




Pathology and molecular diagnosis of canine parvoviral enteritis in Nigeria: case report

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Abstract

This report describes the clinical presentation, pathology and molecular diagnosis of canine parvovirus infection in male Boerboel and female Alsatian puppies. The history of the dogs was considered, examined clinically for vital parameters, haemogram changes and faeces screened for parasites and canine parvovirus faecal antigen. Tissue samples were taken at necropsy for confirmatory diagnosis using histopathology, immunohistochemistry, polymerase chain reaction (PCR) and sequence analysis. There was a severe regenerative anaemia, leucopenia and lymphopaenia. The positive antigen faecal test and pathological findings of haemorrhagic enteritis suggested canine parvoviral enteritis disease. Polymerase chain reaction and sequence analysis confirmed canine parvovirus-2a as the aetiology of the disease. Informed management is important to avoid complications resulting from secondary to severe dehydration, hypovolemia from marked gastrointestinal fluid and protein loss and sepsis from bacterial translocation and leukopenia.

Keywords Canine parvovirus · Infection · Molecular diagnosis · Faecal antigen testing

Introduction

Canine parvoviral enteritis is a highly contagious and fatal disease of dogs and many other carnivores, which results in severe gastroenteritis and myocarditis (Chen et al. 2019). The disease is caused by canine parvovirus (CPV) which is one of the most important causes of mortality in young dogs and that has sustained pandemic circulation in dogs for more than 40 years (Voorhees et al. 2019). Canine parvovirus is a non-enveloped, icosahedral, single-stranded DNA virus belonging to the family *Parvoviridae* and the genus *Protoparvovirus*. The virus genome is about 5200 nucleotides in size containing

two non-structural proteins (NS1 and NS2) and three structural proteins (VP1, VP2 and VP3) at the 3' and 5' ends, respectively (Miranda and Thompson 2016). There are three subtypes of the virus, namely CPV-2a, CPV-2b and CPV-2c (Cotmore et al. 2014), and all these subtypes have been reported in Nigerian (Fagbohun and Omobowale 2018; Ogbu et al. 2019). More so, in Nigeria, the diagnosis of canine parvovirus enteritis had been on histopathological findings and serology while molecular detection of the disease using polymerase chain reaction (PCR) coupled with sequence analysis is not common. This report describes the clinical presentation, pathology and molecular diagnosis of canine parvovirus infection in two different puppies, male Boerboel and female Alsatian puppies.

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Materials and methods

Case history

Two young dogs were presented differently to the Small Animal Clinic. The male Boerboel was 4 months old while the Alsatian was 7 weeks old as at presentation. They were both presented with history of anorexia of about 4 days

(Boerboel) and 2 days (Alsatian) duration. The Boerboel was reported to have been previously vaccinated against parvovirus and dewormed shortly before the onset of the illness, while the Alsatian has no vaccination history.

Clinical and necropsy examinations

Thorough clinical examination was performed on the dogs for vital parameters including body condition, rectal temperature, heart rate and respiratory rate. Their faeces were examined for helminth and protozoal ova and screened for canine parvovirus faecal antigen using the in-house test, FASTest PARVO Card which is a rapid immunochromatographic “lateral flow” test for the detection of canine parvovirus antigen in the faeces of dog and while the blood was evaluated for haematology (Jain 1986). Fluid therapy and intravenous antibiotics (amoxicillin and gentamicin combination) were administered for 3 days. However, the animals died after 3 days of treatment. Both carcasses were sent for detailed post-mortem examination.

Histological and immunohistochemical stainings

Tissues from the intestine, lungs, heart muscle, thymus, liver, kidney and lymph nodes were fixed in 10% neutral buffered formalin for histological processing, stained with haematoxylin and counterstained with eosin for microscopic examination. Six micron (6 μ) thickness sections were further floated on to positively charged slides for immunohistochemical staining for canine parvovirus (CPV) antigens following standard procedures. Briefly, paraffin-embedded tissue sections were deparaffinized before antigen retrieval using heat induction. Endogenous peroxidase activity was quenched. CPV monoclonal antibody at a dilution of 1:200 in PBS was dropped on the tissue and kept in a humidified chamber at 4 °C overnight. Secondary antibody, labelled peroxidase-conjugated streptavidin-boitin complex and 3, 3 diaminobenzidine tetrahydrochloride (DAB) were added respectively following washes. Finally, the sections were washed in running tap water, counterstained with haematoxylin, dried and covered with glass cover slip.

