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Fatal Canine Adenovirus Type 1 Infection in Dhole (*Cuon alpinus*) Pups

V. Manjunatha[1], M. Rout[2], U.K. Srinivas[3], L.M. Kshamaa[3], S. Sujay[3], K. Sripad[4], S.M. Byregowda[4]

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ABSTRACT

Background: Infectious canine hepatitis (ICH) is an uncommonly recognized disease of dogs and wild canids. This study aimed to investigate and report an outbreak of infectious canine hepatitis (ICH) caused by canine adenovirus type 1 (CAV-1) in dhole (*Cuon alpinus*) pups at Bannerghatta Biological Park, Bengaluru, Karnataka.

Methods: During this investigation, four dhole pups succumbed following a short clinical course of ICH. Clinical biochemical and hematological tests were done. Gross and histopathological observations were made along with genome detection through PCR.

Result: Clinical findings comprised of leucopenia, thrombocytopenia and anaemia with high levels of total and direct bilirubin as well as ALT, AST and ALP enzymes. The necropsy findings were sub-mandibular edema, generalised icterus, petechial haemorrhages on liver, spleen, lungs, kidneys and skeletal muscles. Liver was enlarged, haemorrhagic, friable and severely icteric with rounded borders. Histopathologically, intranuclear inclusion bodies typical of CAV-1 infection could be observed in hepatocytes and Kupffer cells in the liver. The disease confirmation was done on histopathological findings and detection of CAV-1 genome through PCR.

Key words: Canine adenovirus type 1, Dhole, Histopathology, Intranuclear inclusion bodies, PCR.

INTRODUCTION

Dhole (*Cuon alpinus*) popularly known as Asiatic wild dog, Indian wild dog, whistling dog, red wolf, red dog, or mountain wolf is enlisted in 'endangered' category by the International Union for Conservation of Nature (IUCN) (Kamler *et al*., 2015). Infectious canine hepatitis (ICH) is a highly contagious, fatal acute liver ailment in dogs caused by canine adenovirus type-1 (CAV-1), a double stranded DNA, non-enveloped icosahedral virus in the genus *Mastadenovirus* in the family *Adenoviridae* (Quinn *et al*., 2005). ICH though first identified in North America in silver foxes (*Vulpes vulpes*) in 1925 (Green, 1925), it got recognized as a specific viral disease of dogs in 1947 (Rubarth, 1947). Besides red and grey foxes and other canids, such as coyotes, jackals and wolves, CAV-1 can also infect wild mammals of the families *Ursidae* (black) (Pursell *et al*. 1983) and polar bears (Chaddock *et al*., *1950*), *Mustelidae* (skunks and otters) and *Procyonidae* (raccoons) (Akerstedt *et al*., 2010; Thompson *et al*., 2010). Reviews of such host range of CAV in wild mammals are described elsewhere (Woods, 2001).

Sporadic spill-over to susceptible unvaccinated domestic dogs may occur from wild species that are likely reservoirs of CAV-1. Though the incidence of acute CAV-1 infection in domestic dogs is presently low, the virus persists in dog populations worldwide as evident from reports from North America (Caudell *et al*., 2005; Wong *et al*., 2012), South America (Oliveira *et al*., 2011; Headley *et al*., 2013), Asia (Kobayashi *et al*., 1993; Hai *et al*., 2009), Middle East (Cheema *et al*., 2012) and Europe (Gleich *et al*., 2009; Muller *et al*., 2010). It has been reported that ICH is most frequently seen in young dogs within 1 year of age, though unvaccinated dogs of all ages can be affected (Greene, 2006). The clinical forms of the disease can evolve rapidly varying from mild, acute, peracute or hyper-acute (Greene, 2006; Stalker and Hayes, 2007). In the present study, we report the clinical, pathological and molecular findings of acute CAV-1 infection in dhole pups providing the evidence of CAV-1 circulation causing disease in unvaccinated captive animal. To the best of our knowledge, it seems to be the first report of CAV-1 in-fection in captive canid in the country.

[1]Wild Animal Disease Diagnostic Laboratory, Institute of Animal Health and Veterinary Biologicals, Bannerghatta Biological Park, Bannerghatta-560 083, Bengaluru, Karnataka, India.
[2]ICAR-National Institute on Foot and Mouth Disease, International Centre for FMD, Bhubaneswar-752 050, Odisha, India.
[3]Zoo Hospital, Bannerghatta Biological Park, Bannerghatta-560 083, Bengaluru, Karnataka, India.
[4]Institute of Animal Health and Veterinary Biologicals, Hebbal, Bengaluru-560 024, Karnataka, India.

