



Detection of Common Bacterial Causes of Otitis in Dogs in Mosul City, Iraq



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Abstract

THE current study included isolation, diagnosis, and testing of antibiotic sensitivity for common bacterial agents that cause otitis in dogs. A total of 65 samples of ear swabs were taken from dogs that were presented at the Veterinary Teaching Hospital of the College of Veterinary Medicine/University of Mosul, as well as from private veterinary clinics in Mosul City, between September 2022 and July 2023. Those animals were 1-3 years of age, from both sexes, and they had clinically shown signs of otitis. Upon the bacterial culture properties, the isolated pathogens were as follows: 17 isolates of *Pseudomonas aeruginosa* (26.1%), 13 isolates of *Streptococcus pyogenes* (20%), and 10 isolates of *Staphylococcus aureus* (15.4%). The bacterial isolates were phenotypically identified by Gram stain and growth characteristics on the special culture media. Also, the biochemical properties were identified via the Vitek 2 Compact system. Furthermore, the diagnosis was confirmed by molecular detection of 16S ribosomal RNA (rRNA) of the bacterial agents by using the conventional polymerase chain reaction (PCR). Following that, these bacteria have shown a wide range of susceptibility according to their antibiotic sensitivity test. To conclude, it has been observed that several bacterial pathogens, including *P. aeruginosa*, *S. pyogenes*, and *S. aureus* were the most common causes of bacterial otitis in dogs in Mosul City, and there was a wide range of antimicrobial sensitivity patterns among these microorganisms.

Keywords: Bacterial agents, Culture isolation, Otitis, Dogs, Polymerase chain reaction

Introduction

Otitis in dogs is considered a common clinical illness that is usually noticed by veterinarians, and dog owners. Generally, there are three types of otitis, which are classified according to their anatomic location in the ear. Firstly, otitis externa occurs in the external ear canal, and it is the most common type of infection in dogs and cats [1]. Itching, fluid secretions, head shaking, and skin abrasions are the most common clinical signs of ear infections. Otitis interna, on the other hand, is an infection of the tympanic membrane (eardrum) and the Eustachian tube, both of which are structural components of the inner ear [2]. In canine, this infection is clinically characterized by secretions of inflammatory serous exudates, painful swallowing, and turning of the head to the affected side, and it might be complicated by the deafness of infected animals. Further, the otitis media which includes inflammation of the vestibular system, this form is rarely seen in dogs, and the infection can extend to cause otitis interna in some cases [3, 4]. The most common bacterial causes of otitis include

Pseudomonas aeruginosa, *Staphylococcus aureus*, and *Streptococcus pyogenes* [5].

Bacterial isolation can be used to make a definitive diagnosis of the disease, which can then be identified using phenotypic and/or genetic methods [6]. Also, the advancement of the laboratory diagnostic tools allows the researchers for accurate identification of the causative agents, such tools as the Vitek 2 Compact test [7]. It can perform all the specific biochemical tests for bacterial isolates and verify identification by the genus and species levels [8].

Pseudomonas aeruginosa is a Gram negative, non-spore-forming bacterium, and it is a causative agent for different diseases in living organisms around the world. It tests positive for the citrate, oxidase, and catalase enzymes. This bacterium is ubiquitous in the environment, and it is considered one of the opportunistic organisms that are causing diseases in immunocompromised hosts [9-11].

Staphylococcus aureus is a Gram positive, non-motile, and coagulase-producing organism. Those bacteria appear on culture media as single, or pairs, in short chains, or mostly as characteristic grape-

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like colonies [12]. *Streptococcus pyogenes* are also Gram positive cocci, non-motile, non-spore forming agents. They grow on culture media as pairs or as variable long chains, and they show Beta-hemolysis on blood agar. These organisms have several virulence factors such as hyaluronidase that can cause damage to the living tissues of hosts [13,14]. Further, they can evade the immune response of the host since they have M-protein on their surface that facilitates bacterial attachment to the tissue and cell membranes. They have been isolated from tonsillitis, otitis, pharyngitis, and other upper respiratory tract infections in different species [15].

