

INVESTIGATING INFECTIOUS AND ZOO NOTIC DISEASES OF STREET DOGS IN THE RESIDENTIAL AREA OF BANGLADESH AGRICULTURAL UNIVERSITY

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ABSTRACT

Dogs are common carnivore and scavenger at Bangladesh Agricultural University (BAU) campus. Dogs may carry many infectious and zoonotic diseases and could play a role in transmitting those diseases in other carnivores and human as well. At day time cats, dogs and golden jackals are found to eat in a same dustbin at BAU campus. So it is possible to share diseases of each other and there are possibilities of crossing species barrier. This study was, therefore, aimed at specific investigation of the occurrence of leishmaniasis, canine distemper (CD), infectious canine hepatitis (ICH) and avian influenza (AI) of dogs at BAU campus. This study was carried out during the period from January to June/ 2012. A total of 10 apparently healthy dogs were euthanized and postmortem examination were carried out. Impression smears were prepared from spleen, liver, bone marrow and stained with Giemsa's stain. Histopathological studies were conducted using routine procedures. For molecular characterization, polymerase chain reaction (PCR) and Reverse Transcriptase polymerase chain reaction (RT-PCR) were adopted for the detection of genomic fragment of visceral leishmaniasis (VL), canine distemper (CD), infectious canine hepatitis (ICH) and avian influenza (AI, viral matrix protein gene). Results of this study showed the presence of leishmania organism in the impression smears of eight dogs. Using PCR amplification technique, *Leishmania donovani* specific genomic DNA (145bp) was detected in two dogs. PCR amplification of genomic DNA of ICH (411bp) and RT-PCR amplification of genomic RNA of CD (287bp) and matrix protein gene of AI (245bp) were not detected in any of the dogs of BAU campus. Future work is needed to explore the presence of sand flies (vector of VL) in the investigating areas and establish role of street dogs of BAU campus towards disseminating leishmaniasis in other animals and human beings.

Key words: Street dogs, visceral leishmaniasis, Canine diseases, Avian influenza, giemsa's staining, PCR

INTRODUCTION

Street dogs are important domestic carnivore and scavenger of the environment of BAU campus. There are huge numbers of street dogs, golden jackals and cats living around BAU campus and often found to play and eat in same tale. Dogs are the usual connectors between the people, animals and nature. Dogs act as reservoirs in the transmission of numerous infectious, emerging and zoonotic diseases, creating risk on to the health of both wildlife and human as well. Among the diseases, leishmaniasis or kala-azar is becoming a common threat for both human and animal welfares. Human and canine visceral leishmaniasis (CVL), a typical rural disease associated with precarious conditions of life (Bevilacqua *et al.*, 2001). It is a chronic parasitic infection that infects approximately 400,000 individuals annually, with a predilection towards early childhood (Grech *et al.*, 2000). The spatial evolution of the epidemic showed that the canine cases had preceded the human cases confirming the importance of dogs as reservoir in urban areas (Bevilacqua *et al.*, 2001).

Canine distemper (CD) on the other hand is a serious, often fatal viral disease in dogs and many other carnivores (Appel, 1987; Yousuf *et al.*, 2014). CD is a highly contagious disease of young dogs, distributed worldwide and infect particularly 3 to 6 months age group with high morbidity and mortality. It is manifested by a biphasic fever, leukopenia, gastroenteritis, respiratory catarrh, frequently pneumonia and neurological complications (Aiello and Mays, 1998). CD was detected in golden jackals of char areas of Brahmaputra river next to Bangladesh Agricultural University (Yousuf, 2008) campus.

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Infectious canine hepatitis (ICH) is an adenoviral disease of young Canidae and Uridae, is characterized clinically by in apparent to rapidly fatal disease (Greene *et al.*, 1990). Canine adenovirus-I (CAV-1) is both endotheliotropic and hepatotropic. In addition to acute hepatocellular necrosis, acute severe hemorrhages are seen on serosal surfaces, within lymphnodes and the liver, and rarely in the brain (Greene, 1990; Kelly *et al.*, 1993).

