CONTENMENT OF DIAGNOSTIC ASSAYS FOR DETECTION OF CANINE PARVOVIRUS AND DETERMINATION OF BACTERIAL CO-INFECTION IN DIARRHOEIC PUPS

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Canine parvovirus-2 (CPV-2) is a serious entero-pathogen that affects the canine population worldwide by causing vomiting and canine parvoviral enteritis (CPVE). In this study, out of the 100 fecalsamples collected in duplicates from diarrheic pups, 10 (10%) and 20 (20%) were found positive by haemagglutination test and antigen detection test. PCR assay of the collected samples revealed that 38(38%) samples were positive for CPV 2. The prevalence of CPV infection concerning various age groups, gender, breed and vaccination status varied significantly. Secondary bacterial co-infection was also investigated primarily caused by *E. Coli* (71.05%) and *Salmonella*. (7.89%). Antibiotic sensitivity test revealed maximum sensitivity towards amikacin (100%) and enrofloxacin (100%) whereas metronidazole was found to be resistant (100%).

Keywords: Antigen test, AST, Canine parvovirus, Co-infection, HA test, PCR.

Canine parvovirus (CPV-2) is a member of the *Parvoviridae* family, *Parvovirinae* subfamily, and *Protoparvovirus* genus (Khatri *et al.,* 2017). It results in acute hemorrhagic gastroenteritis and acute myocarditis in dogs. CPV-2 has a linear, negative-sense, single-strand DNA of approximately 5.2 kb size, and it encodes two structural (VP1 and VP2) and two non-structural (NS1 and NS2) proteins (Chen *et al.,* 2019; Mira *et al.,* 2019). Five different antigenic variants of CPV-2 namely CPV-2a, CPV-2b, new CPV-2a, new CPV-2b, and CPV-2c with mutation in amino acids primarily in the capsid VP2 protein have emerged over the years as a result of the genetic diversity of the virus (Singh *et al.,* 2019). Younger dogs primarily between 6 weeks-6 months of age are affected by CPV-2 infection (Singh *et al.,* 2022).Various diagnostic tests such as hemagglutination tests, antigen detection tests, and PCR assays have been developed to rapidly and accurately diagnose emerging CPV infection in pups. Secondary bacterial infections of the gastrointestinal tract, which may be followed by bacterial translocation, bacteremia, and endotoxemia, play a key role in the pathogenesis of the disease. In the course of canine parvovirus infection, several infections (parasitic, viral, or bacterial intestinal pathogens) are found to be common due to immunosuppressive condition brought on by CPV-2, exacerbating the overall clinical picture (Tuteja *et al.,* 2022; Mylonaki *et al.,* 2016). Among secondary bacteria, entro-pathogens such as *E. coli*are considered one of the major pathogens and are found asnatural commensals in the gastrointestinal tract.This study was based on a set of experiments to detect CPV-2 in diarrhoeic dogs and to detect the presence of secondary bacterial infection if any, responsible for aggravating the prognosis of suffering pups.

Materials and Methods

This study was carried out between 2021-2022 in the laboratory of the Department of Veterinary Microbiology and Animal Biotechnology, T&R Cell, Nagpur Veterinary College, Nagpur.

Haemagglutination test and Antigen detection test:

The HA test was performed as per OIE. HA, titer of 32 and above was considered as positive for CPV antigen. The Ubioquick VET *Canine parvovirus* antigen rapid test kit was used for the detection of CPV-2 in diarrheic dogs.

DNA isolation and Conventional PCR:

DNA was extracted from faecal samples by using an HiMedia DNA isolation purification kit as per manufacturer instructions.The viral DNA extract obtained was amplified by PCR assaying VP2gene-specific primers (Table 1). The reaction was set up in the following manner: 2X PCR master mix, 12.5µl; forward primer and reverse primer, 2µl; NFW, 6.5 µl, and Template DNA, 2 µl followed by cycling conditions as initial denaturation at 94^{o}C for 5 min; denaturation at 94^{o}C for 1 min; annealing at 50□ for 2 min; extension at 72^{o}C for 2 min and final extension at 72^{o}C for 10 min. The PCR products obtained were resolved on 1% agarose gel by electrophoresis.

