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Clinical Signs and Laboratory Markers Compete Favorably with Antigen Detection of Canine Parvovirus-2 in Dogs

Olatunde Babatunde Akanbi[1,2*], Fagbohun Olusegun[3], Aderonke Rachael Kolawole[1], Olanrewaju Samuel Olaifa[1], Victor Aliyu[3], Pius Stephen Ekong[4], Oluwafemi Babatunde Daodu[5], James Olopade[6], Victor Olusegun Taiwo[1].

Corresponding email: akanbi.ob@unilorin.edu.ng

[1]Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria
[2]Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ilorin, Nigeria
[3]Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria
[4]Diagnostic Department, Veterinary Public Health and Preventive Medicine Division, National Veterinary Research Institute, Vom, Plateau State, Nigeria
[5]Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Ilorin, Nigeria
[6]Veterinary Teaching Hospital, Faculty of Veterinary Medicine, University of Ibadan, Nigeria

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Abstract

Thirty dogs (< 1 year) which reported to the Veterinary Teaching Hospital, State Veterinary Hospital and City Veterinary clinic, Ibadan between October 2023 and February 2024 and had clinical signs of depression, vomiting, diarrhea, anorexia, and fever were tested for canine parvovirus-2 by rapid antigen test and confirmed by PCR. Clinical signs (CS) presumptively diagnosed and CPV positive antigen tested dogs were recruited. A 13.33% infectivity rate was recorded in 4-weekolds and 20% in adult dogs between 7 and 12 months of age contrary to belief of parvoviral enteritis in dogs above 6 weeks. A combination of clinical signs in 28 of 30 dogs, 93.3% (95% CI: 77.9-98.2%, $p \leq 0.9918$), were positive for CPV-2, and laboratory markers (LM) including leukopenia seen in 90% of CPV infected and thrombocytopenia observed in 100% of CPV infected dogs (95% CI: 88.4 - 100.0%, $p \leq 1.000$) were confirmed positive for CPE, against 100.0% antigen detection (95% CI: 88.4 - 100.0%, $p \leq 1.000$) in CPV infected. Therefore, the Clinical Signs and Laboratory Markers (CSLM) method for diagnosing CPE competes favorably with rapid antigen detection of Canine Parvovirus-2. The CSLM method can be explored as an alternate diagnostic tool in resource limited environments.

Keywords

Antigen detection, Clinical signs and Laboratory markers, CSLM, Dogs, Parvoviral Enteritis

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Introduction

Canine parvovirus (CPV) type 2, the causative agent of acute hemorrhagic enteritis and myocarditis in dogs, is one of the most important pathogenic viruses (Nandi and Kumar, 2010). It is a highly contagious and often fatal disease of dogs (Chen *et al*., 2019) throughout the world. The disease is highly infectious and is spread from dog to dog by direct or indirect contact with their feces.

Although inactivated and live-attenuated CPV vaccines, both monovalent and along with vaccines against other diseases, have been developed and used for the control of the disease, vaccine failures have been reported due to the presence of maternal antibodies and the emergence of new variants despite proper vaccination of animals (Decaro, 2020). Over the years, several diagnostic assays, both serological and molecular, have been developed for prompt, precise, and sensitive diagnosis of the disease. Rapid immunochromatographic tests for CPV are based on the detection of CPV-specific antigens present in canine fecal samples (Sundaran *et al*., 2015). These tests typically involve the use of monoclonal antibodies conjugated to colloidal gold nanoparticles, which interact with the viral antigens. The appearance of visible colored lines indicates a positive result. The sensitivity of rapid immunochromatographic tests has shown considerable variation across different studies, ranging from 80% to 100%. Factors influencing sensitivity include the viral load in the sample, the stage of infection, and the quality of the test kit (Shahrul *et al*., 2021). The specificity of rapid immunochromatographic tests for CPV is generally high, with minimal cross-reactivity observed with other pathogens.