Corresponding Author: V. Manjunatha, Wild Animal Disease Diagnostic Laboratory, Institute of Animal Health and Veterinary Biologicals, Bannerghatta Biological Park, Bannerghatta-560 083, Bengaluru, Karnataka, India. Email: manjuvet330@gmail.com

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MATERIALS AND METHODS

Case presentations and sampling

On January 2022, one male dhole puppy aged about 4 months was found dead in an enclosure on first day and later within a week remaining 3 puppies of same litter were under treatment succumbed to death. The parents were brought from other Indian Zoo by animal exchange programme and were kept for 7 years, the parents are vaccinated regularly every years with DHPPI and Leptospira vaccine. Before death the puppies showed the clinical signs of fever, lethargy, vomition, dehydration, conjunctivitis, cough and corneal edema *etc*. The post-mortem examination revealed icterus of serous and mucous membranes, enlarged liver with petechial haemorrhages all over the liver parenchyma, fluid accumulation in the abdominal cavity, sub-mandibular edema, petechial haemorrhages on spleen and kidneys, consolidation of lung tissue etc. Within 1-2 weeks, three dhole pups exhibited hind limb ataxia followed by paralysis, seizers and died (Fig 1). All the suspected dhole pups received supportive treatment for viral infections. An epidemiological inquiry revealed that neither the affected pups nor their dams had any history of vaccination against CAV-1.

Sampling for pathological analysis

Whole blood with anticoagulant EDTA and with clot activator was collected for haematological and biochemical analysis, respectively. Haematology was performed using auto-analyzer, while serum was analysed using the ERBA Chem Pro semi-automated analyzer using ERBA biochemical kits as per the manufacturer's instructions. During necropsy, heart blood was collected in vials, while liver and kidneys were collected in labelled ziplock poly-bags and preserved at -20°C. Representative samples from liver, lungs, kidney, spleen, intestine and heart were collected in 10% neutral buffered formalin for histopathology. The tissues were routinely processed for fixation, dehydration, clearing and embedded in paraffin. Sections of 5µm were stained with haematoxylin and eosin (H&E) and examined using light microscopy.

Extraction of DNA and PCR

Different organs (blood, liver and kidney) of four dhole pups were homogenised with phosphate-buffered saline (PBS) for DNA extraction. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Germany; Cat No. 69504) according to the manufacturer's protocol and stored at -20°C. Polymerase chain reaction (PCR) amplification of CAV-1 DNA was performed in tissue samples (liver and kidney) using previously published CAV-1-specific primers 5′-CGC GCT GAA CAT TAC TAC CTT GTC-3′ (ICHF-forward) and 5′-CCT AGA GCA CTT CGT GTC CGC TT-3′ (ICHR-reverse) to amplify 508 base pairs (bp) fragment of the E gene of CAV-1 (Hu *et al*., 2001).

DNA samples were amplified by PCR in a reaction mixture containing (12.5 µl) 2× Taq PCR master mix (Taq PCR master mix kit, Qiagen, Germany; Cat No. 201443), 0.5 mM of each primer and DNA template 1 µg/reaction in a thermocycler (Eppendorf, North America). After initial denaturation at 95°C for 5 minutes, 35 amplification cycles were conducted with each cycle consisting of 94°C for 45 seconds (denaturation), 54°C for 1 minute (annealing) and 72°C for 45 seconds (extension), followed by final extension for 10 minutes at 72°C. PCR products were loaded on 2% agarose gel in electrophoresis unit (Bangalore Genei, India) stained with ethidium bromide stain separated by electrophoresis. Amplicons were observed using gel documentation system (Gel PRIME, Zenith Biozen Labs).