Polymerase chain reaction and sequencing

Similarly, tissues from the heart, thymus, intestine, kidney, liver, spleen, bladder, lung and bone marrow were taken for DNA extraction. The heart, thymus, intestine, kidney, liver, spleen, bladder, lung and bone marrow tissues were homogenized in virus transport medium. Total DNA was extracted from the homogenized samples using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Two pairs of primers: VP2F:5'-GAGC ATGGGCTTACCA-3', VP2R:5'-GCAAGATGCATCAG

GATC-3' and VP2F:5'-GCGCAAACAGATGAAAATCA-3' and VP2R: 5'-CCTTCCACCAAAAATCTGAG-3' were used to amplify a 700-bp and 502 sequences of VP2 genes, respectively.

PCR amplification reaction was carried out in 50 μ l volume containing 5 μ l of total DNA, of 0.2 μ M of each primer, 25 μ l of PCR master mix (10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl₂, 50 units/ml of Taq DNA polymerase, 0.2 mM each dNTP, 5% glycerol, 0.08% IGEPAL®CA-630, 0.05% Tween®20, 0.024% Orange G, 0.0025% Xylene Cyanol FF) and 19 μ l of nuclease free water. The GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) was used for amplification under the following conditions: 94 °C for 2 min for initial denaturation, 35 cycles of 95 °C for 30 s, 43 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 5 min. PCR amplicons were electrophoresed in a 2.0% agarose gel, stained with ethidium bromide and visualized under UV light. The amplified DNA fragments (700 bp) were purified using GeneJET PCR Purification Kit (ThermoSCIENTIFIC®, Pittsburgh, PA). Automated nucleotide sequencing was performed on an ABI 3130XL. The sequences were edited with the Sequence Scanner software, version 1.0 (Applied Biosystems, Foster City, CA), designated NGA8 (intestine), NGA8 (spleen) and NGA8 (thymus). These nucleotide sequences of the partial VP2 gene of the CPV from this study were compared with other published CPV VP2 genes sequences in the GenBank database using BLAST search via the National Center of Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Multiple sequence alignment of the deduced amino acids of the partial VP2 gene of the CPV from this study and those of three reference CPV VP2 genes sequences representing CPV2a, CPV2b and CPV2c retrieved from the GenBank Multiple sequence alignment was carried out with the CLC Main Workbench (Qiagen, Valencia, CA).

