Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 5 Issue 9 September 2023

Research Article

Isolation and Identification of Various Bacterial Species Associated with Cases of Lymphadenopathy in Dogs

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DOI: 10.31080/ASVS.2023.05.0742

Received: June 19, 2023

Published: August 27, 2023

Abstract

Lymphadenopathies are common ailments in companion animals, especially dogs, and can be a result of various factors like bacteria. The current study is directed towards the isolation of bacteria associated with lymphadenopathies, mainly lymphadenitis and to study its antimicrobial resistance patterns. For this, a total of 70 cases from dogs with various lymphadenopathies were studied. Dogs were examined for lymphadenomegaly, irrespective of the disease. Both the peripheral and systemic lymph nodes were considered. A clinical examination of lymph nodes followed by confirmation with an ultrasound examination was carried out. Lymph node aspirates (LNA) were collected aseptically with the guide of ultrasound examination. Obtained LNA was subjected to cytological (Leishman staining) and microscopic examination (using Gram's staining). Cytological examination revealed the presence of inflammatory cells along with bacteria, mostly cocci, some showed the presence of rods. Microscopic examination exhibited Gram-positive cocci in the peripheral lymph nodes and Gram-negative rods in the systemic lymph nodes. The LNA material was inoculated into Brain Heart Infusion agar. Bacteria like *Staphylococcus spp* and *E. coli* were identified based on cultural characteristics and confirmed with MALDI-TOF-MS. The isolated species were subjected to an antibiogram employing 12 antibiotics commonly used for the therapy of clinical infections amongst small animals in India. Azithromycin showed the highest sensitivity (100%) among all the antimicrobials. Ceftriaxone was the second most sensitive drug. Tetracycline exhibited the least sensitivity when compared to others (only 22.22%).

Keywords: Lymph Nodes; Lymph Node Aspirates; Lymphadenopathies; MALDI-TOF-MS

Introduction

Lymphadenitis is classified into various types such as acute, chronic, granulomatous, pyogranulomatous, and caseous. It may result from an infectious agent or an immune-mediated response [3]. It begins when an infectious organism is drained to a lymph node in a remote inflammatory area. The organism invades both the lymph nodes and arteries. The specific inflammatory response depends on the sort of infectious agent present [1]. The bacterial agents isolated and identified so far from lymphadenitis cases in dogs are *Escherichia coli, Serratia marcescens, Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus canis,* and *Prevotella* spp [22]. Additionally, cases of lymphadenitis caused due to the aforementioned organisms have also been reported in humans and swine [16]. Documentation of transmission of some organisms like *E. coli* and *Salmonella* spp. to companion animals via contaminated pet food had been done, where commercial pet food was responsible for mesenteric lymphadenitis [17]. Incidences of some bacteria like *Salmonella* and *Escherichia* translocating from

the intestine to the mesenteric lymph nodes after crossing the mucosal barriers had been documented too [8]. Bacteria could infect a part of an organ or the entire organ through the process of translocation, where they cross the intestinal mucosal barriers and move to the other local organs like the mesenteric lymph nodes and blood vasculature [2]. Staphylococci are a normal component of an animal's microbiota, but depending on the immune status of the host, it can result in transitory, localized or systemic infections [19]. *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subsp. *coagulans* are coagulase-positive Staphylococci (CoPS) members of the canine skin [13,29]. The primary causative agent of canine dermatitis and infection is *S. pseudintermedius*, it had been discovered in both veterinary professionals and canines [23]. *Staphylococcus schleiferi* subsp. *coagulans* were originally reported from canine otitis, but since then it had been isolated from several other infection sites in dogs [5,12]. Cross-infection between humans and animals could also occur. *Staphylococcus intermedius* primarily cause pyoderma in dogs besides causing suppurative infections such as cystitis, endometritis, and otitis externa [21]. *Staphylococcus sciuri*, a coagulase-negative organism is commonly found in different domestic and wild animals and had been isolated from normal dogs from the mucous membrane [28]. Lymphadenitis and other lymphadenopathies associated with other infectious diseases are often overlooked and the diagnosis could be missed. Thus, the present study was envisaged to detect various bacteria associated with lymphadenitis and other lymphadenopathies and to initiate an appropriate therapy after antibiogram profiling.

