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Detection of some genes encoding to produced toxins of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from patients with otitis media using a Multiplex-PCR method

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ABSTRACT

Otitis media (OM) is an infection frequently involving multiple bacterial species. Staphylococcus aureus *and* Pseudomonas aeruginosa *are key pathogens in community and healthcare-associated cases. Precisely identifying these bacteria and their virulence factors is essential for public health. This research used molecular methods to detect specific virulence genes in* S. aureus *and* P. aeruginosa *obtained from OM patients in Basra, Iraq. Among 96 patient samples, 24* S. aureus *and 20* P. aeruginosa *isolates were confirmed using culture, biochemical tests, and the Vitek-2 system. DNA from these isolates was analyzed with multiplex-PCR to target the* S. aureus *genes for staphylococcal enterotoxins A (sea) and B (seb), toxic shock syndrome toxin (tst-1), and exfoliative toxin A (eta). Detection rates were (62.5%) for sea, (41.7%) for seb, (54.2%) for tst-1, and (8.3%) for eta. For* P. aeruginosa, *the exotoxin A gene (exoA) was found in (80%) of isolates. This study demonstrates a high occurrence of these crucial toxin genes in local OM isolates, underscoring their likely contribution to disease development.*

Keywords: otitis media, *Staphylococcus aureus, Pseudomonas aeruginosa,* virulence genes, multiplex PCR, antimicrobial resistance

INTRODUCTION

Otitis media (OM) is a prevalent polymicrobial infection where bacteria cause most instances. Although *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* are recognized primary pathogens [1], bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are now considered increasingly important. *S. aureus* is a formidable pathogen that can produce diseases ranging from minor skin conditions to severe, life-threatening toxic shock [2]. Its disease-causing ability is largely due to an array of virulence factors [3], which include powerful toxins like staphylococcal enterotoxins (SEs) A and B, toxic shock syndrome toxin-1 (TSST-1), and exfoliative toxins (ETs) [4,5]. These toxins can act as superantigens, causing an excessive immune reaction, or function as proteases that damage skin integrity [6].

Likewise, *P. aeruginosa* is a well-known cause of hospital-acquired infections and is especially linked to persistent and hard-to-treat OM cases [7]. A key component of its virulence is exotoxin A, a powerful agent that halts host protein synthesis, resulting in cellular death and tissue destruction [8].

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The worldwide frequency and variety of these virulence genes in clinical samples can differ greatly by geographic region and patient group.In Iraq, particularly in Basra, detailed molecular data on the toxin characteristics of S. aureus and P. aeruginosa from OM patients is lacking. Traditional culture-based techniques can identify the bacterial species but do not uncover their genetic capacity for toxin production, which is vital for comprehending the infection's seriousness and mechanism.

Consequently, this research was designed to address this information gap. Its main goal was to use a multiplex-PCR technique to directly identify the key virulence genes sea, seb, tst-1, and eta in *S. aureus* and the exoA gene in *P. aeruginosa* from OM patients in Basra, Iraq. This molecular strategy yields important information on the local spread of these pathogens and a more detailed understanding of the toxic risks they may present.

MATERIAL AND METHOD

Study population and bacterial isolates

The investigation was carried out at Basra General Hospital in Iraq over a period from November 2019 to February 2021, subsequent to receiving ethical approval (Approval No. 6/2019) from the Basra Health Directorate. We enrolled a total of 96 patients, ranging in age from 1 to 72 years, who presented with ear discharge attributable to otitis media (OM). Following cleansing of the ear with 70% alcohol, a specialist collected swab samples. The sample size of 96 was not determined by a formal power calculation but was instead contingent upon the availability and accessibility of patients throughout the duration of the study.

Identification of bacterial pathogens

Clinical swabs were cultured on Blood, MacConkey, and Mannitol Salt agar plates and subjected to aerobic incubation at 37°C for 24-48 hours. Initial identification of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was conducted by assessing colony morphology, Gram stain characteristics, and standard biochemical profiles. The Vitek-2 system was employed for definitive confirmation of all isolates [9]. From this process, a collection of 24 *S. aureus* and 20 *P. aeruginosa* isolates was established for subsequent molecular analysis.

