

Isolation of Methicillin-Resistant *Staphylococcus Pseudintermedius* Strains from Human Otitis cases in Wasit Province, Iraq

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ABSTRACT

Staphylococcus pseudintermedius, opportunistic pathogen, has protruded as a significant health threat in humans following being zoologically transmitted to humans. This pathogen is reported to cause inflammatory lesions in different sites of human body. Therefore, this study is aimed to identify and evaluate the antimicrobial susceptibility of *S. pseudintermedius* strains isolated from otitis human patients. A total of 84 otitis samples were obtained from ENT clinics in Wasit province, Iraq; and bacteriological identification of *Staphylococcus* species was performed. Identification of *S. pseudintermedius* was evaluated by biochemical tests, API staph, the Vitek®2 compact and detection of some genes. In general, out of 84 samples, 63 samples (75%) were positive for *Staphylococcus* species. Among these 63 positive *Staphylococcus* spp. isolates, only 13 isolates (20.6%) were initially identified as *S. pseudintermedius*. All isolates of *S. pseudintermedius* were identified as methicillin-resistant strains (MRSP) using cefoxitin disc diffusion method. Indeed, MRSP isolates showed high resistance to penicillin (100%), chloramphenicol (100%), erythromycin (100%), and tetracycline (100%). However, the MRSP showed low resistance to gentamicin (23.07 %) and Ciprofloxacin (38.46%). Genetically, all isolates of *S. pseudintermedius* were carry the Staphylococcal Chromosomal Cassette (SCC) element that was concluded by existence of a *mecA* gene. The partial sequence of *S. pseudintermedius* strain SIG 16S ribosomal RNA gene has been deposited in the National Center for Biotechnology Information (NCBI) database under the GenBank accession no. MK968120.1.

Keywords: Methicillin-resistant *Staphylococcus Pseudintermedius*, Otitis, Antibiotic resistance, *mecA* Gene.

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INTRODUCTION

The zoonosis opportunistic pathogen, *Staphylococcus pseudintermedius*, has been recognizing as its responsibility for necrotizing and severe infections in both humans and dogs (1). It was firstly described in 2005 based on 16S rRNA gene sequencing analysis (2) and is the most important opportunistic pathogen in companion animals subject to the different sites of the body. *Staphylococcus pseudintermedius* can cause a variety of diseases such as infected dog bite wounds, bacteremia, pneumonia, brain abscesses and septic arthritis, most of which have been related to dog exposure (3). Somayaji *et al.*, 2016 find that infections disease in human related to this pathogen was increasingly isolated after dogs contact.

The clinical infections in humans caused by *S. pseudintermedius* were wildly misidentified as *S. aureus* (3). *Staphylococcus aureus* is the most isolated bacterium among both community-acquired and nosocomial infections. (4). The latter is routinely diagnosis by bacteriological test namely the production of coagulase or clumping-factor (2). Similarly, the staphylococci pathogens, including *S. pseudintermedius*, are also coagulase positive. So, for proper identification of these pathogens molecular and extensive phenotypic methods have to be use (2).

Similar to *S. aureus*, *S. pseudintermedius* produces numerous virulence factors including toxins, such as haemolysins, exfoliative toxins, coagulase, thermolysin, clumping factor and protein A, enterotoxins and a leucotoxin I (Luk-I) (5).

The other challenge to face veterinary medicine in addition to *S. pseudintermedius* virulence profile is the methicillin-resistance in *S. pseudintermedius* (MRSP). In the last two decades this clinical challenge was emerged worldwide for difficulty of the *S. pseudintermedius* infections treatment.

Due to wide range of infections by *Staphylococcus pseudintermedius* and excessive use of antibiotics, this pathogen develops a resistance to almost all antibiotics classes (6). According to reports of WHO and the United Nations in 2016, Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has protruded as an important health threat in animals, with the potential for transmission to humans (8).

METHODS

The samples included in this study (84) were obtain from ENT clinic attendees in Al-Karama Educational Hospital, Al-Kut city, Wasit province, Iraq. These samples were labeled and transferred aseptically to the microbiology laboratory of medicine college, Wasit university. All isolates were identified in routine diagnostic of the bacteriology basis on a cultural, gram stain, hemolytic on blood agar and biochemical reactions such as catalase, oxidase, coagulase, mannitol fermentation, the confirmation diagnosis was performed according to Api staph and the vitek compact. The final identification of isolates was performed with sequence analysis for the 16S rRNA gene.

