Isolation and Detection of *Mannheimia haemolytica* from otitis affections of dogs

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Journal of Livestock Science (ISSN online 2277-6214) 17: 537-540
Received on 31/7/25; Accepted on 23/7/26; Published on 28/7/26
doi. 10.33259/JLivestSci.2026.537-540

Abstract

In dogs, otitis is one of the commonest problems caused due to bacterial and fungal pathogens. In the present study, a total number of 41 samples from Otitis were collected and processed for the isolation of causal pathogens of Otitis. *Staphylococcus* sps., *Pseudomonas* sps. and *Proteus* sps. were found to be predominant in the samples. *Streptococcus* sps. was found to be at minor extent. Yeasts like *Malassezia* and *Candida* sps. were also identified. However, in two samples bipolar like organisms were observed along with other bacteria. In cultural tests, these isolates produced small grey circle colonies with beta-hemolysis on blood agar, and pink colour colonies on MacConkey agar. Further in biochemical characterisation, these isolates were found to be positive in catalase and oxidase tests. Based on the results a polymerase chain reaction (PCR) test was conducted with *Mannheimia* genus specific (16S) oligonucleotide primers and a specific PCR product of 304bp was yielded with these isolates. Thus, the isolates were provisionally confirmed as *Mannheimia haemolytica*. In Antibiotic sensitivity tests (ABST), these isolates were found to be sensitive to Co-trimoxazole, chloramphenicol and imipenem. Perhaps, this is the first time that *Mannheimia haemolytica* was isolated from canine otitis, as per our knowledge. Further investigation is required on detailed characterisation of these isolates.

Key words: Dogs, *Mannheimia haemolytica*, Co-trimoxazole, Otitis

Introduction

Dogs are the most important companion animal (Kanchana et al., 2025). A dog makes to visit a veterinarian because of the ear infections and it nearly accounts up to 20% (Petrov*et al.,* 2013). Otitis occurrence in dogs is said to have multifactorial reasons making quite complicated in the aspect of treatment. Otitis is the inflammation of the ear canal and the bacteria or yeast associated with this condition are invariably only opportunists and are not the primary pathogens that are solely responsible for that case. The infection may be acute to chronic. It is important to emphasize that under normal circumstances, external ear canal has a low number of resident as well as transient bacteria. The most isolated bacterial pathogens from otitis includes *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Corynebacterium* spp.*, Enterococcus* spp. *and Streptococcus* spp. (Rosser 2004). Nocera *et al.* (2021) found out that otitis externa can affect dogs irrespective of neither age nor gender and most commonly associated pathogen was *Staphylococcus* species. And to minor extent *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus* spp.*, Proteus mirabilis, Enterococcus* spp. and *Corynebacterium* spp. were associated with otitis externa. Chan*et al.,* 2019 reported that yeasts also play a role in the ear infections of dogs. Among the Gram +ve bacteria, *Staphylococcus pseudintermedius* which is a coagulase positive *Staphylococcus* spp. is found to be predominantly associated with the infections in dogs like pyoderma, otitis and urinary tract infections. *Pseudomonas aeruginosa*was found to be destructive pathogen of otitis externa of its multi drug resistant nature (De martino *et al.*, 2016). Occurrence of bipolars in otitis was very rare, and in the present study two clinical samples had a morphological appearance of bipolars on Grams staining. Hence, a detailed study and molecular detection was carried out for the identification of the bipolars present in the otitis infection.

Materials and Methods

Sample collection and processing

Forty one dogs with symptoms of otitis represented to the Veterinary hospitals such as Veterinary Clinical Complex of NTR College of Veterinary Science, Gannavaram, Veterinary Super Speciality Hospital, Vijayawada (VSSH), Private Pet Clinics in Vijayawada, Veterinary Polyclinics (VPCs) in Kakinada and Guntur complaining of ear discharges, pruritis, foul odour in one or both ears were examined and proceeded for collection of auricular swabs during the period June 2023 to September 2023. The present canine population age group varied between 5 months to 11 years and included both male and females. A total number of 41 samples were obtained by using sterile cotton tips and inserted into the ear canal and rotated through 360° and then transferred into the transport broth and maintained at 4°C until further processing. The samples were processed at NTR College of Veterinary Science, Gannavaram.

