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Evaluation of three different methods of viral DNA extraction for molecular detection of canine parvo virus-2 from faecal samples of dogs

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Abstract

The present study was undertaken to evaluate viral DNA extraction methods for fast, rapid and accurate diagnosis of canine parvovirus-2 (CPV). The usual extraction methods are costly and time consuming. CPV is a non-enveloped, single-stranded DNA virus causing hemorrhagic diarrhea in puppies. Three different methods, Phenol-Chloroform Isoamyl alcohol (PCI) extraction method, PureLink™ Viral RNA/DNA Mini Kit and Boiling-Snap chilling methods were evaluated for the DNA extraction, DNA purity, time duration and diagnostic efficacy by CPV specific polymerase chain reaction using extracted DNA. The conventional and kit methods were used by following the conventional protocol and kit manual method respectively. Boiling-Snap chilling method was standardized in laboratory for viral DNA extraction. All three methods were efficiently extracted DNA and detection was made reliable by three methods. The conventional DNA extraction method required more time duration and tedious, whereas kit method comparatively easy and less time consuming. While boiling-snap chilling is far better and best than other both methods, as it is not using any chemical and very quick. It simply followed the boiling and chilling principle for inactivation of inhibitors, nuclease, protease and different enzymes that interfere DNA polymerase. Hence, Boiling-Snap chilling method is concluded to be the best method for CPV DNA extraction and reliable.

Keywords: CPV, boiling-snap chilling, DNA extraction, Kit method, PCI method

Introduction

Canine Parvo virus-2 (CPV) is icosahedral non-enveloped, single stranded DNA virus belong to genus *Parvovirus* under subfamily *Parvovirinae*, family *Parvoviridae* [1]. Genome encodes for two structural proteins VP1 and VP2. VP2 is viral entry ligand and encoded within VP1 gene [7]. The virus was emerged from Feline panleukopenia virus in 1978. CPV is a major causative infectious pathogen of dogs and causes acute gastroenteritis that may worsen to death. Due to high mortality of disease, rapid and molecular diagnosis of the disease is highly required [1]. Diagnosis of CPV now a day more advances in terms of user adaptability, rapidity, accuracy and reliability. Immunochromatography based strip test and dot blot ELISA are mostly preferred. SNAP Parvo Test (IDEXX Laboratories, Inc., USA) and strip test are used in almost established veterinary clinics to make confirmative diagnosis of parvovirus infection in dogs. Even, SNAP Parvo Tests are user friendly and have higher specificity that does not cross react and having significant sensitivity [10].

Rapid methods of diagnosis are only qualitative test that cannot differentiate the antigenic variants. It is required to do confirmative diagnosis of CPV as rapid as possible at molecular level to check the antigenic variants. DNA isolation is critical step in every molecular diagnostic test where quantity and quality is concerned. Conventional and kit methods are time consuming as well as tedious to do and required various chemical. Commercial Kit's method are reliable and faster than the conventional method but yield of DNA is comparatively depend on individual kit, expiration period and supplied solution composition and its durability. However, commercial kits are better in isolation of DNA, they are costlier and usability is time bound. Boiling-Snap chilling method was first introduced by Decaro *et al.* (2005) [2] for CPV but not yet standardized step by step in protocol format. Earlier successful attempt was made for isolation of viral DNA from faecal samples of dog by using chelex resin and it found to be useful for molecular diagnosis making [9]. Considering this, study was undertaken to standardized Boiling-Snap chilling method and evaluates viral DNA extraction methods for fast, rapid and accurate diagnosis of CPV.

Materials and Methods

Three different methods, Phenol-Chloroform Isoamyl alcohol (PCI) extraction method, PureLink™ Viral RNA/DNA Mini Kit and Boiling-Snap chilling methods were evaluated for the viral DNA extraction, DNA purity, Time duration and diagnostic efficacy by CPV based polymerase chain reaction using extracted DNA. Positive samples of CPV that were stored and deposited to department of veterinary microbiology were used in study to determine the efficient method of viral DNA extraction from faecal samples of infected dog.