DNA extraction and PCR amplification

Genomic DNA was purified from all bacterial isolates using the Presto™Mini gDNA Kit from Geneaid, South Korea.

For the *S. aureus* isolates, a multiplex PCR assay was executed to detect the sea, seb, tst-1, and eta genes. The 100 µl reaction mixture comprised 20 µl of PCR PreMix (Biomerieu), 10 µl of DNA template, 2.5 pmol of each primer, and nuclease-free water. The thermocycling protocol consisted of an initial denaturation at 94°C for 5 minutes; followed by 35 cycles of 94°C for 2 minutes, 57°C for 1 minute, and 72°C for 1 minute; and a final elongation step at 72°C for 7 minutes [10].

For *P. aeruginosa,* a simplex PCR targeting the exoA gene was performed. The 20 µl reaction was prepared using i-Taq PreMix (Bioneer), 4 µl of DNA template, 1.5 pmol of each primer, and nuclease-free water. The cycling conditions were set as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 57.2°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 5 minutes.

The sequences for all primers utilized in these assays are provided in Tables 1 and 2 of the original document. The amplified PCR products were subsequently resolved by electrophoresis on 1.5-2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

TABLES 1. *The primers sets to detect virulence genes in* S. aureas *isolates [11-13]*

Primer		Sequences	Size of amplified Product (bp)
Eta	F	GCAGGTGTTGATTTAGCATT	93
	R	AGATGTCCCTATTTTTGCTG	
Sea	F	GGTTATCAATGTGCGGGTGG	102
	R	CGGCACTTTTTTCTCTTCGG	
Seb	F	GTATGGTGGTGTAACTGAGC	164
	R	CCAAATAGTGACGAGTTAGG	
Tsst-1	F	ACCCCTGTTCCCTTATCATC	326
	R	TTTTCAGTATTTGTAACGCC	

Polymerase chain reaction to detect ExoA gene in *P. aeruginosa* isolates

The multiplex polymerase chain reaction (PCR) were performed in a total volume of 20 µl, according to the PCR PreMix master mix (i-Taq for 20 µl rxn including 20 µl of Master mix, Bioneer, Korea), 4 µl of DNA template of *P. aeruginosa* isolate, 13 µl of Nuclease free water, 1.5 pmol of each primers (Table 2). Following the addition of all chemicals to the i-Taq master mix tubes containing the master mix pellet, the PCR tubes were put in a microcentrefuge and centrifuged for around a half minute to thoroughly mix them. The thermal cycler was adjusted as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation (95°C for 30 sec.), annealing (57.2°C for 30 sec.), and extension (72°C for 1 min). A final step (72°C for 5 min) was performed after the completion of the above cycles The PCR product was loaded in 1X TBE buffer and electrophoresed in 2% agarose gel, 5 µl of PCR element was added to each well, and one well received 5 µl of molecular ladder (1000 Kb ladder). After that, the substance was detected by used UV transilluminator.

***TABLE 2.** The sequence of ExoA special primers [14]*

Primer target	Oligonucleotide sequence (5′-3′)		Amplicon size (bp)
ExoA	F	5′ CAG AAC TGG ACG GTG GAG C 3′	535 bp
	R	5′ CCT GTT CCT TGT CGG GGA TG 3′	

RESULTS

Isolate recovery and patient demographics

From the 96 patients with otitis media (OM) enrolled in this study, a total of 44 bacterial isolates were recovered and confirmed. These consisted of 24 *Staphylococcus aureus* and 20 *Pseudomonas aeruginosa* isolates. The patient cohort included 49 males and 47 females, with an age range of 1 to 75 years.

Prevalence of toxin genes in *Staphylococcus aureus*

Multiplex analysis PCR of the 24 *S. aureus* isolates revealed a high prevalence of toxin-encoding genes (Figure 1). The sea gene was the most common, detected in 15 isolates (62.5%), followed by the tst-1 gene in 13 isolates (54.2%). The seb gene was found in 10 isolates (41.7%), while the eta gene was rare, present in only 2 isolates (8.3%).

Representative multiplex PCR results are shown in Figure 2.