Samples for bacteriological tests

Samples of liver, spleen, lung and kidney were also collected maintaining sterile conditions during necropsy for bacteriological examination using standard and selective culture media as per the procedure mentioned earlier (OIE, 2012). Bacteriological culture was done for ruling out the involvement of significant bacterial pathogens such as *Salmonella*, *Campylobacter*, Enterotoxigenic *Escherichia coli* and *Clostridium perfringens etc.*

RESULTS AND DISCUSSION

Two types of adenoviruses CAV-1 and CAV-2 are described in dogs. CAV-1 causes a severe generalized disease called ICH, whereas CAV-2 causes a mild respiratory disease (Zee *et al*., 2004). ICH is transmitted via ingestion of faeces, urine, blood, saliva and nasal discharge of infected dogs (Willis, 2000). Again, animals that survive the infection reportedly carry and shed the virus through nasal discharge and urine, for at least 6 to 9 months and 11 months, respectively (Piacesi *et al*., 2010). After being contracted naso-orally, the virus initially replicates in the tonsillar crypts and Peyer patches, followed by viremia and disseminated infection. CAV-1 is known to have tropism for vascular endothelial cells and hepatocytes (Zee and Maclachlan, 2004), renal parenchyma, spleen and lungs become infected as well.

Haemogram

The haemogram showed lowered white blood cell (WBC) count as leukopenia, neutropenia and lymphopenia. WBC

Fig 1: Carcass of dhole pup submitted for necropsy.

count ranging from 7.1 × 10^3/µl to 4.2 × 10^3/µl showed leucopenia (shift to left). Marked thrombocytopenia (11,000 to 78,000/µl) was evident that could be attributed to platelet clumping as observed during the blood smear examination, but increased consumption due to disseminated intravascular coagulation (DIC) could not be ruled out. There was a moderate left shift (neutrophil count 68% consistent with inflammation and moderate lymphopenia 26% that is attributed to stress and/or exogenous glucocorticoids (Table 1). Blood samples collected during clinical sickness showed a severe anaemia with haemoglobin level ranging from 4.1 g% to 7.5 g% (average 5.8 g%). Red blood cell (RBC) count was 3.0-6.5 × 10^6/µl. Leukopenia and lymphopenia recorded in the study are important paraclinical observations in ICH affected dogs that were also documented earlier (Mosallanejad *et al*., 2010). The finding of anaemia in the pups might also be attributed to severe flea infestation concomitant with acute blood loss due to CAV-1 infection.

Serum biochemical analysis

Serum biochemical analysis showed moderate hyperbilirubinemia (6 ìmol/L against normal range up to 1 to 4 µmol/L); marked increase in alkaline phosphatase (ALP) at 201 to 283 U/L (normal range: 9-90 U/L) and aspartate transaminase (AST) at 42 to 71 U/L (normal range 13-15 U/L). It is noteworthy to mention that all the reference range of values have been considered from adult canine (Table 2). Moderate hyperbilirubinemia, marked increase in ALP and AST are consistent with hepatocellular injury and cholestasis. Activity levels of ALT and AST could be evaluated due to marked haemolysis that might have also contributed to increased bilirubin level. The increased levels of these enzymes are in agreement with those reported previously (Mosallanejad *et al*., 2010) during CAV-1 infection in dog.

Macroscopical findings at necropsy

In our case study, the pups were less than one year of age and external examination of the carcasses revealed mild emaciation and pale mucous membranes. The four dhole pups died with a high case fatality rate after a short clinical course of exhibiting fever, excessive salivation, lymphadenopathy, abdominal pain, loss of appetite, lethargy, severe icterus, neurological signs, paddling of legs, nystagmus, hind limb ataxia, seizures, paralysis, disorientation, anterior uveitis, petechiation. They also showed signs associated with inflammation, liver disease (jaundice) and dysregulation in coagulation, but lacked the classic ocular signs.

The clinical symptomatology described here agreed with those described by earlier workers (Zee and Maclachlan, 2004; Decaro *et al*., 2008; Piacesi *et al*., 2010). Similar signs have also been described by earlier workers (Mosallanejad *et al*., 2010) in a 3-month-old male German shepherd dog. Neonates are usually protected against CAV infection by maternal antibody (Willis, 2000), but in the present study as the adult female dhole was not vaccinated, the pups suffered from acute infection. The source of infection might have been from stray dogs those were traced living around the enclosure. There were many reports of adenoviral infections in dogs in and around the region and these stray dogs were not vaccinated against ICH.

Macroscopical findings during necropsy include severe icterus of visible mucous membranes and the subcutaneous tissue. There was generalized icterus,

Table 1: Haematological parameters of four dhole pups during the clinical illness and the average values.

