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Determining the IL-6 and TNF-α Levels in Staphylococcal infection

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A B S T R A C T

Staphylococci is gram-positive, spherical cells, they are commonly distributed in the environment often part of normal human flora. *Staph. aureus, Staph. saprophyticus,* and *Staph. epidermidis* are the three most significant clinically important pathogens. Cytokines are small secreted proteins, which are produced by nearly every cell to regulate and affect immune response. Proinflammatory cytokines are proteins that have been associated with cell proliferation, differentiation, and synthesis of matrix proteins important to cell growth and tissue repair. These proteins are often released in a cascade, where an initial release of TNF is followed first by IL-1 and then by IL-6. 110 clinical sample including 110 throat swab and 60 saliva samples were collected from tonsillitis patients in Kirkuk of both genders and different age groups. Sterile cotton swab were used for throat swab and sterile container for saliva collection. The results showed that *Staphylococcus aureus* scored the highest percentage, followed by *Staph. hemolyticus* compared to *Staph. epidermidis* that scored the lowest percentage. A sensitivity test was carried out for all bacterial isolates, *Staphylococcus* spp., which showed high resistance to Cefotoxin, Benzyle-pencillin, Ampicillin-Sulbactam, Oxacillin, Ceftriaxone, and Erythromycin, while *Staphylococcus* spp. showed high sensitivity to Linezolid, Teicoplanin, Vancomycin, and Rifampicin. *Staph. aureus* scored high mean levels at IL-6 compared with other *Staphylococcus* spp., while *Staph. aureus* scored low mean levels in TNF-α compared with other *Staphylococcus* spp. All *Staph. aureus* were coagulase positive, the result of coagulase was compatible to VITEK2 COMPACT result, so it can be considered a golden test for *Staph. aureus* identification.

Introduction

Staphylococci are gram-positive, spherical cells, generally arranged in small clusters of grapes, although microorganisms may be single cells, pairs, or short chains in clinical specimens. They are commonly distributed in the environment often part of normal human flora. The Staphylococcus genus contains roughly 21 subspecies and 39 species. Staph. aureus, Staph. saprophyticus, and Staph. epidermidis are the three most significant clinically important pathogens [1]. Staph. aureus is also an important pathogen in humans, it is a common cause of skin infections and foodborne disease (FBD) in people, as well as sepsis in hospitalized patients [2,3]. They were non-motile, capsulated, oxidase negative, catalase positive, and non-spore former. Staphylococcus spp. can grow on several types of media, fermenting carbohydrates and producing various pigments, may be white, yellow or deep yellow. Optimal temperature is between 30–37 ºC. The growing colonies on solid media are round, smooth and raised [4]. The ability to produce coagulase sets these bacteria apart from those belonging to other bacterial genera and species. Staphylococci are mostly divided into two groups: Coagulation-positive and Coagulation-negative staphylococci (CoPS and CoNS). According to coagulase production, coagulase is an enzyme-like substance that causes fibrin to clot and coagulate, a characteristic that is frequently linked to pathogenicity [5]. Approximately 30-50% of the human population carries Staph. aureus, and its main habitat is the nasopharynx, a location where strains can survive as temporary or long-term contributors to the healthy microbiota without generating any symptoms [6]. It contain many important virulence factors, including cell wall-associated proteins, biofilm, polymers, extracellular enzymes, and toxins [7].

Biofilms are accumulations of cells that adhere to surfaces and are surrounded by a matrix of polysaccharides, proteins, and nucleic acids. Bacteria within biofilm exhibit high levels of resistance to biocides and antibacterial agents. These agents are needed at high levels, which can be 1000-fold greater than that required in case of planktonic cells to produce antibacterial effects [8,9].

