Original Article

Isolation and Identification of Bacterial Pathogens from Clinical Samples of Patients with Tonsillitis in the Holy City of Karbala

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Abstract

Background: The drug resistance characteristic of bacteria is one of the most important health problems in world countries. The indiscriminate use of antibiotics without consulting a doctor and for an inappropriate period can lead to high antibiotic resistance of bacteria. In addition, one of the ways to resist bacteria is their ability to produce biofilms. **Objectives:** The study aimed to isolate and identify pathogenic bacteria from clinical samples of patients with tonsillitis in the holy city of Karbala and investigate their resistance to common antibiotics and the relationship of this trait with their ability to build biofilm. **Materials and Methods:** Samples were taken from patients with tonsillitis in the holy city of Karbala, and the obtained isolates were diagnosed by biochemical tests and subjected to antibiotic sensitivity tests by the disc-diffusion method. The VITEK-2 compact system confirmed identification and antibiotic sensitivity. Biofilm building of some selected isolates was tested using microplate assay. **Results:** From 128 swabs, 120 bacterial isolates were obtained, including *Staphylococcus* spp with 19 isolates and *Klebsiella pneumoniae* with 18 isolates. The rest of the numbers included genera *Streptococcus* spp, *Escherichia coli*, *Enterobacter* spp, and *Pseudomonas* spp. All isolates of *Staphylococcus aureus* and *K. pneumoniae* were multidrug resistant. And that many of them had a strong ability to build a biofilm. **Conclusion:** *Staphylococcus aureus* and *K. pneumonia* are among the main causes of tonsillitis for the patients under study. These pathogens are multi-drug resistant, and there is a highly significant relationship between that resistance and the ability of these pathogens to produce a biofilm.

Keywords: Antibiotic susceptibility, bacterial pathogens, biofilm, tonsillitis

Introduction

Tonsillitis is inflammation of the tonsils, which is a common disease of the upper respiratory tract that affects all age groups, particularly, children and adults. It is considered a common clinical condition caused by bacterial or viral infection.[1] The tonsils play an important role in the immune system as a first line of defense against microbes that enter through the nose and mouth. So, the most common symptoms of tonsillitis are sore throat, red, swollen tonsils, pain when swallowing, fever, cough, headache, fatigue, chills, swollen lymph nodes in the neck, and pain in the ears or neck,[2] whereas, the less common symptoms include nausea, stomach pain, vomiting, change in voice, and bad breath.[3] Some of the most causative pathogenic organisms that infect the upper respiratory tract are *Streptococcus pneumonia*, *Hemophilus influenza*, *Chlamydia, Klebsiella pneumonia*, and *Staphylococcus aureus.*[4] *Klebsiella pneumonia* and *S. aureus* are considered the most dangerous and opportunistic pathogens because they cause acquired pneumonia, especially in patients who are hospitalized, chronically ill, or immunocompromised.[5]

These bacteria may invade the skin tissues of the patient causing bacteremia, and severe pulmonary infections by affecting the respiratory system, endocarditis, arthritis, urinary tract, digestive system, or meningitis, and what makes it more dangerous is that it increases the risk of

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death.[6,7] According to data issued by the European Center for Disease Prevention and Control (ECDC), it was found that about 33,000 people in the European Union die annually from infection due to multidrug-resistant bacteria. Many factors contribute to the spread of infection related to health care, such as the contamination of devices and equipment, surgical operations, and indiscriminate use of antibiotics.[8]

Antimicrobial resistance is recognized as an urgent public health problem worldwide. Overprescribing antibiotics without consulting a doctor and for a long time is associated with increased antibiotic resistance among the bacterial population.[9,10] The antibiotic resistance mechanisms are varied. These mechanisms include the production of antibiotic inhibition enzymes, change in antibiotic target, change in the membrane permeability for antibiotics, as well as the ability of bacteria for biofilm formation.[11] In biofilm formation, the bacterial cells aggregate together to form a film layer attached to solid surfaces, via the production of an extracellular matrix. This matrix provides a layer that prevents the antibiotic and immune defense system from reaching the bacterial cells. The chemical composition of the extracellular matrix was polysaccharides, proteins, and DNA.[12]

This study aimed to isolate and identify pathogenic bacteria from clinical samples of patients with tonsillitis in the holy city of Karbala, investigate their resistance to common antibiotics, and study the association of their ability to form a biofilm with their resistance to those antibiotics.

Materials and Methods

Sample collection and diagnosis

A total of 128 clinical samples were isolated from patients with tonsillitis in the holy city of Karbala. In addition, 20 samples were obtained from healthy people (as a control group). All samples were collected by using a sterile cotton swab, after obtaining the patient's consent, knowing the health condition, and taking the information according to a form prepared for this purpose.

