Original Article

Detection of the *Mycobacterium avium* complex in dogs with lymphadenitis

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10.22099/IJVR.2023.45268.6651

(Received 30 Nov 2022; revised version 3 Oct 2023; accepted 16 Oct 2023)

Abstract

Background: *Mycobacterium avium* complex (MAC) is connected to human immunosuppressive diseases, including HIV-AIDS, and may pose a zoonotic threat. MAC causes lymphadenopathy in children, respiratory infection in adults, and generalized infection in immunocompromised individuals. Infection with nontuberculous Mycobacteria (NTM) in humans is now primarily brought on by MAC. Recently, MAC members have emerged as pathogenic organisms for animals and humans. While dogs are generally resistant to mycobacterial infections, there have been some cases of infection that result in systemic or disseminated diseases. The organisms can be transmitted to dogs through oral contact, and their faeces can be a possible source of infection for dog owners. It is important to note that this ailment is zoonotic, especially if infected pet dogs are in prolonged contact with their humans. **Aims:** The study was planned to demonstrate the occurrence of MAC organisms and other Mycobacteria in dogs associated with lymphadenopathy cases with special emphasis on lymphadenitis. **Methods:** A total of 123 samples (100 lymph node aspirates, 15 lymph node tissues, and 8 blood samples) from 83 dogs suspected of lymphadenitis accompanied by gastroenteritis, chronic skin infections, immunosuppression, chronic pulmonary diseases, and other chronic undiagnosed diseases were studied. The samples were processed for cytological and microscopic examination by Ziehl-Neelsen staining. Following the decontamination procedure, the aspiration and lymph node tissue samples were inoculated into Middlebrook 7H11 media for up to 8 weeks. The aspirated material was also directly used for molecular detection by triplex-nested polymerase chain reaction (nPCR) assay. **Results:** A cytological study revealed pyogranulomatous inflammation of the lymph node tissue. Impression smears from lymph node tissues displayed the presence of acid-fast organisms. Out of 83 cases of dogs, 8 were found to be positive for *Mycobacterium* spp. Among those 8 positive cases, 3 were confirmed to belong to MAC, and 5 belonged to the *Mycobacterium tuberculosis* complex (MTB complex). **Conclusion:** MAC and MTB are the underestimated bacteria that could be the causative agents of lymphadenitis in animals.

Key words: Lymph node aspirate, Lymph node tissue, *Mycobacterium avium* complex, *Mycobacterium tuberculosis* complex, Nested PCR

Introduction

The genus *Mycobacterium* is broadly classified into two types namely nontuberculous Mycobacteria (NTM) and *Mycobacterium tuberculosis* complex (MTC) (Hobi *et al*., 2015). NTM are saprophytic organisms, and infection mainly occurs after contact with infected animals (directly or indirectly), the environment or consuming contaminated avian meat or faeces (Campora *et al*., 2011). NTM comprise *Mycobacterium avium* complex (MAC). MAC is further subdivided into *Mycobacterium avium*, and *Mycobacterium intracellulare*. *Mycobacterium avium* includes subspecies *avium* in birds, *hominissuis* in swine and humans, *silvaticum* in pigeons, and *paratuberculosis* in ruminants (Thorel *et al*., 2001). MAC cause disseminated and systemic infections such as lymphadenopathy, alimentary tract infection, respiratory tract infection, and pyogranulomatous inflammation of the liver and spleen, particularly in immuno-compromised and immunosuppressed dogs (Lam *et al*., 2012). The common symptoms include cutaneous lesions and pulmonary and disseminated disease. The specificity of sugar residues in the surface glycopeptidolipids allows the classification of MAC species into 28 serovars as per the seroagglutination test. Serovars 1 to 6, 8 to 11, and 21 have been designated as *M. avium*, whereas serovars 7, 12 to 20, and 25 belong to *M. intracellulare* (Inderlied