A disc diffusion method was used to determine antibiotic susceptibility on Mueller Hinton Agar. A variety of antibiotics were tested including ciprofloxacin (CIP),

Gentamicin (GE), tetracycline (TE), Erythromycin (E), vancomycin (VA), sulfamethoxazole-trimethoprim (SXT), chloramphenicol (C), ceftiofur (FOX), penicillin (PE) and cefotaxime (CTX). The samples that detected with *Staphylococcus pseudintermedius* were moved to detect MRSA. These samples were tested by disk diffusion technique using ceftiofur (FOX; 30 µg) to detect the antimicrobial susceptibility of pathogen based on the Clinical and Laboratory Standards Institute (9).

This new species was registered as Iraqi isolate for *Staphylococcus pseudintermedius* bacteria in National Center for Biotechnology Information (GenBank accession no. MK968120.1).

DNA extraction, amplification, and sequencing

The extraction of *Staphylococcus pseudintermedius* DNA was done by using the Genomic DNA Extraction kit (Geneaid/ Thailand). Amplifying of 16SrRNA, Mec gene and Nuc gene was achieved by PCR technique. The total volume for the reaction was 20µl of PCR PreMix (Bioneer, South Korea) consisting of 1µl from each primer forward and reverse, 3µl of DNA and the volume completed up to 20µl with free nucleases deionized water according to the instructions of the company.

For the optimization program to the primers of every gene the annealing temperature was a gradient in PCR of

many degrees started from 51°C to 65°C). PCR cycling programs were as following: To detect 16srRNA gene fragment, the mixture has a denaturation run in 95°C for 5 min. for just one cycle, then 35 cycles with a temperature of 94°C for 30 sec, annealing was with 58°C temperature for 30 sec. for 35 cycles, then the extension was for 35 cycles in 72°C for 1 minute each, and final extension with 72° C for 7min. for just one cycle and holding done with 4° C for 1 cycle. For mecA gene, denaturation did with 95°C for 5 min. for just one cycle followed by 35 cycles with a temperature of 94°C for 30 sec, for annealing the temperature was 51°C for 30 sec. for 35 cycles, the extension is done with a temperature of 72°C for 1 min. for 35 cycles, for the final extension, the temperature was 72° C for 7min. for 1 cycle and holding for 4° C for 1 cycle. The nuc gene was detected according to Sasaki *et al.*, 2010 where the optimized PCR protocols consisted of an initial denaturation at 94°C for 3 minutes, then 35 cycles of desaturation at 94°C for 30 seconds, the temperature used for annealing was 51-65°C for 30 seconds, for extension was 72°C for 30 seconds and a final extension temperature was at 72°C for 3 minutes. The electrophoresed was used to resolve the products. Table (1)

Table 1. Primers used in this study.

Primer name	Sequence (5' - 3')	Size bp	Source
16S1 16S2	GTGCCAGCAGCCGCGTAA AGACCCGGAACGTATTCAC	886	Poulsen <i>et al.</i> 2003
mecA1 mecA2	GGGATCATAGCGTCATTATTC AACGATTGTGACACGATAGCC	527	
nuc1 nuc2	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG	926	Sasaki <i>et al.</i> 2010

Statistical Analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1) (10). The percentages were compared by using Chi-square test. P < 0.05 is considered statistically significant.

RESULTS AND DISCUSSION

The current study showed that the prevalence of *Staphylococcus* species was 63 isolates (75%) of total samples, from these only 13 isolates (20.6%) were initially identified as *S. pseudintermedius* using a biochemical identification system. Figure (1). Similar

results were recorded by (11) where the prevalence of *S. pseudintermedius* was 18.3% and (12) in Lithuania who obtain (26.6%) *S. pseudintermedius* isolates from the clinical source. The other study in Japan also reported a similar result with (29.82%) prevalence (13).

