Identification of Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolated from Canine Otitis Externa Cases

Fatma T Hadi
Department of Physiology, Biochemistry and Pharmacology College of Veterinary Medicine University of Baghdad, fatima.taleb1206h@covm.uobaghdad.edu.iq

Nibras Naeb Abdulhamza Alabbas
Instructor. Department of Physiology, Biochemistry and Pharmacology College of Veterinary Medicine University of Baghdad.

Abstract

The current study aimed to identify methicillin-resistant Staphylococcus aureus (MRSA) isolated from dogs' ears collected from various locations in Baghdad, Iraq. One hundred and fifty swabs from dogs' with otitis externa were collected from October 2021 to December 2021. Samples were inoculated on mannitol salt agar (MSA) and incubated at 37°C for 24 hrs, then sub cultured on blood agar. Colony characterization, Gram's staining, common biochemical assays, and the analytical profile of VITEK® 2 were used for isolates detection In addition, and the genotypic analysis of the MRSA isolates by using polymerase chain reaction (PCR) assay targeting the mecA gene was performed. Staphylococcus aureus was found in 26.6% (40/150) of dogs 'swabs based on VITEK® 2 system. The aforementioned system revealed that the isolates had an identification level with a probability of 99 percent, according to the manufacturer's technical data sheet and the Genotypes identified by PCR showed that the isolate selected from (40) isolates (100%) harboring mecA genes. It can be concluded from this study that MRSA isolates harboring the mecA gene are associated with the otitis externa cases in dogs.

Keywords: *Methicillin-resistant Staphylococcus aureus, otitis externa, VITEK® 2, PCR, the mec A genes.*

1. Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is an opportunistic invasive pathogen causing major and diverse diseases in humans and animals. This bacterium has been reported to cause an emerging zoonotic infection with public health and veterinary consequences (Algammal et al., 2020; Boswihi & Udo, 2018). It can cause community-acquired infections and it is known to colonize the skin, nose, genitourinary tracts, and pharynx, with the anterior nares as the main reservoir (Taylor &Unakal, 2022). MRSA infections, especially in companion animals, offer a danger of nosocomial transmission in veterinary facilities. Furthermore, companion animals with MRSA infections can transmit the bacterium to human patients who were found to be positive in around 10% of homes, indicating that dogs may be a source of (re)infection for people (Boswihi & Udo,2018; Tong et al., 2015).This bacterium Gram-positive, non-motile, non-spore forming, aerobic, catalase positive (all pathogenic Staphylococcus species), and cocci arranged as pairs, tetrads, or irregular grape- like clusters with 0.5-1.0 μm in dimeter, and can be distinguished by producing yellow or golden pigment (Algammal et al., 2015; Taylor &Unakal, 2022; Gatta & Al-Graibawi, 2021).This pathogen developed the

ability to cause a broad variety of illnesses, from small infections like skin and soft tissue infections (SSTI) to serious infections like bloodstream infections (BSIs), bacteremia, endocarditis, osteomyelitis and pneumonia (Boswihi & Udo,2018; Tong et al., 2015). The pathogenicity of this bacterium is associated with a variety of virulence factors that allow the organism to attach, suppress the immune system and damage the host. Furthermore, this microorganism contains a plethora of pathogenic factors that aid in tissue colonization, tissue damage, and distant illnesses (Tam & Torres, 2019; Markey et al., 2014). Staphylococcus spp. is most frequently isolated from the ear canals of dogs with otitis (Zamankhan et al., 2010). Otitis externa is an inflammatory condition that affects the pinna and external ear canal. Acute or chronic otitis externa are also possible (persistent or recurrent otitis lasting for 3 months or longer). Chronic inflammation can result in changes to the external ear canal, such as glandular hyperplasia (Huang et al., 2009).The massive random antibiotic use, mutations in genes coding for target proteins and the acquisition and accumulation of antibiotic resistance-conferring genes have resulted in the emergence of methicillin-resistant S. aureus (MRSA), which has become a major public health issue, and its strains have developed resistance to all ß-lactam antibiotics including Penicillin and Cephalosporin over time. (Santajit & Indrawattana, 2016; Harkins et al., 2017; Jafar & Abed, 2021).The emergence of bacteria with multiple antibiotic resistance genes, which persist and spread worldwide will compromise the treatment of infections causing clinical failures of these therapies (Schmidt et al., 2015). The main objective of this study was to isolate and characterize methicillin-resistant Staphylococcus aureus, which causes otitis in dogs.

