



Molecular Detection and Pathomorphological Studies of Canine Distemper Virus Infection (CDV) in Dogs

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ABSTRACT

In this study RT-PCR was used to detect CDV NP gene from the whole blood samples of dogs clinically suspected for CD followed by the study of pathomorphological alterations in different tissues. Clinical signs such as respiratory distress, purulent oculo-nasal discharge, biphasic fever, gastroenteritis, pustular dermatitis, prominent hyperkeratosis of the digital pads and nervous disorders were observed in the clinically suspected cases. The nervous disorder includes seizure, convulsion, paddling movement, tremor followed by posterior paralysis. On necropsy, bronchopneumonia, congestion and haemorrhages over the mucosal surface of the urinary bladder, stomach, intestine and brain were prominent lesions. Microscopically, both eosinophilic intranuclear and intracytoplasmic inclusion bodies were recorded in the neurons and glial cells, epithelial cells of the bronchioles, urinary bladder, kidney tubules and gastric glands. Congested vessels with haemorrhages were also found in the brain, lungs, stomach, intestine and lymphoid organs.

HIGHLIGHTS

- Amplification of NP gene was found in 41.67 percent (n=25) cases out of total 60 sample tested by RT-PCR.
- Most prominent clinical manifestations were oculo-nasal discharge, gastroenteritis, biphasic fever, hyperkeratosis, pustular dermatitis and nervous disorders.
- In histopathological studies inclusion bodies were recorded in the lungs, brain, kidneys, stomach and urinary bladder.

Keywords: Canine distemper virus, Dog, Histopathology, RT-PCR

Canine distemper (CD) is a potentially fatal, multi-systemic and highly contagious viral disease of canines, which was recognized as one of the leading cause of high mortality in dogs (Dongre *et al.*, 2013). Canine distemper is an infectious disease, infecting a broad range of animals including terrestrial and aquatic carnivores. The virus is shed primarily in the ocular and nasal secretions of the infected animals (Beineke *et al.*, 2015).

Clinical diagnosis of CD is complicated due to its similar clinical signs with other respiratory and enteric disorders.

Molecular diagnosis gives confirmatory conclusion about the presence or absence of the viral antigen in the animals. Reverse transcriptase-polymerase chain reaction (RT-PCR) is found to be one of the reliable techniques to detect

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canine distemper virus (CDV) antigen in the clinical samples (Kim *et al.*, 2006; Yi and Cheng 2014), though its sensitivity varies with the nucleic acid extraction method and primers used for amplification of the target region (Saito *et al.*, 2006). As the disease is contagious in nature and cause high mortality of young animals, therefore an early diagnosis of the disease is essential for proper treatment and to take care of the animals. The aim of the present study was to molecular detection of CDV at the early stage of the disease and correlate with pathomorphological alterations.

MATERIALS AND METHODS

Collection of samples

The sources of materials were Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Science, Khanapara; State Veterinary Dispensary, Chenikuthi; Govt. of Assam and the other private clinics within the Guwahati city. Dogs with the history of fever, purulent oculo-nasal discharges, pneumonia, pustular dermatitis and neurological disorders were considered as suspected cases of CD for the present study. Samples were collected from a total of 60 clinically suspected cases. Three ml of blood was aseptically collected in EDTA vacutainer and stored at -20°C for further analysis by RT-PCR.

Histopathological examination

Tissue samples from different organs showing specific lesions were collected in 10% formal saline solution at the ratio of 1:10 for histopathological examination. After proper fixation, tissue samples were processed for routine histopathological examination as per standard procedure (H&E) (Culling, 1974).