et al., 1993). Currently, *M. avium-intracellulare* complex from pulmonary infection (Vise *et al*., 2016), *M. avium* subsp. *hominissuis* (MAH) from pleural effusion and disseminated infection have been reported in dogs (Campora *et al*., 2011). Diagnosing a disseminated infection with *Mycobacterium avium* in small animal disease is still overlooked. Identifying a primary infection at necropsy is difficult (Friend *et al*., 1979). Although the period of primary infection and the establishment is long, it is typically followed by rapid spread to all organs. There is an authenticated report of breed predisposition in Miniature Schnauzer and Basset Hound dogs to MAC, and it is caused by a recessively inherited deficiency of an adaptor protein called "Caspase recruitment domain-containing protein 9" involved in the pro-inflammatory and inflammatory response (Ghielmetti *et al*., 2020). In animals, *Mycobacterium avium* subsp *hominissuis* (MAH) has been correlated to lymphadenitis of the mesenteric and cranial lymph nodes (Shin *et al*., 2010); it can also induce systemic infection of parenchymatous organs (Alvarez *et al*., 2008). "*Mycobacterium avium* subsp. *silvaticum*" causes lesions similar to tuberculosis. *Mycobacterium avium* subsp. *silvaticum* has been characterized by its inability to grow on egg-based media, proliferative growth in the presence of pyruvate at pH 5.5, and dependency on mycobactin (a siderophore) for growth (Thorel *et al*., 2001). *Mycobacterium intracellulare*, initially known as *Nocardia intracellularis* is more prevalent in clinical samples and environment, has a wider apparent host range, and contributes exclusively to disseminated MAC diseases. The type strain *Mycobacterium intracellulare* (ATCC 13950) was isolated from a human case and was declared responsible for enlarged lymph nodes, disseminated disease and progressive pulmonary disease (Kyriakopoulos *et al*., 1997). Generally, the signs are seen in senile dogs and are described by anorexia, progressive weight loss, lymphadenomegaly, coughing, dyspnoea, hematemesis, epistaxis, and diarrhoea; sometimes associated with melena (Martinho *et al*., 2013). Reports of *M. avium* infection in dogs involving the systemic lymph nodes as well as peripheral lymph nodes have been well documented (Sharp *et al*., 2019). MAC has been identified as a cause of disseminated lymphadenitis in Australian shepherd dogs occurring concurrently with cases of panniculitis and cutaneous manifestations (Ramos *et al*., 2019). Due to the increased interaction between dogs and humans, there is a likelihood of transmission between the two species (Thorel *et al*., 2001). A MAC-infected dog risks public health since it can spread the disease to humans, especially those with impaired immune systems (Ghielmetti *et al*., 2020). Some transmission cases of *M. tuberculosis* from humans to companion dogs affecting the mediastinal and tracheobronchial lymph nodes accompanying hepatomegaly have also been discovered recently (Hackendahl *et al*., 2004). As the diseases caused by these organisms are unreported and unnoticed, and considering the increasing interaction of humans and dogs as well as the stray dogs population, more emphasis on these organisms is necessary to understand the patterns of infection. The study was designed to identify the causative organisms responsible for lymphadenitis in dogs. Four different methods, i.e., cytological and microscopic examination, bacterial culture, and molecular confirmation, were used for this purpose.

Materials and Methods

Ethics statement

Ethical approval for this study was obtained from Institutional Animal Ethics Committee (GADVASU/ 2022/IAEC/64/03).

Sample collection

The owners were informed about the sample collection and were provided with their verbal informed consent for sampling and information publishing. Sample collection was carried out by veterinarians adhering to the regulation of animal welfare.

A total of 123 samples comprising 100 lymph node aspirates (LNA), 15 lymph node (LN) tissues, and 8 blood samples were collected from 83 dogs. Out of 100 aspirates, popliteal aspirates were 45, prescapular aspirates were 40, submandibular aspirates were 8, mandibular aspirates were 3, and mesenteric aspirates were 3, whereas 1 aspirate was from the inguinal lymph node. Among the 15 lymph node tissue samples, a total of 10 mesenteric lymph node tissues, 3 mediastinal lymph nodes, and 2 popliteal lymph node tissues were collected. According to the literature, dogs with a history of lymphadenitis and clinical signs including diarrhoea (with or without melena), emaciation, lethargy, a body temperature of 38.8° to 40°, splenomegaly, hepatomegaly, chronic pulmonary infections, chronic skin infections, immunosuppressive conditions like lymphoma, and other chronic diseases were chosen. Different samples were collected depending on the severity of the illnesses; for instance, from dogs with more severe illnesses, blood and lymph node aspirates were obtained. There were 52 dogs with serious conditions, the majority of which were severe lymphadenitis. Other conditions included gastroenteritis, tick fever, skin infections, pulmonary infections linked to lymphadenomegaly, immunosuppression from lymphoma, gastroenteritis, and jaundice. Only aspirates were typically used for less critical illnesses including moderate lymph node enlargement and mild skin infection. Dogs that died after receiving therapy, but did not exhibit any improvement in their health condition had lymph node tissue samples collected. These canines primarily had histories of lymphadenitis, an immunosuppressive condition caused by tumors, lymphoma, and in some cases, an undetermined diagnosis. A physical examination of the lymph nodes (Figs. 1a and b) was done followed by confirmation using ultrasound examination (Fig. 2). The LNA samples were collected in a sterile 1000 µL of phosphate buffer