Organism Quantity:
Selected Organism : *Staphylococcus pseudintermedius*

Source: _____ Collected: _____

Comments: _____

Identification Information	Analysis Time: 8.25 hours	Status: Final
Selected Organism	85% Probability <i>Staphylococcus pseudintermedius</i>	
	Bionumber: 07141217773271	
ID Analysis Messages		

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	ILATk	+	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	+															

Figure 1: Results of biochemical tests using the VITEK method with probability number (85%)

Distinguish between these species by conventional microbiological diagnostic techniques was unsuccessful resulting in *S. pseudintermedius* possible misidentification (13) and (14). Therefore, the final specific microbiological identification requires the combination of phenotypic and genotypic tests.

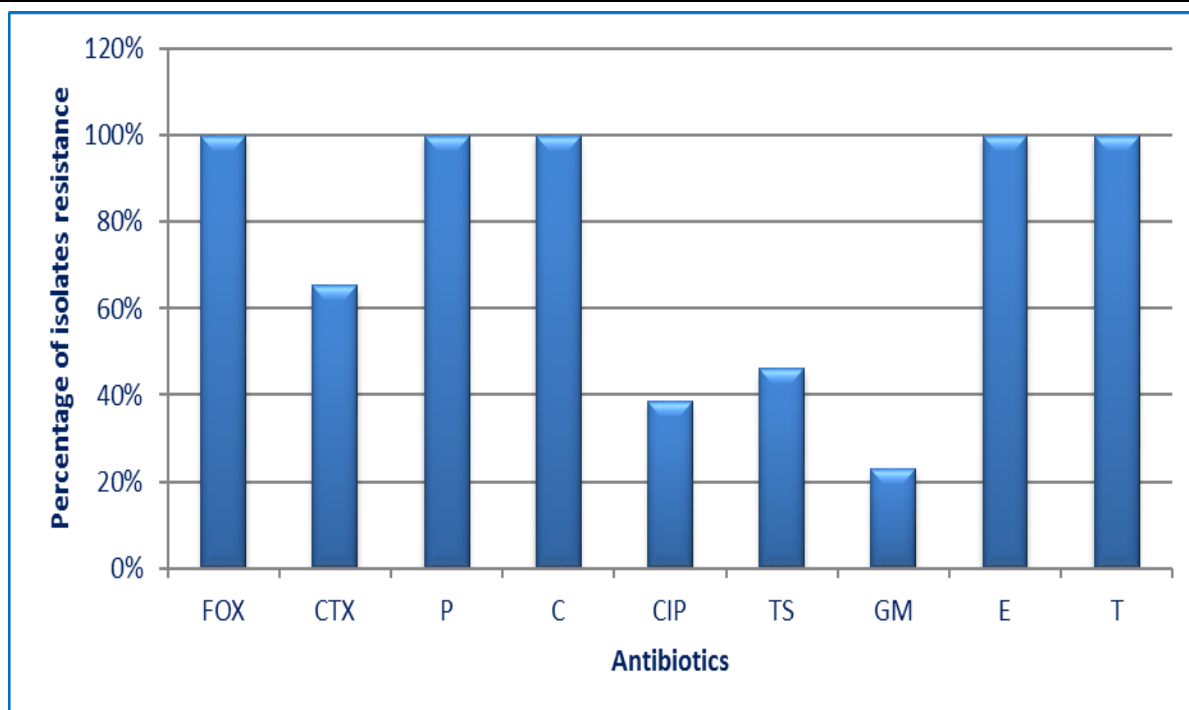
Our results showed that all MRSP were multi-drug resistant to a wide range of antimicrobial classes. The percentage of *S. pseudintermedius* isolates that have a resistant to antimicrobials showed in figure 2. Resistance to cepheems was high and ranged from 100% for *cefoxitin* to 61.5 % for *cefotaxime*. Similarly, (8) in Korea demonstrated that *S. pseudintermedius* isolates have high resistance rate to *cefoxitin* and *cefotaxime*. Furthermore, all *S. pseudintermedius* isolates were resistant to Penicillin, chloramphenicol, tetracycline and erythromycin as a percentage of 100%. In agreement with these results, a study by (7) has reported high resistance of this bacteria to penicillin in Argentina. The resistance of *S. pseudintermedius* to erythromycin was as

100% in our study and was similar to a study reported by (15). A recent study suggested that the increased antimicrobial resistance of MRSP is closely related to the acquisition of multiple resistance genes (16).

Misuse/overuse of antibiotic by public and healthcare professionals among other factors take part in increasing the rates of antimicrobial resistance (17; 18; 19). Furthermore, improper prescription of antibiotics due to insufficient surveillance systems and independence on reliable microbiological techniques (20).