The H5N1 influenza viruses have been isolated from several mammalian hosts (Keawcharoen *et al.*, 2004; Amonsin *et al.*, 2006; Zhu *et al.*, 2008). Pigs, for example, are regarded as a natural influenza virus host that could be infected by an H5N1 influenza virus in the field or an experimental setting (Lipatov *et al.*, 2008; Zhu *et al.*, 2008) and could lead the generation of an H5N1 pandemic strain. Cats and dogs, which come into close contact with humans, may also play a role in the interspecies transmission of influenza viruses. Songserm *et al.* (2006) reported natural infection of dog with a natural outbreak of H5N1 influenza virus and recorded systemic replication of the virus in dog. This study was aimed to adopt protocols for the detection of selected genomic fragments of VL, ICH, CD and AI matrix protein genes and designed strategies to prevent its future dissemination.

MATERIALS AND METHODS

The investigation was carried out in the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh. A total of 10 apparently healthy dogs were caught by the dog catchers of district public health corporation, Mymensingh, euthanatized by intravenous injection of saturated solution of magnesium sulfate and brought for investigation to the Department of Pathology, BAU during the period from January-June, 2012. Systemic dissection and investigation was carried out and liver, lymph node, kidney, spleen, heart, lungs, bone marrow, intestine and skin were collected.

Preparation of impression smears

A thin smear of blood and impression smears from the cut surface of liver, lungs, spleen and bone marrow were made on to clean slides and stained with Giemsa's stain (Luna, 1968). A low (10x) and high power (40x and 100x) microscopic observation were carried out to investigate the promastigote and amastigote stages of leishmanial protozoa in tissue spaces and in macrophages of various organs. Investigation onto the impression smears of liver was also carried out at 100x objective to find out hepatic intranuclear inclusion bodies of ICH and intranuclear and intracytoplasmic inclusion bodies in the cell smears prepared from lungs suggestive for CD.

Histopathological investigation of tissues

During necropsy liver, lungs, spleen, intestine and skeletal muscles from all dogs were collected and fixed in 10% buffered neutral formalin for histopathological studies. Formalin fixed tissue samples were trimmed, processed, sectioned and stained with Hematoxylin and Eosin staining (Luna, 1968). A low (10x objective) and high power (40x, 100x objectives) microscopic examination were carried out to observe the changes related to specific infections.

Detection of VL and ICH by PCR

DNA extraction

Wizard genomic DNA extraction Kit (Promega Inc, USA) was used to extract genomic DNA for the detection of genomic fragments of Leishmania and ICH. Portion of liver and spleen of street dogs were also collected and extracted genomic DNA using manufacturer instruction (Khan *et al.*, 2012). Spectrophotometry (A_{260} and A_{280}) and agarose gel electrophoresis were done for confirmation of the purity and concentration of DNA. Primer sets from published literature (Table 1) were used to identify causal agent of VL (*L. donovani*) and viruses of ICH.

PCR detection of VL and ICH

PCR reactions were adopted for the detection of genomic fragments of *L. donovani* (Khan *et al.*, 2012) and ICH viruses. PCR was carried out in a final reaction volume of 25 μ l in the thin walled PCR tubes. The Promega Inc PCR Master Mix Kit was used to amplify genomic DNA in a programmable thermocycler. The thermal profile used was an initial denaturation for four minutes at 94°C followed by 45 cycles of DNA amplification reaction in a Master Cycler (Master Cycler Gradient, Eppendorf, Germany). The condition of PCR amplifications

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were denaturation for 60sec at 94°C, annealing for 90sec at 61°C and extension for 60sec at 72°C followed by a final extension for 10min at 72°C. The PCR reactions were held at 4°C and finally the reaction was terminated by adding 3µl 50mM EDTA. The PCR products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and examined under UV light using an image documentation system (Cell Biosciences, Alphamager HP, USA).

Table 1. Primers and their sequences used to detect genomic fragments of VL, ICH, CD and AI from the internal organs of street dogs

Primers name	Sequences (5'-3') of primer	Amplicon size/ diseases	References
LDF ₁	CTTTTCTGGTCCCGCGGTAGG	145 bp/ VL	Gomes <i>et al.</i> , 2006
LDR ₁	CCACCTGGCCTATTTTAACCA		
ICHF	CACTCGAGATGGCCATCTTAATAT	411 bp/ ICH	Chouinard <i>et al.</i> , 1998
ICHR	TCCTGCAGTTTAGAGCGGACCCTG		
CDF	ACAGGATTGCTGAGGACCTAT	287 bp/CD	Frisk <i>et al.</i> , 1999
CDR	CAAGATAACCATGTACGGTGC		
MPAIF	CTTCTAACCGAGGTCGAAACG	245 bp/AI	Fouchier <i>et al.</i> , 2000
MPAIR	AGGGCATTGTGGACAAKCGTCTA		

