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Molecular Identification of *Staphylococcus aureus* as causative agent of acute otitis media in Iraqi patients

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Background: *S. aureus* is capable of acquiring resistance to antibiotics through several processes. Acute otitis media (AOM) is a common pediatric bacterial infection affecting approximately 80% of children prior to the age of 3 years Acute otitis media. AOM is the primary reason for the prescription of antibiotics in children Understanding the epidemiology and the etiology of AOM is important for the clinical selection of empiric treatment.

Materials and Methods: This study was carried out at University of Al-Qadsiyah, Iraq. One hundred samples were collected from children Patients (3 years old) that has been diagnosed by using Vitek and PCR technique. The study was conducted to investigate the prevalence of *Staph. aureus* bacteria in AOM child patients, multiplex PCR was done to test the prevalence of resistance genes then sensitivity test was done.

Result: After collecting 100 samples 60 (65%) were *S. aureus*, while 25 (25%) were as *E.coli* and 10 (10%) *Streptococcus* bacteria.Antibiotic sensitivity testwas done using 19 antibiotics, *S. aureus* bacteria were sensitive to Amikacin, Ciprofloxacillin, Clindamycin, Gentamycin, Levofloxacin, Meropenem, Linezolid, Vancomycin, Rifampicin, Tetracycline and Tobramycin. While*S. aureus* bacteria were resist to Flomoxef, Nitrofurantoin, Olfofloxacin,Benzylpenicillin, Oxacillin, Trimethoprem, Trimethoprem –Sulfamethoxazole and Mezlocillin.twenty isolates of *S. aureus* were examined for the presence of mecA , ermA, ermB, and ermC gene, which coding for bacterial resistance. The positive mecA ,

ermA, ermB, and ermC were showed in percentage of 100%, 35%, 20% and 15% respectively.

Conclusion: Our outcomes showed high prevalence of *mecA* gene in *S. aureus* which may play a significant role in antibiotic resistance.

Key words:*S. aureus*, , PCR, resistance genes, Antibiotics and Iraq

1-Introduction

Staphylococcus aureus is a Gram-positive that belong to the Micrococcaceae family and the diameter that ranges between 0.5 – 1.5 µm. The first report on S. aureus dates back to 1880, when the surgeon Sir Alexander Ogston discover it (1). However, it was not until 1884 that Friedrich Julius Rosenbach distinguished S. aureus from the closely related Staphylococcus epidermidis, based on the golden color of its colonies (2). S. aureus can survive for extended periods both on human hands and on surfaces and cross-transmission among individuals or from the environment contributes to nasal carriage (3). facultative anaerobic bacterium commonly found as a human commensal in the anterior nares, throat, and on the skin. Although this bacterium is part of our microbiota, it is better known for being the causative agent of several diseases ranging from mild skin infections or food poisoning, to life-threatening conditions, like sepsis or necrotizing pneumonia. More importantly, its capability to swiftly develop resistances to antibiotics makes this bacterium a public health threat in the community, as well as in hospitals (4).

During the first half of the 20th century, in fact, S. aureus infections were still considered highly fatal. It was only around 1940 that these infections started to be treated with penicillin, reducing the mortality rates. S. aureus isolates that developed resistance to this antibiotic were noticed shortly after the discovery of penicillin (5), and within a few years, penicillin resistant lineages had emerged in the clinic (6)Later on, methicillin was introduced as treatment for staphylococcal infection, but resistant strains (all defined as methicillin resistant Staphylococcus aureus(MRSA) emerged already in 1961, only one year

after its introduction, 1970s and 1980s there were several MRSA outbreaks worldwide, but these were limited to hospital settings (7). S. aureus is capable of acquiring resistance to antibiotics through several processes, which include mutations in the core genome, as well as the acquisition of exogenous resistance genes carried by plasmids and other mobile genetic elements. These mechanisms started to become better understood after publication of the first whole genome sequences of two isolates of this bacterium in 2001 (8).