Multiplex PCR reaction was performed for all isolates of *S. aureas* using specialized primers targeting the specific sequence of genes: enterotoxin A (Sea) and B (Seb), toxic shock syndrome toxin (Tsst-1) and exfoliative toxin (Eta).

Furthermore, the analysis revealed that most isolates carried multiple toxin genes. Only three *S. aureus* isolates (12.5%) were negative for all four tested genes. The distribution showed that four isolates harbored three different toxin genes, eleven isolates carried two genes, and six isolates possessed a single toxin gene.

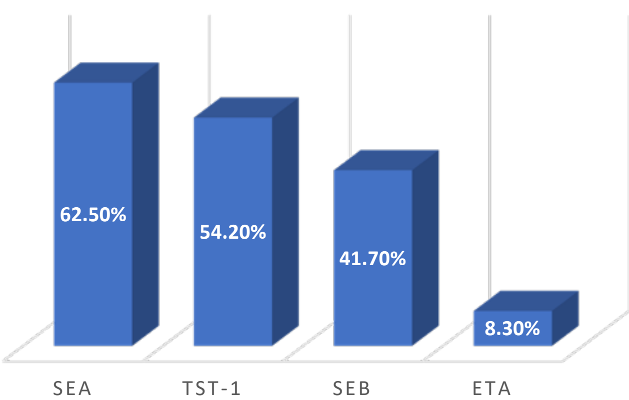

***FIGURE 1.** Prevalence of toxin-encoding genes (sea, tst-1, seb, and eta) among Staphylococcus aureus isolates obtained from patients with otitis media (n = 24)*

***TABLE 3.** The order of appearance of genes in S. aureas isolates according to their numbers in each isolate*

No. S. aureus isolates	Isolates number	Toxins encoding genes			
		Sea	Seb	Tsst	Eta
3	4,7,22	+	+	+	-
1	6	+	+	-	+
5	5,9,15,19,24	+	+	-	-
5	1,8,10,13,17	+	-	+	-
1	11	-	-	+	+
1	21	+	-	-	-
1	18	-	+	-	-
4	2,3,12,20	-	-	+	-
3	14,16, 23	-	-	-	-
24		15	10	13	2

Prevalence of the exoA gene in *Pseudomonas aeruginosa*

PCR screening of the 20 *P. aeruginosa* isolates for the exoA gene, which encodes exotoxin A, showed that 16 isolates were positive, yielding a prevalence of 80% (Figure 3).

DISCUSSION

This research presents a molecular analysis of principal virulence genes in *S. aureus* and *P. aeruginosa* obtained from otitis media (OM) patients in Basra, Iraq. The results demonstrate a substantial occurrence of toxin-encoding genes, highlighting the significant pathogenicity of these clinical isolates.

A particularly striking finding was the elevated frequency of the sea (62.5%) and tst-1 (54.2%) genes among the *S. aureus* isolates. The dominance of the sea gene is in agreement with data from other investigations on human infections [15,16] but diverges from studies on foodborne strains, which frequently report a higher prevalence of seb [17]. Such differences underscore that the distribution of toxin genes is shaped by the bacterial ecological niche and geographic source. The rate of tst-1 detected in our patient group was markedly greater than that described in numerous other clinical reports [18,19], potentially suggesting the circulation of a unique local clonal variant. The low detection rate for the eta gene (8.3%) aligns with its established rarity in the literature [20,21].

Regarding *P. aeruginosa,* the high identification rate (80%) of the exoA gene is congruent with other studies on clinical specimens, including those from OM [22], and confirms its role as a principal virulence determinant in local strains.