RT-PCR

Reverse transcription and amplification of NP gene

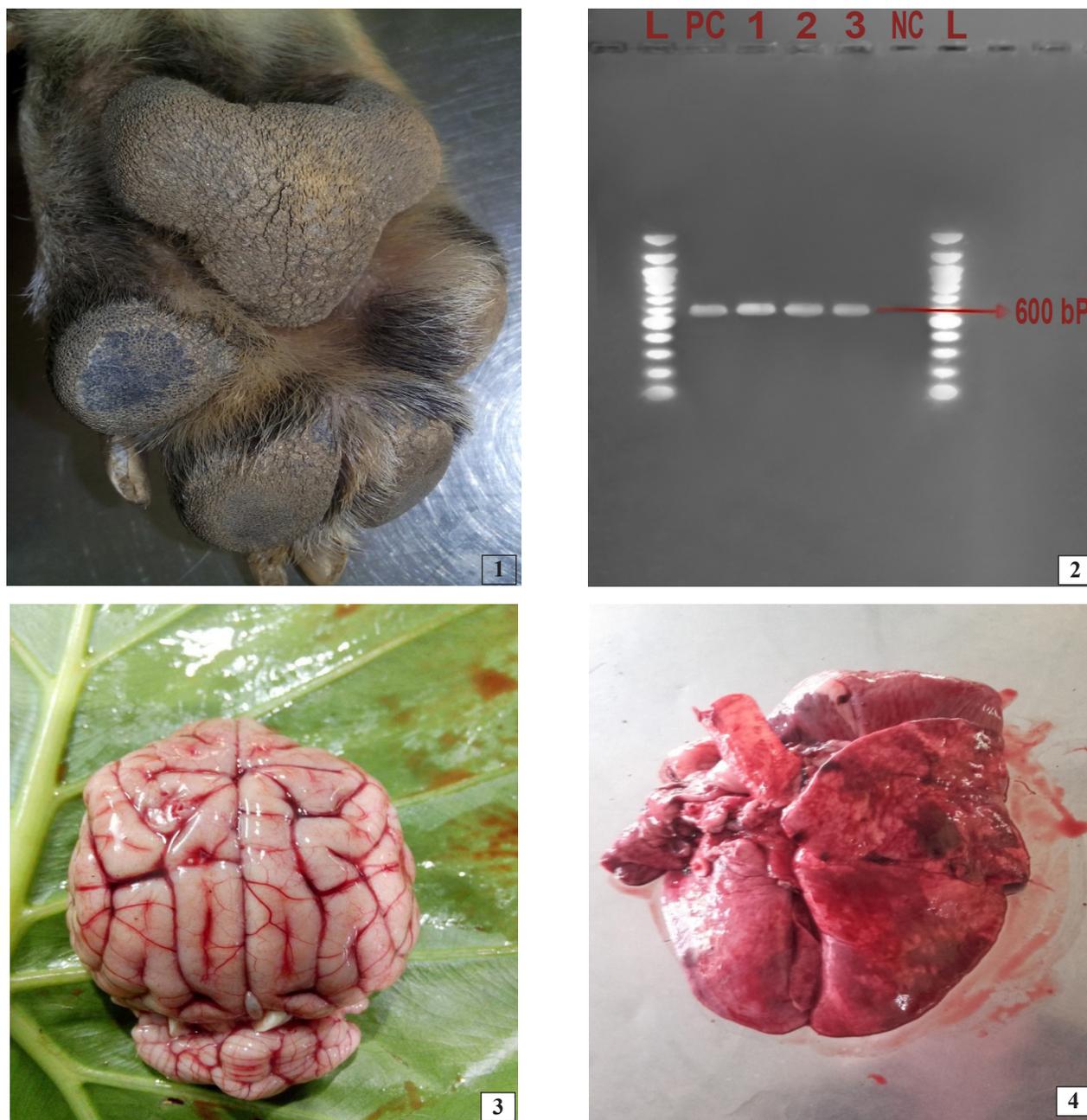
Total RNA was extracted from 250µl of whole blood samples via TRIzol™ (Thermo Scientific, Massachusetts, USA), following standard protocol. The cDNA was produced with Revert Aid™ M-MuLV reverse transcriptase (Thermo Scientific). Reverse transcription

was carried out at 70°C for 5 min, followed by 25°C for 10 min and hold at 4°C. Amplification of CDV nucleoprotein (NP) gene was obtained from the cDNA template with a NP gene specific primer pair (GenBank accession no.EF375619) along with the CDV vaccine (Nobivac DHPPi) as positive control. The forward primer was 5'-GAAACTATGTATCCGGCT-3' and the reverse primer was 5'-TGACTCACTCCATTCGGA-3'. The 25µl reaction mixtures contained 0.5µl (10pmol/µl) of each of the forward and reverse primer, 12.5µl of master mix, 1µl of template DNA and 10.5µl of nuclease free water. The reactions were performed in a PCR Thermal Cycler S1000™ (Bio-Rad, USA). Amplification was conducted by a temperature cycling protocol consisting of initial denaturation at 95°C for 5 min, 30 cycles of 30s for denaturation at 95°C, 1min of primer annealing at 45°C, and 1min of extension at 72°C, followed by 5 min of final extension at 72°C. Following RT-PCR, 7ml of the amplicons were electrophoresed in a 1% Tris acetate-EDTA agarose gel and electrophoresis was carried out at 80 volts for 60 minutes. The gel was stained with ethidium bromide and the bands were visualized at 312nm using a gel documentation system (DNA Bio-Rad imaging system). Product sizes were determined with reference to a 100bp DNA ladder.