S. aureus is generally acknowledged for its role as a pathogen, carriage of this bacterium is generally asymptomatic. The 'harmless' commensal state of S. aureus was acknowledged in the mid-1940's (2). The pandemic caused by this clone lasted from 1954 to 1957, starting in Australia and expanding to several countries, including the USA and the UK, where this clone was responsible for several outbreaks (9). At that time, the incidence of S. aureus-caused infections was 3 per 100.000 person-years but increased to approximately 20 per 100.000 person-years during the next 30 years. This rise has been attributed to nosocomial infections, acquired after invasive medical interventions. Nowadays, the rate of S. aureus infections appears to be stable, but the actual incidence varies over time and is dependent on geographical location. Of note, less affluent regions of the world exhibit higher rates of the infections caused by S. aureus than the wealthier regions (10). the last 20 years, MRSA strains have adapted to spread among the community, causing infections in healthy individuals and displaying a more virulent behavior. efforts have been undertaken to develop vaccines against this pathogen but, unfortunately, none of the tested candidates has so far passed the stage of clinical trials (11).

Acute otitis media (AOM) is a common pediatric bacterial infection affecting approximately 80% of children prior to the age of 3 years Acute otitis media (AOM) is a common pediatric bacterial infection affecting approximately 80% of children prior to the age of 3 years Acute otitis media (AOM) is a common pediatric bacterial infection affecting approximately 80% of children prior to the age of 3 years AOM is the primary reason for the prescription of antibiotics in children (12). Understanding the epidemiology and the etiology of AOM is important for the clinical selection of empiric treatment. It was reported that the incidence of pediatric AOM and the causative pathogens varied among different regions and geographic settings. Although Streptococcus pneumoniae (S. pneumoniae),

Haemophilusinfluenzae (H. influenzae), and Moraxella catarrhalis (M. catarrhalis) are the three leading causes of AOM in children (13).

2-Materials and methods:

Sample collection

This study was carried out at University of Al-Qadsiyah, College of Biotechnology, Department of Health AL-Qadsiyah. One hundred samples were collected from children (3 years old) that has been diagnosed by using Vitek and PCR technique, the samples collected from Efaq, Aldiwniyah Hospitals and private clinic. The study was conducted during the period from September 2022 to Feberuary, 2023 to investigate the prevalence of *Staph. aureus* bacteria in Al-Hussein Hospital and from Child and pregnancy women Hospital. Samples were transported to laboratory using special transport media, Samples were cultured on Bloog agar, MaCconkey agar and Mannitol salt agar in order to detect presence of *Staph. aureus* bacteria, then sensitivity test was done.Growing samples were preserved after that at -20 °C in deep freeze.

Molecular study

Multiplex PCR

The m PCR technique was performed for detection of *E. coli* bacteriafrom samples. This method was carried out according to following steps:

DNA Extraction (14)

DNA from samples were extracted by using Presto™ Bacteria DNA Extraction Kit

Genomic DNA estimation

The extracted genomic DNA from sool samples was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm). After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system. The sampling arm

was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of DNA was added to measurement (15).

Preparing of the Primers Suspension

The PCR primers for detection of *Staph. aureu*weres designed in this study using NCBI-Gene bank (AB608092.1- MK635343.1) and primer 3 plus design. These primers was provided from Scientific Reserch. Co. Ltd, Iraq as shown, table (1).

Table (1): primers used in this study

Primers	Sequence 5'-3'		Product size
erm A gene	F	**GTTCAAGAACAATCAATACAGAG**	421 bp
	R	**GGATCAGGAAAAGGACATTTTAC**	
erm B gene	F	**GTTTACGAAATTGGAACAGGTAAAGG**	359 bp
	R	**GAATCGAGACTTGAGTGTGC**	
erm C gene	F	**GCTAATATTGTTTAAATCGTCAAT**	642 bp
	R	**GGATCAGGAAAAGGACATTTTAC**	
mec A gene	F	**AAAATCGATGGTAAAGGTTGGC**	533 bp
	R	**AGTTCTGCAGTACCGGATTTG**	

3- Results

Samples collection

According to the present study outcomes, after collecting 100 samples 60 (65%) showed positivity as *S. aureus*, while 35 (%) of these samples were negative to *S. aureus* species represented by 25 (25%) as *E.coli* bacteria and 10 (10%) as *Streptococcus* species, as showed in (2)table.