The VITEK 2 System is a new diagnostic technique that could be used for bacterial identification, and also it can detect bacterial sensitivity for antimicrobials. This automatic system relies on the fluorescence-based techniques for diagnosis [16, 17]. The results of this system can be obtained within 1-3 hours for bacterial identification, and up to 18 hours for antimicrobial sensitivity testing [16].

However, the bacterial culture is considered a gold standard test for bacteriology. Molecular tools such as conventional and real-time polymerase chain reaction (PCR) could be useful for the diagnosis and precise detection of bacterial virulence genes. [18,19]. Additionally, they are used for validating the bacteriological findings, and genotyping of various microorganisms. These advanced tools can target the presence of housekeeping, virulence, and/or other vital genes that are important for the identification and classification of pathogens [20-22].

The aims of the present study were to isolate and identify the most common bacterial causes of otitis in dogs, and screening of these pathogens for the antimicrobial sensitivity test.

Material and Methods

Ethical approval

The animals were fairly handled according to the approved protocol (UM.VET.2022.076) by the Animal Care and Use Committee organized by the College of Veterinary Medicine / University of Mosul.

Samples collection

The ear swab samples of 65 dogs were collected from different regions in Mosul city. The study was started from September 2022 to the end of July 2023.

Laboratory investigation

All of the dogs included in the present study had clinical signs of otitis, and the collected swabs were

transported to a diagnostic laboratory in nutrient broth for further analysis. Then, the collected swabs were grown on the Edwards medium, Mannitol salt agar (MSA), and Cetrimide agar, which are selective media for *S. pyogenes*, *S. aureus*, and *P. aeruginosa* respectively. The cultures were incubated at 37 °C for 24-48 hours and following that, smears were made for Gram staining, and the phenotypic and biochemical characteristics were evaluated. The antimicrobial sensitivity testing for bacterial isolates was done according to [23]. In brief, the bacterial isolates were cultured on Mueller-Hinton agar, and 6 antibiotic discs including: (Azithromycin, Norfloxacin, Chloramphenicol, Amoxicillin, Trimethoprim, and Ceftriaxone) were used in this test (Bioanalyse Inc., Turkey).

The isolated bacteria were identified based on the morphological traits, post incubation on their selective media for 24 hours, such as the shape of growing colonies, colors, surface appearance, texture, transparency and presence of special odors [24]. Further, the microscopic features of the growing bacterial colonies were revealed by the Gram staining.

The Vitek® 2 system (bioMérieux, Inc., USA) was used to provide the biochemical profile of bacteria, and it is used according to the manufacturer's instructions. In that, the selected bacterial colonies were resuspended in 3 ml of a special reagent, and then samples were aspirated into a chamber containing a special cassette. The principle of this technique is based on the colorimetric evaluation of the bacterial suspensions, and comparing the reads with the software database.

Molecular detection

It is noteworthy that seven of positive bacterial isolates for each species were processed in this step. In detail, the genomic bacterial DNA was extracted from the grown colonies using a highly pure PCR template preparation kit (Roche Life Science, Switzerland). The process was done following the provided protocol by the manufacturer. The PCR master mix (Takara, Japan), the primers were used for the amplification of the 16S rRNA segment, and they were used for molecular detection of *P. aeruginosa* [25], *S. aureus* [26], and *S. pyogenes* [27]. The designed primers are listed in Table 1. The PCR conditions were as follows: Initial denaturation at 95 °C for 5 minutes, denaturation at 94 °C for 1 minute, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, and then final extension at 72 °C for 5 minutes. The denaturation, annealing, and extension steps were run for 30 cycles. After that, the PCR amplicons were visualized on 1% agarose gel.

Results

Bacterial isolation

The outcomes of bacterial culture from ear swabs were as follows: the highest number were showed the presence of *P. aeruginosa* in 17 (26.1%), *S. pyogenes* in 13 (20%), and *S. aureus* in 10 (15.4%) cultures out of 65 samples as mentioned in (Table 2).