RESULTS AND DISCUSSION

Clinical signs

The most frequently observed clinical signs were respiratory distress characterized by pneumonia, coughing, laboured breathing and ocular and nasal discharge. Initially, nasal and ocular discharge was watery in nature, which later becomes mucopurulent leading to attachment of both the eyelids. This reports correlate with the findings of Mortella *et al.* (2006) and Zhao *et al.* (2015). High rise of body temperature from 102.8-105°F, anorexia, lethargy, dehydration and weight loss were noticed in all the animals at the initial phase of the disease. Similar findings were reported by Elia *et al.* (2015). Biphasic fever was recorded as a prominent clinical signs in almost all the affected animals. The initial rise of temperature was seen at the viraemic stage when virus was circulating in the blood, following rise of temperature was reported due to the secondary bacterial infections as CDV results



Figs. 1-4: Photographs showing (1) Hyperkeratosis of the digital pads, (2) RT-PCR amplification of the NP gene (L = Standard 100bp DNA ladder, PC = Positive control, 1,2,3 = Positive samples, NC = Negative control), (3) Congestion in the brain, (4) Patchy areas of emphysema, congestion and haemorrhages in the lungs indicating bronchopneumonia

immunosuppression by affecting lymphoid organs. In young animals, pustular dermatitis was noticed in the ventral abdominal wall, inner side of the thigh and ears. Both hyperkeratosis and parakeratosis of the skin around the eyes and nose were observed in some of the dogs more than

two years of age. Foot pads were hard to touch and painful due to hyperkeratosis (Fig. 1) which supports the findings of Carvallo *et al.* (2012). In some animals, neurological disorders were evident, which includes salivation, partial to generalized seizure, paddling movements and tremor.



Few chronic cases were reported with posterior paralysis. Neurological signs appear as CDV may affect the grey and white matter of the central nervous system (Amude *et al.*, 2007).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used as a diagnostic tool for the detection of CDV in the present study. Amplification of NP gene was found in 41.67 percent (n=25) cases out of total 60 sample tested. The positive samples and control showed clear band at 600bp against absence of band in negative control (Fig. 2). Since the NP gene is required for replication of the virus, the mRNA of the NP gene is transcribed more extensively in the infected cells (Shin *et al.*, 1995) and appears to be a good marker of infection (Alcalde *et al.*, 2013; Yi and Cheng 2014).