The tonsillar swabs were transferred to the laboratory and cultivated on blood agar base, MacConkey agar and Mannitol salt agar for diagnostic purposes. The agar plates were incubated overnight aerobically at 37°C.[13] A microscopical examination with staining was performed to determine Grams Staining reaction, shape, and cell arrangement. ADditionally, biochemical tests) catalase test, oxidase test, coagulase test, indole production, methyl red, Voges-Proskauer, and Bacitracin susceptibility tests were performed according to Tille and van Wincoop.[14]

Antibiotic sensitivity test

The susceptibility of isolated bacteria was determined by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute, CLSI, 2022. In detail, *S. aureus* isolates were activated using Mannitol salt agar and *K. pneumonia* isolates were activated on MacConkey agar and incubated at 37°C for 24 h. Bacterial colonies from each isolate were transferred into a suspension of sterile normal saline and adjusted to 0.5 McFarland turbidity standards. Aseptically, 100 µL of bacterial suspension was spread on the surface of Mueller-Hinton agar plates followed by the distribution of antibiotic disks that were specific for each isolate using sterile forceps (5 disks per plate). The plates were incubated invertedly at 37°C for 24 h. The inhibition zone was determined according to the standards outlined by the CLSI. antibiotics used include Amikacin (30 mg), Azithromycin (15 mg), Ciprofloxacin (5 mg), Gentamicin (10 mg), Doxycycline (30 mg), Rifampicin (30 mg), Penicillin (10 mg), Trimethoprim sulphamethoxazole (25 mg), Tetracycline (30 mg), Levofloxacin (5 mg), Cefotaxime (30 mg), Imipenem (10 mg), Ceftriaxone (30 mg), and Cefoxitin (30 mg).

Investigation of a biofilm formation

Screening the ability of bacteria to form a biofilm, the micro-titter-plate assay was performed according to the method described by Nicolau Korres *et al.*[15] The test was visually scored as (strong, moderate, or none).

Quantification of biofilm formation was read using the spectrophotometer of ELISA reader at optical density 630 nm wavelength. The data were calculated according to the following equation:

Mean (OD) control-Mean (OD) test=(OD) bioflim.

VITEK-2 system

The VITEK-2 system was used to confirm the biochemical and antibiotic sensitivity test, which was performed according to the method described by Gherardi *et al.*[16] and De Cueto *et al.*[17]

Ethical approval

The study was conducted by the ethical principles that have their origin in the Declaration of Helsinki. The study protocol, the subject information, and consent forms were reviewed and approved by a local ethics committee according to the document number 4087(including the number and the date in November 29, 2021) to get this approval.

Results

During a period from December, 2021 to February, 2022, smears were collected from patients with tonsillitis in Karbala holy city. One hundred and twenty-eight clinical samples were collected from 80 females and 48 males and for different age groups as shown in Figure 1. A total

of 120 bacterial isolates were collected from tonsillitis patients' swabs, 70 isolates from females, and 50 isolates from men. The percentage of infection in female patients is higher than in male patients, that is, 58.33% and 41.66%, respectively. The results are also analyzed according to patient age into five groups. Figure 1 shows that, 38% in the age group 5–15, 27% in the age group 16–25, 21% in the age group 26–35, and 14% in the age group 36–45.

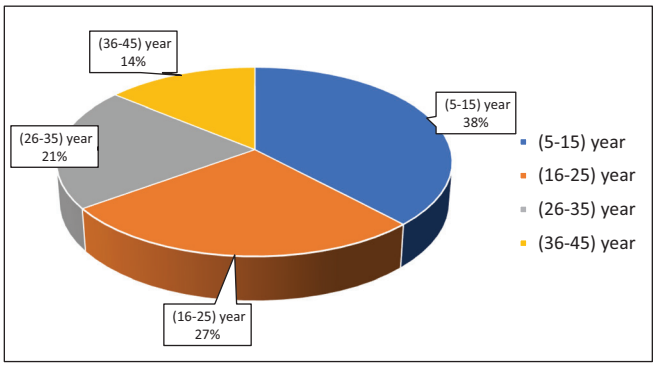

Figure 1: Age groups distribution of the 120 bacterial isolates from a tonsillitis patient

Table 1: The percentage of bacterial isolates

Type of bacteria	Insulation ratio	Number of isolates
Streptococcus spp	24.16%	29
Staph. aureus	29.16% 15.83%	19
Staph. spp	13.33%	16
K. pneumoniae	15%	18
E. coli	12.50%	15
Enterobacter spp	10.83%	13
Pseudomonas spp	8.33%	10
Total	100%	120

Morphological and biochemical results exhibited that a total of 35 isolates belonged to *Staphylococcus* spp. (from these isolates, 19 isolates were identified as *S. aureus*), 29 isolates were *Streptococcus* spp, 18 isolates of *K. pneumonia*, 13 isolates of *Enterobacter* spp, and 10 isolates of *Pseudomonas* spp. The *S. aureus* and *K. pneumonia* are more predominant than the other isolates, of tonsillitis. Table 1 summarizes the percentage of each bacteria species.