CPV rapid antigen testing was necessary in the recruitment of CPV-infected dogs from the Veterinary Teaching Hospital, University of Ibadan, for this study because clinical signs alone are inconclusive in the diagnosis of canine parvoviral enteritis (CPE), as several other viral pathogens, including coronaviruses, adenoviruses, morbilliviruses, rotaviruses, reoviruses, and noroviruses, can also cause diarrhea in dogs (Nicola and Canio, 2012); however, canine parvovirus is the number one viral cause of puppy enteritis and mortality (Shabbir *et al*., 2009). Polymerase chain reaction (PCR) is known to show the highest sensitivity compared to traditional methods of hemagglutination or virus isolation (Desario *et al*., 2005), but is expensive to run, thereby increasing diagnostic cost. While quantitative real-time (q)PCR is the gold standard in the diagnosis of CPV infection (Desario *et al*., 2005; Decaro *et al*., 2005; Decaro *et al*., 2013), qPCR can only be performed in specialized laboratories, which are oftentimes not available in the developing world and in the developed world not easily found nearby, hence this delays the diagnosis of CPV. This study, therefore, aims to establish a combination of sensitive and specific clinical and laboratory markers to predict the occurrence and diagnose canine parvovirus infection in dogs, which is comparable to the rapid tests and cost-effective for resource-limited countries.

Materials and Methods

Clinical data collection and consent

Upon the arrival of the pet to the hospital, all vital parameters were taken. These included temperature, history, and clinical signs of CPE-suspect dogs with the consent of the pet owners.

Rapid immunochromatographic antigen test

The CPV rapid antigen test kit (Anigen Rapid®) used was from Bionote, South Korea,

and a pack contains test kits comprising: 1. 10 test devices, 2. 10 assay diluent tubes, 3. 10 disposable droppers, 4. 10 disposable swabs, and 5. Instructions for the user. The rapid antigen swab stick was gently inserted into the rectum of the dogs and swabbed gently, after which it was inserted into the buffer. This was done 3--4 times and mixed thoroughly, then a few drops of the mixed buffer assay were pipetted and placed on the sample well and allowed to stay for 30-60 seconds, which shows positive double lines in these suspected cases and confirms canine parvovirus presence.

DNA extraction and polymerase chain reaction

Total DNA was extracted from 10 samples using the QIAamp DNA stool mini kit (Qiagen, Germany) following the manufacturer's instructions as previously described (Fagbohun and Omobowale, 2018). Briefly, a pair of primers: VP2F: 50-GCGCAAACAGATGAAAATCA-30 and VP2R: 50-CCTTTCCACCAAAAATCTGAG-30 used in this study were designed using Primer 3 from JustBio (www.justbio.com/hosted-tools.html). Polymerase chain reaction (PCR) amplification was carried out under the following conditions: 94°C for 2 min for initial denaturation, 35 cycles of 95°C for 30 s, 46.7°C for 1 min, 72°C for 1 min, and final extension at 72 °C for 5 min.

Blood collection for hematology

Blood was collected in a heparinized bottle, and a thin smear was made from it, allowed to air dry, and then viewed under a binocular microscope. Blood was aspirated into a capillary tube, sealed at an end, and placed in a micro-hematocrit centrifuge for 5 minutes to spin. Then, the packed cell volume (PCV) was read, and other blood parameters were read for a complete blood count.

Results and Discussion

Thirty dogs of six different breeds from seven of the 11 local government areas of Ibadan in Oyo State in southwest Nigeria between 1 and 12 months of age reported to the Veterinary Teaching Hospital (VTH) of the University of Ibadan (23/30), State Veterinary Hospital (SVH), Ibadan (2/30), and City Veterinary Clinic (CVC) (5/30) between October 2023 and February 2024 were recruited for the study. Parvoviral enteritis was diagnosed by three methods. These are clinical signs of anorexia, vomiting, and diarrhea (30/30), antigen detection tests including CPV rapid test (23/30) and PCR (10/30), and clinical pathology laboratory markers including leukopenia, lymphopenia, and thrombocytopenia. German Shepherd breed was most represented, followed by Boerboel, Rottweiler, and Eskimo, while mixed breed and Cane Corso were least represented (Table I). Four of the dogs (13.3%) contracted canine parvovirus type 2 at 4 weeks old (preweaning period). Twenty percent (6/30) of the dogs were vaccinated at least once and a maximum thrice and were found to be infected with CPV as detected by the CPV rapid immunochromatographic antigen test kit or PCR, based on the detection of CPV-specific antigens or DNA present in canine fecal samples. Seven of the dogs' fecal samples were not tested by the rapid antigen test kit from the SVH and CVC, and three fecal samples from VTH tested by the rapid antigen test kit were all confirmed using PCR (10/10). Twenty percent 20% parvoviral infectivity rate in dogs between 7 and 12 months of age was recorded (Figure I), and 6.6% (2/6) of these dogs had 1-2 shots of CPV vaccination. The notable clinical signs of vomiting were observed in 66.7% (20/30) of studied dogs, diarrhea in 70.0%