Table 1: PRIMERS TARGETING THE VP2 GENE OF CPV-2

Name of Primer	Primer sequence (5'-3')	Product size
CPV-2ab-F	GAAGAGTGGTTGTAAATAATT	681bp
CPV-2ab-R	CCTATATAACCAAAGTTAGTAC	

Bacteriological isolation and biochemical characterization:

Following confirmation for CPV by PCR, positive swab samples were inoculated in sterile nutrient broth and incubated at 37□ for 18 hours. For culturing of the enriched samples, MacConkey agar was utilized for differentiation of lactose fermenting and lactose non-fermenting bacterial species followed by selective isolation on EMB agar and brilliant green agar.

Polymerase Chain Reaction of E coli isolates:

Following DNA isolation from pure culture colonies by the kit, the DNA samples were subjected toPCR targeting the *stx2* gene of *E. coli.* The forward and reverse primers utilized in this study are depicted in (Table 2).The reaction was set up in the following manner: PCR master mix, 12.5µl; forward primer and reverse primer, 1µl; NFW, 7.5 µl, and Template DNA, 3 µl followed by cycling conditions as initial denaturation at 94^{o}C for 5 min; denaturation at 94^{o}C for 1 min; annealing at 54□ for 1 min; extension at 72^{o}C for 2 min and final extension at 72^{o}C for 10 min. The PCR products obtained were resolved on 1% agarose gel by electrophoresis.

Table 2: PRIMERS TARGETING *STX2* GENE OF *E. coli*

Name of Primer	Primer sequence (5'-3')	Product size
LP43	ATCCTATTCCCGGGAGTTTACG	584 bp
LP44	GCGTCATCGTATACACACAGGAGE	

Antibiotic Sensitivity test:

The pure colonies were inoculated on the nutrient broth and incubated at 37□ for 18-24 hours after determining growth with the help of and thereafter was subjected to inoculation on MHA agar (Muller-Hinton) followed by placement of antibiotic discs. The plates were then incubated at 37□ for 24 hours followed by an investigation of zones of inhibition i.e.,

Senstive (S), Resistant (R), or Intermediate (I) following the ranges given in the chart in accordance with CLSI standards. The different antibiotics used in this study were as follows: Amoxycillin-Sulbactam (30/15 mcg), Amikacin (10 mcg), Cefotaxime-Clavulanic acid (30/10 mcg), Ceftriaxone-Tazobactam (80/10 mcg), Ceftriaxone (10 mcg), Enrofloxacin (10 mcg), Furazolidone (50 mcg) and Metronidazole (5mcg).

Results and Discussion

In this test, 100 samples collected from diarrhoeic pups were subjected to the Haemagglutination test out of which (10/100; 10%) were found to be positive with a titre range of 32-256 HAU depicted in (Fig 1). Similar results were obtained by Nair, 2022, who screened a total of 50 samples by HA test, of them 05 (10%) samples agglutinated porcine RBCs with a titer ranging from 32-256. The results obtained by the Antigen detection kit in our study revealed that 20 (20%) samples were positive for CPV-2 and the findings obtained (Fig 2).

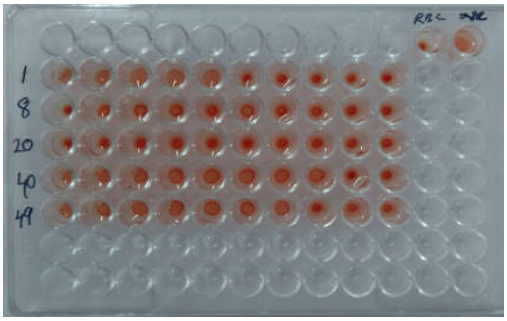


Fig 1: Haemagglutination Test of CPV with porcine RBCs
Lane 1:RBC and virus control
Lane 2:Sample showing matt formation with positive titre of 32 HAU
Lane 3,4:Sample showing matt formation with positive titre of 64 HAU
Lane 5:Sample showing matt formation with positive titre of 256 HAU
Lane 6:Sample showing matt formation with positive titre of 128 HAU

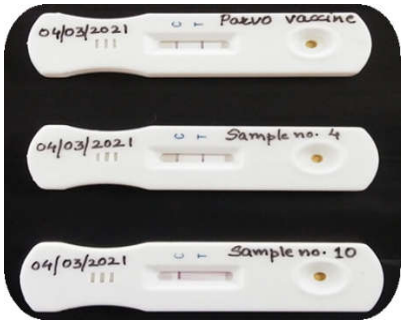
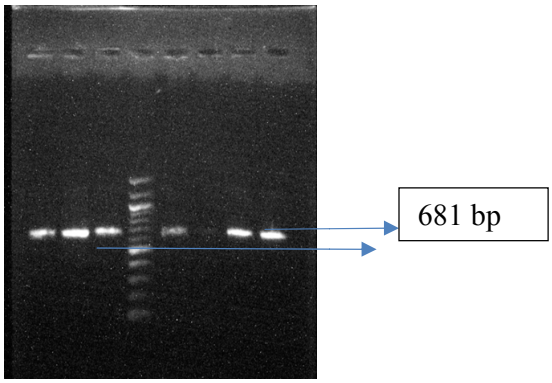