Table (2): Collected samples (N=100)

Total	**Number**	**Percentage**
Bacterial species		
S. aureus	65	**(65%)**
E.coli	25	**(25%)**
Streptococcus sp.	10	**(10%)**
Total	100	**100%**

Antibiotic sensitivity test results of *Staphylococcus aureus*

Disc diffusion test was done in order to examine the susceptibility of 65 isolates of *Staphylococcus aureus* to 19 antibiotics. The results showed that*Staphylococcus aureus* were more sensitive to the following antibiotics Amikacin60(92.3%), Ciprofloxacillin55 (84.6%), Clindamycin 35 (53.8%), Gentamycin50 (76.9%), Levofloxacin50 (76.9%), Meropenem61 (93.8%), Linezolid 47 (72.3%), Vancomycin 60(92.3%), Rifampicin 60(92.3%), Tetracycline 63 (96.9%) and Tobramycin 57 (87.7%). While*Staphylococcus aureus* bacteria were more resistance to Flomoxef 40 (61. 6%), Nitrofurantoin 39 (60%), Olfofloxacin 41 (63.1%),Benzylpenicillin59 (90.7%), Oxacillin45 (69.3%), Trimethoprem42 (64.6%), Trimethoprem –Sulfamethoxazole41 (63.1%) and Mezlocillin 38(58.5%) respectively, table (3).

Table (3): Antibiotics sensitivity test of ***Staphylococcus aureus***

No	Antibiotic disc	Sensitive No (%)	Resist No (%)

.١	Amikacin	60 (92.3%)	5 (7.6%)
.٢	Ciprofloxacillin	55 (84.6%)	10 (15.4%)
.٣	Clindamycin	35 (53.8%)	30 (46.2%)
.٤	Flomoxef	25 (38.4%)	40 (61. 6%)
.٥	Gentamycin	50 (76.9%)	15 (23.1%)
.٦	Levofloxacin	50 (76.9%)	15 (23.1%)
.٧	Meropenem	61 (93.8%)	4 (6.2%)
.٨	Linezolid	47 (72.3%)	18 (27.7%)
.٩	Vancomycin	60 (92.3%)	5 (7.6%)
.١٠	Nitrofurantoin	26 (40%)	39 (60%)
.١١	Olfofloxacin	24 (36.9%)	41 (63.1%)
.١٢	Oxacillin	20 (30.7%)	45 (69.3%)
.١٣	Rifampicin	60 (92.3%)	5 (7.6%)
.١٤	Tetracycline	63 (96.9%)	2 (3.1%)
.١٥	Tobramycin	57 (87.7%)	8 (12.3%)
.١٦	Benzylpenicillin	6 (9.2 %)	59 (90.7%)
.١٧	Trimethoprem	23 (35.4%)	42 (64.6%)
.١٨	Trimethoprem –Sulfamethoxazole	24 (36.9%)	41 (63.1%)
.١٩	Mezlocillin	27 (41.5%)	38 (58.5%)

Determine of mecA , ermA, ermB, and ermC gene in *Staphylococcus aureus* isolates using multiplex PCR

In current study twenty isolates of *Staphylococcus aureus* were examined for the presence of mecA , ermA, ermB, and ermC gene, which coding for bacterial resistance, results indicated that from those tested 20 isolates, agarose gel electrophoresis image that showed multiplex PCR product analysis of antibiotics resistance gene in Staphylococcus auras isolates. The positive mecA , ermA, ermB, and ermC lanes were showed in percentage of 100%, 35%, 20% and 15% respectively, figure (1).

