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Full Length Research Paper

Detection of canine parvovirus 2: A comparison of conventional polymerase chain reaction and haemagglutination test

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Several laboratory techniques have been adopted for detection of canine parvovirus (CPV) but the big question has been on their accuracy, speed, cost and availability. This study was meant to compare conventional polymerase chain reaction (PCR) and haemagglutination (HA) test in the detection of CPV-2. PCR targeted the VP2 gene while HA targeted the haemagglutinin all in the virus. 113 samples comprising 80 rectal swabs and 33 necropsy tissues from dogs showing symptoms that were suggestive of parvovirus enteritis were collected from veterinary clinics in Jos. HA test detected the virus in 86 (76.11%) of the samples while PCR confirmed the virus in 62 (54.87%) of the total samples. Information from questionnaires administered revealed that only 23 dogs had been vaccinated against the agent, 79 dogs had not been vaccinated while 11 were uncertain about the vaccination status of the dogs. Interestingly, HA test revealed that 17 (73.91%) of the already vaccinated dogs had the virus while PCR detected the agent in 14 (60.87%) of these dogs. All PCR-positive samples were also positive by HA test but not all HA-positive tests were positive by PCR technique. Chi-square analysis showed that there was no significant difference (P > 0.05) between the results of HA test and PCR. These findings have confirmed that HA test could be employed for the preliminary screening of the agent in less endowed facilities in view of its low financial cost, turnover time and sensitivity.

Key words: Haemagglutination (HA) test, polymerase chain reaction (PCR), dogs.

INTRODUCTION

Canine parvovirus (CPV) is a highly contagious virus mainly affecting dogs. The disease, commonly called parvovirus enteritis is highly infectious and is transmitted from dog to dog by direct or indirect contact by contaminated feaces (Appel et al., 1979).

The virus is a small, non-enveloped single stranded deoxyribonucleic acid (DNA) virus belonging to the parvoviridae family and parvovirus genus under the parvovirinae subfamily (Hong et al., 2007). The virus, which first appeared in 1977/ 1978, probably arose from a very closely related virus in cats, feline panleukopaenia virus (FPLV) through a small number of mutations in the single capsid protein; a species jump which may have involved intermediate passage in other carnivores such as mink or raccoons (Truyen et al., 1996). As early as 1979, the first variants of CPV2 appeared, termed CPV2a, and they were quickly followed by the appearance of CPV2b in 1984 (Parrish et al., 1985, 1991). The original type 2 virus has now disappeared widely having been replaced by the 2a and 2b variants; although the relative proportions of these two types varies from country to country (Truyen et al., 1996;

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Table 1. Distribution of samples as collected from various sampling points.

Vet clinic	Tissue	Swab	Total
NVRI	11	29	40
ECWA	07	28	35
State	08	12	20
Kufang	03	03	06
Venus	04	03	07
Market	NIL	05	05
Total	33	80	113

Chinchkar et al., 2006; Pereira et al., 2007).

Laboratory diagnosis is made through detection of CPV-2 in the feces by either an enzyme immunoassay (EIA) or a hemagglutination test, or by electron microscopy. PCR has become available to diagnose CPV-2, and can be used later in the disease when potentially fewer viruses are being shed in the feces that may not be detectable by enzyme immunoassay (Silverstein, 2003).

The simplest procedure for the laboratory diagnosis of canine parvovirus infection is haemagglutination of pig or rhesus monkey erythrocytes (pH 6.5, 4°C) by virus present in feacal extracts (Kapil et al., 2007). The specificity of this haemagglutination is determined by titrating the fecal specimen in parallel in the presence of normal and immune dog serum. Fecal samples from dogs with acute enteritis may contain up to 20,000 haemagglutination units of virus, equivalent to about 10^9 virions, per gram. Electron microscopy, virus isolation, enzyme immunoassay, and amplification of viral DNA using the polymerase chain reaction are also used for laboratory confirmation of clinical diagnosis (Decaro et al., 2005, 2006). Serological diagnosis is also used in some settings, with the IgM-capture immunoassay used to determine recent infection (Parker et al., 2001).