Fig 2: Ubioquick VET *Canine parvovirus* antigen rapid test kit showing positive and negative reaction for CPV antigen along with commercially available parvo vaccine.
Both "C" and "T" line show- Positive
Only "C" line show – Negative

Conventional PCR revealed that (38/100; 38%) samples were positive for PCR targeting the VP2 gene confirming the presence of Canine parvovirus depicted in (Fig 3). It was observed that the sensitivity of molecular assays such as PCR was greater for the detection of CPV-2 in comparison to HA and Antigen detection tests. Similar findings were observed in studies by Dhas, 2023. Prevalence detected by PCR assay based on age, sex-wise, breed and vaccination status revealed to be 0-3 (48%) age group followed by 3-5 (33%) and then above 5 months of age (20%), males (45%) were more infected than females (27.50%), crossbreed (49%) having higher CPV infection than others and unvaccinated (48%) dogs were found to be more positive than vaccinated dogs (22.50%) in the current study (Fig. 5).

1 2 3 4 5 6 7 8

1 2 3 4 5 6 7 8



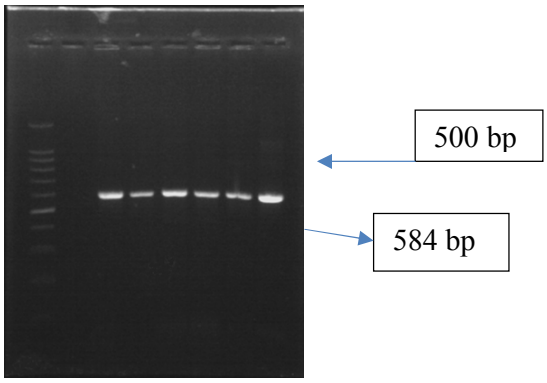


Fig.3: Amplification of VP2 gene of *Canine parvovirus* -2 by PCR. **Lane 1:-Sample No.24** **Lane 2:-Positive control (Vaccine)** **Lane 3:- Sample No.37** **Lane 4:- Ladder 100 bp**	**Fig. 4: Amplification of *stx*2 gene of *E.coli*by PCR.** **Lane M :- Ladder 100 bp** **Lane 1 :- Blank** **Lane 2 :- *E.coli* reference strain *stx*2 (584 bp)** **Lane 3 :- Sample no.24**
Lane 5:- Sample No.45 **Lane 6:- Negative control** **Lane 7:- Sample No.51** **Lane 8:- Sample No.70**	**Lane 4 :- Sample no.37** **Lane 5 :- Sample no.45** **Lane 6 :- Sample no.5**