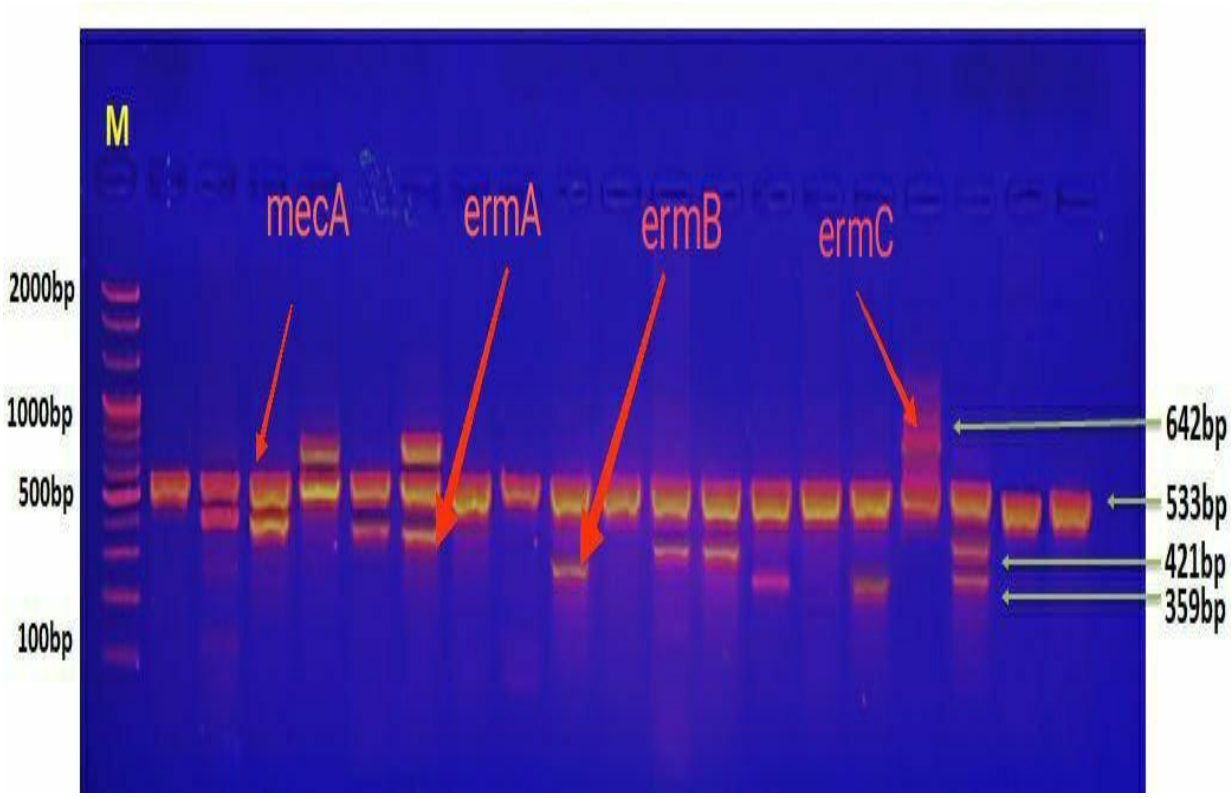

Figure (1): Agarose gel electrophoresis image that showed multiplex PCR product analysis of antibiotics resistance gene in Staphylococcus auras isolates. M (Marker ladder 2000-100bp). The positive mecA , ermA, ermB, and ermC lanes were showed at (533bp, 421bp, 359 and 642bp PCR product size) respectively.

4- Discussion

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of bacteremia and invasive diseases that include pulmonary, skin, and soft tissues and musculoskeletal infections in children (16, 17).

Our finding in present study showed that from 100 samples 65 (65%) showed positivity as *S. aureus*, while 35 (%) of these samples were negative to *S. aureus* species represented by 25 (25%) as *E.coli* bacteria and 10 (10%) as *Streptococcus* species, this outcome were agreed to Mirsoleymani et al., (2014) study related to pediatric could not isolate gram negative bacteria, but they isolated Staphylococcus coagulase positive. Byrd et al., (2017) showed greater *Staphylococcus aureus* predominance in patients with more severe disease and *Staphylococcus epidermidis* predominance in patients (18). Also the fecal carriage rates were 20.0% for *S. aureus* and 4.5% for methicillin-resistant *S. aureus* (MRSA). Moreover, *S. aureus* fecal carriage was positively correlated with outpatient diagnosis (19).

While our data were disagreed with Salman et al., (2022) mentioned that urinary tract infections (UTIs) and AOM are one of the most common infections in different age groups, including children. Bacteria are the main etiological agents. The study was to isolate, identify, and determine the antibiotic susceptibility of bacteria isolated from children from Baghdad, Iraq, of which, 267 were gram-negative bacteria, and 32 were gram-positive bacteria. *Escherichia coli* (56%) was the most commonly isolated gram-negative bacteria followed by Staphylococcus aureus (37.5%). *E. coli* and *P. aeruginosa* were the most antibiotic-resistant bacteria (20). Similar findings in different countries were observed in recent studies where the E. coli was found to be the primary etiological agent of UTI in children (21-24).