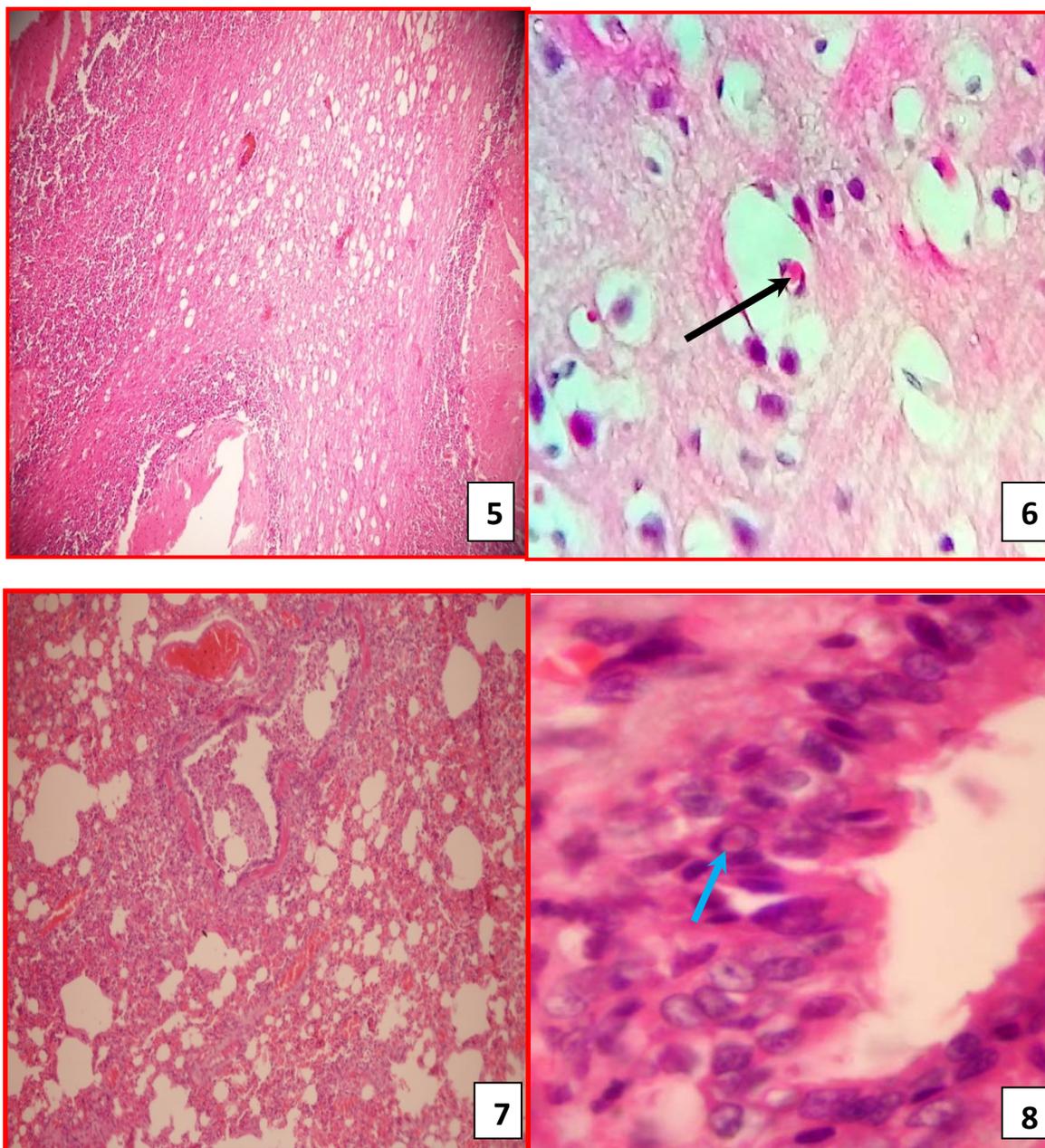
Gross pathology

Necropsy was performed in six animals found positive in RT-PCR. The brain lesions observed were haemorrhages, congestions and meningitis (Fig. 3). Similar findings were also reported by Machida *et al.* (1993). The virus has the affinity towards central nervous system (Jones and Hunts, 2006) that could be the cause of gross alteration in the CNS. Lungs of the affected animals showed congestion, focal emphysema, dark patchy pneumonic areas in all the lobes especially in the diaphragmatic lobes indicate bronchopneumonia (Fig. 4). The affected lungs were dark in colour and heavy. Squeezing of the cut section of the lungs revealed frothy exudates coming out particularly from the bronchi and bronchioles. The tracheal lumen also contains mucopurulent exudates and the mucosa was congested. These findings correlate with the reports of earlier workers (Lan *et al.*, 2006; Kubo *et al.*, 2007). From the previous literature it was revealed that lung was the target organ for CDV and initial replication of the virus take place in the macrophages of the lungs (Beineke *et al.*, 2009). The stomach was found to be empty with some watery contents in all the 6 positive cases. Haemorrhages were observed across the mucosal folds in the fundic and pyloric junction of the stomach. The intestinal mucosa showed congestion and haemorrhages with thickening of the intestinal wall in some cases. Similar findings were recorded by Galan *et al.* (2014) and Zhao *et al.* (2015).

The kidneys were congested and haemorrhagic with presence of necrotic foci over the cortex. This finding is correlates with Jones and Hunts (2006). The urinary bladder was found distended and on opening blood tinged urine was came out. The mucosal surface of the bladder revealed petechial to diffuse ecchymotic haemorrhages spread over the whole mucous membrane. Congestion with mild to moderate enlargement of the lymphoid organs including spleen was noticed. The lesions were marked in the mesenteric lymph nodes. This gross alteration may be due to the affinity of the CDV to the lymphoid organs (Kumagai *et al.*, 2003).

