus infection by agar gel precipitation test and fluorescent antibody techniques. Cheiron. 1982;11:323-325.
3. Biswas S, Das PJ, Ghosh SK, Pradhan NR. Detection of canine parvovirus (CPV) DNA by polymerase chain reaction assay and its prevalence in dogs in and around Kolkata, West Bengal. Indian Journal of Animal Sciences. 2006;76(4):324.
4. Deepa PM, Saseendranath MR. Serological studies on canine parvoviral infection. Indian Veterinary Journal. 2000; 77(11): 927-928.
5. Phukan A, Deka D, Boro PK. Occurrence of canine parvovirus infection in and around Guwahati. Indian Journal of Animal Sciences. 2004;74:930-931.
6. Parrish CR. Host range relationships and the evolution of canine parvovirus. Veterinary Microbiology. 1999;69(1-2):29-40.
7. Reed AP, Jones EV, Miller TJ. Nucleotide sequence and genome organization of canine parvovirus. Journal of Virology. 1988;62(1):266-276.
8. Tsao J, Chapman MS, Agbandje M, Keller W, Smith K, Wu H, Compans RW. The three-dimensional structure of canine parvovirus and its functional implications. Science. 1991;251(5000):1456-1464.
9. Agbandje M, McKenna R, Rossmann MG, Strassheim ML, Parrish CR. Structure determination of feline panleukopenia virus empty particles. Proteins: Structure, Function, and Bioinformatics. 1993;16(2): 155-171.
10. Decaro N, Martella V, Elia G, Desario C, Campolo M, Lorusso E, Buonavoglia C. Tissue distribution of the antigenic variants of canine parvovirus type 2 in dogs. Veterinary Microbiology. 2007;121(1-2): 39-44.
11. Phromnoi S, Sirinarumitr K, Sirinarumitr T. Sequence analysis of VP2 gene of canine parvovirus isolates in Thailand. Virus Genes. 2010;41(1):23-29.
12. Sambrook J, Russell DW. Molecular Cloning: A laboratory Manual. 3rd Edn. Cold Spring Harbor Laboratory Press, New York; 2001.
13. Mizak B, Rzezutka A. Application of nested PCR for detection of canine parvovirus in faeces. Bulletin of the Veterinary Institute in Pulawy. 1999;43(1):19-24.
14. Pollock RV, Carmichael LE. Maternally derived immunity to canine parvovirus infection: transfer, decline, and interference with vaccination. Journal of American Veterinary Medical Association. 1982;180(1):37-42.
15. Pollock RV. Experimental canine parvovirus infection in dogs. The Cornell Veterinarian. 1982;72(2):103-119.
16. Carmichael LE. Canine parvovirus type-2-an evolving pathogen of dogs. Annales de Médecine Vétérinaire 1994;138:459-464.
17. Mochizuki M, San Gabriel MC, Nakatani H, Yoshida M, Harasawa R. Comparison of polymerase chain reaction with virus isolation and haemagglutination assays for the detection of canine parvoviruses in faecal specimens. Research in Veterinary Science. 1993;55(1):60-63.
18. Buonavoglia C, Martella V, Pratelli A, Tempesta M, Cavalli A, Buonavoglia D, Carmichael L. Evidence for evolution of canine parvovirus type 2 in Italy. Journal of General Virology. 2001;82(12):3021-3025.
19. Decaro N, Elia G, Martella V, Desario C, Campolo M, Di Trani L, Buonavoglia C. A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. Veterinary Microbiology. 2005;105(1):19-28.
20. Sakulwira K, Oraveerakul K, Poovorawan Y. Detection and genotyping of canine parvovirus in enteritic dogs by PCR and RFLP. Science Asian. 2001;27:143-147.
21. Schmitz S, Coenen C, Matthias K, Heinz-Jürgen T, Neiger R. Comparison of three rapid commercial canine parvovirus

antigen detection tests with electron microscopy and polymerase chain reaction. Journal of Veterinary Diagnostic Investigation. 2009; 21(3): 344-345.
22. Kaur G, Chandra M, Dwivedi PN, Sharma NS. Isolation of Canine parvovirus with a view to identify the prevalent serotype on the basis of partial sequence analysis. Veterinary World. 2015; 8(1): 52.
23. Kushwaha SS, Kaur G, Chandra M, Dwivedi PN. Identification of the Prevailing Antigenic Types of Canine Parvovirus in Northern and Central India. International Journal of Current Microbiology and Applied Science. 2018; 7(8): 3881-3889.
24. Kumar M, Chidri S, Nandi S. A sensitive method to detect canine parvoviral DNA in faecal samples by nested polymerase chain reaction. Indian Journal of Biotechnology. 2011, 10: 115-118.
25. Banja BK, Sahoo N, Das PK, Swain P, Panda HK. Comparison of different laboratory tests for diagnosis of parvo and corona viral infections in dogs. Indian Veterinary Journal. 2002; 79(5):425-428.
26. McCaw DL, Hoskins JD, Greene CE. (Ed.) Infectious Diseases of the Dog and Cat, 3rd Edn., p. 63, Saunders Elsevier, St. Louis, 2006.
27. Deepti S, Verma AK, Amit K, Mukesh S, Singh SK, Tripathi AK, Iftekhar A. Detection of Canine Parvo Virus by polymerase chain reaction assay and its prevalence in dogs in and around Mathura, Uttar Pradesh, India. American J. Biochem Mol Bio. 2013; 3(2): 264-270.
28. Streck AF, Rüster D, Truyen U, Homeier T. An updated TaqMan real-time PCR for canine and feline parvoviruses. Journal of Virological Methods. 2013; 193(1): 6-8.
29. Kumar M, Nandi S. Molecular typing of canine parvovirus variants by polymerase chain reaction and restriction enzyme analysis. Transboundary and Emerging diseases. 2010; 57(6): 458-463.
30. Decaro N, Desario C, Elia G, Martella V, Mari V, Lavazza A, Buonavoglia C. Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. Microbiologica-Quarterly J. Microbiol. Sci. 2008; 31(1): 125-130.
31. Kaur G, Chandra M, Dwivedi P N, Narang D. Multiplex real-time PCR for identification of canine parvovirus antigenic types. Journal of Virological Methods. 2016; 233: 1-5.
32. Das H, Kaur G, Chandra M, Dwivedi P N. Detection of CPV 2a Antigen Type of Canine Parvovirus in the States of Punjab and Assam, India. Current Journal of Applied Science and Technology. 2019; 38(2):1-7.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/72480