(21/30), and anorexia in 86.66% (26/30). A combination of vomiting and diarrhea was observed in 50% (15/30) of studied dogs, anorexia, and diarrhoea in 66.7% (20/30), and anorexia and vomiting in 60.0% (18/30). A CPE clinical diagnosis was defined based on the presence of at least one of the clinical signs. Based on clinical signs, 28 of 30 studied dogs, 93.3% (95% CI: 77.9-98.2%, $p \leq 0.9918$), were positive for CPE and were classified as CPE suspected dogs (Table 1 and Figure 2). Two dogs without a positive clinical diagnosis were in the same litter with a dog that showed vomiting. Other clinical signs observed in the CPE dogs were pyrexia (hyperthermia) in 3.3% (2/30) dogs, hypothermia in 43.3% (13/30), and normothermia in 43.3% (13/30).

Lymphopenia was observed in 53.3% (16/30) of studied dogs, leukopenia in 90.0% (27/30), and thrombocytopenia in 100.0% (30/30) of studied dogs. A combination of leukopenia and lymphopenia, thrombocytopenia and lymphopenia was observed in 53.3% (16/30) of studied dogs, while thrombocytopenia and leukopenia were observed in 90.0% (27/30) of studied dogs. A CPE laboratory diagnosis was defined based on the presence of both thrombocytopenia and leukopenia in the blood sample. In this study, our defined method of clinical signs and laboratory markers (CSLM) for the diagnosis of CPE was based on the combination of clinical diagnosis and the presence of laboratory markers. Based on the CSLM method, of all 30 studied dogs, 100.0% (95% CI: 88.4 - 100.0%, $p \leq 1.000$) were confirmed positive for CPE. Based on the antigen detection method, all 30 studied dogs, 100.0% (95% CI: 88.4 - 100.0%, $p \leq 1.000$) were confirmed positive for CPE.

A combination of vomiting and bloody diarrhea was observed only in 40% (12/30) of the CPV-infected dogs, in 61.5% (8/13) of non-anemic dogs, and only in four (4) anemic dogs. In this study, 56.6% (17/30) of all the parvovirus-infected dogs recruited were anemic (PCV ≤35%), while 43.33% (13/30) were non-anemic (PCV ≥35%). Leukopenia was seen in 27 (90%) of the 30 positive dogs tested by rapid antigen or PCR, while more than half (60%) of the CPV-infected dogs recruited for this study had neutropenia. Fifty-three percent of the CPV-infected dogs showed lymphopenia, and 100% (30/30) had thrombocytopenia (Figure 2). The breed, sex distribution, rapid antigen test and PCR results (Figure 3), clinical signs, and laboratory marker findings are presented in Table 1.

Table 1. Breed, Sex, Clinical Signs, Hematological Markers, and Antigen Detection in the Diagnosis of Canine Parvovirus Enteritis

S/N	Breed	Sex	Clinical signs diagnosis	CPV Antigen test	≠PCR CPV+ test	CPE Clinical signs			CPE Laboratory markers			CSLM* Diagnosis
						Anorexia	Vomiting	Diarrhea	Leukopenia	Lymphopenia	Thrombocytopenia	
1	German Shepherd	M	+[a]	NT	+	+	-	+	+	-	+	CPE
2	German Shepherd	M	+	+		+	-	+	+	-	+	CPE
3	German Shepherd	M	+[a]	NT	+	+	+	+	+	-	+	CPE
4	Boerboel	M	+	+		+	-	+	+	-	+	CPE
5	German Shepherd	M	+	+		+	-	+	+	-	+	CPE