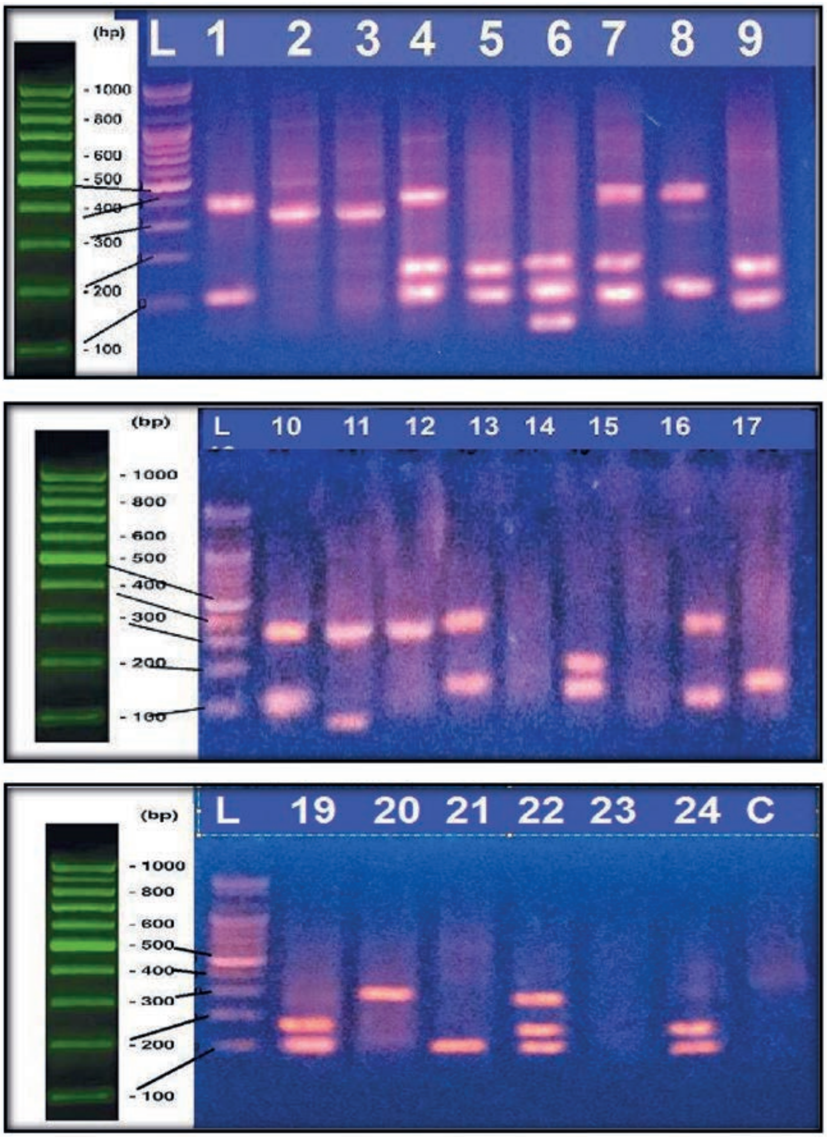

FIGURE 2. *Agarose gel electrophoresis of multiplex PCR products detecting toxin-encoding genes (sea, seb, tst-1, and eta) in* Staphylococcus aureus *isolates. Lane L: DNA molecular weight marker; lanes 1–24: S. aureus isolates obtained from patients with otitis media; lane C: negative control (PCR reaction without DNA template). Expected amplicon sizes were 102 bp (sea), 164 bp (seb), 326 bp (tst-1), and 93 bp (eta)*

Clinical and epidemiological implications

The frequent presence of potent superantigen genes such astst-1 and sea implies that OM resulting from these *S. aureus* strains may lead to heightened inflammatory responses and a greater likelihood of complications. The co-occurrence of multiple toxin genes within a single isolate is an alarming discovery that could potentially hinder treatment and lead to poorer patient outcomes. From an infection control perspective, these findings underscore the necessity for diligent surveillance to identify and prevent the dissemination of these virulent clones in healthcare environments. Moreover, this work provides essential baseline information on the molecular epidemiology of these pathogens in Southern Iraq, creating a reference point for subsequent regional monitoring and comparative research.

CONCLUSIONS

This investigation confirms that clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from OM patients in Basra, Iraq, are frequently carrying key virulence genes. The high prevalence of sea and tst-1 in *S. aureus* and exoA in *P. aeruginosa* is especially concerning, given their association with increased virulence, significant tissue injury, and perturbed host immunity.