2. Materials and Methods

Samples collection

Totally, 150 dogs' ear swabs were collected from various locations across Baghdad city, including Baghdad Veterinary Hospital and some private veterinary clinics during the period extending from October to December 2021- Most of the dogs belonged to K9 or security dogs. The samples were collected under the supervision and control of the owner, and the sampling process was carried out through the use of swab gel tubes that were transported to the laboratory by cool box.

Routine diagnosis

In the laboratory, the swabs were cultured on a selected medium, Mannitol Salt Agar (MSA, Oxoid /England), for 24 hours at 37°C. The suspected S aureus colonies were cultivated on blood agar (Oxoid /England) for 24 to 48 hours at 37°C, and their morphology was assessed by shape, size, color, and microscopic inspection following Gram's staining (Markey et al., 2014; Harley J&P Harley,2002). Moreover the suspected colonies were confirmed by the biochemical reactions such as coagulase, catalase, oxidase, DNase, urase, carbohydrate fermentation tests along with the beta hemolysis on the blood agar (Markey et al., 2014).

VITEK®2 identification

VITEK®2 Compact (Biomerieux, USA) was also used as an automated system as another procedure for identifying S. aureus isolates. It is an automated microbiology system utilizing growth based technology. It employed to confirm the identification of both Gram-positive and Gram-negative bacteria by using colorimetric reagent cards that are incubated and interpreted automatically. This system is characterized by being rapid, accurate and

efficient diagnostic system (Pincus, 2006). An overnight subculture of suspected S. aureus colonies was suspended in 2.5 ml of 0.45% NaCl to generate a 1.5 x10 CFU/ml (0.5 McFarland) bacterial solution in a 12 x75 mm transparent polystyrene test tube; the bacterial density was then adjusted using a densitometer (Biomerieux). The generated bacterial solution was put into the VITEK®2 system using a Gram-positive cocci card (GPC) to read the kinetic fluorescence measurements, and the findings were available within 4-8 hours.

Molecular diagnosis

For the genotypic identification of S. aureus isolates by PCR, the bacterial DNA was extracted to kit (ABIO pureTM, USA) Cells pellets, were made from 1ml of overnight bacterial culture centrifuged for 2min at 13000 rpm. The Supernatant was then discarded. For gram-positive bacteria, 100µl of Nuclease-free water plus 100µl from Lysozyme solution were added to the cell pellet, vortexed and Incubated in water bath for 30min at 37ºC. After incubation, the samples were centrifuged for 2min at 13000 rpm, and the supernatant was then discarded. For protein digestion and cell lysis, 20µl of Proteinase K solution (20 mg/ml) and 200µl of Buffer BL was added to sample then the tube was mixed vigorously using vortexed and incubated at 56˚C for 30 min. For further lysis, it was incubated for further 30 min at 70 ºC. From absolute ethanol, 200µl was added to the sample, pulse-vortexed to mix the sample thoroughly, all of the mixture was transferred to the mini column carefully, and then centrifuged for 1 min at 8,000 rpm, and the collection tube was replaced with a new one, 600µl from Buffer BW was added to the mini column, and then centrifuged for 1 min at 8,000 rpm. Then, the collection tube was replaced with a new one; 700µl from Buffer TW was applied, and centrifuged for 1 min at the same speed mentioned above. The pass-through was inserted, the mini-column back into the collection tube, and the mini-column was centrifuged at full speed (>13,000 x g) for 1 min to remove residual wash buffer. Later, the mini-column was placed into a fresh 1.5 ml tube, 100µl from Buffer AE was added and incubated for 1 min at room temperature, then centrifuged at 5,000 rpm for 5min.

Quantitation of DNA

Quantus Fluorometer (Promega, USA) was used to detect the concentration of extracted DNA in order to detect the quality of samples for downstream applications. For 1 µl of DNA, 200µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Primers preparation

The primers were supplied by Macrogen Company in lyophilized form. The primers were dissolved in the required amount of nuclease free water to give a final concentration of 100 pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10 µl of primer stock solution (stored at -20ºC) to 90 µl of nuclease-free water to obtain a working primer solution of 10 pmol/µl. The primers used in this study were designed by (Macrogen, Korea) and are mentioned in Table 1 below.