6	German Shepherd	**F**	+	+		+	+	+	+	+	+	**CPE**
7	Cane Corso	**F**	+	+		+	+	+	+	-	+	**CPE**
8	German Shepherd	**F**	+	+		+	+	+	-	-	+	**CPE**
9	Mixed	**F**	+ᵅ	**NT**	+	+	+	+	+	+	+	**CPE**
10	Boerboel	**F**	+	+		+	+	+	+	+	+	**CPE**
11	Rottweiler	**F**	+	+		+	+	-	+	+	+	**CPE**
12	Rottweiler	**F**	+	+		+	+	-	+	+	+	**CPE**
13	German Shepherd	**M**	+ᵅ	**NT**	+	+	+	+	+	+	+	**CPE**
14	German Shepherd	**M**	+ᵅ	**NT**	+	-	+	+	+	+	+	**CPE**
15	German Shepherd	**M**	+	+		+	+	+	+	+	+	**CPE**
16	Rottweiler	**F**	+	+		+	+	-	+	+	+	**CPE**
17	Rottweiler	**M**	+ᵅ	**NT**	+	+	+	-	+	-	+	**CPE**
18	Boerboel	**F**	+ᵅ	**NT**	+	+	-	+	+	+	+	**CPE**
19	Mixed	**M**	+	+		+	+	+	+	+	+	**CPE**
20	Boerboel	**M**	+	+		+	+	+	+	+	+	**CPE**
21	Boerboel	**F**	+	+		+	+	+	+	-	+	**CPE**
22	Boerboel	**M**	+	+		+	+	+	+	+	+	**CPE**
23	Boerboel	**M**	+	+		+	-	-	+	+	+	**CPE**
24	German Shepherd	**M**	+	+	+	+	+	+	+	+	+	**CPE**
25	German Shepherd	**M**	+	+	+	+	+	+	+	-	+	**CPE**
26	Boerboel	**F**	+	+	+	+	-	-	-	-	+	**CPE**
27	Eskimo	**M**	-	+		-	-	-	+	-	+	**CPE**
28	Eskimo	**M**	+	+		-	+	-	-	-	+	**CPE**
29	Eskimo	**M**	-	+		-	-	-	+	-	+	**CPE**
30	German Shepherd	**F**	+	+		+	-	+	+	+	+	**CPE**

Note: Seven of the dogs' fecal samples not tested by rapid antigen test kit from the SVH and CVC (ᵅ); only clinical signs were used to diagnose CPE. ≠ Seven of the dogs' fecal samples not tested by rapid antigen test kit from the SVH and CVC; and three fecal samples from VTH tested by rapid antigen test kit were all confirmed using PCR (10/10). * A combination of clinical signs and laboratory markers were used to diagnose Canine Parvoviral enteritis (CPE), NT- not tested

Table 2. Comparison of the Clinical Signs and Laboratory Markers and Antigen Detection in the Diagnosis of Canine Parvovirus enteritis

S/N	Breed	Clinical Signs	Antigen Detection	Clinical Signs and Laboratory Markers
1	German Shepherd	+	+	+
2	German Shepherd	+	+	+
3	German Shepherd	+	+	+
4	Boerboel	+	+	+
5	German Shepherd	+	+	+
6	German Shepherd	+	+	+
7	Cane Corso	+	+	+
8	German Shepherd	+	+	+
9	Mixed	+	+	+
10	Boerboel	+	+	+
11	Rottweiler	+	+	+
12	Rottweiler	+	+	+
13	German Shepherd	+	+	+
14	German Shepherd	+	+	+
15	German Shepherd	+	+	+
16	Rottweiler	+	+	+
17	Rottweiler	+	+	+
18	Boerboel	+	+	+
19	Mixed	+	+	+
20	Boerboel	+	+	+
21	Boerboel	+	+	+
22	Boerboel	+	+	+
23	Boerboel	+	+	+
24	German Shepherd	+	+	+
25	German Shepherd	+	+	+
26	Boerboel	+	+	+
27	Eskimo	-	+	+
28	Eskimo	+	+	+
29	Eskimo	-	+	+
30	German Shepherd	+	+	+