Puppy/ Parameters	WBC (×10^3/µl)	RBC (×10^6/µl)	Hb (g/dl)	PCV %	MCV (fL)	MCH (pg/cell)	Granulocytes %	Lymphocytes %	Monocytes %	Platelets (lakhs/µl)
Normal values	7.91± 4.79	7.49± 0.05	16.69± 0.26	46.88± 1.31	62.50	24- 28	56- 72	21-.25	1-4	2.0 to 4.0
Dhole puppy 1 (Male)	7.1	6.5	7.5	25.8	39	11.4	66.3	28.1	5.6	0.25
Dhole puppy 2 (Female)	4.8	5.4	6.1	22.6	45	11.8	66.5	26.2	7.3	0.11
Dhole puppy 3 (Female)	4.2	3.4	4.1	14.8	43	11.9	66.8	26.8	6.4	0.34
Dhole puppy 4 (Female)	5.1	3.0	5.5	24.0	81	18.2	72.5	23.5	5.0	0.78
Average values	5.3	4.575	5.8	21.8	52	13.325	68.025	26.15	6.075	0.37

Table 2: Serological parameters of four dhole pups during clinical illness and the average values.

Puppy/ Parameters	Total protein	SGPT/ ALT	SGOT/ AST	Total bilirubin	Direct bilirubin	Indirect bilirubin	CRT	BUN
Normal values	6.09	10-109	13-15	1.0 to 4.0			0.5-1.44	20.56
Dhole puppy 1 (Male)	4.8	283	71	5.3	5.0	0.3	0.6	15
Dhole puppy 2 (Female)	3.4	264	65	5.6	5.2	0.4	0.7	14
Dhole puppy 3 (Female)	3,9	238	53	6.1	5.8	0.3	0.7	12
Dhole puppy 4 (Female)	4.5	201	42	6.6	6.2	0.4	0.8	9
Average values	4.23	246.5	57.75	5.9	5.55	0.35	0.7	12.5

Fig 2a and b: Sub-mandibular region of the carcass showing icterus, edema, gelatinization and/or marked hemorrhage in the thymus and lower neck region.

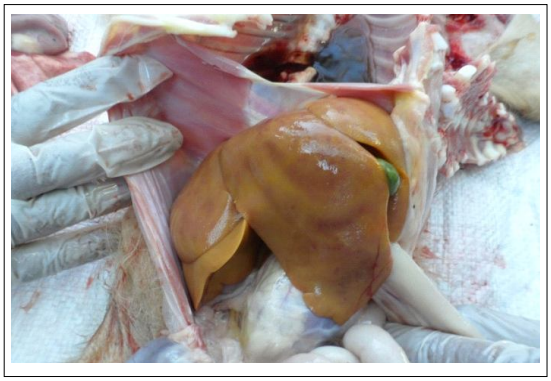


Fig 3: Liver lobes enlarged, streaks of icteric and haemorrhagic areas clearly visible on surface.

marked haemorrhage in the oral mucosa, subcutaneous tissues in the sub-mandibular region extending from tip of the lower jaw up to the thoracic inlet. Yellow gelatinous material was present in the sub-mandibular area with oedema in 3 out of 4 pups (Fig 2). Liver showed the petechial to ecchymotic haemorrhages, yellowish discoluration with mottled appearance, diffusely enlarged with round borders having a zonal pattern with alternating red and yellow areas due to the haemorrhages and icteric changes on the surface of the liver (Fig 3). Lungs were severely congested with patchy areas of consolidation. (Fig 4). Kidneys were normal in size but yellowish in colour. The pelvis region was more yellowish might be due to soft tissue and urine accumulation (Fig 5). Among lymphoid organs, spleen showed slight enlargement in three pups

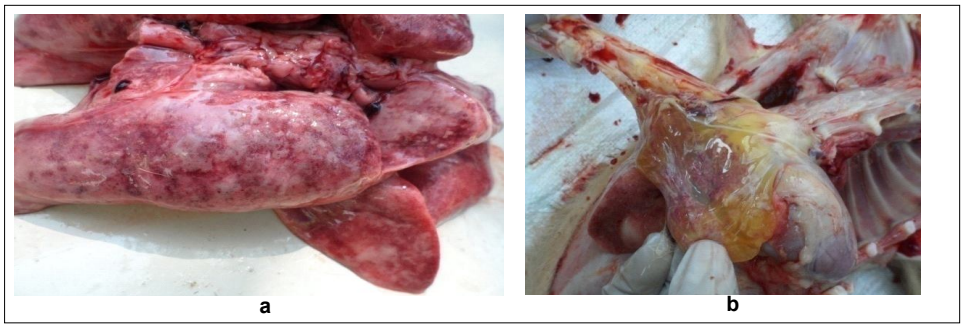


Fig 4a and b: Lungs enlarged, emphysematous, edematous, icteric with surface showing patches of haemorrhages.