Results

Clinical examination

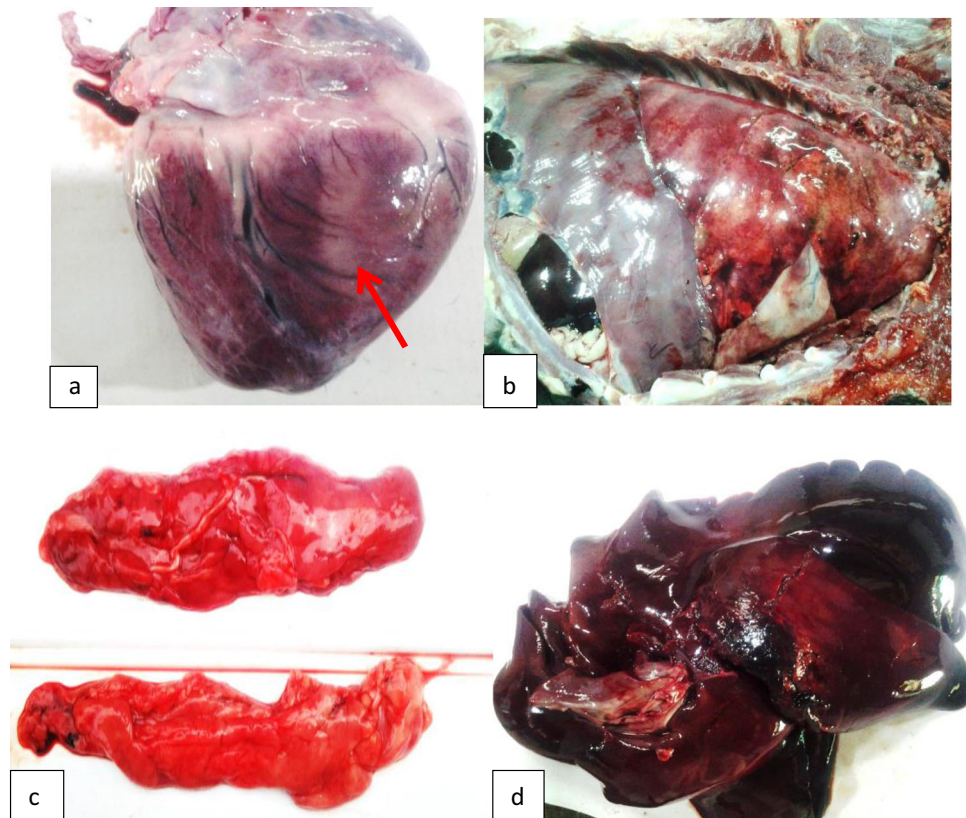
On examination, both dogs were dull, weak and dehydrated due to the many episodes of frequent stooling. Their rectal temperatures were 40.4 °C and 39.6 °C in the Boerboel and Alsatian, respectively. Heart rates were 96/min and 98/min in the Boerboel and Alsatian, respectively, while the respiratory rates were 68/min and 63/min in the Boerboel and Alsatian, respectively. The Boerboel passed out reddish-yellow, foul smelling stool while the Alsatian passed out bloody watery diarrhoea (melena). Their stools both tested negative for helminth and protozoal ova tests, however, were positive for canine parvovirus faecal antigen test. Haematological

Table 1 The haematologic parameters in the Boerboel puppy at clinical examination

Parameter		Values
Total protein (g/dl)		6.9
Fibrinogen (mg/dl)		200
Haemoglobin (g/dl)		5.6
PCV (%)		18
RBC ($\times 10^6/\mu\text{l}$)		2.98
MCV (fl)		60
MCHC (g/dl)		31
Reticulocytes (%)		0.2
Platelets ($/\mu\text{l}$)		225,000
Total white blood cell count ($/\mu\text{l}$)		5150
Differential	%	Absolute ($/\mu\text{l}$)
Segmented neutrophils	85	4377
Band neutrophils	3	155
Lymphocytes	4	206
Monocytes	4	206
Eosinophils	4	206
Basophils	0	0

evaluation for the Boerboel revealed severe anaemia, leucopenia characterized by severe lymphopaenia (Table 1); the Alsatian died before haematology workup.

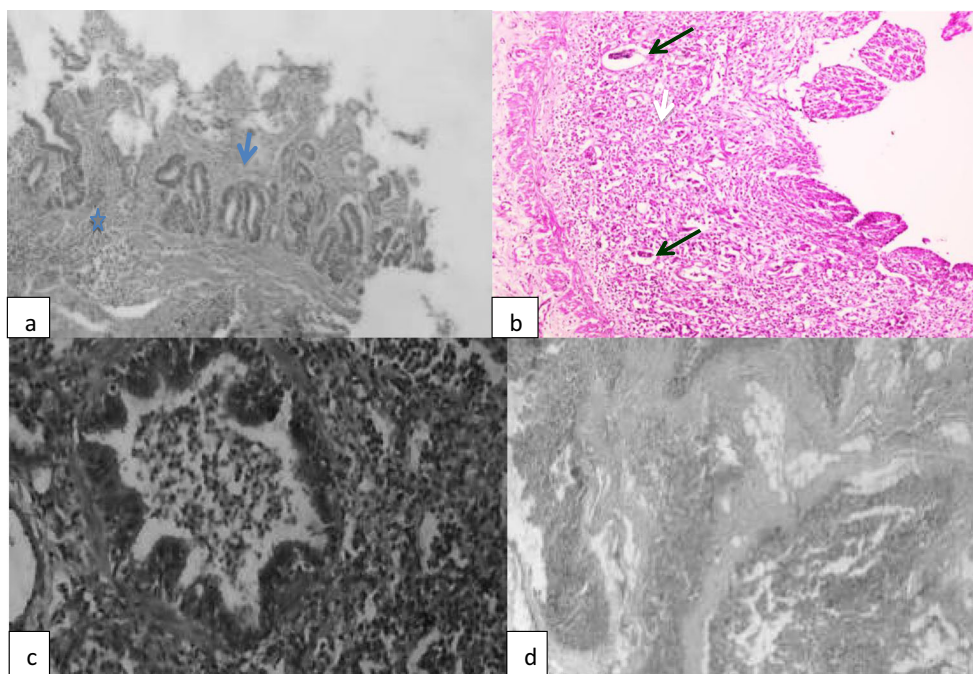
Fig. 1 **a** Myocardial necrosis evident as pale streaks on the myocardium (red arrows). **b** Lung consolidation, haemorrhage and hyperaemia. **c** The thymus showing severe congestion. **d**. The liver showing severe congestion