Cytokines are small secreted proteins (<40 kDa), which are produced by nearly every cell to regulate and affect immune response [10]. Proinflammatory are proteins that have been associated with cell proliferation, differentiation, and changes in gene expression, and synthesis of matrix proteins important to cell growth and tissue repair. They are also crucial in preventing infections and responding to injuries. These cytokines perform the function that led to their designation as "pro-inflammatory" because they coordinate the first immune response to infection or damage by recruiting immune cells to the location and activating them. These proteins are often released in a cascade, where an initial release of TNF is followed first by IL-1 and then by IL-6 [11]. TNF- α a cytokine with pleiotropic effects on many cell types, has been discovered as a significant regulator of inflammatory reactions and is known to play a role in the pathogenesis of some inflammatory and autoimmune illnesses. Structurally, TNF-α is a homotrimer protein consisting of 157 amino acids, mainly generated by activated macrophages, T-lymphocytes, and natural killer cells [12]. It is functionally known to trigger a series of various inflammatory molecules, including other cytokines and chemokines [13]. TNF-α is a powerful pro-inflammatory agent that regulates many facets of macrophage function. It is rapidly released after infection, trauma or exposure to bacterial-derived lipopolysaccharide (LPS) and has been shown to be one of the most abundant early mediators in inflamed tissue [14]. Interleukin-6 (IL-6) is a pro-inflammatory cytokine that plays a significant role in the proliferation and differentiation of human cells. It promotes the production of numerous proteins involved in acute inflammation [15]. It has been identified as a 26-kD secreted protein that stimulates B cells to produce antibodies [16]. IL-6 is secreted by a wide range of cell types under the conditions of infection, bacterial infections, inflammation, or neoplastic disease. IL-6 is essential for innate and adaptive immunity, is required for efficient pathogen clearance [17]. In infection caused by Staph. aureus, generally, immune cells are stimulated to promote pro-inflammatory cytokines (such as: IL-6), IL-6 production immediately increases in the acute inflammatory condition. These cytokines retain intracellular and extracellular growth from Staph. aureus but excessive production can lead to systemic inflammation with damaging effects rather than protection of the host [18].

Aim of study

1-Isolation of Staphylococcus spp. from clinical samples and determining their antibiotic resistant
2-Studying the immunological parameters of patients with Staphylococcal infections.

Materials and Methods

Study design A 110 clinical samples include 110 throat swab and 60 saliva samples were collected from tonsillitis patients in Kirkuk General Hospital and Al Children hospital in Kirkuk city of both gender and different age groups (4-55 years) at period from (27/11/2022) to (30/2/2023).

Sample collection Sterile cotton swabs were used for throat swabs and sterile container for saliva collection, then the swabs were cultured on blood and Mannitol agar by striking method and incubated at 37°C for 24-48 hours. While the saliva was centrifuged for 10 minutes and the supernatant was separated and kept in Eppendorf tube in freezer until it diagnosed by Elisa (SUNLONG kit) for detecting of IL-6 and TNF-α levels. The organisms were identified by direct Gram staining, culture methods on Blood Agar, mannitol salt agar at 37 for 24-48 hours. Different biochemical tests like catalase test and coagulase were performed for the identification of the various Staphylococcal pathogens after their isolation, and the conformation and antibiotic sensitivity test is done by the VITEK 2 COMPACT system.

Statistical analysis

The normality of variables was first determined (Kolmogorov-Smirnov and Shapiro-Wilk test). The variables that pass the normality of tests (no significant different) which were presented as Mean± St. deviation, with student t test to detect the difference significance (comparison two groups) and ANOVA tests used to comparison more two groups. Other variables were showed as percentages numbers, and Pearson-Chi-square test was used to reveal significant differences in frequency.