These results possess direct implications for the clinical management of OM in the region. The existence of these toxin genes could lead to more chronic infections, exacerbated inflammation, and a greater potential for complications, which may affect treatment strategies and patient monitoring. While empiric antibiotic treatment remains essential, it does not counteract the injury mediated by these pre-formed toxins. Conse-

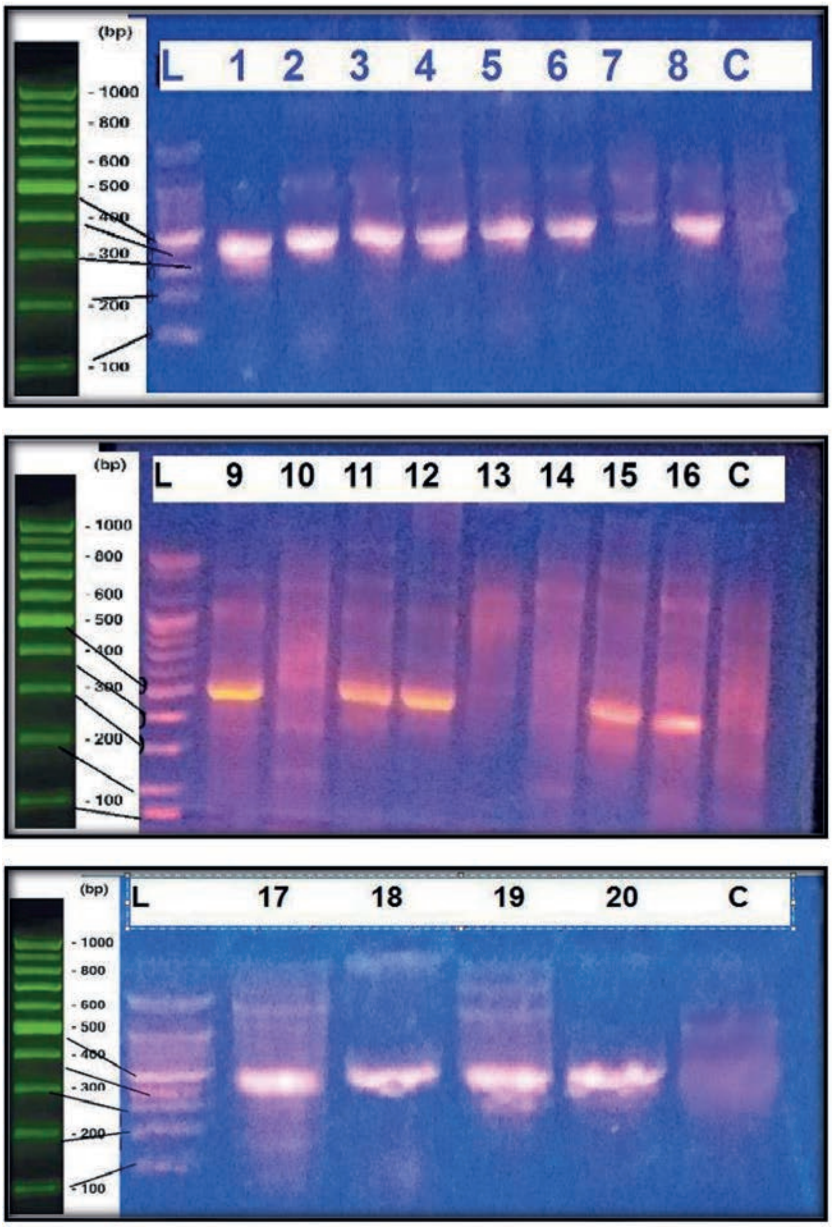

FIGURE 3. *Agarose gel electrophoresis of PCR products detecting the exoA gene in* Pseudomonas aeruginosa *isolates. Lane L: DNA molecular weight marker; lanes 1–20:* P. aeruginosa *isolates obtained from patients with otitis media; lane C: negative control (PCR reaction without DNA template). The expected amplicon size for exoA was 535 bp*

quently, comprehending the local toxigenic landscape of these bacteria is a vital move towards more effective and individualized patient care.

Furthermore, this research underscores the urgent requirement for the ongoing surveillance of virulence genes as a component of regional antimicrobial and epidemiological monitoring programs in Iraq. Creating baseline data on the molecular features of prevalent strains is fundamental for identifying emerging hypervirulent clones, informing hospital-based infection control measures, and ultimately enhancing public health responses to these prevalent and therapeutically difficult pathogens.

Conflict of interest

The authors declare no conflict of interest.

Financial support

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