Resistance to sulfamethoxazole-trimethoprim was 46.15% in agreement with what observed in the study done in Brazil by (21). *Staphylococcus pseudintermedius* is a critical human pathogen in light of rising levels of methicillin and multidrug resistance (22).

Aminoglycoside showed low resistant 23.07 %. Gentamicin was showing similar resistance rates in a study done in Brazil by (23). Indeed, the most effective antibiotic against *S. pseudintermedius* was gentamicin followed by Ciprofloxacin.



FOX: *cefoxitin*, CTX: *cefotaxime*, p: *penicillin*, C: *chloramphenicol*, CIP: *ciprofloxacin*, TS: *sulfamethoxazole- trimethoprim*, GM: *gentamicin*, E: *Erythromycin*, T: *tetracycline*.

Figure 2: Antibiotics tested against *S. pseudintermedius* isolates

DNA of all isolated were extracted, purity and concentration were confirmed with Nanodrop. The purity of *S. pseudintermedius* isolated was (1.7-2). The molecular detection of 16SrRNA gene revealed that 13/13 (100%)

of isolates contained this gene (Fig3). The occurrence of the *MecA* gene was detected in all *S. pseudintermedius* isolates 13/13(100%) Fig 4.

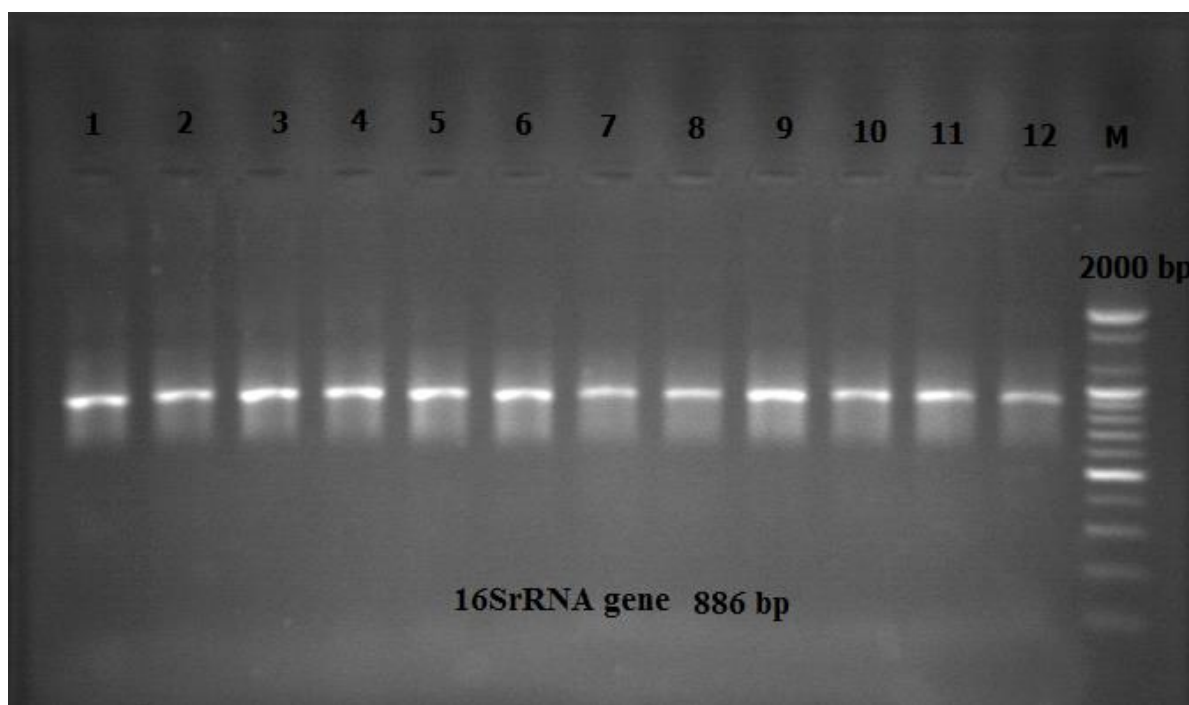


Figure 3: Agar gel electrophoresis showing PCR amplification at 886 bp fragment for 16SrRNA gene, M: 2000 bp DNA ladder.