Results

Morphological identification of bacteria

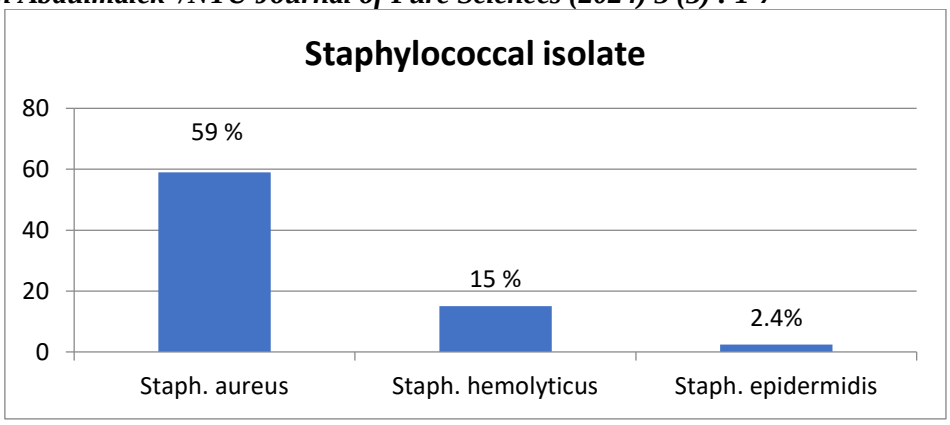
Beta hemolysis (due to complete hemolysis of RBCs in the medium) surrounded the colonies were observed after 24 hours of incubation. On mannitol salt agar, golden yellow or white colonies are seen after 24 hours of incubation, figure (1).

- A - **-B-**

Figure (1) A-Beta hemolysis of Staphylococcus on blood agar
B-*Staphylococcus aureus* and Coagulase negative Staphylococci on mannitol agar

The catalase test was preformed to differentiate *Staphylococcus* spp. which were catalase positive, about 33 isolate were identified as suspected *Staphylococcus* spp. depending on coagulase slide method test in which they grouped into coagulase negative (9 isolates) and coagulase positive *Staph. aureus* (24 isolates). All isolates were subjected to the VITEK-2 system in AL-BALSAM hospital for confirmation of the identification of the bacterial isolates. The result of coagulase test for coagulase positive *Staph. aureus* was compatible to the VITEK 2 COMPACT results.

The results showed there is significant differences (P<0.001) among percentages of bacterial isolates in patients with tonsillitis, *Staphylococcus aureus* scored highest percentage (59%),followed by *Staph. hemolyticus* (15%) compared to *Staphylococcus epidermidis* that scored lowest percentage (2.4%), figure (2).



Figure(2): Percentage of *Staphylococcal* spp. isolated from patients with tonsillitis

A sensitivity test was carried out for all bacterial isolates, *Staphylococcus* spp. showed high resistance to Cefotoxin (≥ 85%), Benzyle-pencillin (100%), Ampicillin-Sulbactam(≥ 85%), Oxacillin (≥ 85%), Ceftriaxone (≥ 85%), and Erythromycin(≥ 62%), while *Staphylococcus* spp. showed high sensitive to Linezolid (100%), Teicoplanin (100%), Vancomycin (100%), and Rifampicin (100%), table (1).

Table (1) Antibiotic resistance of *Staphylococcus* spp. that isolated from patients with tonsillitis.

Staphylococcus spp.				
Antibiotics	*Staph. aureus*	*Staph. epidermidis*	*Staph. hemolyticus*	*Staph. hominis*
Penicillin				
Benzyl-pencillin	100%	100%	100%	100%
Oxacillin	84%	100%	100%	100%
Ampicillin- Sulbactam	84%	100%	100%	100%
Cephalosporin				
Cefoxitin screen	84.6%	100%	100%	100%
Ceftriaxone	84%	100%	100%	100%
Aminoglycosides				
Gentamycin	7.7%	0%	40%	0%
Lincosamide				
Ciprofloxacin	15.4%	0%	40%	0%
Rifampicin	0%	0%	0%	0%
Trimethoprim	19.2%	0%	20%	0%
Vancomycin	0%	0%	0%	0%
Clindamycin	26.9%	100%	0%	100%
Macrolides				
Erythromycin	61.5%	100%	100%	100%
Tetracyclines				
Tetracycline	26.9%	100%	100%	100%
Fluoroquinolones				
Levofloxacin	15.3%	0%	15.4%	0%
Moxifloxacin	15.4%	0%	0%	0%
Oxazolidinones				
Linezolid	0%	0%	0%	0%
Glycopeptide				
Teicoplanin	0%	0%	0%	0%
Glycylcyclines				
Tigecycline	0%	0%	100%	0%
Fusidane				
Fusidic acid	3.8%	100%	60%	100%

IL-6 and TNF-α results

Figure (3) shows the result of IL-6, the result revealed that *Staph. aureus* scored high mean levels at IL-6 (14.51±13.1 ng/L) compared with other *Staphylococcus* spp. (0.52±0.1 ng/L).