The results in this study reported that*Staphylococcus aureus* were more sensitive to the following antibiotics Amikacin60(92.3%), Ciprofloxacillin55 (84.6%), Clindamycin 35 (53.8%), Gentamycin50 (76.9%), Levofloxacin50 (76.9%), Meropenem61 (93.8%), Linezolid 47 (72.3%), Vancomycin 60(92.3%), Rifampicin 60(92.3%), Tetracycline 63 (96.9%) and Tobramycin 57 (87.7%). While*Staphylococcus aureus* bacteria were more resistance to Flomoxef 40 (61. 6%), Nitrofurantoin 39 (60%), Olfofloxacin 41 (63.1%),Benzylpenicillin59 (90.7%), Oxacillin45 (69.3%), Trimethoprem42 (64.6%), Trimethoprem –Sulfamethoxazole41 (63.1%) and Mezlocillin 38(58.5%).

These results were compatible toa study conducted by Godfrey et al., (2022) and showed that there was predominance of male participants (67.5%) with a median age was 2 years and an interquartile range (IQR) 10 months to 4 years. Staphylococcus (35.6%). All bacteria showed a high resistance to ampicillin (80%- 100%) followed by ceftriaxone (40 - 70%). The *S. aureus* isolates showed good sensitivity of about =80% to a clindamycin, meropenem, co-amoxiclav and ciprofloxacin; but had a high resistance to ampicillin (83%), ceftriaxone (69%) erythromycin (62%) and vancomycin (44.8%) (25). A study conducted by Joya et al., (2022) in Afghanistan found increasing antibiotic resistance in staphylococci to ampicillin, amoxicillin, and erythromycin (26).In Behzadnia et al., (2014) Zingg et al., (2017) and Alvares et al., (2019) study Ciprofloxacin, Gentamycin Levofloxacin Ciprofloxacillin can be recommended as an alternative 2nd line antibiotic in *Staphylococcal spp* isolated showed good sensitivity to clindamycin (27)(28)(29).

While our findings were disagreed to studies indicated that there was an increased resistance to the commonly used antibiotics which are, vancomycin, erythromycin, co-amoxiclav, gentamycin and ciprofloxacin and this was similar to what has been observed in several studies in developed and developing countries [(30)(31).

There was notably an increase in resistance to antibiotics up to 50% compared to previous studies which reported up to 40% (32). This is possibly due to overprescription of the drug since more than 50% of the participants used ceftriaxone prior to admission. This trend of increased resistance was also noted with meropenem whereby previous studies had reported a 100% sensitivity (33). However, there was less resistance to gentamycin and co-amoxiclav compared to a previous study done at Bugando in Western Tanzania (34). The same resistance pattern has also been reported among neonates (31, 35). The differences in these outcomes reported in various studies may be attributed to the smaller sample size used in this study compared to present study.

In present study the positive mecA , ermA, ermB, and ermC genes were showed in percentage of 100%, 35%, 20% and 15% respectively.

More and more attention has been devoted to the rapid detection platform for antimicrobial susceptibility testing, which is well established to have an impact on the clinical outcome of severe infections. For instance, rapid and sensitive detection of MRSA strains

using PCR for the mecA gene coding for methicillin resistance has long been known and confirmed (36). In addition, detection of genes conferring clinically relevant resistance to the antimicrobial agents like aminoglycoside, tetracyclines, macrolides, and lincosamides is extremely useful to succeed in antimicrobial therapy and infection control policy. Several authors have reported the use of multiplex PCR for the detection of S. aureus isolates, in which these techniques are designed to detect virulence and resistance genes of S. aureus simultaneously, in just a few reactions.(37).