The sensitivity of all *S. aureus* isolates against (10) antibiotics were tested according to the Kirby-Bauer method, and the sensitivity was determined based on measuring the diameter of the inhibition zone of the antibiotic [Figure 2]. The results showed that all isolates of *S. aureus* obtained in this study was multidrug-resistant (MDR) as its resistance to Penicillin was 100%. It was 100% resistant to Tetracycline, Rimpicin, Doxycycline, and Cefoxitin, whereas it was 50% resistant to Trimethoprim-sulfamethoxazole (SXT), and its resistance was 83.33% to Ciprofloxacin. The isolates of *S. aureus* showed a susceptibility to Azithromycin rate of 83.33%, *S. aureus* was able to resist Tetracycline by 100%, isolates resistant to Ciprofloxacin are about 83.33%, *S. aureus* bacteria were 100% sensitive to levofloxacin and Gentamicin.

As for *K. pneumoniae*, a sensitivity test was conducted for the 18 bacterial isolates against 12 types of antibiotics, as shown in Table 2. The results showed, as in Figure 3, that most of the bacterial isolates are widely resistant to multiple antibiotics (XRD) with a resistance rate of 100% towards the antibiotics: Cefoxitin, Penicillin, Cefotaxime, Gentamicin, Tetracycline, Ciprofloxacin, and Ceftriaxone, and it showed a resistance of 57.14% toward the antibiotic Amikacin, Doxycycline, Azithromycin, and Levofloxacin, whereas its resistance rate was 71.42% against Imipenem.

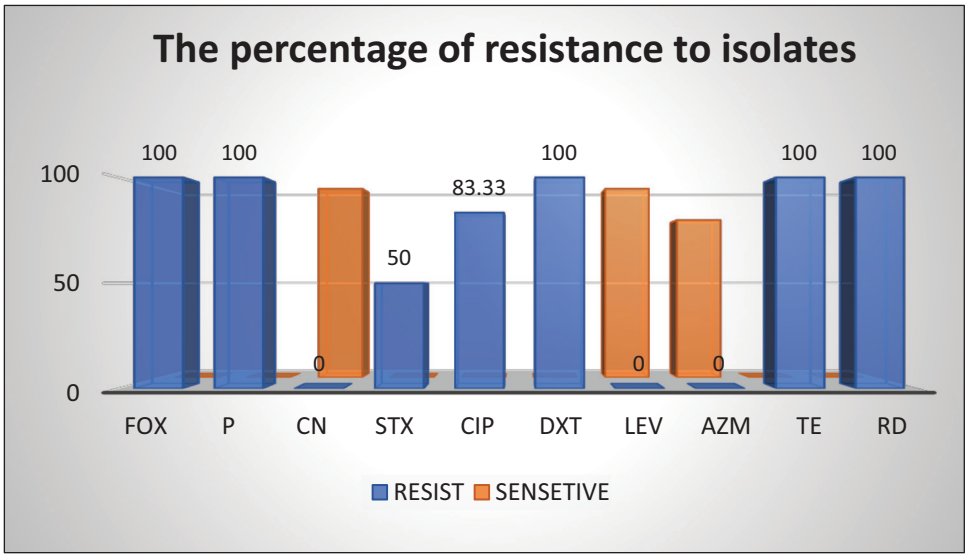

Figure 2: Percentages of resistance and sensitivity of *S. aureus* isolates to antibiotics

Table 2: Pattern of *S. aureus* and *K. pneumoniae* biofilm formation by using the micro-titer plate method

***S. aureus* biofilm formation**			
The total number of isolates	**Biofilm strength**	**Number of isolates**	**Percentage**
19	Strong	6	31.57%
	Moderate	8	42.12%
	Weak	5	26.31%
***K. pneumoniae* biofilm formation**			
The total number of isolates	**Biofilm strength**	**Number of isolates**	**Percentage**
18	Strong	7	38.88%
	Middle	7	38.88%
	Weak	4	22.22%