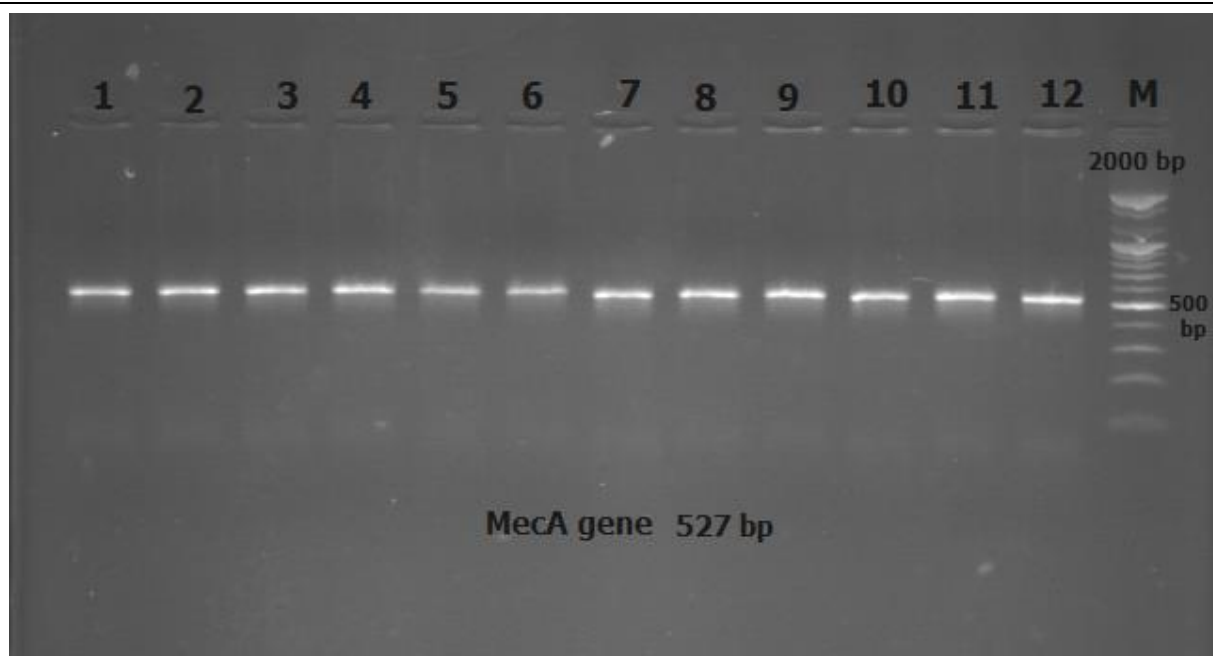


Figure 4: Agar gel electrophoresis showing PCR amplification at 527 bp fragment for MecA gene, M: 2000 bp DNA ladder.

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged as an important threat to human health in last year and associated with increased mortality. (24) have reported the critical of MRSP in human and animal health worldwide due to its multidrug resistance phenotype. Moreover, (8) raising a similar issue about the prevalence of MRSP as it rapidly increased in the past few years.

The Nuc gene was absent among all *S. pseudintermedius* isolates. This result is consistent with other studies. (25) showed that PCR for nuc can generate false-negative results because of variations in the DNA sequence of the nuc gene. Also, (26) show amplification of thermonuclease (nuc) gene is used to detect and quantify *S. aureus* and known as *S. aureus* species-specific gene. (27). The present study suggests that the absence or presence of the nuc gene is not a reliable method for the identification of *S. pseudintermedius*. Efficiency of genetic methods in the detection of accurate and rapid bacterial isolates thus shortening the time and cost (28).

CONCLUSION

Staphylococcus pseudintermedius has announced as a transmitted disease from dogs to human and has a significant health threat. This finding reports as the first study for isolation and identification of *S. pseudintermedius* which was first isolated in Al-kut city as its responsibly for infections in humans. 16S rRNA sequencing provides more confident detection of *S. pseudintermedius*. Methicillin-resistant *Staphylococcus pseudintermedius* could be a threat to human health due to its concerning level of antimicrobial resistance. The results of antibiotic sensitivity test revealed that the most active compound against *Staphylococcus pseudintermedius* isolates was gentamicin followed by ciprofloxacin.

Ethical Clearance:

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and

higher education and scientific research ministries in Iraq.

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