Cultural and biochemical studies

Samples were further inoculated in Brain Heart Infusion broth and incubated at 37°C for 24h. On observing the turbidity, the BHI broth culture was inoculated on nutrient agar. The plates were incubated at 37°C for 24 h to 48 h, and were further employed to Gram's staining for morphological identification of various bacterial pathogens. Further the isolates were tested for IMViC test as per Quinn *et al.* (2011) for the biochemical characterization. The suspicious bipolars were further streaked on blood agar and MacConkey's agar.

Molecular confirmation of *Mannheimia haemolytica* isolates by PCR

Bacterial DNA extraction

For genotypic detection of *Mannheimia haemolytica,* DNA was extracted by simple boiling and snap chilling method from the isolates (Ponnusamy *et al*., 2017).

Detection of 16S rRNA gene specific for *Mannheimia haemolytica* by PCR

All the presumptively positive *Mannheimia haemolytica* isolates were subjected to PCR for the detection of the 16S rRNA gene (304 bp) specific for *Mannheimia haemolytica* as described by (Sahay*et al.*, 2020). The cyclical conditions were as follows with an initial denaturation at 95ºC for 5 min, denaturation at 94ºC for 30 sec, annealing 56ºC for 60 sec, extension at 72ºC for 60 sec and final extension at 72ºC for 8 min. The cycle was carried for 30 cycles. After completion of the cycles, the PCR product obtained was subjected to 1.7 % agarose gel electrophoresis.

Table 1 Nucleotide sequences and amplicon sizes of *Mannheimia haemolytica* (Sahay *et al.*, 2020)

	Target gene	Primer sequence (5'-3')	Amplicon size(bp)
Mannheimia haemolytica	*16S rRNA*	F- GCTAACTCCGTGCCAGCAG R - CGTGGACTACCAGGGTATCTAATC	304

Antimicrobial susceptibility test

Mannheimia haemolytica antibiotic resistance pattern was investigated using the Kirby Bauer disc diffusion method (Bauer et al., 1966) against ten distinct and regularly used antibiotics in veterinary medicine. *Mannheimia haemolytica* susceptibility patterns were investigated using zone diameter and interpretation break points from the Clinical and Laboratory Standards Institute (CLSI, 2007). Table 2 lists the antimicrobial discs utilised in this study, their concentrations, and the inhibition zone widths used to infer resistance.

Mannheimia haemolytica isolates were first subcultured in BHI broth and incubated for 24 hours at 37℃. The turbidity was adjusted to 0.5 McFarland units (corresponding to an approximate cell density of 1.5×10^8 CFU/ml and absorbance of 0.132 at 600 nm). To obtain a lawn culture, 200 L of each inoculum was planted on Mueller Hinton agar using a sterile cotton tipped swab. Plates were allowed to dry before six antibiotic discs (six discs per plate, one in the centre and five at the periphery) were placed equidistantly aseptically with sterile forceps. The plates were incubated at 37℃ for 18 hours under favourable conditions, and the diameter of the inhibition zones was quantified to determine the antibiotic susceptibility/resistance patterns for each strain.