MATERIALS AND METHODS

Samples and sample collection

Samples were collected from veterinary clinics in Jos, Plateau State in Nigeria. The sampling points are as presented in Table 1. A total of 113 were collected for the research, 80 of which were rectal swabs while thirty 33 were necropsy tissues. The rectal swabs were collected from dogs presenting with signs suggestive of parvovirus enteritis. Swab sticks were inserted into the anal cavity of the dog and turned clockwise. It was gently withdrawn and transferred into a virus transport medium. Necropsy tissues of target organs such as intestine, heart and lymph nodes were harvested from dogs that died with signs suggestive of parvovirus enteritis. The harvested organs were placed in universal sample bottles. Samples were labeled appropriately and conveyed to the laboratory in cold packs. Dogs of all age and breed were included in the study.

Questionnaires

A questionnaire was prepared and presented to owners of dogs included in this study which they completed on an interview basis. This was meant to retrieve information on age and breed of dog, onset of illness, clinical presentation as well as vaccination history.

Haemagglutination (HA) test

10% tissue suspension was made with phosphate buffered saline pH 7.4 as earlier described by Kapil et al. (2007). They were spun along with rectal swabs suspended in VTM at 14000 rpm for 3 min. The supernatant was then collected and used for haemagglutination test. Porcine erythrocytes were collected from healthy pigs from the ear vein into an anticoagulant container. The cells were washed by adding normal saline and centrifugation at 3,000 rpm for 5 min. This was repeated two times and the cells were constituted to a final concentration of 10% from which 1% was reconstituted for the HA test (Kapil et al., 2007).

DNA extraction

Total DNA was extracted using the QIAamp® DNA Mini Kit according to manufacturer's specifications. Different procedures were adopted for DNA extraction from rectal swabs and necropsy tissues as specified by the manufacturers.

CPV-2 specific master mix

The master mix contained 13.88 µl of nuclease free water (Promega®), 2.5 µl of 10 × PCR reaction buffer, 0.62 µl of dNTP mixture (10 mM) (Fermentas®), 1.5 µl of magnesium chloride (25 nM), 0.5 µl each of forward and reverse primers (555 forc and 555 revc, respectively) for CPV2 (Inqaba Biotech SA®) at 20 pmol/µl and 0.5 units of Taq DNA polymerase (Qiagen®). This gave a total volume of 20 µl master mix.

5 µl of the CPV-2 master mix (above) was added to 5 µl extracted DNA in a new tube for amplification.

Amplification of DNA extracts

The mixture was submitted to a thermal cycling profile of initial denaturation at 94°C for 5 min. This was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 2 min, extension at 72°C for 2 min and 72°C for 10 min in an AppliedBiosystem® 9700 PCR machine.

The primer sequences used were designed by Inqaba Biotech SA®. The sequences are follows:

555forc 5'-CAGGAAGATATCCAGAAGGA-3'
555rev 5'- GGTGCTAGTTGATATGTAATAAACA-3'

Table 2. Percentage positivity of samples based on breed of Dog.

BREED	HA Positive (%)	PCR Positive (%)
Rottweillers (*n*=28)	26 (92.86)	20 (71.43)
Doberman pinchers (*n*=36)	33 (91.67)	24 (66.67)
Pit bull (*n*=14)	10 (71.43)	07 (50.00)
Tan coloured (*n*=04)	03 (75.00)	02 (50.00)
Local (*n*=28)	11 (39.29)	07 (25.00)
Unidentified (*n*= 03)	03 (100)	02 (66.67)

Table 3. Percentage prevalence according to sample type.

Sample type	HA positive (%)	PCR positive (%)
Swabs (*n*=80)	55 (68.75)	36 (45.00)
Tissues (*n*=33)	31 (93.94)	26 (78.79)
Total (T= 133)	86 (76.11)	62 (54.49)

$\chi^2 = 1.159$, DF= 1, P > 0.05.