Table 1. The primers used in the PCR assay.

Primer Name	Seq.	Annealing Tem. (°C)	Product Size (bp)
mecA-F	5`-GTAGAAATGACTGAACGTCCGATAA-3`	53	310
mecA-R	5`-CCAATTCCACATTGTTTCGGTCTAA-3`		

Polymerase chain reaction:

The PCR reaction was done using Thermal cycler (Thermo Fisher Scientific, USA)

Reaction Volume /run	**20 µl**
Safety Margin	5 %
Length of PCR product (bp)	310
Annealing temperature of primers	53
No. of PCR Cycles	30

Table 2.PCR Component Calculation

Table 3.PCR Component Calculation

Master mix components	Stock	Unit	Final	Unit	Volume
Master Mix	2	X	1	X	10
Forward primer	10	µM	1	µM	1
Nuclease Free Water					6
DNA	10	ng/µl	10	ng/µl	2
Total volume					20
Aliquot per single rxn	18µl of Master mix per tube and add 2 µl of Template				

Table 4: PCR Program

Steps	**°C**	**m: s**	**Cycle**
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	
Annealing	53	00:30	30
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Agarose gel electrophoresis

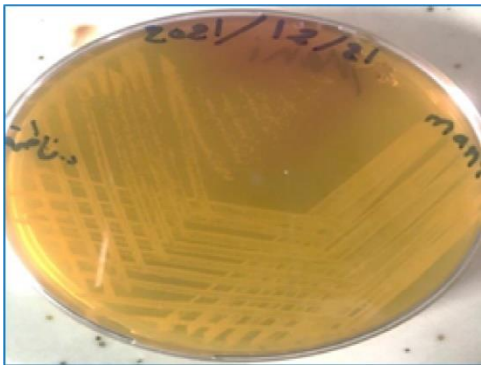
Following PCR amplification, agarose gel electrophoresis was used to confirm amplification. For the 1.5% agarose gel preparation, 100 ml of 1× TAE buffer was used for dissolving 1.5 gm of the agarose powder in a flask, and the solution was heated to boiling using a Microwave until all the gel particles were dissolved. Then,1µl of Ethidium Bromide (10mg/ml)) was added to the melted agarose, which was stirred in order to be mixed. The solution was left to cool down at 50-60°C. The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes, and the agarose was left to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel tray was moved to in the electrophoresis tank.

The tank was filled with 1× TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel. The PCR product was loaded directly into the gel well along with 10 ng/ µl in another well. Electrical power was turned on at 100v/mAmp for 60min. The Ethidium bromide-stained bands in the gel were visualized using Gel imaging system (Javid et al., 2018).

3. RESULTS

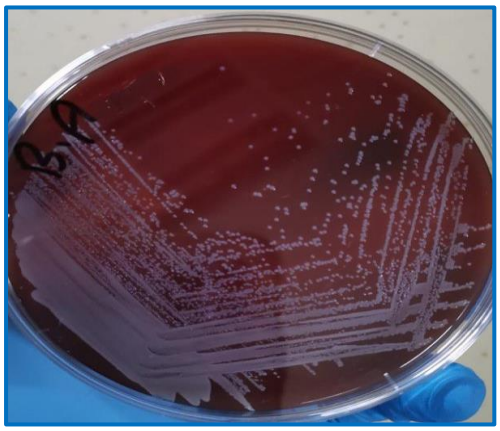
Staphylococcus aureus isolates fermented mannitol and were able to generate acidic compounds that lowered the pH of the medium, turning the phenol red indicator to yellow as shown in Figure 1.

Figure 1. Growth of S. aureus on MSA with appearance of golden-yellow pigment colonies (mannitol fermentation).



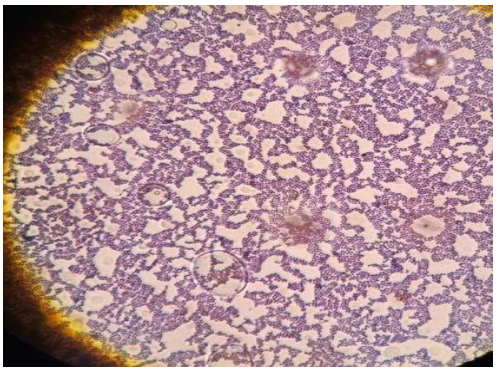
On the blood agar, the colonies of S. aureus appeared large, round, smooth, and white to gray gleamy (Figure 2) as well as α and β hemolysis.