Necropsy findings

Both carcasses were fresh upon presentation. The eyes were sunken in the Alsatian carcass and with rough dry hair coat (dehydration) in both carcasses. The oral and ocular mucous membranes were moderately pale in both carcasses (anaemia). The perineum was pasted with dark red watery faeces in both carcasses. The heart was moderately enlarged (globosed), with pale streaks on the myocardium (Fig. 1a) and blood clot in the left ventricle in the Alsatian carcass. The trachea contained frothy exudate in the Alsatian carcass, while the right lungs were dark red and consolidated (pneumonia acute severe lobar) with a few petechial and ecchymotic haemorrhages (Fig. 1b). The thymus was diffusely haemorrhagic in both carcasses (Fig. 1c). The liver was moderately congested with a few ecchymosis (hepatomegaly, congestive, moderate, haemorrhagic (Fig. 1d)) in both carcasses. The spleen was mildly enlarged in both carcasses with multiple capsular hematomas in the Boerboel. The small intestinal mucosal surface was bright red, haemorrhagic, thickened and lined by foul smelling diphtheritic membrane covered with excessive mucus in both carcasses. The content of the entire large intestine was muco-gelatinous and dark red in both carcasses. The kidneys were grossly normal. The diaphyseal bone marrow of the femur was bright red and gelatinous (regenerative anaemia). Tentative post-mortem diagnosis of canine parvoviral enteritis was made.

Fig. 2 Histopathological findings. **a** Intestine, enteritis; subacute; moderate; necrotizing with GALT hyperplasia. **b** Intestine showing absence of villi (villous collapse) and glandular necrosis. **c** Lung, bronchopneumonia; acute; severe; suppurative; diffuse. **d** Thymus, thymic atrophy with depleted cortical follicles (arrow). Haematoxylin & Eosin stain, $\times 100$ –400



Microscopic findings

Microscopically, the intestinal mucosa had stunted and denuded villi devoid of enterocytes. There was moderate cryptal hyperplasia and infiltration chiefly by mononuclear cells (lymphocytes, macrophages) and a few neutrophils in the crypts and lamina propria in both carcasses (Fig. 2a). The gut-associated lymphoid tissue was remarkably hyperplastic

in the Boerboel (Fig. 2b). There was bronchiolar epithelial necrosis and hyperplasia with marked neutrophilic infiltrate (Fig. 2c) in the bronchiolar lumen and alveoli of the lung in the Alsatian carcass. There was collapse of thymic cortex, atrophy of thymic follicles (Fig. 2d) that was replaced by fat cells, loss stroma cells and epithelial cells (Hassal's Corpuscles) in both carcasses, and their blood vessels were remarkably congested and accentuated. There was moderate

Fig. 3 Immunohistochemical staining. **a** Immunostaining of CPV-2 antigens in the intestine. SBC HRP DAB/HX $\times 100$. **b** Immunostaining of CPV-2 antigens (arrows) in the intestine. SBC HRP DAB/HX $\times 400$. **c** Immunostaining of CPV-2 antigens (arrows) in the muscle. SBC HRP DAB/HX $\times 400$. **d** Immunostaining of CPV-2 antigens (arrows) in the thymus. SBC HRP DAB/HX $\times 400$