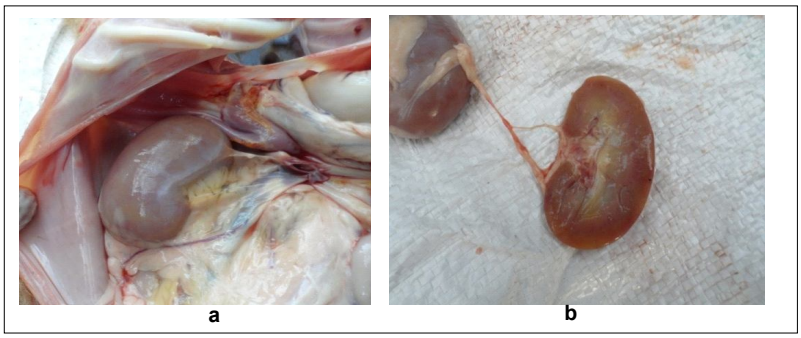


Fig 5a and b: Moderately enlarged kidneys, capsule difficult to peel off, cut surface and medulla yellowish in colour.

with black areas on the surface indicating the sub-capsular haemorrhages (Fig 6). Other organs such as lymph nodes, stomach, intestine and hearts were also congested.

Histopathological observations

Light microscopic examination of HandE-stained sections of liver showed marked multifocal hepatocellular necrosis, severe congestion in sinusoids and around the central vein. The hepatocytes in the necrotic zone showed marked changes such as more eosinophilic cytoplasm with karyorrhexis and karyolysis of nuclei. (Fig 7, 8, 9). Hepatocytes and Kupffer cells contained round to ovoid, magenta coloured basophilic intranuclear inclusion bodies measuring 4-8 µm in diameter with marginated chromatin (Fig 10).

The most significant lesion reported in literature and observed in this study was necrohemorrhagic hepatitis (Chouinard *et al*., 1998) along with hepatomegaly has been compatible with CAV-1 infection. Gross necropsy findings of all the dead animals are suggestive of ICH (Greene, 2006). The basophilic intranuclear inclusion bodies observed in the study have earlier been reported within the hepatocytes and also in CNS vascular endothelium (Caudell *et al*., 2005). However, both eosinophilic and basophilic (amphophilic) inclusions in peracute ICH were earlier documented in young Alsatian and Labrador male dogs (Cheema*et al*., 2012). Based on all these histopathologic findings, the diagnosis was indicative of ICH.

Although viral detection by classical or molecular methods demonstrated the presence of CAV-1 in tissues, the observa-tion of nuclear inclusion bodies in the liver through histopathological examination (Duarte *et al*., 2014), which was satisfied in the study. Significant histopathological alterations were observed within most tissues collected during necropsy. Finally, the systemic ICH with involvement of CAV-1 was presumptively diagnosed by the characteristic clinical manifestation of necrotizing hepatitis associated with intranuclear inclusion bodies predominantly within Kupffer cells and hepatocytes (Brown *et al*., 2007; Greene, 2012). Similar histopathological findings along with intranuclear inclusion bodies in hepatocytes were also reported in three cases of ICH in free-ranging red foxes (*Vulpes vulpes*) in England satisfying the isolation of CAV-1 (Thompson *et al*., 2010). Liver and kidney have significantly been classified as the 'sentinel' organs for CAV-1 infection. Liver is main organ affect during the acute stage of ICH in dogs (Decaro*et al*.,2012), while the kidney is involved in the 'chronic' and the virus shed in the urine (Baker *et al*., 1954). Focal mesangial sclerosing glomerulonephritis and interstitial nephritis were reported in acute spontaneous cases of ICH (Wright, 2008). Example of CAV-1 infection involving brain lesions (encephalopathy) has also been documented earlier in Labrador retriever pups (Caudell *et al*., 2005). The precise cause of death in ICH is uncertain though liver is the primary site of viral injury. However, severe organ damage or the development of DIC might also play role.