Figure 2: Growth of S. aureus on the blood agar shows α and β hemolysis to the blood cells.



Microscopic examination under a light microscope showed that S. aureus was Gram-positive pairs, cocci, single cells, and appeared as grape-like clusters after incubation for 18–24 h at 37 °C, as shown in Figure 3.

Figure 3. Staining of S. aureus with ram's stain and examination under the light microscope shows Gram-positive, cocci, single-cells, pairs, or grape–like clusters.



Catalase test indicated that these microorganisms are positive to this test due to production of the catalase enzyme, and the result appeared as bubbles formation (Figure 4).

Figure 4: Staphylococcus aureus shows positive catalase test as indicated by air bubble formation.

Table 5. The results of VITEK® 2 system for the identification and confirmation of S. aureus.

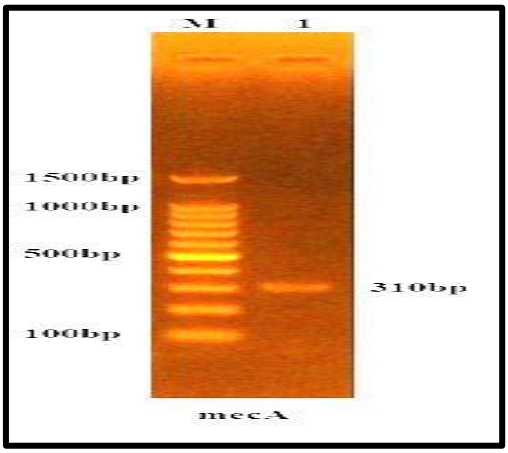
Identification Information	Card: GP	Lot Number: 2421836403	Expires: Dec 6, 2022 12:00 CST
	Status: Final	Analysis Time: 3.80 hours	Completed: Oct 22, 2021 14:49 CDT
Organism Origin	VITEK® 2		
Selected Organism	99% Probability Staphylococcus aureus Bio number: Confidence: 050402023663231 Excellent identification		
Analysis Organisms and Tests to Separate:			

Biochemical Details16																	
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	lLATk	+	42	LAC	-	44	NAG	-	45	dMAL	+	46	BACI	-
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	+
64	OPTO	+															

PCR result analysis

The DNA sample of an isolate diagnosed as S. aureus by the VITEK®2 was also confirmed by PCR based on the mecA gene. Figure 5 shows the PCR product of the expected size (310 bp) of the partially amplified mecA gene of S. aureus.

Figure 5: Agarose gel electrophoresis for the PCR product shows a band of approximately 310bp of the partially amplified mecA gene of a S .aureus isolate. The DNA sample amplified by PCR was fractionated on 1.5% agarose gel stained with Eth.Br. M: 100bp DNA ladder. Lanes 1: 310bp of the expected band of the PCR product.



4. DISCUSSION

Staphylococcus aureus is the most significant human and animal pathogen among the Staphylococci spp. And it is distinguished by the development of golden pigment. The coagulase test is the most basic laboratory approach for distinguishing S. aureus from coagulase-negative staphylococci. Gram staining, standard biochemical assays, and colony characterization used in this study are comparable to S. aureus phenotypic characteristics described by (Markey et al., 2014). Blood agar is usually used to distinguish pathogenic bacteria based on their hemolytic power to the red blood cells (Gatta & Al-Graibawi, 2021; Ballhausen et al., 2014). Colony morphology and all biochemical tests (Gram's staining, coagulase, catalase, urease, hemolysis, and DNase tests) are distinguishing characteristics of S. aureus (Habib et al., 2015).