solution (PBS) in a microcentrifuge tube, and stored at -20°C. Similarly, lymph node tissues were collected in sterile sample containers and kept at -20°C. The blood samples were collected in EDTA vials.

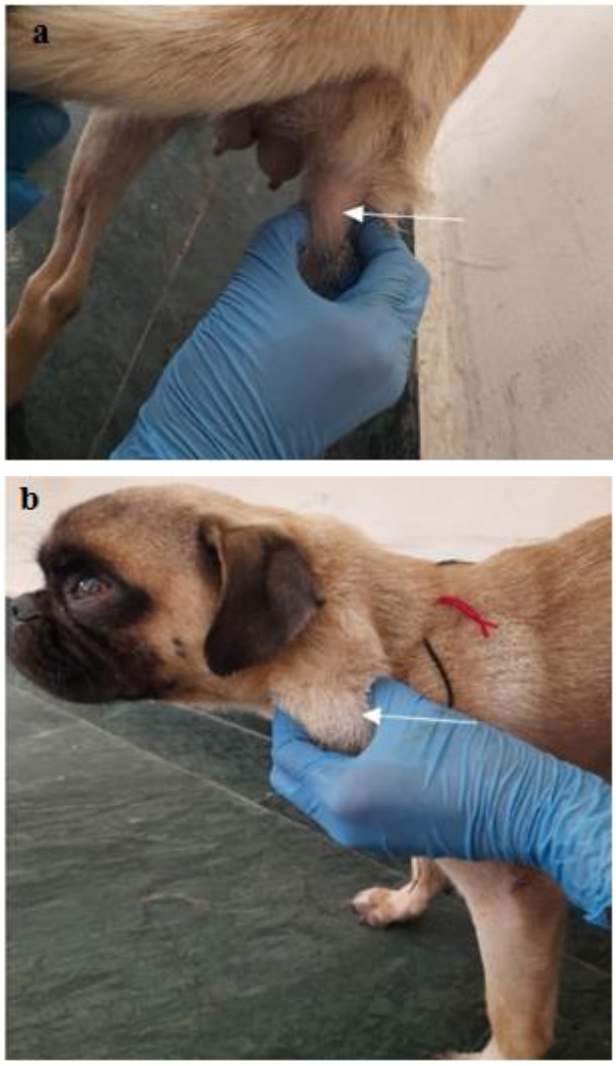

Fig. 1: (**a**) Physical examination of right popliteal lymph node (white arrow), and (**b**) Physical examination of left prescapular lymph node (white arrow)

Fig. 2: Ultrasound examination. Enlarged lymph node (left popliteal lymph node, long axis) with irregular borders - hyperechoic - length, 3.68 cm × width, 1.32 cm

Cytological and microscopic examination

The LNA was smeared over a clean glass slide in duplicate. One slide was subjected to Leishman staining (Gajendra *et al*., 2015) and the other to Ziehl-Neelsen staining (Chen *et al*., 2012) to study the cytological changes and detect the target organisms.

Decontamination and inoculation of the LNA and lymph node tissues into the media

The LNA decontamination procedure (Petroff's method) was carried out as stated by Gopinath and Singh (2009) and was as follows: 100 µL of the sample was treated with 100 µL of 4% NaOH. After 30 min of incubation at 37°C, the sample was neutralized with phosphate buffer (pH 7.2). After centrifugation, the supernatant was decanted. The resulting pellet was used for inoculation after resuspension with phosphate buffer, and 100 µL of this resuspended solution was inoculated into Middlebrook 7H11 media. Lymph node tissues were decontaminated by 0.75% Hexadecylpyridinium chloride (HPC) method as described by Nasr *et al*. (2016). A volume of 200 µL resuspended material was inoculated into Middlebrook 7H11 media in duplicate, one media with Mycobactin J (40 µL/100 ml of media) (iD.Vet MYCO 2 mg vial freeze-dried) and one without Mycobactin J. The inoculated media was incubated at 37°C with regular monitoring for 8 weeks (OIE. 2018).