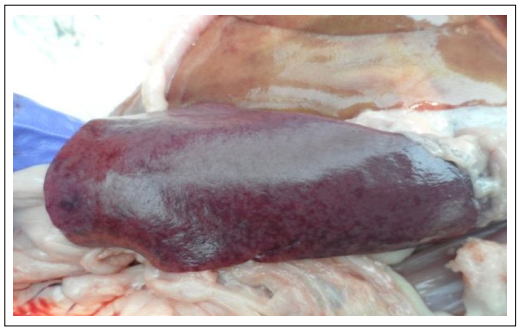


Fig 6: Spleen enlarged with sub-capsular haemorrhages.

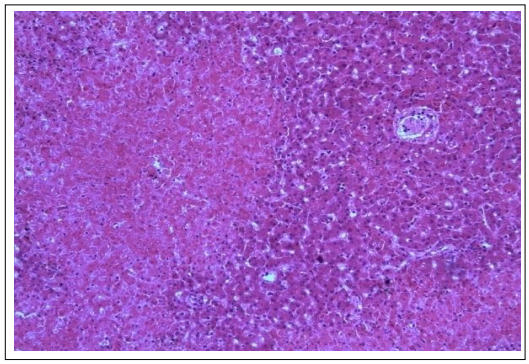


Fig 7: Section of liver showing focal areas of necrosis (Magnification 10×).

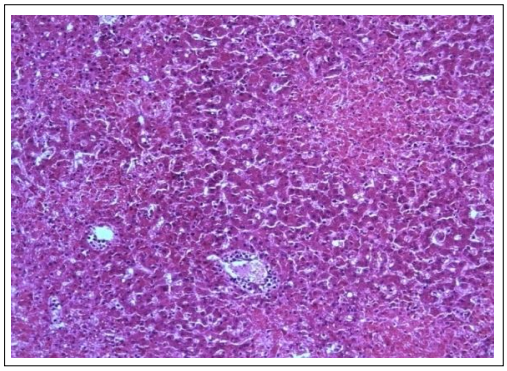
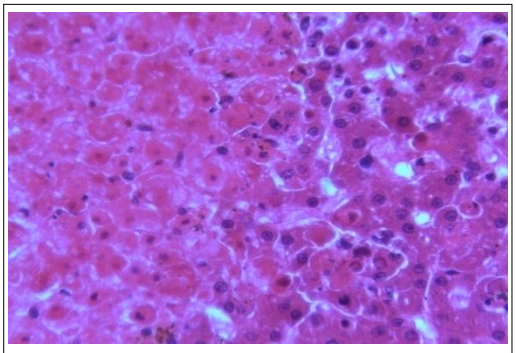
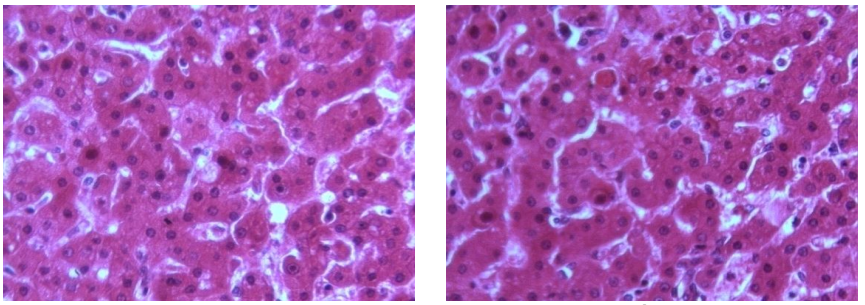


Fig 8: Section of liver showing focal areas of necrosis and congested central veins (Magnification 10×).



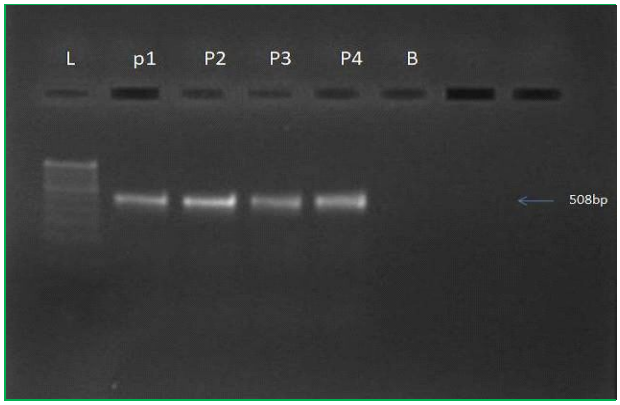
Necrotic hepatocytes revealing loss of nucleus and highly eosinophilic cytoplasm (Magnification 40×).