This study showed a percentage 26.6% (40/150) of dogs infected with S. aureus. This result was higher than the records of (Tarazi et al., 2015) who reported 12.7% (19/150) dogs in Jordan, and higher than records of (Christiane et al., 2022) that record 7.8% of (10/112) in central and north Germany, and Less than (Ma et al., 2020) in New South Wales, Australia that records 67.3% of dogs (204 of 303). S. aureus may also be detected or verified using VITEK®2 and the PCR technique (Paterson et al., 2014). The isolates obtained an identification by the VITEK®2 system with a probability of 99 % .This study supports previous investigations on the capacity of the VITEK®2 technology to identify S. aureus isolates. (Delmas et al., 2008).Several strategies for detecting MRSA have been developed, including phenotypic and genotypic methods, such as the mecA gene, which is a genetic marker utilized for the quick and direct identification of MRSA (Ostojić, & Hukić,2015). Carrying the mecA gene on the staphylococcal cassette chromosome mec (SCCmec) gives resistance to all β-lactam antimicrobials (methicillin-resistant) (Habib et al., 2015; Delmas et al., 2008).Genotypes identified of the selected isolate from (40/150) 26.6 % MRSA isolated from dogs by biochemical and morphological identification also VITEK®2 technology show that the isolate harboring mec A gene or have the same size of expected band 310 bp. The present study is comparable to another performed on dogs, pets, and pet owners in Malaysia, which found that 20% of canines were infected with MRSA (Chai et al., 2021).

Reference

Alani HA, Hassawi DS, Flayih MT. Patterns of antibiotic resistance in Staphylococcus aureus isolates and detection the Heteroresistance to vancomycin by

population analysis method. JUAPS. 2017;11(3):26-33.

Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007 Mar 23;128(6):1037-50.

Algammal AM, Hetta HF, Elkelish A, Alkhalifah DH, Hozzein WN, Batiha GE, El Nahhas N, Mabrok MA. Methicillin-Resistant Staphylococcus aureus (MRSA): one health perspective approach to the bacterium epidemiology, virulence factors, antibiotic-resistance, and zoonotic impact. Infection and Drug Resistance. 2020;13:3255.

Alwash SJ, Aburesha RA. Detection of toxin-associated genes in seven spa-types of Staphylococcus aureus in Iraq. Indian Journal of Forensic Medicine & Toxicology. 2021 Jul 1;15(3):4157.

Avberšek J, Papić B, Kušar D, Erjavec V, Seme K, Golob M, Zdovc I. Feline Otitis Externa Caused by Methicillin-Resistant Staphylococcus aureus with Mixed Hemolytic Phenotype and Overview of Possible Genetic Backgrounds. Antibiotics. 2021 May 18;10(5):599.

Ballhausen B, Kriegeskorte A, Schleimer N, Peters G, Becker K. The mecA homolog mecC confers resistance against β-lactams in Staphylococcus aureus irrespective of the genetic strain background. Antimicrobial Agents and Chemotherapy. 2014 Jul;58(7):3791-8.

Boswihi SS, Udo EE. Methicillin-resistant Staphylococcus aureus: An update on the epidemiology, treatment options and infection control. Current Medicine Research and Practice. 2018 Jan 1;8(1):18-24.

Chai MH, Sukiman MZ, Liew YW, Shapawi MS, Roslan FS, Hashim SN, Mohamad NM, Ariffin SM, Ghazali MF. Detection, molecular characterization, and antibiogram of multi-drug resistant and methicillin-resistant Staphylococcus aureus (MRSA) isolated from pets and pet owners in Malaysia. Iranian Journal of Veterinary Research. 2021;22(4):277.

Christiane ,C., Franziska, L. , Robert W. , Robin, K., and Wolfgang, W.(2022) Colonization of Dogs and Their Owners with Staphylococcus aureus and Staphylococcus pseudintermedius in Households, Veterinary Practices, and Healthcare Facilities. https://doi.org/10.3390/microorganisms10040677.

Delmas J, Chacornac JP, Robin F, Giammarinaro P, Talon R, Bonnet R. Evaluation of the Vitek 2 system with a variety of Staphylococcus species. Journal of clinical microbiology. 2008 Jan;46(1):311-3.

Gatta MN, Al-Graibawi MA. Phenotypic and Genotypic Detection of Biofilm Producing Methicillin Resistant Staphylococcus aureus (MRSA) Isolated from Human. Annals of the Romanian Society for Cell Biology. 2021 Aug 5;25(6):19047-58.

Habib F, Rind R, Durani N, Bhutto AL, Buriro RS, Tunio A, Aijaz N, Lakho SA, Bugti AG, Shoaib M. Morphological and cultural characterization of Staphylococcus aureus isolated from different animal species. Journal of Applied Environmental and Biological Sciences. 2015 Jan 1; 5(2):15-26.