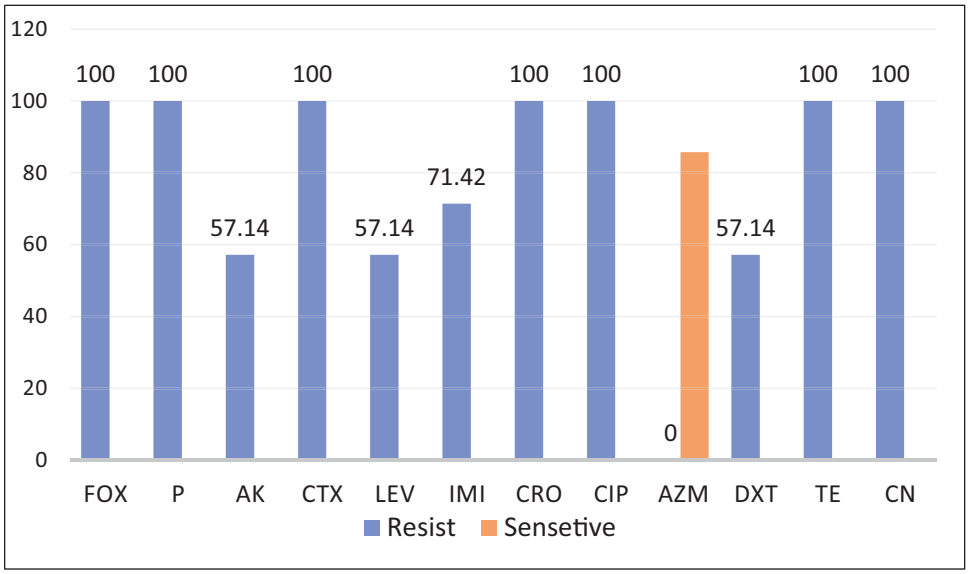

Figure 3: Resistance ratios of *Klebsiella pneumoniae* isolates for antibiotics

The results of biofilm formation showed that for *S. aureus* isolates, as shown in Table 2, where five isolates, with a rate of 26.31%, had a weak production of biofilm, and eight isolates with a rate of 42.12%, had an average productivity of a moderate biofilm, and six isolates with a rate of 31.57%, strong adherent biofilm production, whereas for the *K. pneumoniae* bacteria, the results were four isolates of 22.22% with weakly adherent biofilm and seven isolates of 38.88% of moderately adherent biofilm and seven isolates of 38.88% produced strong adherent biofilm formation.

The VITEK-2 compact system was used to confirm the final diagnosis of *S. aureus* and *K. pneumonia*, as shown in Table 3 which proved their high ability to build a biofilm to obtain a more accurate diagnosis of the selected bacterial isolates and to identify their sensitivity to antibiotics.

Table 3: Results of identification of *Staphylococcus aureus* and *Klebsiella pneumonia* bacteria isolates that possesses a strong ability to produce biofilms using VITEK-2 compact system

Klebsiella pneumonia	
Diagnosis probability percentage	**Isolate number**
99%	22, 83, 91, 110, 112
97%	76, 17
Staphylococcus aureus	
Diagnosis probability percentage	**Isolate number**
98%	10, 50, 67, 101
96%	33, 89

Discussion

Considering the antibiotic susceptibility tests, the results showed that all *S. aureus* isolates recorded a complete resistance (100%) against penicillin, which is in agreement with the findings of Gitau *et al.*[18] and Chigbu and Ezeronye.[19] In addition, all *S. aureus* isolates were resistant to tetracycline (100%), which agrees with Kumar and Varela,[20] also these bacteria showed a complete resistance against Rifampicin (100%), which belongs to the group of cephalosporins, and Doxycycline (100%)

agrees with the finding of Agrawal *et al.*[21] as well as Cefoxitin (100%), Whereas resistance to Trimethoprim-sulfamethoxazole was recorded (50%). The mechanism action of Trimethoprim-sulfamethoxazole is inhibiting the synthesis of enzymes that are necessary for bacterial growth and activity requirements, the results agreed with Coombs *et al.*[22] Also, the percentage of resistance to ciprofloxacin was 83.33%, which is close to what was obtained by Reygaert,[23] who found the percentage of resistance was 88%.

Moreover, *S. aureus* isolates showed a sensitivity (83.33%) against Azithromycin, which belongs to the group of macrolides. As mentioned by Kumar and Varela,[20] this antibiotic prevents the growth of bacteria after a short period of exposure by inhibiting the synthesis of protein through binding to the 50S subunit of bacterial ribosomes.