Fig 9: Section of liver showing focal areas of necrosis.



a b

Fig 10a and b: Section of liver showing degenerating hepatocytes with intranuclear inclusions and margination of chromatin (Magnification 40×).



Here, the positive amplification of E gene of CAV-1 (508-bp) from liver samples of four infected dhole pups is shown in lanes P1 to P4; Lane L: 100-bp molecular weight marker; Lane B: Negative control.

Fig 11: Figure of PCR assay showing the amplified product 508-bp of E gene segment corresponding to CAV-1 analyzed by 2% electrophoretic agarose gel stained with ethidium bromide.

Bacteriological culture

Bacteriological culture was found to be negative for significant bacterial pathogens such as *Salmonella*, *Campylobacter*, enterotoxigenic *Escherichia coli* and *Clostridium perfringens* in all four cases.

PCR findings

Additional degree of confidence towards confirmatory diagnosis was obtained from the conventional PCR assay that successfully amplified the 508 bp fragment of the E region of CAV-1 (Fig 11) in hepatic tissue homogenates of the suspected pups submitted for necropsy.

Previously, CAV-1 causing ICH was detected using PCR (Kiss *et al*., 1996). CAV-1 was reported in a 10-month-old female fennec fox (*Vulpes Zerda*) further confirmed by virus isolation and nucleotide sequencing (Choi *et al*., 2014). ICH was also reported in juvenile red foxes in wildlife rescue centres in the United Kingdom (UK), where CAV-1 was detected in tissues from affected foxes by PCR and sequencing (Walker*et al*., 2016). Nucleic acid-based technique has proved to be most useful in detecting ICH in many other reported instances. Four outbreaks of ICH occurring in Italy between 2001 and 2006 confirmed by PCR have been reported (Decaro *et al*., 2007). For the first time, Pizzurro *et al.* (2017) reported the isolation and whole genome sequence of a CAV-1 isolate from the liver of a free-ranging wolf (*Canis lupus*) in Southern Italy. Duarte *et al.* (2014) described fatal CAV-1 acute infection in an unvaccinated 56-day-old Yorkshire terrier pup in Portugal, where this is considered a rare infection. Knowles *et al.* (2018) have recently detected ICH in a free-ranging brown bear (*Ursus arctos horribilis*) cub from Alaska, USA with demonstration of both intranuclear inclusion bodies in hepatocytes and immunohistochemical reactivity to adenoviral antigens along with sequencing of the hexon protein gene that showed 100% identity to CAV-1.

The infection of the dhole pups in our study could likely be due to a combination of factors. Firstly, the parent dholes were not vaccinated due to practical difficulties. Secondly, the pups could not be vaccinated at 4 and 6 weeks of age, because of the constraint in their handling in free range condition without any restraining cages in the enclosure. Thirdly, there are no guidelines for vaccination schedule and dosage for dholes in India. Since dhole is a canid, zoo vets feel that the vaccination schedule might be similar to dogs and follow the dog vaccination schedule and dog vaccines, since no separate vaccines are available for the said species. Fourthly, it is known that CAV-1 is endemic in the domestic and stray dogs in the nearby places of the Biological Park at Bengaluru. Being housed indoors, the pups could have acquired the infection via contaminated fomites from the stray dogs roaming around their enclosure, for which the entire litter was exposed to infection leading to fatal ICH within a short period of time. The incidence of CAV-1 infection in dogs ap-pears to have decreased worldwide since the 1950's due to vaccination of dogs with CAV-2, which is antigenically similar to CAV-1 (Philippa, 2010). Modified live vaccines (MLVs) induce specific antibodies persisting for as long as 14 years conferring full protection against ICH. Recently, a canine hepatitis DNA vaccine has also been developed (Liu *et al*., 2008).

CONCLUSION

The present study reported clinical signs, gross and microscopical lesions compatible to ICH in the affected dhole pups with simultaneous profiling of hemogram and biochemical parameters followed by identification of inclusion bodies and genome of CAV-1 through PCR. This report showed that CAV-1 is currently circulating in the captive dhole population in the country and causing mortality, hence a suitable vaccination schedule and hygienic procedures has to be followed for the susceptible animals.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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