Extraction of DNA from LNA samples

The DNA from LNA was extracted using the CTAB method as described by Tadele *et al*. (2014). To ensure the highest yield and purity, the extracted DNA was eluted in 30 µL of Tris-EDTA buffer and stored at -20°C for later use.

Extraction of DNA from lymph node tissues

Extraction of DNA from LN was done using NucleoSpin Tissue, Mini kit (Macherey-Nagel for DNA from cells and tissue kit) method as per the manufacturer's instructions. Similarly, blood samples were used directly for DNA extraction using QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. Slight modifications were done in both procedures that is after the addition of lysis buffer the samples were incubated at 100°C in a dry bath for efficient lysis of the cells.

Oligonucleotide primers

The primers (procured from bio serve) used for triplex-nested PCR to detect species of *Mycobacterium* were referred to as described by Park *et al*. (2006). The primer sequence was as follows: ITS-F1: 5´-CGA AGC CAG TGG CCT AAC CC-3´, ITS-R: 5´-TGG ATC CTG CCA AGG CAT CCA CCA T-3´, TB-F1: 5´-GGT GCA TGA CAA CAA AGT TGG-3´, MAC-F1: 5´-ACA ACA CTC GGT CCA TCC GT-3´, and mycom-2: 5´-ATG CTC GCA ACC ACT ATC CA-3´.

Triplex-nested PCR assay for the detection of *Mycobacterium* spp.

The triplex-nested PCR assay was performed as developed and described by Park *et al*. (2006) which specifically targeted the unique region called the internal transcribed spacer region between the *16S* and *23S rDNA* gene of the genus *Mycobacterium*. It was employed directly on the extracted DNA from blood samples, lymph node aspirates, and lymph node tissues. Two types of PCR assay were performed viz., primary and secondary PCR. For the primary assay, 25 µL volume of the reaction mixture was prepared by the addition of 5 µL of test DNA template, 12.5 µL of GoTaq® Green Master Mix, 10 pmol (1 µL) of each primer, ITS-F1 and 1 µL of ITS-R. The reaction volume was made up to 25 µL by adding 5.5 µL of NFW (nuclease-free water). Amplification of DNA was performed at an initial denaturation of 94°C for 3 min, followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min, additionally a final extension for 10 min at 72°C. For the secondary reaction, 12.5 µL of GoTaq® Green Master Mix, 2 µL of the primary amplified product as a template DNA, 1 µL of ITS-F1 (2.5 pmol), TB-F1 (5 pmol), and MAC-F1 (10 pmol) as forward primers, and 3 µL of mycom-2 (15 pmol) as reverse primer was mixed. The reaction volume was made up to 25 µL by adding 4.5 µL of NFW. Amplification of DNA was performed at an initial denaturation of 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, also a final extension for 10 min at 72°C. Gel electrophoresis was done with 1.5% agarose (prepared in 100 ml of 1X TBE solution with the addition of 0.5 µg/ml of ethidium bromide). The amplified product was visualized in a gel documentation system (Syngene, USA).

Results

Cytological examination

The LNA and the blood smears subjected to Leishman staining, examined under ×100 magnification demonstrated the presence of activated macrophages and degenerated neutrophils accompanied by occasional lymphocytes. This was suggestive of pyogranulomatous inflammation of the lymph nodes (Fig. 3).

Microscopic examination of LNA smears and LN tissues

The LNA smear collected from popliteal, prescapular, and submandibular lymph nodes, stained with Ziehl-Neelsen stain could not demonstrate the presence of acid-fast bacilli. The reasons may be the scanty quantity of aspirate, and the paucibacillary condition. Out of 15 LN tissues, 2 mediastinal LN tissue and 1 mesenteric LN tissue impression smear upon acid-fast staining demonstrated scanty acid-fast, rod-shaped bacilli (Figs. 4a and b).