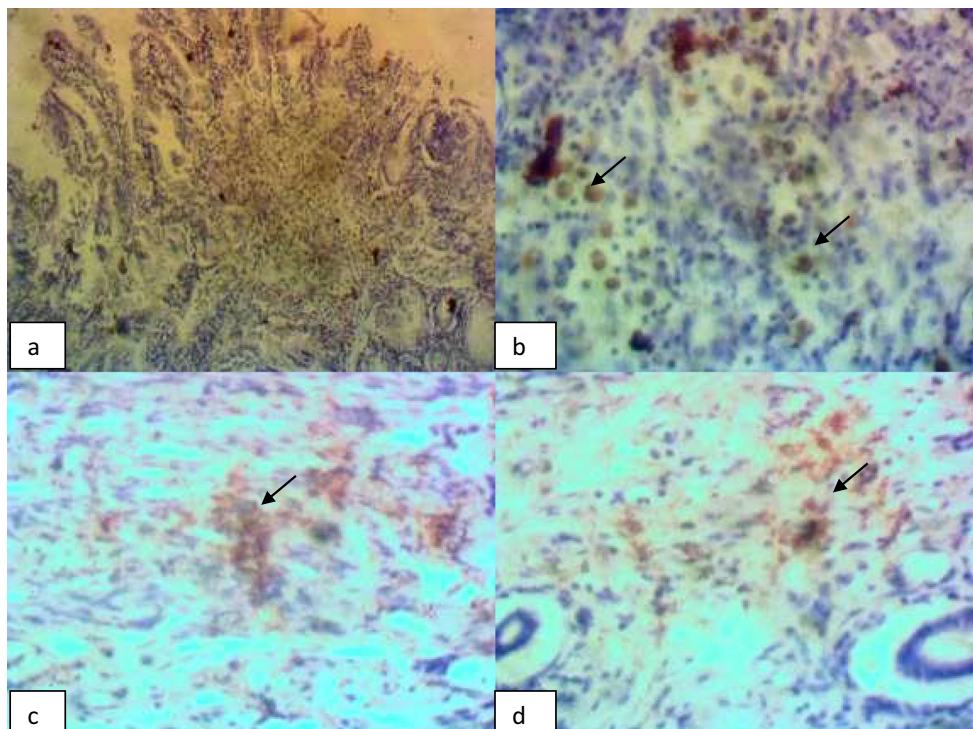
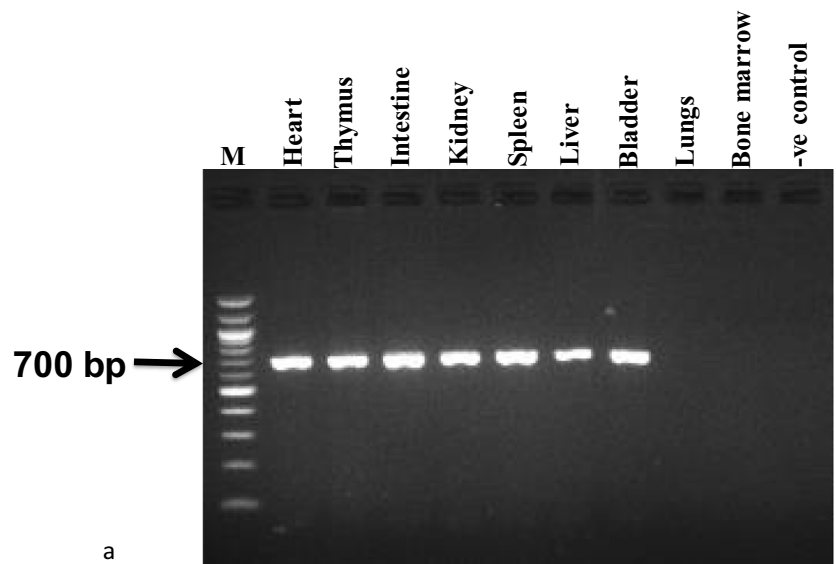
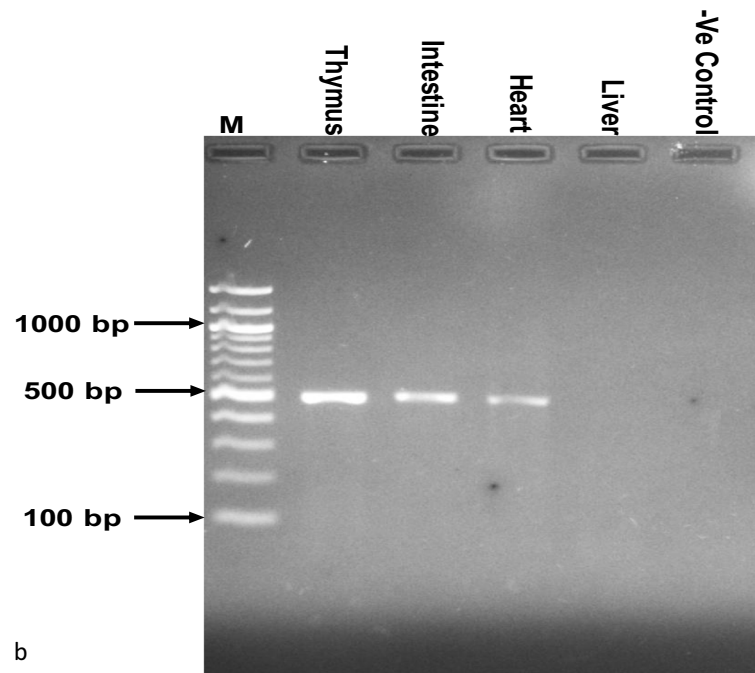