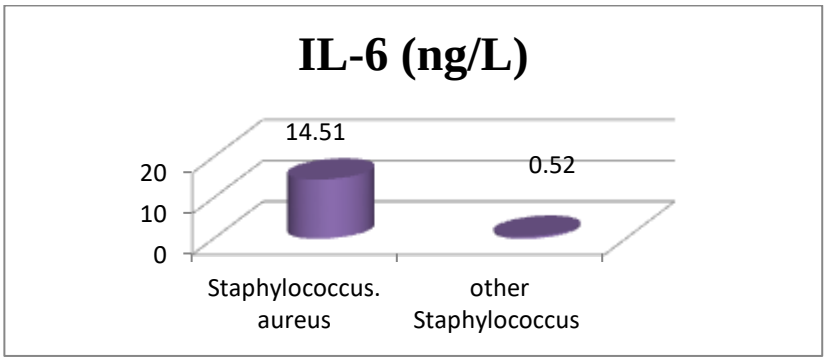


Figure (3): Saliva levels of IL-6 in *Staphylococcus aureus* infection compared with other *Staphylococcus* spp.

Figure (4) shows the result of TNF-α, the result revealed that *Staph. aureus* scored (90.47±77.79 pg/ml) compared with other *Staphylococcus* spp. (113.0±79.9 pg/ml).

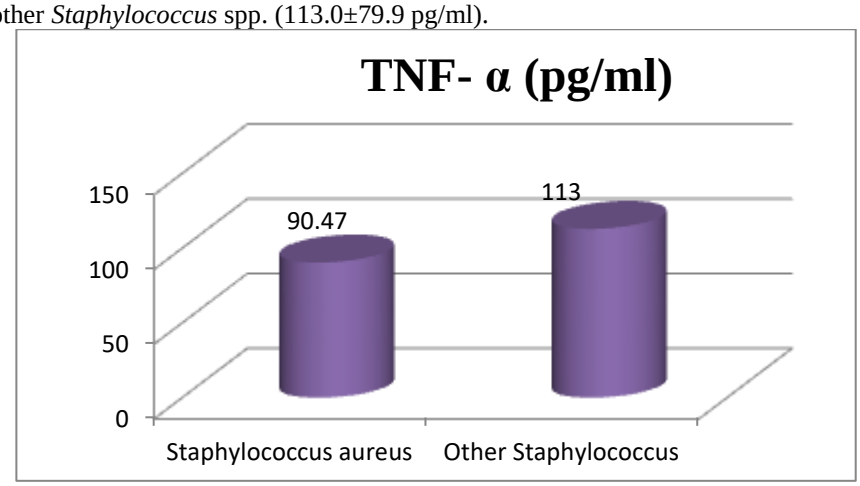


Figure (4): Saliva levels of TNF- α in *Staphylococcus aureus infection compared with other Staphylococcus spp.*

Discussions

According to the results obtained in this study, *Staph. aureus* was the most prevalent microorganism and its colonization was mainly in the oropharynx, the biofilm presence in cases of *Staph. aureus* infection is described as a factor that makes the treatment difficult [19].The most commonly isolated bacteria from tonsillitis patients were *Staph. aureus* in the Katkowska, *et al.* study [20]. Also *Staph. aureus* had the highest frequency with 50% in the tonsillitis patient [21].

CoNS possess numerous diverse strategies to both cause infection and survive in the host. In comparison to *Staph. aureus*, this group of bacteria exhibits lower pathogenic potential, but less is known about CoNS virulence mechanisms. Recent studies have focused on the ability of CoNS to produce a variety of extracellular enzymes, toxins, the enzymes and toxins discussed play significant roles in several of the impacts staphylococci have on their hosts, including virulence factors that cause tissue death and spreading factors that make it easier for the bacteria to invade neighboring tissues [22].