Moreover, *P. aeruginosa* colonies were grown on cetrimide agar with a metallic green color and a characteristic odor. Examination under the light microscope was revealed the presence of Gram negative bacilli of the stained smears from the above grown colonies. The colonies of *S. aureus* on MSA were observed in a golden-yellowish color. After staining with Gram stain, they were noticed as Gram positive grape-like clusters. In addition, our study showed the presence of *S. pyogenes* bacteria as a causative agent for otitis in dogs. The colonies of *S. pyogenes* were grown as translucent and pinpoint colonies on Edwards agar. The stained smears of these colonies with Gram staining

showed Gram positive cocci, which appeared as short chains under the light microscope. The above descriptions are depicted in (Figures 1, 2).

Biochemical tests by Vitek® 2 Compact

The bacterial isolates were confirmed by the advanced biochemical profile tests via the Vitek® 2 system. Their biochemical properties were then compared with the software database, and the final outputs were matched with the compatible readings regarding each bacterial agent.

Molecular detection of pathogens

Regarding molecular detection, the expected PCR amplicon size was successfully retrieved for each pathogen. The band size of 1351 bp was confirmed the presence of *P. aeruginosa* in the extracted bacterial isolate, whereas the size of 996 bp confirmed confirmation of the infection with *S. pyogenes*. Finally, the bacterial isolate of *S. aureus* yielded 228 bp. The results are shown below (Figures 3-5).

TABLE 1. Designed primers used for molecular detection of pathogens

Pathogen	Targeted segment	Primer	Primer sequence	Amplicon size (bp)
<i>P. aeruginosa</i>	16S rRNA	F	AGAGTTTGATCCTGGCTCAG	1351
		R	CTACGGCTACCTTGTTACGA	
<i>S. aureus</i>	16S rRNA	F	GTAGGTGGCAAGCGTTACC	228
		R	CGCACATCAGCGTCAG	
<i>S. pyogenes</i>	16S rRNA	F	CCAGCAGCCGCGGTAATACG	996
		R	

TABLE 2. The number of total and positive samples for bacterial isolation

Bacteria	Number of samples	Positive samples	Percentage
<i>P. aeruginosa</i>	65	17	26.1%
<i>S. pyogenes</i>	65	13	20%
<i>S. aureus</i>	65	10	15.4%

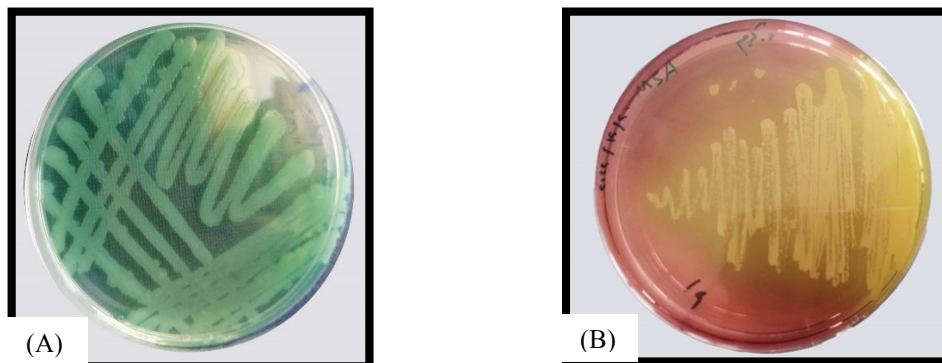


Fig. 1. (A) Colonies of *P. aeruginosa* were grown on Cetrimide agar with a metallic green color. (B) Colonies of *S. aureus* on MSA showed a golden-yellowish color.

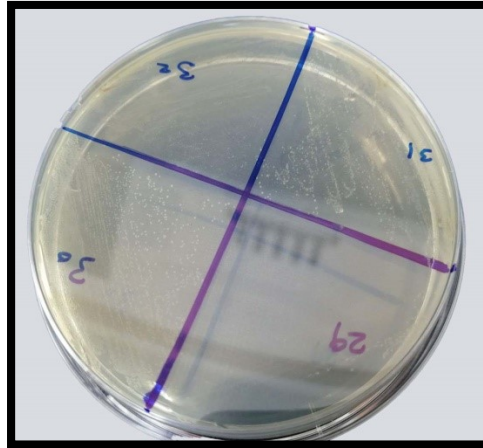


Fig. 2. Culture of *S. pyogenes* grown as translucent and pinpoint colonies on Edwards Agar