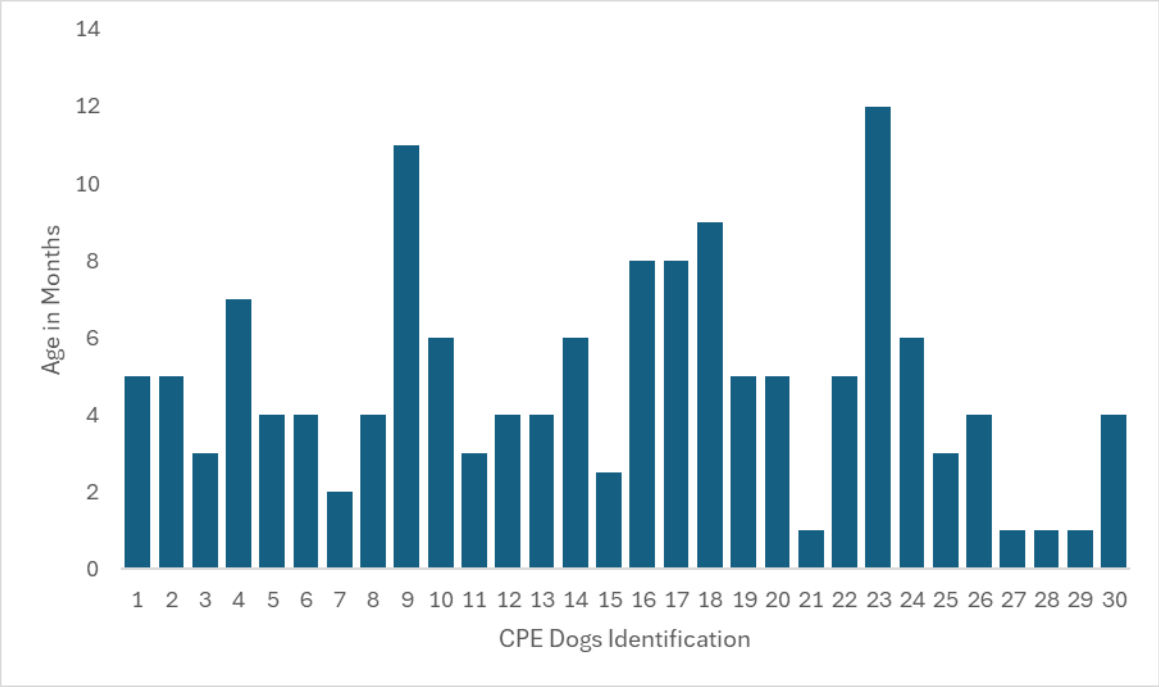

Figure 1. Age distribution of Canine Parvovirus infected dogs

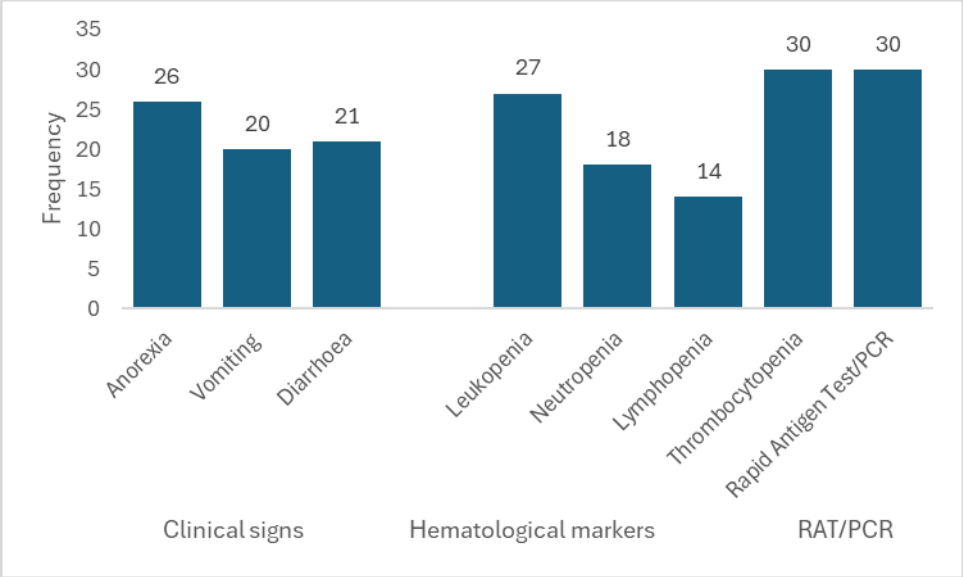

Figure 2. Comparison of clinical signs, hematological markers, and rapid antigen test result of Canine Parvovirus infected dogs.

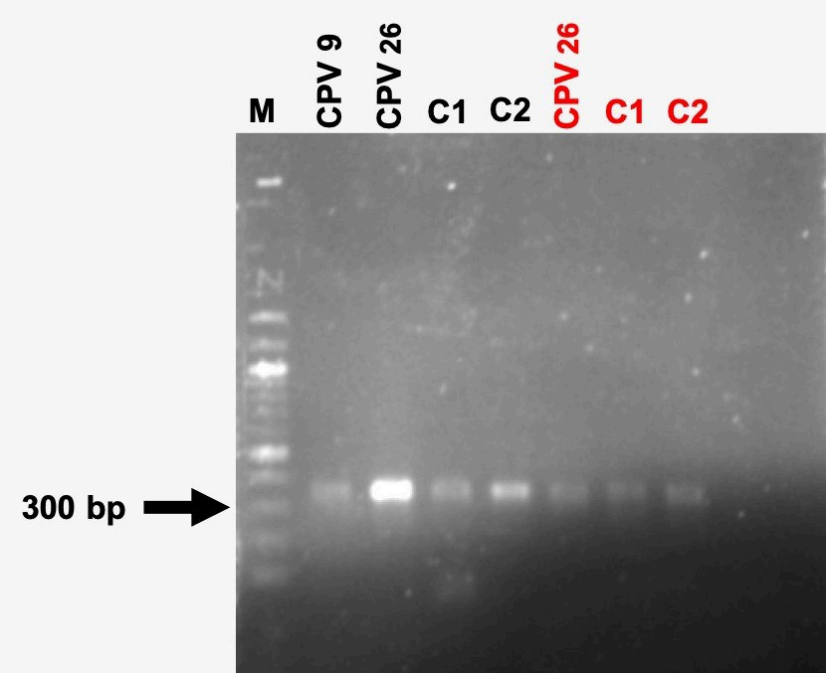

Figure 3. Agarose Gel Electrophoresis of VP2 Proto SC gene. Lane 1: Molecular marker, lane 2: clinical sample 9, lane 3: clinical sample 26, lane 4: first control, lane 5: sample control, lane 6: clinical sample 26, lane 7: first control, lane 8: second control

The canine parvovirus (CPV) rapid antigen test kit, a rapid immunochromatographic test for CPV based on the detection of CPV-specific antigens present in canine fecal samples (Sundaran *et al*., 2015), was used to detect CPV-2 antigen in the feces of 23 dogs (23/30), while PCR was used to detect CPV DNA in 10 dogs (10/30) hitherto tested with rapid antigen test (3/30) or presumptively diagnosed by clinical signs (7/30) for canine parvoviral enteritis (CPE) and recruited for this study. These dogs were naturally infected with CPV and were in the virus-shedding phase of the infection, during which infected dogs shed the virus in their feces within 4-5 days of post-exposure and throughout the duration of the illness and approximately for an additional 10 days during recovery, as previously reported by Apoorva *et al*. (2022). The German Shepherd breed was the most represented of the 30 dogs that tested positive for Canine parvovirus type 2, and this is because it is the most kept age-long breed in this part of Nigeria. Canine parvoviral enteritis is reported to be a disease primarily affecting unvaccinated dogs between ages 6 weeks and 6 months (Nandi and Kumar, 2010). Contrary to this belief age-long, we observed in this study that 20% (6/30) of the dogs were vaccinated at least once and maximum thrice, yet still came down with canine parvovirus infection. Thirteen-point three percent (4/30) of these dogs contracted canine parvovirus type-2 at 4 weeks of age during their pre-weaning period, contrary to the general belief that most puppies get infected around the weaning age of 6-8 weeks (Buonavoglia *et al*., 2001), and 20% parvoviral infectivity rate in dogs between 7 months and 12 months of age was recorded despite 6.6% (2/6) vaccination rate in these dogs. This