DNA isolation by Phenol Chloroform Isoamyl alcohol (PCI) method

It was carried out as per protocol described by Nandi and Kumar (2010) [6]. Briefly, the 200 µl of faecal sample was treated with SDS (10% W/V) and proteinase K (250 µg/ml) and kept on dry bath at 56 °C for 30 min. Then 200 µl PCI mixture was added and vortexed. The mixture was centrifuged at 10,000 rpm for 5 min. The upper aqueous layer was carefully aspirated and further Sodium acetate (10% of aspirate volume) was added. This was added with 1ml of chilled ethanol and kept overnight at – 20 °C. Next day, tube was centrifuged at 12000 rpm for 15 min and supernatant was discarded. The obtained pellet was suspended in 500 µl of 70% ethanol and after mixing, centrifuged again at 12000 rpm for 2 min. The pellet was dried at 37° C for about 20-30 min then resuspended in nuclease free water till further use.

DNA isolation by DNA isolation PureLink™ Viral RNA/DNA Mini kit

It was done as per manufacturer's instructions from PureLink™ DNA/RNA mini kit (Invitrogen). In this method, 200 µl faecal samples and 25 µl Proteinase K with 200 µl lysis Buffer was added in micro centrifuge tube and vortexed for 5 min. Tube was incubated at 56 °C for 15 minutes. Further 250 µl of 96–100% absolute ethanol was added to it and vortexed for 15 second and the lysate was kept for 5 minutes at room temperature. Manufacturer's supplied viral spin column in a collection tube was further used by adding the lysate and centrifuged at 6800 ×g for 1 minute and flow through was discarded. The spin column was washed with 500 µl Wash Buffer (WII) with ethanol. Then it was centrifuged at 6800 ×g for 1 minute repeated for twice. Then the spin column was centrifuged at maximum speed (11000 ×g) for 1 minute to give dry spin and removal of remaining any solution. Finally, DNA was eluted with 50 µl sterile nuclease free water (E3) by centrifuging the spin column at maximum speed for 1 minute.

DNA isolation by Boiling-Snap chilling method

It was proceed as per protocol of Decaro *et al.* (2005) [2] with modification. Time and temperature for boiling step was evaluated for different combination to get best result. Similarly, Dilution of samples, centrifugal force and snap chilling time were evaluated to get possible outcome.

DNA purity and concentration

The characteristics of DNA isolation procedure were checked by evaluating the purity and concentration of extracted DNA. The quantity of DNA was measured in spectrophotometer by

recording the concentration and the ratio of 260/280 nm wavelength for single stranded DNA. Qualified samples only used for further experiment.

Time duration

The whole procedures of DNA isolation methods were noted with time duration requirement from faecal sample processing to DNA extraction. The average time taken in the above protocols was recorded to summarize the rapid method.

Polymerase Chain Reaction (PCR) to check diagnostic efficacy

PCR reaction was carried out with primer set used by Perriera *et al.* (2000) [8] where obtaining of 681 bp amplicon by using forward primer (F) 5'-GAAGAGTGGTTGTAAATAATA-3' and reverse primer (R) 5'-CCTATATCACCAAAGTTAGTAG-3' was considered as positive reaction. The reaction mix contain 12.5 µl master mix (*Taq* PCR master mix kit, Qiagen), one µl of each forward and reverse primer (10 pmol/ µl, working concentration of primers), 2 µl of DNA isolated by conventional and kit method whereas 4 µl DNA from Boiling-Snap Chilling method and remaining nuclease free water to make 25 µl. The initial denaturation on 95 °C for 7 min, denaturation, annealing and extension temperature/time combinations was 94 °C/30 sec, 48 °C/1 min and 72 °C/1 min for 35 cycles. Then final extension was carried out at 72 °C for 10 min. The gel electrophoresis was done by using 1.5% agarose, stained by ethidium bromide dye, DNA ladder as a marker and run at constant 80 V for 1 hour.

Results and Discussion

Boiling-Snap chilling method is standardized in department of veterinary microbiology. Systematically protocol is following. The heating and chilling plate first set on 95 °C. faecal samples were processed first by diluting in 1:5 with Phosphate buffer saline (PBS) and vortexed well for 2 min. Then after, a volume of 200 µl processed faecal sample in PBS/ Hank's Balance salt solution (HBSS) was heated at 95° C for 5 minutes and snap chilled on ice for 5 min. Then the tube was centrifuged for 5 min at 2500 rpm. Supernatant was used as DNA template for molecular detection.