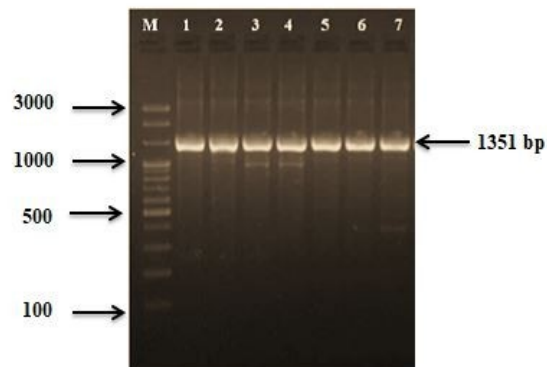


Fig. 3. Molecular detection of *p. aeruginosa* isolate which showed PCR product of size 1351 bp, loaded on 1% agarose gel. (M: DNA marker, 1-7: Samples of extracted DNA from bacterial cultures).

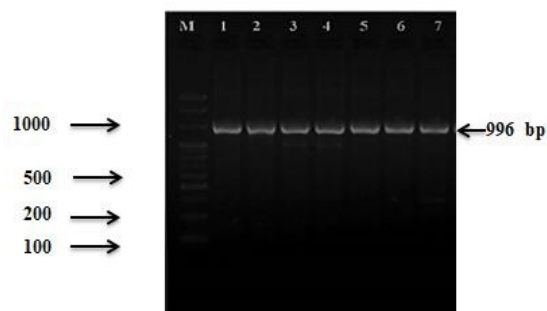


Fig. 4. Molecular detection of *S. pyogenes* isolate which showed PCR product of size 996 bp, loaded on 1% agarose gel. (M: DNA marker, 1-7: Samples of extracted DNA from bacterial cultures).

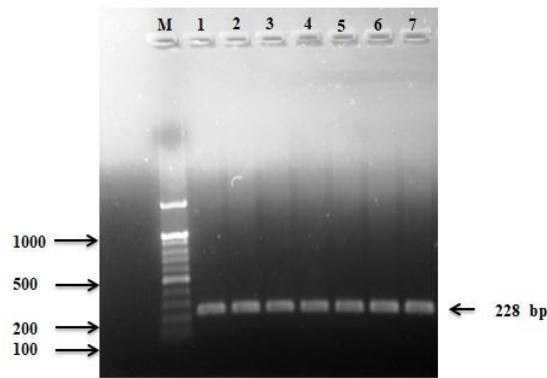


Fig. 5. Molecular detection of *S. aureus* isolate which showed PCR product of size 228 bp, loaded on 1% agarose gel. (M: DNA marker, 1-7: Samples of extracted DNA from bacterial cultures).

Antimicrobial sensitivity test

The results of the antibiotic sensitivity test showed that there was a variance in the resistance and sensitivity of the bacterial isolates. The bacteria of *p. aeruginosa* showed 100% resistance to

Amoxicillin, Azithromycin, and Trimethoprim. Contrarily, they exhibited a 50% resistance to chloramphenicol. However, these isolated bacteria showed 100% sensitivity for both Ceftriaxone and Norfloxacin, as showed in (Table 3).

TABLE 3. The antimicrobial sensitivity test for *p. aeruginosa*

Antibiotic	Percentage %		
	Sensitive	Intermediate	Resistant
Amoxicillin	0	0	100
Norfloxacin	0	0	100
Azithromycin	0	0	100
Ceftriaxone	100	0	0
Chloramphenicol	0	50	50
Trimethoprim	0	0	100

The results of antibiotic sensitivity for *S. aureus* were revealed that these bacteria were 100% resistant to Amoxicillin and Ceftriaxone, whereas their resistance reduced to 57% for Chloramphenicol. In the contrast, they had been

showed 100% and 85% sensitivity for Norfloxacin and Azithromycin respectively, while it had been noticed 43% sensitivity for both Chloramphenicol and Trimethoprim, as mentioned in (Table 4).