finding means that parvoviral enteritis disease can occur in dogs less than six weeks old and in vaccinated adult dogs above six months old. This calls for further studies on maternal immunity and vaccination to improve protection of dogs against CPV. Several reasons bordering on vaccine quality, vaccine break, CPV-2 cross-strain protection, or the immune status of the dogs in question may be responsible for disease outbreaks in vaccinated dogs. In addition, there are variations in the amount of antibodies and induction of active immunity after vaccination due to the genetics of the puppies (Buonavoglia *et al*., 2001). Moreover, there have been questions regarding the effectiveness of current vaccine usage and their impact on the local canine population (Amina *et al*., 2022).

The use of CPV rapid antigen testing was necessary in the recruitment of CPV-infected dogs for this study because clinical signs alone are inconclusive in the diagnosis of canine parvoviral enteritis (CPE), as several other viral pathogens, including coronaviruses, adenoviruses, morbilliviruses, rotaviruses, reoviruses, and noroviruses, can also cause diarrhea in dogs (Nicola and Canio, 2012), but canine parvovirus is the number one viral cause of puppy enteritis and mortality (Shabbir *et al*., 2009). Therefore, the clinical signs of depression, vomiting, diarrhea, anorexia, and fever may suggest a preliminary diagnosis of CPV enteritis (Mathios *et al*., 2016).

These clinical signs were varied in our study; for example, fever during CPV infection, which indicates viral spread in the bloodstream (viremia stage), which should last three to five days post-infection with CPV and be noticeable in plasma (Elisa, 2020), was only seen in 3.3% (2/30), as more CPV-infected dogs were hypothermic (43.3%) and the rest (43.3%) were normothermic. It can be deduced that most of the infected dogs (86.66%) reported to the hospital after the viremic stage of the infection, while only 3.3% were in the viremic stage and overlap with the shedding phase of the infection. This finding is as previously observed by Troy *et al*. (2019), in which a slight rise in temperature in the initial stage of the disease is seen before a gradual return to a subnormal level with the advancement of vomiting and diarrhea. The positive CPV rapid antigen test was seen in all dogs in the study and in line with the pathogenesis of CPV infection which follows lymphoid tissue viral replication (oral cavity and pharynx, mesenteric lymph nodes, and thymus), a viremic stage (up to 5 days), and rapidly dividing cells (epithelial cells of the intestinal crypts, bone marrow, spleen, thymus, and various lymph nodes), which occurs for a period of three to five days post-infection (Mathios *et al*., 2016, Elisa, 2020). The destruction of rapidly dividing cells of the bone marrow (Mathios *et al*., 2016; Elisa, 2020; Decaro *et al*., 2005), a common hematological consequence of CPV-2 infection, is responsible for 56.6% (17/30) of all the parvovirus-infected dogs being anemic. The virus's impact on the bone marrow, coupled with gastrointestinal bleeding, leads to decreased red blood cell production and increased destruction, contributing to the development of anemia (Decaro *et al*., 2006). Anemia per se, is not a predictive biomarker for CPV but the degree of anemia can assist veterinarians to tailor supportive care, such as blood transfusions, accordingly (Decaro *et al*., 2006). Following fluid loss, hemoconcentration occurs, and this may be responsible for the 43.33% (13/30) non-anemic CPV-infected dogs. The combination of vomiting and bloody diarrhea which was observed only in 40% (12/30) of the CPV-