RT-PCR detection of CD and AI viruses

RNA extraction protocol

RNA from the host tissues including viral RNA was extracted from liver and spleen of street dogs using commercially available RNA extraction Kit (Promega INC, USA) and used the protocol as described by the manufacturer. The purity and concentration of RNA was determined by using spectrophotometry (A_{260}/A_{280}) and agar gel electrophoresis.

RT-PCR detection of CD and AI viruses

One step RT-PCR was carried out with a total of 25 µl of reaction volume. The 2x master mix was dispensed to each PCR tubes containing 20pmol primers of each and 100-150ng/ 5µl extracted RNA. The RT-PCR protocols were adopted and carried out in a Master Cycler (Master Cycler Gradient, Eppendorf, Germany). The thermal profile used for CD Viruses comprised a reverse transcription for 30min at 50°C (42°C for AI). The subsequent PCR were carried out with an initial denaturation for 4min at 94°C followed by 40 cycles of amplification reaction. The condition of PCR amplifications were denaturation for 60sec at 94°C, primer annealing for 2min at 59.5°C (60sec at 45°C for AI) and extension for 3min at 72°C followed by a final extension for 10min at 72°C. The RT-PCR reactions were held at 10°C and the reaction was terminated by adding 3µl 50mM EDTA. The RT-PCR products were analyzed by electrophoresed in 1.5% agarose gel, stained with ethidium bromide and examined under UV light using an image documentation system (Cell Biosciences, Alphamager HP, USA).

RESULTS AND DISCUSSION

Dogs are the carrier of many infectious and zoonotic diseases, hence investigation were carried out on to randomly selected 10 dogs of BAU campus to identify the presence of *Leishmania donovani*, *Canine distemper*, *Infectious canine hepatitis* and *Avian influenza* (matrix protein) organisms. Necropsy, staining of impression smear with Giemsa's, histopathological examination, PCR and RT-PCR protocols were adopted. Physical examination of the dogs revealed rough hair coats and alopecia (Fig. 1a). Spleens were enlarged and lungs were irregularly consolidated and blackened (N=02). There was splenomegaly and enlarged liver in two dogs and it could be due to subclinical form of leishmaniasis (Petersen, 2009).

Leishmanial promastigote like organisms were seen in the impression smears prepared from spleen, liver and bone marrow (Fig. 2) stained with Giemsa's stain. Protozoa similar to promastigotes phases of *Leishmania* in the organ smears was seen and indicated infection with *Leishmania spp* (Al-Shamahy (2003). Small oval shape parasites with dark nucleus and a small kinetoplast were seen in the impression smear of liver and spleen (Noli, 1999). Out of 10 dogs examined, eight showed the presence of promastigote stage of *leishmania* in liver, spleen and bone marrow.

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About 83.4% infectivity of dogs with the promastigotes of *Leishmania* spp in either liver or spleen was reported with reactive cellular infiltration (Al-Shanawi, 1975). However, using impression smear staining species of *Leishmania* involved could not be identified.



Fig. 1. Investigating street dogs (a) for the detection of diseases. At necropsy dominant lesion was only seen in the skin and internal organs and were apparently healthy (b). Following histopathology, nodular lesion in liver (c, arrow, 10x) characteristics of Visceral leishmaniasis was seen in a dog.