Materials and Methods

The dogs having lymphadenomegaly accompanied by various disease conditions like tick fever, gastroenteritis, pulmonary infection, diarrhoea (with or without melena), pyoderma, immunosuppression due to lymphoma, tumour metastasis, hyperthermia and chronic cases were studied. A physical examination of the lymph node was done followed by an ultrasound examination. Lymph nodes showing hyperechoic structure and enlargement were chosen (Figure 1).

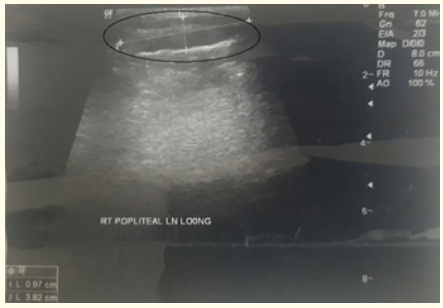

Figure 1: Ultrasound examination-right popliteal lymph node - Enlargement with irregular borders – Hyperechoic - Length-3.82 cms x width-0.97 cms.

A total of 70 lymph node aspirates (LNA) were collected after proper sterilization of the examination site from the dogs irrespective of their age, breed, and sex (the study took place in the Department of Veterinary Microbiology, GADVASU, Ludhiana, India for a period of 12 months, the clinical samples were obtained from the small animal multi-speciality clinic, GADVASU). The LNA material was diluted in 1000µL of phosphate buffer saline solution as the content obtained was scanty. The LNA was processed for microscopic and cytological examination by preparation of smear on a glass slide in duplicate. One smear was stained first with Leishman staining technique [10] and the other smear was subjected to Gram's staining [15]. The LNA was inoculated onto Brain Heart Infusion (BHI) agar (HiMedia Laboratories Pvt. Ltd) and kept at 37°C for incubation till visible colonies appeared. The colonies were further subjected to Gram's stain for confirmation; additionally, the selected colonies were streaked on Mannitol salt agar (MSA), MacConkey's lactose agar (MLA) and Eosin methylene blue agar (EMB) (HiMedia Laboratories Pvt. Ltd) and incubated for 12 to 24 hours at 37°C. The pure colonies that grew on BHI agar in their log phase of growth (12 hours old) were selected for identification using MALDI-TOF-MS (Matrix-assisted laser desorption/ionization-time-of-flight-Mass Spectrometry). The procedure was followed as per the manufacturer's instructions. A single bacterial colony was smeared onto the target plate using a sterile wooden toothpick. A volume of 1µL of 70% formic acid was added and allowed to dry at room temperature. Further, the addition of 1µL of a matrix HCCA (consisting of α-Cyano-4- hydroxycinnamic acid dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid) onto the smeared bacteria was done and allowed to dry at room temperature. The target plate was placed in the chamber of the spectrometer and analysis was done using MALDI Biotyper® Sirius system, 4.1.100 software (Bruker Daltonics, Germany) (30). The antibiotic sensitivity testing was performed according to the Clinical & Laboratory Standards Institute (CLSI, 2020) guidelines. The following antibiotics were used for sensitivity profiling: amikacin (AK 30), ampicillin (AMP 25), azithromycin (AZM 15), cefotaxime (CTX 30), cefoxitin (CX 30), ceftriaxone (CTR 30), ciprofloxacin (CIP 30), doxycycline (DO 30), erythromycin (E 15), gatifloxacin (GAT 30), ofloxacin (OF 5) and tetracycline (TE 30) was used to test the antimicrobial sensitivity profiling. The bacterial colony was initially inoculated in test tubes containing BHI broth and incubated at 37°C for 6 hours till the development of visible growth (light to moderate turbidity).

The turbidity was compared with 0.5 McFarland standard and used for the sensitivity test (by Kirby-Bauer Disk diffusion method).

Results

Leishman stain demonstrated the presence of degenerative neutrophils with numerous macrophages engulfing the bacterial colonies, and some occasional lymphocytes (Figure 2).