Cavalcanti *et al*., [23] reported that 83.6% of *Staph. aureus* isolates from tonsil tissue was resistant to penicillin and 13.6% to amoxicillin . Additionally, the present study demonstrated that *Staph. aureus* isolates were resistant to penicillin (100%) and Ampicillin (84%). The ineffectiveness of penicillin has led to increased use of other antibiotics such as β-lactam inhibitors and cephalosporins. Antibiotic resistance can be explained by the impossibility of antibiotic penetration and action in the tonsil core, (especially if the bacterial cells are covered with an biofilm extracellular matrix), the resistance of strains to typical antibiotic treatments due to the constant use of antibiotics due to recurrent infections and the prevalence of biofilm formation (21). Additionally,

one of key factors to antibiotic resistance against penicillin group of antibiotics is beta-lactamase production [24].

In study carried out in Kirkuk city by (Mohammed SA) that showed *Staphylococci* isolates was Methicillin-resistant *Staphylococcus aureus*(MRSA) which was resistance to all other resident agents, including ampicillin, azithromycin, erythromycin, gentamicin, and ciprofloxacin. Vancomycin resistance was not found. This could be a result of both the extensive use of antibiotics and the general lack of health knowledge among the population [25].

In another study in Kirkuk city oxacillin resistance was found in (27.3%) of the isolates, followed by penicillin G resistance in (24.3%), amoxicillin resistance in (15.2%), erythromycin resistance in (12.1%), and tetracycline resistance in (6.1%), and there was a (9.1%) resistant rate to each of clindamycin [26].

There are many mechanisms by which bacteria confer resistance to the antibiotic agents including intrinsic impermeability, as well as acquired resistance as mutations, plasmids, and transposons. Furthermore, the tonsils with chronic inflammation, and adenoid contain more scar tissues following each infection. In turn causing an impairment of antibiotic penetrating into the core and become more resistant to antibiotic therapies [27].

Mutations in genes coding for penicillin-binding protein (PBP) can generate structural modifications, which alter the binding of these proteins to β-lactams antibiotics by decreasing their affinity and determining antimicrobial resistance. Mutations can also lead to overexpression of PBPs and produce a small but significant increase in resistance to β-lactam antibiotics. These mutations can be generated by the selective pressure exerted by excessive use of β-lactams [28].

In addition to the changes in PBPs, *Staph. aureus* may develop resistance to β-lactams due to hyperproduction of β-lactamases. Authors shows that *Staph. aureus* producing large amounts of β-lactamases can also inactivate more slowly penicillin resistant to penicillinases. In this study, the production of large amounts of β-lactamases may have influenced the cefoxitin disk test and determined phenotypic resistance (29).

Study results showed the all *Staphylococcus* species are sensitive Trimethoprim, and these results compatible to results Cavalcanti *et al*., [23],

Katkowska *et al*., [20] showed the Gentamycin is best antibiotic towards *Staphylococcus* spp. , where this antibiotic scored sensitivity 77% against this bacterial species. Above results matched with our results.

IL-6 was the most prominent and significantly upregulated cytokine detected by ELISA in the 24-hour supernatant following stimulation with viable *Staph. aureus* [30]. AL-Dulaimi *et al*., In his study agreed with our study in which patients with tonsillitis showed increased concentration of IL-6 for all patients infected with *staph. aureus* compared to control groups [31].

The result of current study was agreed with Chen *et al.* [32], which showed in their study that saliva level of TNF- α increase in different bacterial infections such as *Staph. aureus* and *Strep. pyogenes* infections.

Conclusions

All *Staphylococcus* spp. isolates showed high resistant to benzyl-penicillin, ampicillin-sulbactam, oxacillin, ceftriaxone, and erythromycin. All *Staphylococcus* bacterial isolates except *Staph. aureus* were resistant to tetracycline and fusidic acid, and *Staph. aureus* was the most prevalent bacteria among tonsillitis patients. All Staph. aureus were coagulase positive, the result of coagulase was compatible to VITEK2 COMPACT result, so it can be considered a golden test for Staph. aureus identification. In Staphylococcal infection the proinflammatory cytokines levels increased during the acute infection.

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