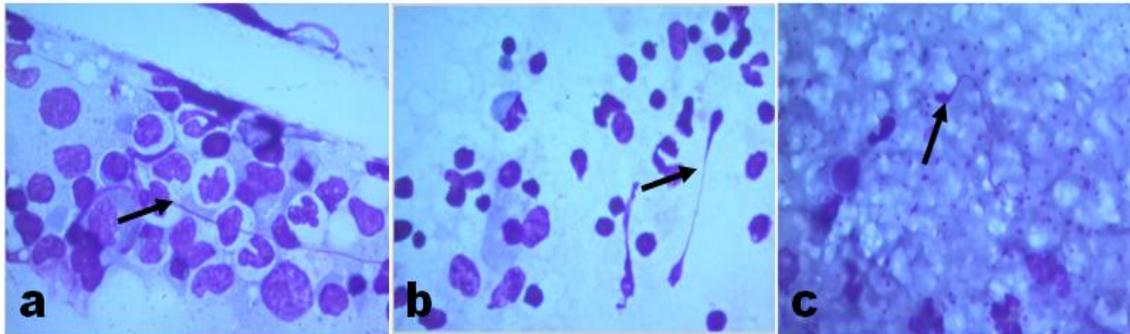


Fig. 2. Impression smears prepared from bone marrow (a), spleen (b) and liver (c) stained with Giemsa's stain. Promastigote (black arrow, 100X) phases of the *Leishmania* protozoa was seen in the smears prepared from bone marrow, spleen and liver.

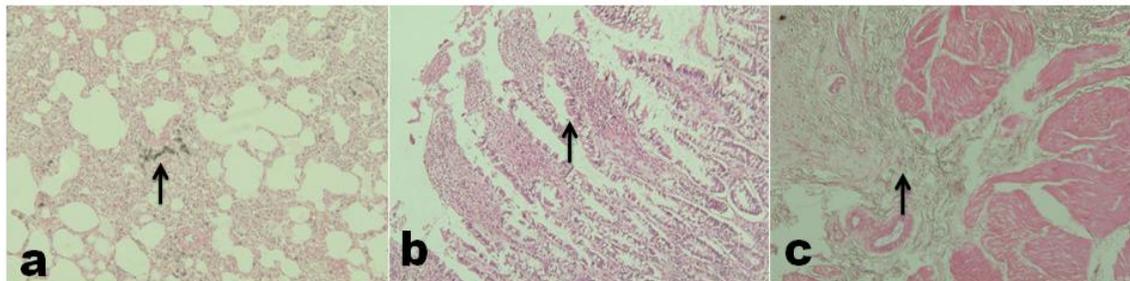


Fig. 3. Section of lungs, intestine and skeletal muscle stained with H&E. Deposition of black coloured carbon particle (Carbon - Anthracosis) in the alveolar septal wall and macrophages (black arrow, 10X) of lungs (a) was seen. There was catarrhal enteritis and sloughing of mucosal epithelium (black arrow, 10X) in intestinal mucosa (b). Focal necrosis in the skeletal muscle (c) and deposition of reticular and elastic fiber in the space between necrotic muscle bundles (black arrow, 10X) were seen.

In histopathological examination, leishmanial amastigote and promastigotes were found only in the spleen. The presence of large number of promastigote in spleen without tissue reaction and lacking of inflammation incriminated dogs as asymptomatic carrier of VL. Granuloma formation and presence of necrosis or proliferation of connective tissues and giant cells in chronically infected liver can be seen (Tryphonas *et al.*, 1977). Early lesions with high parasitic load can show granuloma at the edge of lesions and deeper sections of the biopsy (Mansur *et al.*, 1970). Nonspecifically, there was deposition of black colored carbon particle (anthracosis) in the alveolar epithelium and macrophages of lungs (Fig. 3a) and these could be due to living ecology of dogs close proximity of rice mills or other carbon sources. Catarrhal enteritis and sloughing of mucosal epithelium of intestine was seen in three cases (Fig. 3b). In a case there was necrosis of skeletal muscle and excessive deposition of reticular and elastic fiber in the space between necrotic muscle bundles was seen (Fig. 3c). The reason of necrosis in skeletal muscle could not be explained but the necrosed area was replaced by reticular fiber. Histopathological examination of liver section did not reveal major alteration. Intracytoplasmic inclusion body in liver and intranuclear and intracytoplasmic inclusion body in lungs specific to ICH and CD respectively were not seen. So the presence of ICH and CD in the liver of dogs was not documented by histological technique.