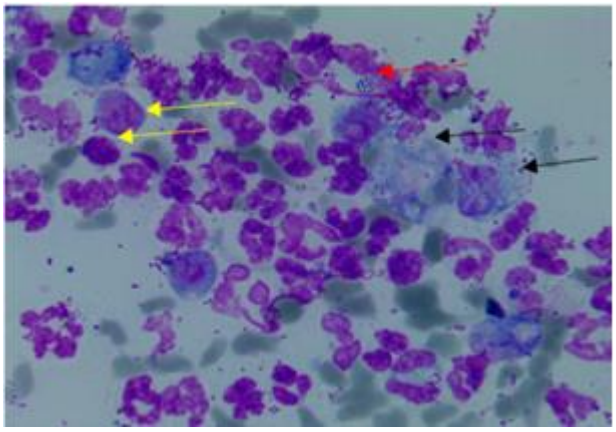

Fig. 3: Cytology - popliteal lymph node - pyogranulomatous inflammation - numerous macrophages (black arrows) engulfing bacteria (red arrow) with moderate neutrophils and occasional lymphocytes (yellow arrows), (Leishman stain, ×100)

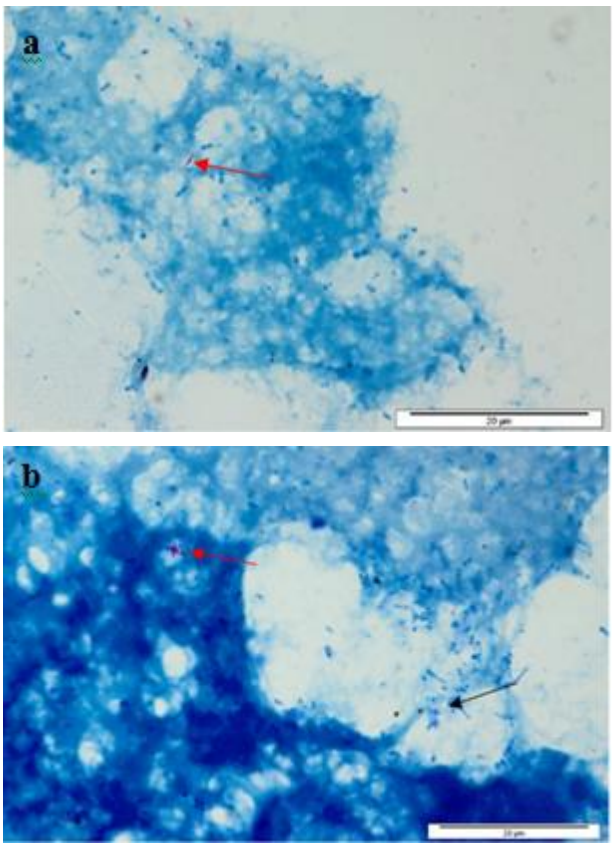

Fig. 4: (**a**) Impression smear - mediastinal lymph node - acid-fast *Bacillus* (red arrow). Ziehl (Neelsen staining, ×100), and (**b**) Impression smear - mediastinal lymph node - acid-fast bacilli (red arrow) with few non-acid-fast cocci (black arrow), (Ziehl-Neelsen stain, ×100)

Culture

Only one LNA sample out of 115 samples (100 LNA and 15 LN tissues) inoculated onto Middlebrook 7H11 media showed successful development of growth after 8 to 9 weeks of inoculation (Fig. 5). Blood samples were directly used for molecular detection and not for culture.

The colonies were small, non-pigmented, and wrinkled. Confirmation was done by colony staining with Ziehl-Neelsen stain where the colonies demonstrated acid-fast properties (Fig. 6).

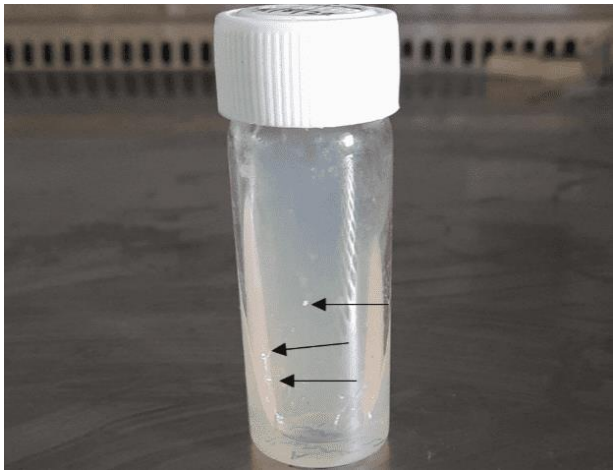

Fig. 5: Culture - lymph node aspirate - *Mycobacterium avium*-Middlebrook 7H11 media