Histopathology

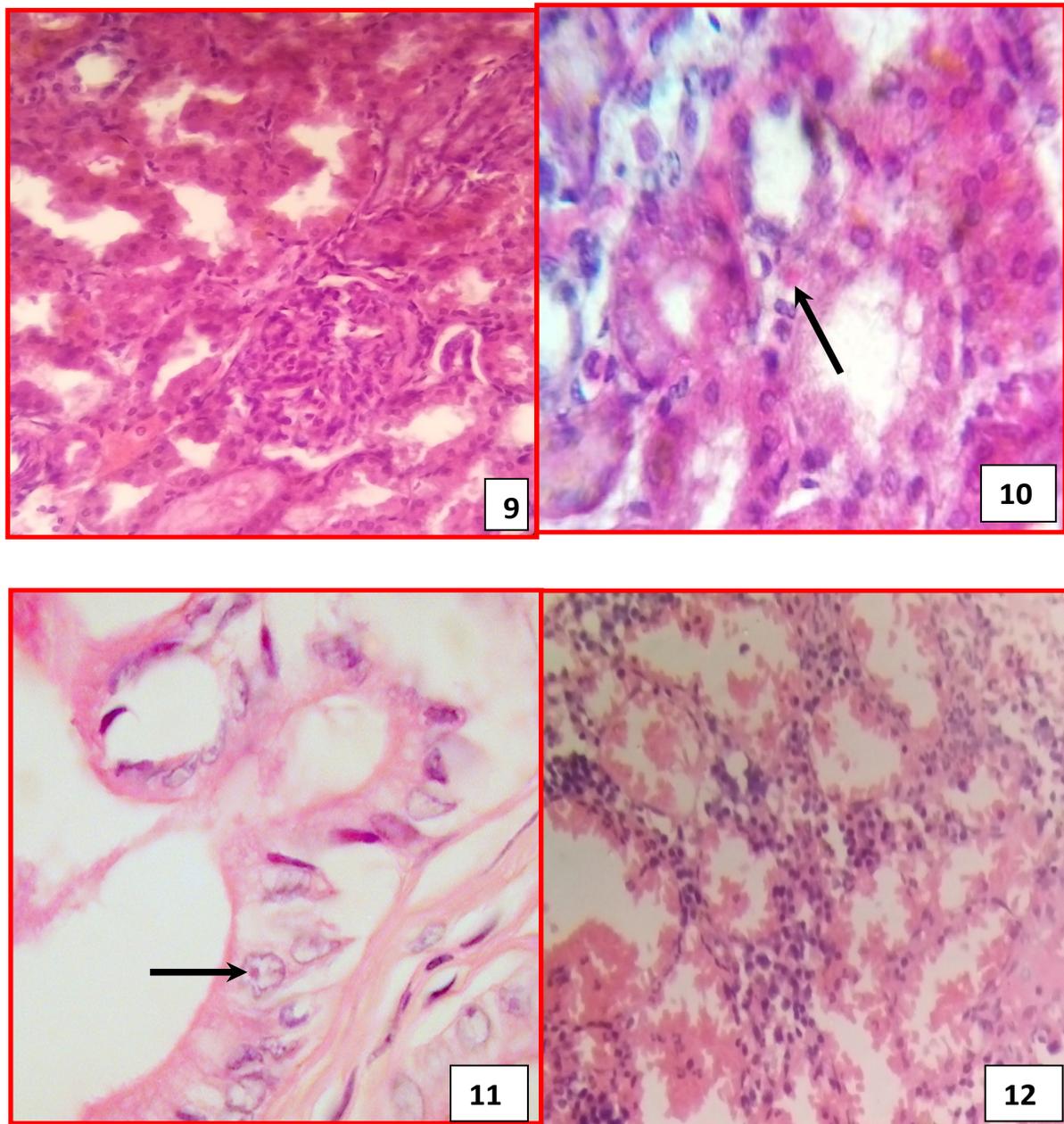
Non-suppurative encephalomyelitis was the most prominent microscopic change in the brain. Other changes in the brain were multifocal areas of demyelination in the cerebrum and cerebellum (Fig. 5). Oligodendrocytes were metabolically active cell in the central nervous system and play an important role for myelin renewal and when brain tissues were infected by CDV, degeneration of oligodendrocyte was often occurred because of metabolic impairment which may be the cause of demyelination (Yao-qian *et al.*, 2013). Most of the neurons were degenerated and marked perivascular cuffing predominantly with lymphocytes and plasma cells. Degeneration of the Purkinje cells seen in few cases of this study was also reported by Lisiak *et al.* (1979). Several eosinophilic intranuclear and intracytoplasmic inclusion bodies were observed in the neuron and glial cells (Fig. 6) which are characteristic histopathological changes recorded by various workers (Lan *et al.*, 2006; Kubo *et al.*, 2007; Renteria-Solis *et al.*, 2014). Alveolar septa showed marked thickening due to presence of oedematous exudates, haemorrhage and infiltrating cells indicative of interstitial pneumonia (Fig. 7). The alveolar lumen was occluded with serofibrinous exudates, polymorphonuclear cells and mononuclear cells with hyperplasia of the alveolar lining epithelium. Squamous metaplasia of bronchiolar epithelia were occasionally seen in the lungs. These findings are in accordance with Apple *et al.* (1994). Inclusion bodies, both intranuclear and intracytoplasmic type were also observed in the alveolar macrophages, epithelial cells of the bronchioles, kidney, stomach and urinary bladder (Fig. 8,9,10,11), which were considered as a characteristic histopathological lesions in CD cases (Lan *et al.*, 2006; Zhao *et al.*, 2015; Green and