Results of evaluation of three methods for Viral DNA extraction in terms of repetition, time duration, average DNA concentration and PCR positivity by three methods were described in table 1, 2 and 3. Comparison in terms of duration is mention in table 4. PCR positivity comparison of samples depicted in figure 1.

Table 1: PCI method of DNA isolation parameters

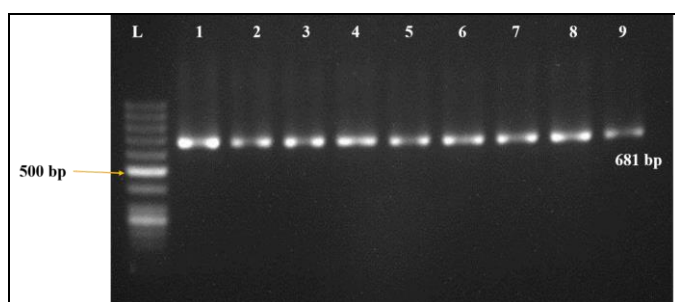
S. no	Attempt No	Time taken	Avg. DNA Conc. (ng/µl)	PCR Positivity
1	1	17 hours 50 min	40.774	3/3 samples
2	2	17 hours 20 min	28.53	3/3 samples
3	3	18 hours 30 min	55.51	3/3 samples
4	4	17 hours 10 min	47.61	3/3 samples
5	5	17 hours 35 min	48.26	3/3 samples
6	6	17 hours 55 min	45.23	3/3 samples

Table 2: Kit method of DNA isolation parameters.

S. no	Attempt No	Time taken	Average DNA Conc.(ng/ul)	PCR Positivity
1	1	1 hour 10 min	Insignificant	0/3 samples
2	2	57 min	Insignificant	0/3 samples
3	3	1 hour 2 min	8.59	1/3 samples
4	4	55 min	53.95	3/3 samples
5	5	1 hour 10 min	16.94	3/3 samples
6	6	1 hour 20 min	23.14	3/3 samples

Table 3: Boiling-Snap chilling method of DNA isolation parameters.

S. no	Attempt No	Time taken	Average Conc.(ng/ul)	PCR Positivity
1	1	25 min	29.45	3/3 samples
2	2	20 min	58.45	3/3 samples
3	3	19 min	88.34	3/3 samples
4	4	24 min	82.334	3/3 samples
5	5	20 min	65.42	3/3 samples
6	6	25 min	72.37	3/3 samples

**Fig 1:** CPV positive samples amplicon size 681 bp from three methods. L: 50 bp Ladder, 1-3: CPV DNA isolated by PCI method, 4-6 CPV DNA isolated by Boiling-Snap chilling method and 7-9 CPV DNA isolated by Kit method.**Table 4:** Comparison of three methods

Average time taken in DNA isolation		
PCI method	Commercial kit method	Boiling-Snap chilling method
17 hours 34 min	67 min 28 sec	22 min 8 sec

PCI method is time consuming, on an average it takes 17 hours 34 min to isolated DNA. Whereas, 67 min 28 sec and 22 min 8 sec in commercial kit method and Boiling-Snap chilling method respectively. Average concentration of DNA yield is high in PCI method^[5] because of isolation of DNA from faecal samples, hence it isolate all faecal microbial DNA^[3]. The same way in Boiling-Snap chilling method, whereas DNA concentration and 260/280 ratio varied due to faecal microbial DNA, protein and other interference. Kit method has relatively optimum level of DNA concentration as well as ratio because of purity. Kit method extract highest pure DNA, but yield may be less or vary due to the loss during steps^[4]. Boiling-Snap chilling method is very cost effective and rapid. Faecal samples have various digestive enzymes residues, bile salts, bile pigments and impurities of faeces. Hence, enzymes might be interfering to the viral DNA as well as DNA polymerase enzyme, which might not allow amplifying in PCR. For the inactivation of the enzymes, it must require to boil the samples. Boiling will do the inactivation of enzymes; even further dilution will not interfere with the DNA polymerase enzyme. Sometimes, if there is presence of faecal residues while performing the DNA isolation method, it may cause variation in result due to

interference. That's why initial dilution must be required. In conclusion, Boiling-Snap chilling method is best suitable among all method for the CPV DNA isolation from the faecal sample. It is very easy, rapid and cost effective method to do molecular based confirmative diagnosis.

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