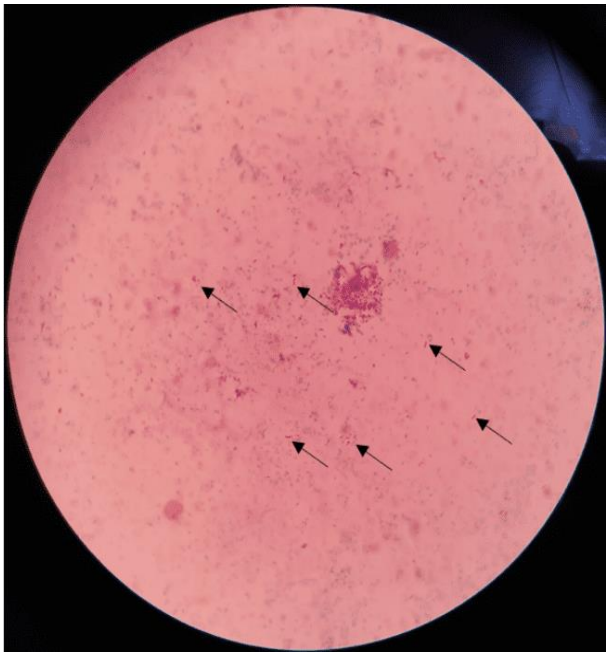

Fig. 6: Culture - lymph node aspirate - *Mycobacterium avium* (Ziehl Neelsen staining, ×100)

Triplex-nested PCR

Two types of lymph nodes were included in the study viz., peripheral lymph nodes (submandibular, prescapular, inguinal, and popliteal lymph nodes) and systemic lymph nodes (mesenteric and mediastinal lymph nodes). Out of 100 LNA and 15 LN tissue samples obtained from 83 dogs, 4 LNA samples (2 out of 45 from popliteal and 2 out of 40 from prescapular lymph node) and 7 LN tissue samples (3 out of 10 from mesenteric, 1 out of 2 from popliteal and all the three from mediastinal lymph node) displayed the amplicon size of 322 bp, specific for pan mycobacterial segment. Additionally, 4 of the LNA exhibited an 84 bp amplicon that confirms *M. avium* complex. The rest of the 7 LN tissues revealed the species-specific amplicon size of 133 bp, confirmatory of *M. tuberculosis* complex. In total, 11 samples were positive for *Mycobacterium* spp. (Figs. 7 and 8). None of the blood samples showed a positive amplicon for *Mycobacterium* spp. With respect to the cases of dogs, out of 83 cases of dogs, 3 cases of dogs were found to be positive for *M. avium* complex, and 5 dog cases were positive for *M. tuberculosis* complex. Further sequencing of the samples belonging to MAC revealed that the organisms belonged to *M. avium* subspecies *avium* although the samples were suggestive of *M. tuberculosis* complex. In one 7-year-old male Labrador, an aspirate sample from the prescapular and popliteal lymph node was positive for MAC. In another 6-year-old male Labrador, one aspirate sample from the prescapular lymph node was found to be MAC positive. One Gaddi dog was MAC positive after examination of the popliteal lymph node. Among the lymph node tissue samples, a 6-year-old female Labrador showed positive results for MTB complex from the popliteal lymph node. Using triplex-nPCR, a 9-year-old female mongrel dog was confirmed infected with MTB complex in a mesenteric lymph node tissue sample. In addition, another 3 mongrel dogs (2 males and 1 female) tested positive for MTB complex from both mediastinal and mesenteric lymph node tissue samples. As for the breeds in India, mongrels showed the highest positivity (4 out of 24) followed by Labrador retrievers (3 out of 26) and a