TABLE 4. The antimicrobial sensitivity test for *S. aureus*

Antibiotic	Percentage %		
	Sensitive	Intermediate	Resistant
Amoxicillin	0	0	100
Norfloxacin	100	0	0
Azithromycin	85	0	15
Ceftriaxone	0	0	100
Chloramphenicol	43	0	57
Trimethoprim	43	57	0

In this study, the results of *S. pyogenes* isolate were shown that these bacteria had 100% and 62 % resistance to Amoxicillin and Azithromycin respectively. Further, they were shown 50% of both sensitivity and resistance activities for

Trimethoprim and Ceftriaxone. In addition, these microorganisms had presented a sensitivity of 100% for Norfloxacin, and they were shown 87% sensitivity for Chloramphenicol, as illustrated in (Table 5).

TABLE 5. The antimicrobial sensitivity test for *S. pyogenes*

Antibiotic	Percentage %		
	Sensitive	Intermediate	Resistant
Amoxicillin	0	0	100
Norfloxacin	100	0	0
Azithromycin	13	25	62
Ceftriaxone	50	0	50
Chloramphenicol	87	13	0
Trimethoprim	50	0	50

Discussion

The bacteriological examinations of 65 ear swab samples from infected dogs with otitis were revealed the presence of *P. aeruginosa*, *S. Pyogenes* and *S. aureus*. The results of this study were in agreement with previous reports that were described the isolation of these pathogens [28, 29].

The biochemical properties of these microorganisms were validated by using the Vitek® 2 system. Generally, the common causative bacterial agents of otitis in animals include *Pseudomonas* spp., *Streptococcus* spp., and *Staphylococcus* spp. [30, 31].

Furthermore, it has been reported that bacteria of *P. aeruginosa* were the most prevalent agent among the other bacterial causes of otitis in dogs [32, 33]. This result is similar to the finding of the current study, this might be attributed that these organisms have high adhesion ability to the endothelial cells lining the auditory canal [34, 35].

The results of the isolation of *S. aureus* from cases of otitis are due to these bacteria being normally present on different body surfaces and they are considered opportunistic microorganisms that could incidentally cause a disease [36].

The colonies of *P. aeruginosa* were grown on Cetrimide agar, and they were appeared greenish in color since they have the ability to produce pyocyanin and pyoverdin. These pigments are water-soluble and they can yield a fluorescent light when the colonies are exposed to ultraviolet light [37]. On the other hand, the colonies of *S. aureus* were grown on MSA with a characteristic golden color since that medium has a high concentration of Sodium Chloride salts which inhibit the growth of other bacteria. Also, these bacteria have the ability to synthesize Staphyloxanthin, which is responsible for the golden color and it is considered as a virulence factor as well [38]. The bacteria of *S. pyogenes* were grown on Edward's media as colorless pinpoint colonies because of the presence of Esculin in those media [39].

The results of the antimicrobial sensitivity test for different bacterial isolates in the current study were showed that *P. aeruginosa* isolates were reported 100% resistance to Amoxicillin [40]. Also, they

showed the same resistance to the Azithromycin and Trimethoprim, whereas they showed 50% resistance to Chloramphenicol. The mechanism of antimicrobial resistance mostly used by these pathogens is the production of Beta-lactamase enzymes that play a major role in that resistance [41]. In addition, the resistance may occur due to changes in the permeability of the porins that are located within the bacterial outer membranes [42]. On the other hand, these bacterial isolates were found to be highly sensitive (100% sensitivity) to both Ceftriaxone and Norfloxacin. That finding is in agreement with what has been reported previously by [43]. This may occur due to the fact that these antibacterial agents are classified as broad-spectrum agents that target both Gram positive and negative microbes [44].

The bacteria of *S. aureus* were resistant 100% to Amoxicillin and Ceftriaxone, whereas they were sensitive 100% and 85% to Norfloxacin and Azithromycin respectively. The production of Beta-lactamase enzymes is responsible for the resistance of these microorganisms to antimicrobials [45]. These results were in harmony with the findings of [46], and this might be associated with the emergence of new generations that develop antibiotic resistance through the synthesis of Beta-lactamase enzymes [45].