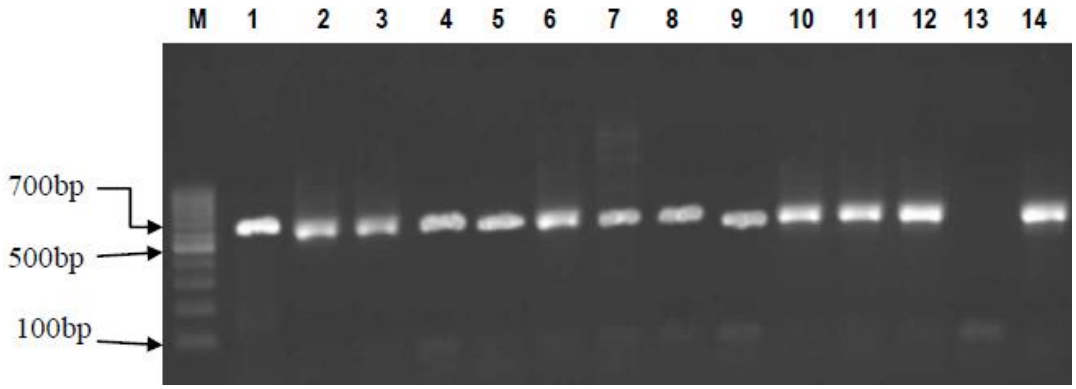

Figure 1. Gel electrophoresis of amplicons of cpv-2 dna from tissue samples. KEY: M = marker, 1-12 = field samples, 13 = negative control, 14 = positive control.

The master mix composition and PCR program was as described by Streck et al. (2009) but with minor modifications.

Gel electrophoresis

10 µl of PCR amplicons were electrophorosed in a 1.5% agarose gel stained with ethidium bromide in the presence of 2 µl of loading buffer (Fermentas®). Positive and negative controls were included. The electrophoresis was carried out at 80 volts for 50 min.

Statistical analysis

Chi-square was used to test for significance in prevalence of CPV-2 among breed of dogs and between the two diagnostic tools under investigation with P-value at 0.05.

RESULTS

HA test showed that 86 (76.11%) of the samples were positive for CPV while conventional PCR revealed that 62(54.49%) of the samples were positive for the virus. Analyses of the results based on breed of the examined dogs showed that the foreign breed of dogs had higher positivity for the virus than the local breeds. Rottweillers, Doberman pinchers and Tan coloured dogs all showed higher carriage capacity of the agent than the local breed of dogs. In each case, HA test gave higher rate of detection of the agent than conventional PCR (Table 2).

Analysis of the various samples showed that 55 (68.75%) of rectal swabs were positive by HA while 36 (45.00%) were positive by conventional PCR. Also, HA test revealed that 31 (93.94%) of necropsy tissues were positive for the agent as against 26 (78.79%) by conventional PCR (Table 3). However, the bands of PCR products from tissues were sharper than those from rectal swabs (Figures 1 and 2).

Responses from questionnaires revealed that only 23 (20.35%) of the dogs had history of previous vaccination

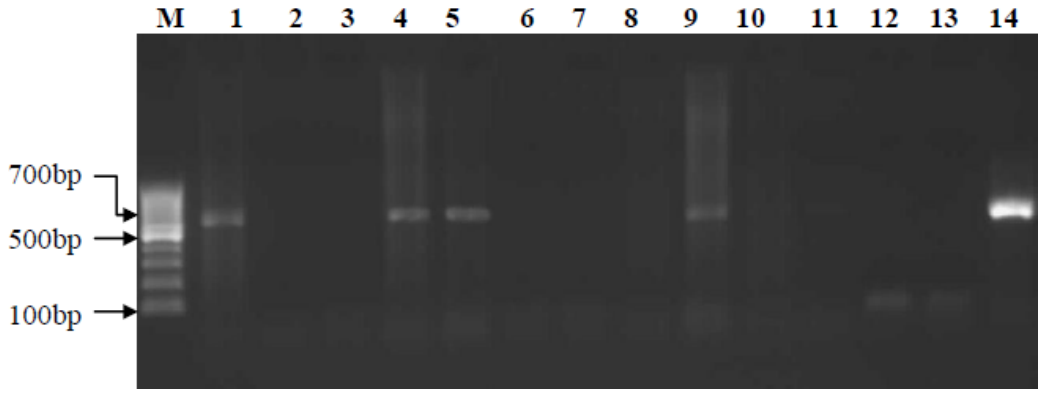

Figure 2. Gel electrophoresis of Amplicons of CPV-2 DNA from rectal swabs. KEY: M = marker, 1-12 = field samples, 13 = negative control, 14 = positive control.

Table 4. Percentage prevalence accorging to vaccination history.