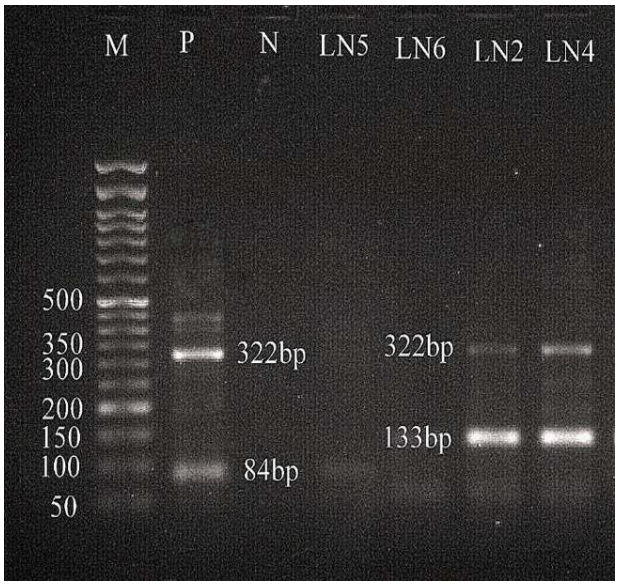

Fig. 7: Agarose gel electrophoresis showing an amplicon of 322 bp (panmycobacteria) (primers: ITS-F1 and ITS-R), 84 bp (species-specific for *Mycobacterium avium* complex) (primers: MAC-F1 and mycom-2), and 133 bp (species-specific for *Mycobacterium tuberculosis* complex) (primers: TB-F1 and mycom-2) from lymph node tissue. M: Marker (50 bp DNA ladder [DM1100] ExcelBand™ SMOBIO Technology Inc., US), P: Positive control (*M. avium*), N: Negative control, LN5 and LN6: Negative for target organisms, and LN2 and LN4: Positive for MTB complex

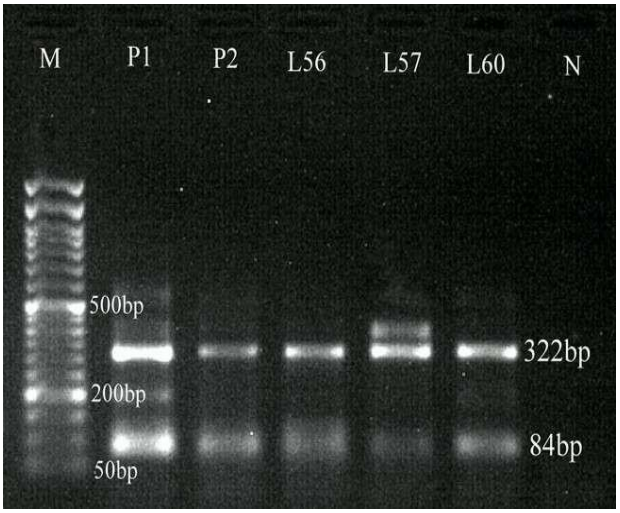

Fig. 8: Agarose gel electrophoresis showing an amplicon of 322 bp (panmycobacteria) and 84 bp (primers: MAC-F1 and mycom-2) [species-specific for *Mycobacterium avium* complex (MAC)] from lymph node aspirates. M: Marker (50 bp DNA ladder [DM1100] ExcelBand™ SMOBIO Technology Inc., US), P1: Positive control (*M. avium*), P2: Positive control (*M. intracellulare*), L56, L57, L60: Positive for MAC, and N: Negative control

Gaddi dog (1 case positive). With respect to the types of different diseases, chronic undiagnosed cases were detected to be more positive, it was found that 4 such cases were indicative of mycobacteriosis. In addition to 2 cases suspected of lymphadenitis, 1 case of lymphoma and 1 case of tick fever were all tested positive. Dogs between the ages of 6 to 9 years revealed more positive incidences as compared to other age groups.