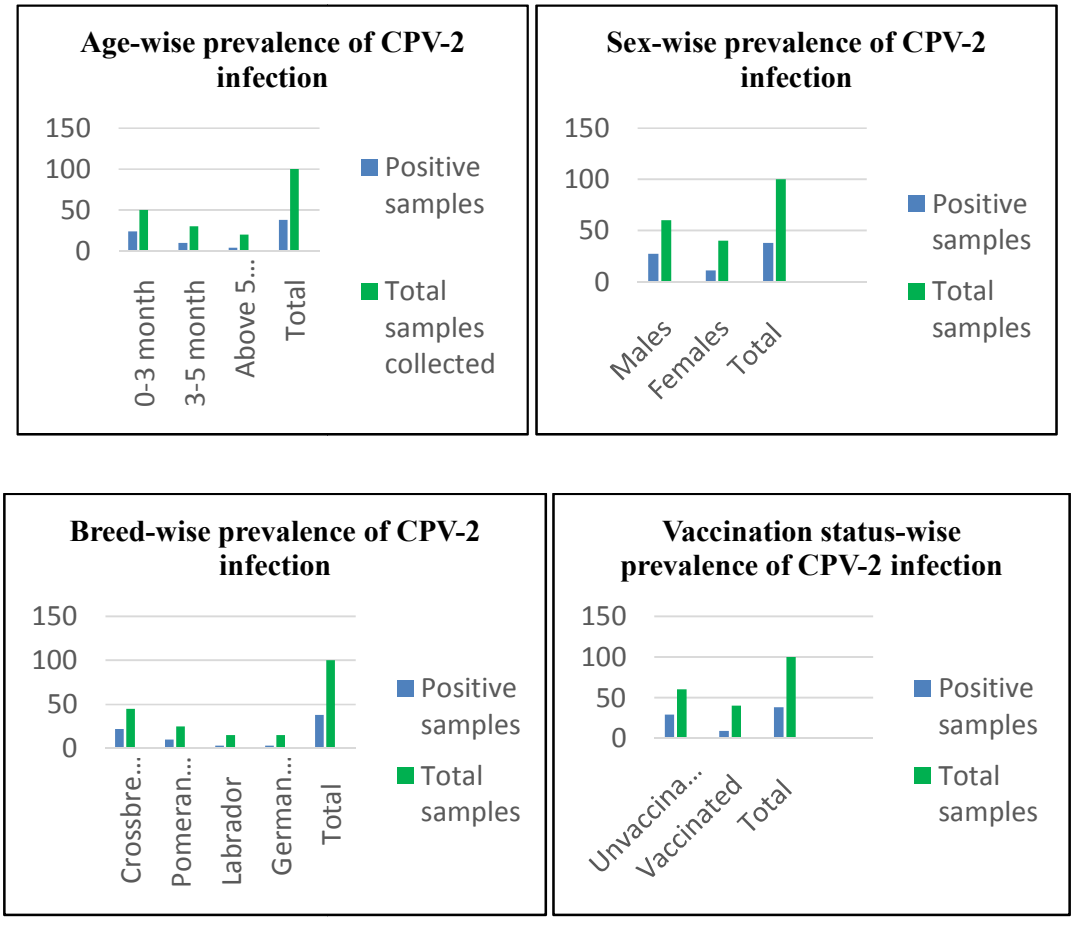


Fig.5: Prevalence of *Canine parvovirus* based on age, sex, breed and vaccination status

Bacteriological evaluation of possible co-infection aggravating the cases of *Canine parvovirus* showed that the major microorganisms arising as a factor influencing the health status of pups were *E. coli* (27/38; 71.05%) followed by *Salmonella* spp. (3/38, 7.89%) and solo infection with CPV as (8/38; 21.05%). The colony morphology of bacterial isolates is depicted in (Fig.6 and 7). The gene-specific PCR targeting the *stx2* gene revealed that (15/27; 55.55%) samples were confirmed positive for the same (Fig. 4). Findings by Magar *et al.,* 2020, were in agreement with the results obtained in our study,.

Antibiotic sensitivity testing of the isolates revealed that the most sensitive drugs showing significant zones of inhibition were amikacin (100%) and enrofloxacin (100%) followed by ceftriaxone-tazobactam (80%), cefotaxime-clavulanic acid (60%) and furazolidone (50%). The least sensitivity was seen by ceftriaxone (30%) and amoxycillin-sulbactam (20%) antibiotics, while the bacterial isolates were resistant to metronidazole (100%) depicted in (Fig.8).Similar results were seen in study by Schiro *et. al.*, 2022.

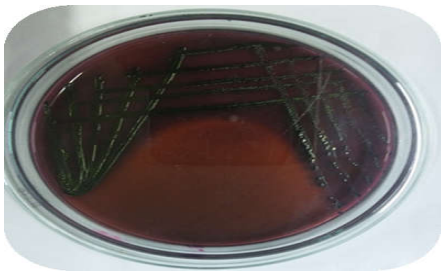


Fig.6: Colonies of *E. coli* exhibiting greenish metallic sheen on Eosin Methylene Blue agar

Fig. 7: Colonies of *Salmonella spp.* on Brilliant green agar

Fig.8: Antibiotic sensitivity depicting zones of inhibition

Conclusions

This study concluded that the molecular assay such as PCR was more sensitive compared to conventional assays such as HA and Antigen detection tests. Moreover, a bacterial co-infection was detected in diarrheic pups suffering from CPV infection, revealed to be caused predominantly by *E. coli* followed by *Salmonella spp*. implying the positive co-relation of CPV with secondary bacterial co-infection due to immunosuppression. AST results revealed amikacin as well as ciprofloxacin to be the effective treatment strategy for secondary bacterial co-infection while metronidazole was revealed to be resistant.

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