Hana DB, Kadhim HM, Jasim GA, Latif QN. Antibacterial activity of Commiphora molmol extracts on some bacterial species in Iraq. Sch. Acad. J. Pharm. 2016;5:406-12.

Harkins CP, Pichon B, Doumith M, Parkhill J, Westh H, Tomasz A, de Lencastre H,

Bentley SD, Kearns AM, Holden MT. Methicillin-resistant Staphylococcus aureus emerged long before the introduction of methicillin into clinical practice. Genome biology. 2017; 18(1):1-1.

Harley J, P Harley J. Laboratory exercises in microbiology. The McGraw− Hill; 2002.http://125.212.201.8:6008/handle/DHKTYTHD_123/3050

Huang HP, Little CJ, McNeil PE. Histological changes in the external ear canal of dogs with otitis externa. Veterinary dermatology. 2009 Oct;20(5‐6):422-8.

Jafar Alwash S, Abed Aburesha R. The Differences in Antibiotic-resistance among Several Staphylococcus aureus strains in Iraq. Medico-legal Update. 2021 Jul 1;21(3).

Javid F, Taku A, Bhat MA, Badroo GA, Mudasir M, Sofi TA. Molecular typing of Staphylococcus aureus based on coagulase gene. Veterinary world. 2018 Apr;11(4):423.

Lafi ZK. Determination of Antimicrobial Resistance blaZ and mecA Genes and Immunotherapeutic Efficacy of Transfer Factor to Staphylococcus Aureus Isolated from Human and Buffalo. MS. C thesis. College of Veterinary Medicine-Unversity of Baghdad. 2019.

Ma GC, Worthing KA, Ward MP, Norris JM. Commensal staphylococci including methicillin-resistant Staphylococcus aureus from dogs and cats in remote New South Wales, Australia. Microbial ecology. 2020 Jan;79(1):164-74.

Markey, B.K.; Leonard, F.C.; Archambault, M. Cullinane, A. and Maguire, D. (2014). Clinical Veterinary Microbiology. 2nded. Mosby Elsevier. Pp.161.

Ostojić, M., & Hukić, M. (2015). Genotypic and phenotypic characteristics of Methicillin-resistant Staphylococcus aureus (MRSA) strains, isolated on three different geography locations. Bosnian journal of basic medical sciences, 15(3), 48.

Paterson, G.K.; Harrison, E.M. and Holmes, M.A. (2014). The emergence of mecC methicillin-resistant Staphylococcus aureus . Trends in microbiology, 22(1), 42-47.

Pincus DH. Microbial identification using the bioMérieux Vitek® 2 system. Encyclopedia of Rapid Microbiological Methods. Bethesda, MD: Parenteral Drug Association. 2006:1-32.

Santajit S, Indrawattana N. Mechanisms of antimicrobial resistance in ESKAPE pathogens. BioMed research international. 2016 Oct;2016.

Schmidt T, Kock MM, Ehlers MM. Antimicrobial Resistance in Staphylococci at the human–-animal interface. Antimicrobial Resistance: An Open Challenge. 2015 Nov 26; 85.

Tam K, Torres VJ. Staphylococcus aureus secreted toxins and extracellular enzymes. Microbiology spectrum. 2019 15; 7(2):7-2.

Tarazi YH, Almajali AM, Ababneh MM, Ahmed HS, Jaran AS. Molecular study on methicillin-resistant Staphylococcus aureus strains isolated from dogs and associated personnel in Jordan. Asian Pacific Journal of Tropical Biomedicine. 2015 Nov 1;5(11):902-8.

Taylor TA, Unakal CG. Staphylococcus aureus. InStatPearls [Internet] 2022 Jul 18. StatPearls Publishing.

Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler Jr VG. Staphylococcus aureus infections: epidemiology,

pathophysiology, clinical manifestations, and management. Clinical microbiology reviews. 2015; 28(3):603-61.

Zamankhan Malayeri H, Jamshidi S, Zahraei Salehi T. Identification and antimicrobial susceptibility patterns of bacteria causing otitis externa in dogs. Veterinary research communications. 2010 Jun;34(5):435-44.