Figs. 5-8: Photomicrograph showing. **(5)** Empty spaces in the cerebellum indicating demyelination (H.&E.x100), **(6)** Intranuclear inclusion in the microglial cells (arrow) of the cerebrum (H.&E.x1000), **(7)** Congestion, haemorrhages, emphysema, bronchopneumonia with exudates in the bronchiolar lumen (H.&E.x400), **(8)** Bronchiolar hyperplasia with intranuclear inclusion (arrow) in the bronchiolar epithelial cells (H.&E.x1000)

Vandevelde, 2011; Kubo *et al.*, 2007). This is in agreement with the present findings where inclusion bodies could be demonstrated in all the carcasses. The inclusion bodies recorded in both the cells were found to be round with intense eosinophilic appearance. The stomach revealed

congestion, haemorrhage, degeneration and desquamation of lining epithelial cells which is also recorded by Galan *et al.* (2014). The stomach showed few necrotic cells with eosinophilic intranuclear inclusion bodies in the epithelial cells of intestinal crypts and gastric glands (Kubo *et al.*,



Figs. 9-12: Photomicrograph showing. **(9)** Tubular degeneration, haemorrhage and increase hypercellularity of the glomerulus (H.&E. x400), **(10)** Presence of intracytoplasmic inclusion body (arrow) in the tubular epithelium of kidney (H.&E.x1000), **(11)** Intranuclear acidophilic inclusion body (arrow) in the mucosal epithelium of the stomach (H.&E.x1000), **(12)** Haemorrhages with marked lymphoid depletion in the lymphoid follicles of lymph node (H.&E.x400).

2007). The CD virus is pantropic and has special affinity to the epithelial cells of the body where virus replicates, might damage to the stomach mucosa (Jones and Hunts, 2006). Intestine revealed necrosis and detachment of the villus epithelium and infiltration of mononuclear cells in

the lamina propria. Similar findings reported by Zhao *et al.* (2015). The epitheliotropic nature of the virus might be the reason of histopathological alterations found in the present study (Green and Vandavelde, 2011). Degeneration, necrosis and sloughing of transitional epithelium with

congestion and haemorrhages in the submucosal layer were noticed. Mild infiltration with mononuclear infiltrating cells was observed in the sub mucosa and muscularis layer. These findings correlate with Zhao *et al.* (2015). Spleen and lymph nodes revealed congestion and haemorrhages with mild to moderate depletion of lymphocytes (Fig. 12) in the splenic white pulp and in the follicles as well as. The canine distemper virus has affinity towards the lymphoid organs and replicates in the lymphoid cells, so, it could be the cause of lymphoid depletion (Lan *et al.*, 2006).

CONCLUSION

The CDV NP gene was detected in the whole blood samples of infected animals with the help of RT-PCR which can be considered as a sensitive, reliable and fast method for early diagnosis of CDV infection. Most common clinical signs manifested by the animals were biphasic fever, oculo-nasal discharge, hyperkeratosis, gastroenteritis and nervous disorders. The gross lesions and histopathological demonstration of inclusion bodies in the different tissues recorded in this study were correlates with the clinical signs manifested by the animals found positive in RT-PCR.

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