Fig. 4 a Agarose gel electrophoresis of the PCR amplicons of the canine parvovirus. Lanes 1–11: M, molecular weight marker, samples from the heart, thymus, intestine, heart, kidney, liver, spleen, bladder, lung, bone marrow and the negative control. Amplicons are 700 bp in size. **b** Lanes 1–6: M, molecular weight marker, sample from the thymus, intestine, heart, liver and the negative control (–ve Control). Amplicons are 502 bp in size



a



b

follicular lymphoid hyperplasia with congestion of vascular sinuses and a few haemosiderin-laden macrophages in the spleen from the Boerboel carcass. There was lymphoplasmacytic myocarditis in the heart of both carcasses, and no visible lesions were observed in the kidneys from both carcasses. There was immuno-localization of CPV antigens in the intestines (Fig. 3a, b), heart muscles (Fig. 3c) and thymuses (Fig. 3d) of both carcasses.

PCR and sequencing

Seven of the organs, the heart, thymus, intestine, kidney, liver, spleen and bladder, were positive for the virus from the

Boerboel whereas PCR were unable to detect the virus in the lung and bone marrow (Fig. 4a). However, only three of the organs were positive in the Alsatian (Fig. 4b). BLAST analysis and multiple sequence alignment revealed the sequences to be CPV-2a based on the presence of asparagine as position 426 of the VP2 protein with a T440A in the sequence (Fig. 5).

Discussion

Canine parvovirus causes a highly contagious and fatal disease, developing into acute haemorrhagic enteritis and myocarditis, in dogs (Miranda and Thompson 2016). Though CPV

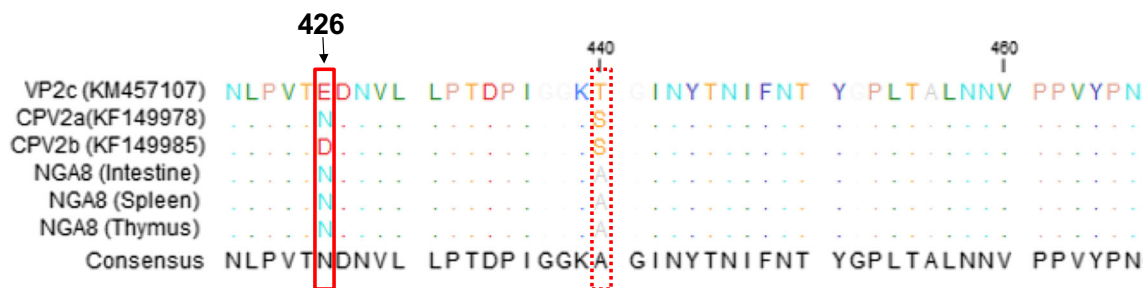


Fig. 5 Multiple sequence alignment of the deduced amino acid sequences of VP2 protein of CPV subtypes. The VP2 of CPV sequences here analysed are represented by the sequence of sample NGA8 (intestine), NGA8 (spleen) and NGA8 (thymus). Reference strains are CPV-2a