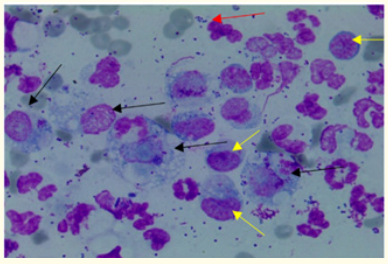

Figure 2: Cytology - Popliteal Lymph node - Pyogranulomatous inflammation - Numerous macrophages (Black arrow) engulfing bacteria (Red arrow) with moderate neutrophils and occasional lymphocytes (Yellow arrow). Leishman stain. 100X magnification.

Gram`s stain demonstrated Gram-positive cocci in the aspirated material (Figure 3).

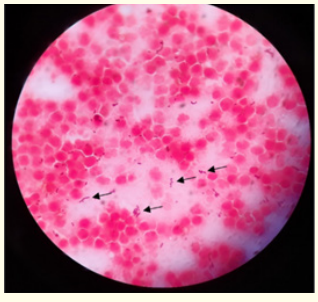

Figure 3: Fine needle aspiration cytology - Lymph node – Gram-positive cocci – Gram's stain. 100X magnification.

The bacterial colonies grown on BHI agar were examined with Gram's staining technique. Two types of colonies were noted viz., Gram-positive (in cocci) and the other Gram-negative (coccobacilli). The bacteria showing Gram-positive colonies were streaked onto MSA, whereas Gram-negative colonies were streaked onto MLA and EMB agar. On MSA, the colonies demonstrated the development of yellow colour suggestive of *Staphylococcus* spp [20]. On the other hand, colonies streaked on EMB agar showed a typical greenish metallic sheen and on MacConkey's agar, it demonstrated pink colonies as a result of lactose fermentation, indicating *Escherichia* spp (20). Further, *S. schleiferi* (3/70), *S. pseudintermedius* (2/70), *S. sciuri* (1/70) *S. xylosus* (1/70), *E. coli* (2/70), *Brevundimonas diminuta* (1/70), *Microbacterium keratinolyticum* (2/70), *M. foliorum* (1/70), and *M. aurum* (1/70) was confirmed with MALDI-TOF-MS; as *Staphylococcus* and *Escherichia* spp. are clinically more relevant, they were further subjected to sensitivity testing commonly used antibiotics. Testing was done for each of the isolates (given in Table 1), and it turned out that *S. sciuri* was sensitive to most of the antimicrobials (ampicillin, amikacin, azithromycin, gatifloxacin, ofloxacin, tetracycline, ciprofloxacin, ceftriaxone, cefotaxime and doxycycline). Besides, it showed intermediate sensitivity towards erythromycin and resistance to cefoxitin. *Staphylococcus xylosus* was resistant to ampicillin, amikacin, gatifloxacin, ofloxacin, ciprofloxacin and cefoxitin; sensitive towards azithromycin, ceftriaxone and doxycycline, whereas it showed intermediate sensitivity for tetracycline, erythromycin and cefotaxime. Both strains of *S. pseudintermedius* were sensitive to ampicillin, amikacin, gatifloxacin, ofloxacin, azithromycin, ciprofloxacin, erythromycin, and ceftriaxone; resistant to cefoxitin, doxycycline, ceftriaxone, and tetracycline. For *S. schleiferi*, the highest sensitivity was observed towards azithromycin, two strains were sensitive to ampicillin and one strain was resistant. Similarly, two strains were sensitive and the other was resistant to amikacin. Two strains showed resistance towards Gatifloxacin and the other was sensitivity. Tetracycline, ofloxacin, erythromycin and cefotaxime were mostly resistant for all three strains. Ciprofloxacin, ceftriaxone, doxycycline, and cefoxitin were sensitive against two strains but were unable to work against the third strain. As far as *E. coli* is concerned, one strain showed sensitivity towards all the antibiotics, but the second strain was resistant towards tetracycline of all the eleven antimicrobials used.