Vaccination history	HA positive (%)	PCR positive (%)
Vaccinated dogs (*n*=23)	64 (81.01)	14(60.87)
Unvaccinated dogs (*n*=79)	5 (45.45)	53(67.09)
Uncertain (*n*=11)	64 (81.01)	6 (54.55)
total (T=113)	86 (76.11)	73 (64.60)

against the agent, 79 (69.91%) had not been vaccinated while 11 (9.73%) had uncertain vaccination history. However, HA test detected the virus in as much as 17 (73.91%) of the already vaccinated dogs while PCR detected the agent in 14 (60.87%). The rate of detection of the agent in all categories of dogs and samples was higher by HA test than it was by PCR technique with the exception of the outcome from dogs with uncertain vaccination history (Table 4).

Although all PCR positive samples were also positive by HA test, not all HA positive samples were positive by PCR technique. In all, chi square analysis showed that there was no significant difference ($P > 0.05$) in the number of positive samples obtained by the two diagnostic tools.

DISCUSSION

Canine parvovirus continues to be an important pathogen of dogs and is responsible for serious occurrences of morbidity and mortality, despite the availability of safe and effective vaccines (Decaro et al., 2006a, b).

The two diagnostic tools gave different values of the prevalence rate of the disease in the area. HA test showed a prevalence of 76.11% while conventional PCR gave a prevalence rate of 54.49%. However, chi-square analysis showed that there was no significant difference between the two values. The seeming high rate of detection of the agent by HA compared to PCR technique is largely due to the specificity of the former against the later. This is because of all the diagnostic tests available, PCR has been found to be more specific (Harasawa et al., 1993; McColl et al., 1993). As such, it is safe to conclude that HA test over-diagnosed the agent leading due to its low specificity.

Foreign or improved breeds of dogs were more susceptible to the disease when compared with their local counterparts. This fact has already been established (Nelson and Couto, 1998).

Again, HA test detected that 31 (93.94%) of tissues were positive for the virus while conventional PCR detected the agent in 26(78.78%) of tissue samples. This is higher when compared with 55(68.75%) HA positive and 36(45.00%) PCR - positive rectal swabs. This discovery is not surprising considering the fact that the targeted organs had earlier been fingered as the predilection sites of the agent (Lobetti, 2003). Also, the fact that the tissues were from animals suspected to have died of the disease means the concentration of the viruses in these sites will naturally be higher than those from rectal swabs collected from dogs with gastroenteritis.

Another interesting part of the result is the questionnaire information. Only 23 (20.35%) of the dogs sampled had been vaccinated against CPV. A whooping 79 (69.91%) had not had vaccination while 11(9.75%) were not sure if the vaccinations they received earlier was against the agent or another agent entirely. These findings are not encouraging at all. The responses

showed that more than half of the population sampled did not know of the vaccination schedules while about ¾ of dog owners thought issues of animal health were the least in their priority list. The implication of this is that zoonotic diseases can easily spread within the population while at the same time maintaining a perfect ecology for emerging and re-emerging infectious diseases. The government and health extension officers also have not done much in public awareness programs.

From the 23 vaccinated dogs, HA test revealed that 17(73.91%) while conventional PCR showed that 14(60.87%) still came down with the wild virus. Although this sample size is too small to analyze vaccine failure, it will also be safe to say that most of the vaccines in use around here were formulated with the original CPV-2 type. These have been found to confer protection against the variants of CPV-2(Parrish et al., 1991). Also, the possibility that the vaccines in circulation could be contaminated by wild type CPV should not be overlooked (Senda et al., 1988; Sweet and Hilleman, 1960; Yuasa et al., 1976). As a live vaccine, CPV-2 vaccines could easily lose their potency due to poor handling.

In general, all PCR-positive samples were also positive by HA test but not all HA-positive samples were confirmed positive by conventional PCR. This indicates to a large extent the high sensitivity but low specificity nature of the HA test when compared to the highly sensitive and highly specific nature of the PCR technique.

The interesting side of the story is that statistically, there was no difference in the values obtained from the two diagnostic tools investigated. The findings therefore infer that the two diagnostic tools could be used interchangeably for detection of CPV for purposes of research since the variations in sensitivity and specificity to the agent are acceptable within scientific limits. However, in diagnosing CPV in clinical cases, the HA test should not be used all alone to avoid incidences of false negativity in results. Rather, it should be run as a preliminary investigation to define a line of treatment before confirmation with a more specific technique such as PCR.

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