Our data regarding mecA , ermA, ermB, and ermC genes were in accordance with Kotey et al., (2022) recorded infants are at risk of *Staphylococcus aureus* (*S. aureus*) colonization and infection. MRSA was confirmed by polymerase chain reaction (PCR) of the *mecA* gene. Colonization with coagulase-negative *Staphylococci* (CoNS) was protective of both *S. aureus* (OR = 0.008; $p < 0.001$) and MRSA (OR = 0.052; $p = 0.005$) carriage. Maintenance of good hand hygiene prevented *S. aureus* carriage (OR = 0.16; $p < 0.001$). *S. aureus* resistance to antibiotics decreased across penicillin (96%), trimethoprim-sulfamethoxazole (61%), tetracycline (61%), erythromycin (39%), gentamicin (39%), fusidic acid (26%), rifampicin (17%), clindamycin (7%), and linezolid (0%); 68.8% *S. aureus* were multidrug resistant. Concluded that *S. aureus* and MRSA prevalence were high among the infants due to resistance gene especially mecA gene (38).

Also Lim et al., (2012) reported no significant correlation between methicillin resistance and the presence of erm genes in this study. However, these findings were in contrast to previous reports which indicated that erm genes are mostly spread in MRSA strains (39).

In the same way Williams et al., (2020) showed that *mecA* gene is commonly used to identify resistance in *Staphylococcus aureus*, but historically is not used for coagulase-negative staphylococci (CoNS). Analysis of 412 staphylococcal blood cultures revealed that the absence of *mecA* had high concordance (100%) with oxacillin susceptibility for *S. aureus* and CoNS alike (40).

Generally, the results of the cefoxitin disk diffusion assay correlated highly with *mecA* gene PCR; it was found to be 100% specific and demonstrated higher sensitivity (98.4%) in detecting *mecA* positive isolates.(41).

High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2a. Multiresistant MRSA strains are found in

hospitals worldwide, while unrelated and more susceptible strains represent less than 1% of the MRSA population. This supports the hypothesis that horizontal transfer plays an important role in the dissemination of the *mecA* gene in the *S. aureus* population (42).

Whilethese data regarding mecA , ermA, ermB, and ermC genes were not in consistence with Iranian study conducted by Houri et al., (2020) revealed that in eighty clinical isolates of S. aureus were obtained from patients referred to three hospitals in Tehran. A multiplex PCR assay was used for detection of enterotoxins A to D (sea, seb, sec, and sed,), toxic shock syndrome toxin 1 (tsst), and exfoliative toxins A and B (eta and etb), as well as antibiotic resistance genes, including mecA, aacA-aphD, erm(A), erm(C), tetK, tetM, vat(A), and vat(C). The frequency of MRSA strains was 63.7% (n = 51). The prevalence of sea, sec, seb, sed, tsst, and eta among S. aureus isolates was 51.2%, 23.7%, 15%, 3.7%, 33.7%, and 3.7%, respectively. None of the isolates possessed etb. The frequency of the resistance genes, was determined as follows: mecA (63.7%), erm(A) (33.7%), erm(C) (45.6%), tetK (33.7%), tetM (23.7%), aacA-aphD (21.2%), vat(A) (0%), and vat(C) (0%). Findings of Houri et al., (2020) indicated a high incidence of sea- and tsstpositive and MRSA strains with higher rates of antibiotic resistance (43).

Additionally Liang et al., (2018), Chen and Huang, (2014) and Champion et al., (2014) has been indicated that for the detection of antibiotic resistance genes in S. aureus isolates. In our study, more than 63% of the S. aureus isolates carried mecA gene, and thus considered as MRSA strains. MRSA strains have disseminated worldwide and remain a global public health problem. The prevalence and characterization of clinical isolates of MRSA in community and healthcare settings have been reported from different parts of the world (44-46).

Moreover, the growing increase in multidrug resistance among MRSA strains aggravates this problem and represents a great challenge for the management of severe MRSA infections. In the current study, the prevalence of S. aureus isolates carried erm (A), erm(C), and a combination of erm(A) plus erm(C) were 26.5%, 45.6%, and 13.2%, respectively, and similar rates of the erm genes have been reported in other Iranian study (47).

In general, various surveys conducted in different parts of the world have indicated that ermA and ermC were responsible for the majority of resistance to erythromycin and clindamycin among S. aureus isolates (48).

The differences in virulence gene percentage may belongs to the site of infection in which sample was taken (49, 50). Or the differences in data recorded in each study may belongs to the research package (questions, methodology, analytical procedures) may be the same but factors such as the time of the research, the depth of the research probing, and the level of dedication to unearth the truth regarding the research questions may vary the findings in most cases.

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