Discussion

In the present study, it was observed that species of *Staphylococcus* and *Escherichia* were mostly associated with lymphadenopathies as suggested by the cytological and microscopic examination as there was the presence of inflammatory cells with the bacteria. In addition, the isolation and identification of these bacteria and further confirmation with MALDI-TOF-MS also supported the results. It is known that *Staphylococcus* spp. is involved in several infections like dermatitis, cystitis, osteomyelitis, metritis, discospondylitis, encephalitis, necrotizing fasciitis, urinary tract infections, respiratory tract infections, secondary bacterial infections and nosocomial infections. One type of study was conducted [18] concerning organisms associated with dog livers. It was found that *Staphylococcus xylosus* was isolated from 3 out of the total 20 cases from dogs. It was also observed that the liver had undergone some changes like discrete hepatocyte vacuolization with mild inflammatory infiltration, as a result, a conclusion was made that *S. xylo-*

Antimicrobial agent	*S. schleiferi*			*S. xylosus*	*S. pseudintermedius*		*S. sciuri*	*E.coli*	
	a	b	c		a	b		a	b
Amikacin	S	R	S	R	S	S	S	I	S
Ampicillin	S	S	R	R	S	S	S	S	S
Azithromycin	S	S	S	S	S	S	S	S	S
Cefotaxime	I	R	I	I	R	R	S	I	S
Cefoxitin	S	R	S	R	R	R	R	S	S
Ceftriaxone	S	S	I	S	S	S	S	S	S
Ciprofloxacin	R	S	S	R	S	S	S	S	S
Doxycycline	S	R	S	S	R	R	S	S	S
Erythromycin	I	S	I	I	S	S	I	-	-
Gatifloxacin	R	R	S	R	S	S	S	S	S
Ofloxacin	R	I	S	R	S	S	S	S	S
Tetracycline	I	R	I	I	R	R	S	R	S

Table 1: Antimicrobial resistance profiling.

[a] - bacteria from 1st sample, [b] - bacteria from 2nd sample, [c] - bacteria from 3rd sample.

sus was responsible for the infection. *Staphylococcus* species is also associated with pyoderma cases [9]. As described in a particular study [9], out of 54 pyoderma cases in dogs, 4 dogs had *Staphylococcus schleiferi* infection. Out of those 4 cases, 2 organisms were identified as *S. schleiferi* subspecies *coagulans* and the rest of the 2 were *S. schleiferi* subspecies *schleiferi.* One of the dogs had a mixed infection with *Staphylococcus schleiferi* and *Staphylococcus intermedius*. In a study involving allergic dermatitis in dogs, Cain., *et al*. [5] found that all of the 225 dogs screened had *S. schleiferi* infection. Out of the 225 cases, 102 (45%) isolates were from the ear sample and 95 (42%) isolates were from the skin. In an experiment designed by Schmidt et. [24] including 73 Labrador retrievers, it was found that 72 dogs were infected with *S. pseudintermedius* and its presence was confirmed by isolation, PCR, and MALDI-TOF-MS. De Martino., *et al*. [9] also found that *S. pseudintermedius* though being commensals can cause infection in dogs. He discovered that out of 122 cases of otitis externa, 91 cases were positive for *S. pseudintermedius* infection. Coagulase-positive (CoPS) and coagulase-negative (CoNS) Staphylococci being common in dogs as stated by Bertelloni., *et al*. (2021). Besides *S. aureus*, *S. pseudintermedius* and *S. sciuri* have been recognized as infectious agents despite the low rate of detection. Although *S. sciuri* is reported to be associated with nosocomial infections and is also present as a commensal in dogs and other animals [28], considering the findings from this study, it is difficult to state the absence of infection caused by these species. Moreover, an assumption could be made that the organisms were responsible for establishing the infection based on improvement in the health status of animals following antibiotic treatment in the dogs included in the study. Although it has been documented that these organisms are commensals, it is still possible that they may initiate the infection. The possibility that these bacteria are the cause of lymphadenitis or other lymphadenopathies cannot be understated, highlighting the findings presented above. Additionally, due to the resemblance between these organisms and *Staphylococcus aureus*, many cases go undetected or are reported incorrectly. *Escherichia coli* had also been associated with granulomatous colitis in dogs, and such cases were reported by Simpson., *et al.* [28]. The adherent and invasive nature of *E. coli* was observed in the biopsy obtained from the intestines. Previously, Cochran., *et al*. [7] reported *Escherichia* in the regional lymph nodes of dogs with granulomatous colitis. It was further confirmed with immunohistochemistry, culturing, and 16S rRNA gene detection. Of all the 86 dogs investigated, a total of 5 dogs (4 boxer dogs and 1 French Bulldog) were infected with *E. coli*. Confirmation of the cases was also done by demonstrating the organisms via intra mucosal route in the colon. Antibiotic sensitivity testing employing the Kirby-Bauer disk diffusion method is one of the simplest and most reliable techniques used for routine testing of the resistance-sensitivity pattern of antibiotics against many bacteria. In the current study, it was found that azithromycin and ceftriaxone were more effective against the bacteria isolated from dogs. A study was conducted by Ganiere., *et al*. [12]. on canine pyoderma cases where the causative agent *S. intermedius* was studied for resistance patterns against certain antibiotics. Most of the clinical strains were resistant to Penicillin-G and the related β-lactamase antibiotics like ampicillin and amoxicillin. Similar findings were observed in the