Table 2: Antibiotic discs used in disc diffusion test for *Mannheimia* isolates

S.N.	Antimicrobial agent	Symbol	Concentration	Sensitive (mm)	Intermediate (mm)	Resistant (mm)
1	Ampicillin	AMP	10µg	≥17	14-16	≤13
2	Chloramphenicol	C	30µg	≥18	13-17	≤12
3	Imipenem	IPM	10µg	≥23	20-22	≤19
4	Co-Trimoxazole	COT	25µg	≥16	11-15	≤10
5	Enrofloxacin	EX	10µg	≥18	15-17	≤14
6	Gentamicin	GEN	10µg	≥15	13-14	≤12

Results

In the present study, 41 samples from otitis were processed for the isolation of causal bacterial pathogens present in the infection. Based on the morphological and cultural identification tests, predominantly *Staphylococcus* spp. (30 of 41, 73%) *Pseudomonas* spp. (27 of 41, 66%) and *Proteus* spp. (11 of 41, 27%) were observed and *Streptococcus* spp. (6 of 41, 14%) was found in few samples. *Malassezia* spp. (5 of 41, 12 %) and *Candida* spp. (2 of 41, 5%) were also identified. Bizarre, in two samples bipolars were observed along with other bacterial pathogens (Fig.1). The two bipolar isolates produced small pin point grey coloured colonies with beta-hemolysis on blood agar (Fig.2) and pink colour colonies on MacConkey agar (Fig.3). Further on biochemical tests, these isolates were found to be positive in catalase and oxidase tests and negative for indole, methyl red, voges proskaeur and citrate tests (Fig.4). Polymerase chain reaction (PCR) test with *Mannheimia* genus specific oligonucleotide primers yielded a specific PCR product of 304bp with these two isolates (Fig. 5). In Antibiotic sensitivity tests (ABST), these isolates were found to be sensitive to Co-trimoxazole, chloramphenicol and imipenem and resistant to ampicillin, enrofloxacin and gentamicin (Fig.6). May be this was the first report of *Mannheimia haemolytica* being isolated from canine otitis infections.

Discussion

Tackling the cases of otitis can be quite frustrating for a animal practitioner as it is difficult to manage the disease due to its multifactorial nature. Several microbial pathogens act as a cause for the otitis infections in animals. A clinician could successfully control the chronic otitis infection if all the factors were addressed properly. De cecco *et al.* (2021) described *Pasteurella multocida* as a versatile, Gram-negative bacterial pathogen and morphologically appears as rod or coccobacillus and is frequently observed in the oropharyngeal microbiota of cats and dogs with the highest carrier rate in cats (70–90%) and dogs (20–55%), respectively. Besides, it is also responsible for important diseases like atrophic rhinitis in swine, pneumonia in ruminants, snuffles and septicaemia in rabbits and fowl cholera in poultry Harper *et al.* (2006). In immunocompromised patients and children *P. multocida* is said to cause septicaemia and suppurative leptomeningitis. *P. multocida* penetrates the mucous membranes of the host via the upper respiratory tract, conjunctival membranes and skin when any accidental scratch or bite wounds occur on skin. It is an opportunistic bacterium and could cause mucopurulent rhinitis and sinusitis. Chances of infection to the CNS could occur due to the extension of bacterial infection from nearby tissues, like the middle ear or the nasal cavity. Similar reports about *Pasteurella* infections were reported by Paul*et al.* (2012) and stated that the infection resulted in pneumonia along with septicaemia, arthritis, and otitis media.

De ceccoet *al.* 2021 reported the isolation of *Pasteurella multocida* in three domestic cats and resulted with meningoencephalitis infection with varied severity. In the present study, identification of *Mannheimia haemolytica* in

dogs as one of the pathogens for causing otitis infection and the clinical symptoms like shaking the head clearly resembles *P.multocida* as they both belong to the same family. Thus, *Mannheimia haemolytica* could also be responsible for causing the neurological symptoms in dogs and thus its presence in otitis infections should not be neglected and routine supervision of *Mannheimia haemolytica* should be carried out.

Vaccinations are not available for the prevention of the disease, thus maintaining good oral hygiene of the dogs and regular supervision and prompt treatment of the infections of the ear and respiratory tract infections may minimize the occurrence.

Acknowledgement: Our sincere gratitude to NTR CVSc., Gannavaram, Sri Venkateswara Veterinary University for providing the facilities to carry out the research work.

Conflict of Interest: Authors have no conflict of interest in this study.

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