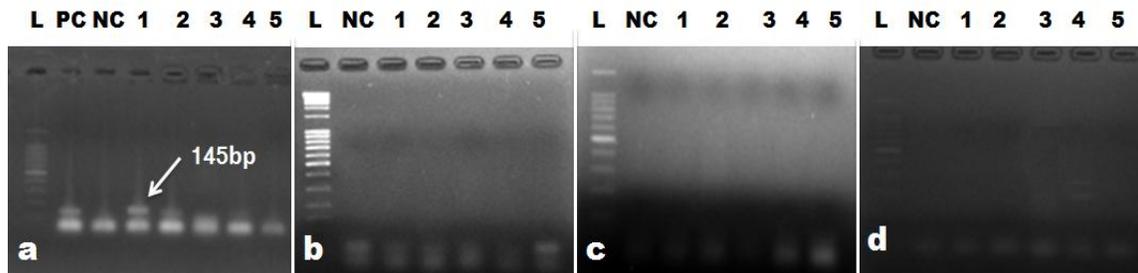


Fig. 4. PCR detection of visceral leishmaniasis (a, 145bp) and infectious canine hepatitis (b, 41bp) from the genomic DNA of liver of dogs. RT-PCR detection of canine distemper (c, 287bp) from the liver and matrix protein gene of avian influenza (d, 245bp) from lungs of street dogs. Lane L is for 100bp ladder, PC is for positive control, NC is for negative control and lane 1 to 5 are for genomic DNA or RNA used in PCR or RT-PCR amplification reaction. Out of ten dogs examined visceral leishmania specific amplicon (145bp, a) was generated in two cases.

It is often difficult to diagnose species of *Leishmania* involved in impression smear staining due to lack of technology of morphologic differentiation of the parasites and often presences of non pathogenic form of *Leishmania* as well. PCR is a highly sensitive and specific technique used to detect Canine leishmaniasis (Torres, 2007). This study used species specific primers in PCR against *L. donovani* and successfully amplified 145bp products from the template DNA obtained from the liver of two dogs (2/10) indicated the presences of *L. donovani* (Fig. 4a). The serological and molecular tests often used to detect leishmaniasis in canids. Cortes *et al.* (2004) conducted a study on 20 dogs and four dogs were positive to VL using serology and 16 using PCR suggesting the higher sensitivity of the PCR test protocol. In this study direct smear staining revealed the presence of leishmanial pro-mastigote or a-mastigote stages of protozoa in eight cases but using PCR only two dogs were positive to VL. The increase reactivity of the dogs in impression smears technology could be due to lack of specific staining reactivity and could also be due to the presence of non-pathogenic leishmania like protozoa in smears.

Characteristics lesions selective for ICH in internal organs during necropsy and histopathological investigation was not seen, PCR assay was, therefore, used for the detection of fragments of ICH. The PCR assay was done with the genomic DNA from the liver of 10 dogs using published primer sequences (Chouinard *et al.*, 1998) but did not amplify desired (41bp) band in gel documentation (Fig. 4b). It was assumed that dogs of BAU campus were not infected with ICH virus. RT-PCR was, therefore, used to detect genomic content of CD virus in clinical specimens of street dogs but did not develop desired (287bp) amplicon (Fig. 4c). A definitive diagnosis of canine distemper virus by isolation on canine cells is time consuming; taking several days to weeks, notably when applied to clinical specimens (Frisk *et al.*, 1999; Kim *et al.*, 2001 and Shin *et al.*, 2004). RT-PCR assays for the detection of CD virus offer substantial improvements in virus detection and thus may render easier

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the control of CD in dogs (Frisk *et al.*, 1999; Von Messling *et al.*, 1999; Rezk, 2000; Mizak *et al.*, 2002; Shin *et al.*, 2004 and Saito *et al.*, 2006, Yousuf *et al.*, 2014). Wild carnivores are at high risk of catching highly pathogenic avian influenza (HPAI) virus (H5N1) due to eating habit of infected dead birds either domesticated or wild. AI virus specific RT-PCR was adopted targeting MP gene but did not amplify desire amplicon (Fig.4d). AI viral infection was not detected in any of the 10 dogs in this study.

This study provides evidence that *Leishmania* like protozoa was present in the internal organs of eight street dogs of BAU campus. Results of PCR (145bp amplicon) showed the presence of *L. donovani* in the liver of two dogs. Canine distemper, infectious canine hepatitis and avian influenza viral genomes were not detected in any of the dogs of BAU campus. It needs to test large number of sample using wider primers pair to confirm the presence of other species of *Leishmania* and detection of vector available at BAU campus.

ACKNOWLEDGEMENT

Thanks are due to SPGR, PIU-BARC, NATP Phase-1 (April 2010 to June 2014) for funding the research.

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