Discussion

Members of *M. avium* complex are the leading cause of disseminated disease among animals and humans. Though an opportunistic pathogen, these organisms can cause serious life-threatening diseases. Infections caused by MAC in dogs are documented occasionally all over the world. This could be due to a lack of disease awareness, and improper, time-consuming, and tedious detection methods like bacterial culture. Rapid detection of the organism is required to establish a suitable therapeutic measure. Thus, the purpose of this study was to identify the causative organism by the use of numerous techniques such as microscopic and cytological examination, as well as nucleic acid detection to confirm bacterial culture. Fine needle aspirate cytology is considered to be one of the rapid diagnostic techniques. In the present study, fine-needle aspirate cytology demonstrated the presence of activated macrophages, degenerative neutrophils, and occasional lymphocytes but Ziehl-Neelsen staining could not detect the presence of acid-fast bacilli. In contrast, triplex-nPCR assay could successfully amplify the targeted gene. The sensitivity of the PCR assay (8.94% positive) was higher than aspirate staining (2.60% positive), and bacterial culture (0.86%). The PCR assay found more positive LN tissue (46.66%) than LNA (4%). The failure to detect bacteria of interest using cytology or aspirate could be due to the low concentration of bacilli in the clinical sample (also known as the "paucibacillary" condition). A similar condition was reported by Hsiao *et al*. (2003) where Ziehl-Neelsen staining could not detect acid-fast bacilli but the samples were found to be positive upon PCR assay. It was observed that the presence of 10^4 bacilli/ml of the tissue or fluid sample is necessary to demonstrate the organisms in the samples. In another investigation of mycobacterial infection in cats with pyogranulomatous inflammation with foci of necrosis and neutrophil infiltration, Laprie *et al*. (2013) came to the same conclusion that the low amount of microorganisms made it difficult to distinguish *Mycobacteria* spp. in the sections stained with haematoxylin and eosin. Also, Ziehl-Neelsen staining did not show many acid-fast bacilli. According to Mayskaya *et al*. (2014), *Mycobacteria* spp. were discovered in patients with paucibacillary disease. In this case, acid-fast bacilli (AFB) were not detected in the Ziehl-Neelsen stained clinical samples but the histological lesion was consistent with mycobacteriosis. Further, the culture of blood sample also revealed systemic infection with the *M. avium* complex. Ziehl-Neelsen staining is not a reliable method for detecting acid-fast bacilli because, according to Reddy *et al*. (2008) the bacilli are only present in small amounts in the aspirate smear. In order to prove the presence of bacteria in samples at low concentrations, it is crucial to employ alternate and multiple detecting procedures. As found in the current study, even though the culture of the microorganism is considered to be a gold standard test, many factors can contribute to the failure of bacterial growth. Some of such reasons are the presence of other bacterial contaminations and procedures employed to reduce these contaminants besides the low concentration of organisms in the sample, and the degradation of samples due to improper storage, the time of sample collection and its processing. In a similar study, *Zanini et al*. (2001) collected 54 suspected lymph node biopsy samples for the examination and the isolation of *Mycobacterium* species from animals showing tuberculous lesions. Upon isolation, only 23 samples (42.6%) of the total 54 samples showed mycobacterial growth after 45 days of inoculation. Fifteen samples (27.7%) failed to grow in culture, while 16 samples (29.6%) had significant bacterial contamination that slowed the growth of *Mycobacterium* spp. Generally, the other bacteria could grow more quickly, and consume the necessary growth supplements for the development of fastidious *Mycobacterium* spp. The use of 4% sodium hydroxide to treat the contaminated specimens resulted in a minimal recovery of pure cultures of *mycobacteria* spp. A higher concentration of sodium hydroxide treatment could harm the *Mycobacteria* spp. and substantially reduce their viable numbers, while the use of 2% sodium hydroxide resulted in the insufficient recovery of *Mycobacterium* spp. This is according to a comparative study on

decontamination processes for recovering *Mycobacterium* spp. for culture conducted by Chatterjee *et al*. (2013). The employment of triplex-nPCR assay resulted in the maximum detection of the organisms in the samples compared to the traditional methods of staining and isolation. The developed triplex-nested PCR approach for the detection of *Mycobacterium* spp, as utilized by Park *et al*. (2006), effectively amplified 322 bp pan-mycobacterial and 133 bp species-specific bands in *M. tuberculosis* complex, while amplifying 322 and 84 bp, respectively, in MAC. Nested PCR targeted the ITS (Internal Transcribed Spacer region between 16S rDNA and 23S rDNA) sequence that is more suitable for differentiating the species of *Mycobacteria* than other sequences because contains genus- and species-specific variable sequences.

The present study indicated that lymphadenopathies were associated with *Mycobacterium* spp. even though some of the cases might be unnoticed. Molecular methods like the nested PCR assay detected more incidences of positive cases than conventional methods like staining and isolation. In comparison with lymph node aspirate and blood samples, lymph node tissue samples were more indicative and provided more confirmatory diagnoses, though preceding methods are more rapid. Mycobacteriosis is a potential risk to companion dogs and their owners. When a companion animal's owners have a history of tuberculosis or mycobacteriosis, the animal should be tested for the presence of MAC, MTB complex, NTM and other species of *Mycobacterium*. Lymphadenopathies and other persistent infections should not be disregarded, and appropriate diagnostic and therapeutic procedures should be developed.

Acknowledgements

The authors are thankful to DBT and the Director of Research for funding the project and providing all the help to carry out this research work under the scheme "Mycobacterial diseases in animal network (MyDAN) Programme". No funding was received to assist with the preparation of this manuscript.

Conflict of interest

The authors have no conflicts of interest to be declared with respect to this article.

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