The isolates of *S. pyogenes* were showed 100%, and 62% resistance to Amoxicillin and Azithromycin respectively, and these outcomes were in common with the study of [47]. These pathogens have the ability to evade the phagocytic white blood cells and invade host tissues since they have M-protein molecules [48]. In contrast, these microbes were showed 100% and 87% sensitivity for Norfloxacin and Chloramphenicol respectively. These results were in agreement with [49], and these observations were occurred due to that these are broad spectrum, and they are classified as new generations of antimicrobials.

The findings of molecular detection were validated the results of bacterial isolation for each pathogen. The expected band size of the PCR products was identical for each microorganism as previously mentioned in the scientific literature. In that, it was showed that amplification of 16s-rRNA

segments for *P. aeruginosa*, *S. pyogenes*, and *S. aureus* were showed 1351 bp, 996 bp, and 228 bp respectively. These results were in agreement with previous reports regarding the band size of the targeted gene in the isolated pathogens including: *P. aeruginosa* [25], *S. pyogenes* [27], and *S. aureus* [26].

Conclusions

To conclude, it was observed that bacteria of *P. aeruginosa* were the most prevalent causative agent for otitis in dogs, followed by *S. pyogenes*, and *S. aureus* agents. Moreover, it was reported that development of antimicrobial resistance phenomena by the new generations of bacteria could have major consequences on public health in general.

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Conflict of Interest

The authors had no conflict of interest in this work.

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الكشف عن المسببات الجرثومية الشائعة لالتهاب الأذن في الكلاب في مدينة الموصل ، العراق

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تضمنت الدراسة الحالية عزل، تشخيص واختبار حساسية المضادات الحيوية للعوامل البكتيرية المسببة لالتهاب الأذن الوسطى في الكلاب. تم أخذ 65 عينة من مسحة الأذن من الكلاب التي كانت متواجدة في المستشفى البيطري التعليمي التابع لكلية الطب البيطري/ جامعة الموصل، وكذلك من العيادات البيطرية الخاصة في مدينة الموصل، خلال الفترة ما بين أيلول 2022 وتموز 2023. الحيوانات المشمولة بالدراسة كانت باعمار تتراوح بين 1-3 سنوات ومن كلا الجنسين، وكانت تلك الحيوانات تعاني سريريا من علامات التهاب الأذن. اعتمداً على الخصائص الزرعية للبكتريا ، تم عزل العوامل المرضية الاتية: 17 عزلة من جراثيم الزوائف الزنجارية *P. aeruginosa* (26.1%) ، 13 عزلة من جراثيم المكورات السبحية القحجية *S. pyogenes* (20%) و 10 عزلات من جراثيم المكورات العنقودية الذهبية *S. aureus* (15.4%). تم تشخيص الجراثيم مظهرياً بالاعتماد على صبغة الكرام وعلى خصائص النمو على الأوساط الزرعية الخاصة . ايضاً تم التعرف على الخواص الكيموجيوية بواسطة جهاز الفانينك. بالإضافة الى ذلك، تم تأكيد التشخيص عن طريق الكشف الجزيئي لـ 16sRNA للبكتريا عن طريق استخدام تفاعل البلمرة المتسلسل التقليدي (PCR). بعد ذلك، اظهرت هذه البكتيريا اختلاف واسع في مدى التأثير بالمضادات الحيوية وبالاعتماد على نتائج اختبار الحساسية لتلك المركبات. في الخلاصة، تمت ملاحظة ان عدة مسببات بكتيرية تضمنت: الزوائف الزنجارية، المكورات السبحية القحجية والمكورات العنقودية الذهبية هي الاكثر شيوعاً كمسببات لالتهاب الأذن في الكلاب في مدينة الموصل ، وكان هناك تباين كبير لحساسية المضادات البكتيرية لهذه الكائنات المجهرية.

الكلمات الدالة: الجراثيم ، الزرع الجرثومي ، التهاب الأذن ، الكلاب ، تفاعل البلمرة المتسلسل.