Levofloxacin showed 100% sensitivity, as in agreement with Drago *et al.*,[24] and Gentamicin (100%) belongs to Aminoglycosides as in agreement with Agrawal *et al.*[21]

While for *K. pneumoniae* (18 isolates), it was performed an antibiotic sensitivity test against 12 types of antibiotic disks. The results showed that most of the isolates demonstrated a multi-drug resistance in a wide spectrum with a complete resistance (100%) against Cefoxitin, Penicillin, Cefotaxime, Gentamicin, Tetracycline, Ciprofloxacin, and Ceftriaxone, whereas the resistance percentage of *K. pneumoniae* against Amikacin, Doxycycline, Azithromycin, and Levofloxacin was 57.14% and against Imipenem was 71.42%.

The results of Cefoxitin and Penicillin were in agreement with the finding,[25] where the resistance of *K. pneumoniae* against Cefoxitin and Penicillin was 97% and 100%, respectively, whereas the results of the Tetracycline antibiotic exhibited a complete resistance (100%) that disagreed with the finding of Fluit *et al.*,[26] who recorded a percentage of 81.8% and was in agreement with it. Similarly, Gentamicin showed a complete resistance (100%) that agreed with the finding of Elmer *et al.*,[27] and Ciprofloxacin (100%), which was in agreement with Masadeh *et al.*[28] The reason for the multi-resistant of Ciprofloxacin is the presence of resistance genes that are located on bacterial chromosomes. Cefotaxime antibiotic exhibited complete resistance (100%) and this result is in agreement with Zaidane[29] who recorded a percentage of 95.5%, whereas Levofloxacin showed 57.14% resistance against *K. pneumoniae*, which disagrees with the finding of Mohamed *et al.*,[30] who recorded a percentage of 83%. ADditionally, *K. pneumoniae* showed resistance against Imipenem antibiotic at a percentage of 71.42% which agreed with the finding of Chen *et al.*,[31] who recorded 73.3%.

In contracts, *K. pneumoniae* exhibited a sensitivity toward Azithromycin at a percentage of 85.71%, which agreed with Parnham *et al.*[32] who recorded a sensitivity of 75%.

This study also revealed the resistance of *Klebsiella pneumoniae* against Amikacin at a percentage of 57.14%, which agreed with the finding of Shakil *et al.*,[33] where the resistance was 60.18%.

Staphylococcus aureus isolates showed three patterns of the adherent. In which five isolates showed a poor biofilm formation (weak adherent) at a rate of 26.31%, eight isolates showed a moderate production of biofilm (moderate adherent) at a rate of 42.12%, and six isolates exhibit a heavy biofilm formation (strong adherent) at a rate of 31.57%. All these results agree with the findings of Mohammed *et al.*,[34] who demonstrated the rate of isolates with weak productivity of biofilm formation was 27%, whereas the rate of moderate was 42% and the rate of strong production was 31%.

Klebsiella pneumoniae isolates showed four isolates with a poor biofilm formation (weak adherent) at a rate of 22.22%, seven isolates showed a moderate production for biofilm (moderate adherent) at a rate of 38.88% and seven isolates exhibit a heavy biofilm formation (strong adherent) at a rate of 38.88%. These results were close agreement with the findings of Abdul-Razzaq *et al.*,[35] who demonstrated the rate of isolates with weak productivity of biofilm formation was 23%, whereas the rate of moderate was 38% and the rate of strong production was 39%.

In order to confirm the diagnosis of isolates of *S. aureus* and *K. pneumoniae* which revealed a strong biofilm formation, a VITEK test was performed,[36] where the level of confidence was 96%–99%. The result of the diagnosis using the VITEK-2 device is that all the isolates have belonged; hence the bacterium under study is one of the causative agents of tonsillitis.[37] The increased percentage of tonsillitis with these bacteria is due to several reasons including the human being considered a major source of pulmonary infection,[38] that is originally located in the pharynx, the time of the collected isolates from patients was in the winter season, as well as the epidemic spread of the COVID-19, and the deterioration of the health situation and its repercussions and pressure on the health sector as a result of the increasing number of visitors to hospitals and health centers. All these factors contribute to an increase in the rate of infection with the bacteria under study.[39,40]

Conclusions

The main causative pathogenic bacteria that cause Tonsillitis in patients under study were *S. aureus* and *K. pneumoniae.* The age group 5–15 years is more susceptible to tonsillitis compared to other age groups. The isolates of *K. pneumoniae* exhibited a higher MDR compared with *S. aureus* which makes it difficult to treat the patients under study. It appeared that the bacterial isolates that were resistant to antibiotics were characterized by their strong

production of biofilm, which indicates its main role as a virulence factor against antibiotics.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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