present study, where the clinical isolates were resistant to these two antibiotics and the pattern was observed in other *Staphylococcus* species also. The third-generation cephalosporins used in this study were found to be effective against some isolates. In general, when compared to first-generation drugs of the cephalosporin class, third-generation drugs are less effective against Gram-positive cocci. But in contrast, the present study documented that third-generation drugs were effective against *Staphylococcus* species. Most of the canine Staphylococcal isolates showed resistance towards tetracycline, whereas doxycycline was found to be sensitive. One strain of *E. coli* was resistant to tetracycline but not doxycycline. Resistance to tetracycline is based on the presence of the tet(M) gene located in the chromosome, mostly encoding for ribosome protection protein [24,25]. The genes can be easily transferred with mobile elements as they are located on the conjugative transposons [27]. Thus, there is a possibility of cross-resistance between the tetracycline classes of drugs. In dogs, the infection caused by *Staphylococcus* spp. employs some common antibiotics including the fluoroquinolone class of antimicrobials (ciprofloxacin, ofloxacin, levofloxacin, etc). Culture and isolation of bacteria is not considered as a standard practice even now and antibiotics are most often used empirically [14]. Fluoroquinolone resistance occurs basically by alterations in the penetration of bacterial cell walls (mutations in bacterial DNA gyrase can occur but this is a rare event). This alteration in permeability can occur due to decreased permeability of the hydrophilic pores (OMP) or through alteration of the active transport (efflux) pump, leading to decreased intracellular concentration of fluoroquinolones [4].

Conclusion

To conclude, *Staphylococcus* species (*S. pseudintermedius, S. schleiferi, S. scuiri*, and *S. xylosus*) and *Escherichia coli* are reported from lymphadenopathy cases in dogs; as per the documented literature, it could be considered as a causative agent. Azithromycin (100%) followed by ceftriaxone (88.88%) showed the highest sensitivity against the isolated bacteria. Other antibiotics like ciprofloxacin (77.77%) followed by ampicillin, amikacin, gatifloxacin, ofloxacin, and doxycycline (66.66%) were also effective. Cefotaxime (33.33%), cefoxitin (28.57), and tetracycline (22.22%) were comparatively resistant. Most of the isolates were multidrug resistant except three, one strain of *S. schleiferi* and *E. coli* and *S. sciuri,* which were sensitive to many of the antimicrobial compounds. Thus, the most important step in the control of the emergence of antibiotic resistance is the prudent use of antibiotics. An important issue in all fields of medicine (including veterinary medicine) is the emergence of antibiotic resistance. Proper and timely management of recurrent clinical infection is essential to reduce antibiotic resistance. The identification and treatment of underlying anatomical or metabolic issues should always be prioritized. The virulence mechanisms of bacteria promoting chronic infection should be the focus of novel therapeutic techniques.

Acknowledgements

Authors' Contributions

Prerna Tikute: Collection of relevant literature, sample collection, laboratory work and manuscript preparation. Deepti Narang: Conceptualization of study design and interpretation. Mudit Chandra: Identification of the isolates. Sujata Turkar: Sample collection and ultrasound examination. Kuldip Gupta: Cytological examination and interpretation.

Fundings

The authors are thankful to DBT & 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 the animal network (MyDAN) Programme". No funding was received to assist with the preparation of this manuscript.

Approval of Animal Ethics Committee

The present study was conducted under the approval of IAEC (GADVASU/2022/IAEC/64/03).

Conflict of Interest Statement

The authors declare no conflicts in any form.

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