(KF149978), CPV-2b (KF149985) and CPV-2c (KM457107). Dots indicate position where the VP2 sequences are identical to that of the consensus sequence. The sites of mutations of VP2 protein in the Nigerian sequences are at positions 426 and 440

was first recognized in 1978 as a new pathogen of dogs (Zhao et al. 2011), a detailed approach to confirmatory diagnosis of the disease based on pathology, immunohistochemical and molecular diagnosis is seldom employed in Africa. As such, these case reports describe the pathology, immunohistochemical and molecular diagnosis of the disease in dogs. There is plethora of reports on serologic detection of the disease in dogs in Western Nigeria (Babalola et al. 2016), Ghana (Folitse et al. 2018) and presence of CPV variant in dog faeces with acute gastroenteritis in northern Nigeria (Ezeokoli et al. 1985; Apaa et al. 2016). The description of the pathology of the intestinal form of the disease was also described by Akpavie and Alaka (1996). Recently, the co-existence of subtypes CPV-2a and CPV-2b and CPV-2a and CPV-2c was found in some dogs in Nigeria with sequence analysis revealing the occurrence of three mutations D413 N, N426D/E and T440A (Geng et al. 2015; Fagbohun and Omobowale 2018), which may be responsible for vaccine failure (Fagbohun and Omobowale 2018).

Clinically, dogs often present acute respiratory distress, cardiopulmonary arrest and cardiomegaly and diffuse unstructured pulmonary infiltrate as observed in these cases. However, the lymphoplasmacytic myocarditis with demonstration of positive CPV immunohistochemically within the myocardium and in the intestine was prominent in these cases. It is clear that the most common enteric form of CPV is what was observed as the virus was detected in the thymus, which was its initial site of replication (Mietzsch et al. 2019). The virus had also been described to disseminate through the body haematogenously and ultimately destroys intestinal villi (Goddard et al. 2010) as observed in these puppies. These haematogenous spread of the virus is further corroborated by the PCR detection of the virus in the heart, thymus, intestine, kidney, spleen, liver and bladder samples. A detailed confirmatory diagnosis of this nature is not only paramount for a precise diagnosis of disease but also corroborates the established pathogenesis of the disease. CPV remains a cardinal causative agent of gastroenteritis in puppies over the age of 2 months, since the virus has tropism for rapidly dividing cells in the S phase of the cell cycle (Appel et al. 1979). Steinel et al. (2001) reviewed infections of the

different antigenic types of CPV-2a and -2b. The tropism of the virus often determines the pathological manifestation. CPV-2a is capable of causing myocarditis depending upon timing of infection. Infection in utero or in first few weeks of life allows the virus to attack most rapidly dividing cells in the body including cardiac myocytes (Prittie and Barton 2004; Sime et al. 2015). The virus also infects the intestinal epithelium, resulting in crypt necrosis, crypt dilatation and villous atrophy, which is diagnostic of the disease (Cooper et al. 1979).

The most common enteric form of CPV targets the lymphoid tissue of the oropharynx, the mesenteric lymph nodes and/or the thymus as its initial site of replication. The virus then disseminates through the body haematogenously and ultimately destroys intestinal villi (Goddard et al. 2010). Given the short intestinal epithelial cell turnover time of 1–3 days, recovery is possible with intensive supportive care even with severe villus damage (Prittie 2004).

Death, however, can occur secondary to severe dehydration, hypovolemia from marked gastrointestinal fluid and protein loss and sepsis from bacterial translocation and leukopenia. All these were observed in the two puppies examined. This shows the virulence of the virus irrespective of the breed; however, it seems younger puppies succumb to the infection more easily considering the incidence in this report and findings of Ezeokoli et al. (1985) and Fagbohun and Omobowale (2018).

In conclusion, these reports confirm the existence of CPV infection in our environment. It also underscores the importance of diagnostic tools in identifying the nature and pattern of disease conditions for quick management response.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval “All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.”

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