infected dogs and was found to be in 61.5% (8/13) of non-anemic dogs, suggests that vomiting and bloody diarrhea in parvoviral enteritis did not affect hematocrit value. Anemia, which is an absolute decrease in hematocrit, hemoglobin concentration, or red blood cell count, in which, in parvoviral enteritis, there is hemorrhage leading to a normocytic normochromic and microcytic hypochromic anemia, was observed in this study. This non-regenerative anemia may be due to the lack of erythroid response seen in early blood loss or because of bone marrow suppression leading to the non- release of reticulocytes into circulation. There is a possibility that, in the non-anemic CPV-infected dogs, the dogs were very healthy and had a high PCV value prior to the infection with CPV, or it was a matter of hemoconcentration. The destruction of rapidly dividing cells of the bone marrow (Decaro *et al*., 2005) also compromises the immune response, leading to general leukopenia. More than half (60%) of the CPV-infected dogs had neutropenia; this was corroborated with hypothermic dogs (43.3%), which implies that the dogs were already in a state of shock. Septic toxins produced by intestinal bacteria circulating in the bloodstream (Decaro *et al*., 2006) can bring about neutropenia following a systemic inflammatory response (Englelbrecht *et al*., 2021) and hypothermia leading to shock, as was the case in these CPV-infected dogs. The neutropenia observed can be part of the general bone marrow cell suppression or bacterial co-infection or translocation following CPV destruction of the epithelial cells of the intestinal crypts (Englelbrecht *et al*., 2021), thereby giving access to bacteria. Fifty-three percent of the CPV-infected dogs showed lymphopenia. Lymphopenia can be attributable to viral infection, stress leukogram, or bone marrow suppression as the virus kills rapidly dividing cells in the body, including hematopoietic cells of bone marrow.

The 100% (30/30) thrombocytopenia observed in these dogs was because of the consumptive coagulopathy due to hemorrhage and bone marrow suppression. This general leukopenia represents a strong laboratory marker for the diagnosis of canine parvoviral enteritis disease, as 27 (90%) of the 30 positive rapid antigen tested dogs recruited for this study had leukopenia.

The result of the rapid immunochromatographic tests kit correlate with a combination of clinical signs and laboratory findings in canine parvovirus (CPV) infection and were effective in the recruitment of 23/30 infected dogs for this study, while PCR was used as the gold standard to confirm CPV in 10 dogs (10/30) hitherto tested with rapid antigen tests (3/30) or presumptively diagnosed by clinical signs (7/30) recruited for this study. Therefore, a combination of clinical signs that may suggest a preliminary diagnosis of CPV enteritis (Mathios *et al*., 2016), leukopenia seen in 90% of CPV-infected dogs, and thrombocytopenia observed in 100% of CPV-rapid antigen-tested dogs recruited for this study compete favorably with rapid antigen diagnosis of Canine Parvovirus-2 infection in dogs. Hence, general leukopenia and thrombocytopenia are strong laboratory markers for the diagnosis of canine parvoviral enteritis disease, and this can be explored in a limited environment.

We found, therefore, that canine parvoviral enteritis was caused by CPV-2 in both vaccinated and unvaccinated dogs of up to 12 months old in this study, and the infection was characterized by general anorexia,

occasional vomiting, or diarrhea, or both, with oftentimes regenerative anemia or hemoconcentration, general thrombocytopenia, and leukopenia.

Conclusion

This study, therefore, showed that general leukopenia (lymphopenia and neutropenia) and thrombocytopenia are effective markers for canine parvoviral enteritis disease. A larger natural and experimental study with more recruited CPV-infected dogs is therefore advocated to validate this finding.

Approval of Ethical Commission

Ethical approval for this research was given by the ACUREC of the University of Ibadan, Ibadan, Nigeria.

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Author's Contribution

AOB: Conceptualized the research project. AOB, KAR, OOS, FO, AV, EPS, DOB: Contributed to the methodology. DOB, FO and OJ: Provided the reagents and tests kits. AOB, KAR, OOS: Carried out the Investigation. AOB: Supervised the research project. AOB, EPS: Carried out the Data analysis. AOB: Wrote the draft manuscript. OJ, TVO: Reviewed and Edited the manuscript. Media Kedokteran Hewan: Peer reviewed and published the manuscript free of charge.

Conflict of Interest

The authors have no financial, personal, or professional conflicts of interest that could influence the research or its outcomes.

Data Availability Statement

Data used in this study are available on request from the corresponding author.

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