In vitro Conjugation between Bacteria Isolated from Acute Pharyngitis and Others from Buccal Cavity

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ABSTRACT

Background: Bacteriological diagnosis of pharyngitis is complicated by the fact that the mouth and pharynx contains a heavy, mixed, normal flora of aerobic and anaerobic bacteria. The normal flora generally outnumbers the pathogens and the role of the laboratory is to distinguish between the commensals and the pathogens.

Objectives: To isolate and identify the aerobic bacterial causes of pharyngitis and that colonize the buccal cavity, and determine the antibiotic resistance.

Methods: Many patients visited the ENT outpatient clinic at Tikrit Teaching Hospital in Tikrit city with acute pharyngitis infection from 12/12/2008 to 22/2/2009. Throat and buccal cavity swabs were collected from one hundred twenty two samples by a physician from each patient that was only infected by acute pharyngitis. After culture on sheep blood agar plate, only bacterial etiology (positive culture result) cases were selected, then swabs were cultured for identification.

Results: There were different types of bacteria isolated from infected throats in this study. The results showed that Streptococcus pyogenes was the most commonly isolated bacteria (37.7%), followed by Staphylococcus aureus (33.6%).While other bacteria came in lesser frequencies. Whereas buccal cavity bacterial isolates showed that Viridans Streptococci were most commonly isolated bacteria (71.3%), then coagulase-negative Staphylococci (21.3%), Pseudomonas aeruginosa (4.1%), and Proteus mirabilis (3.3%). The results of antibiotic sensitivity after curing showed that cured isolates of Pseudomonas aeruginosa and Staphylococcus aureus lost their resistance to Cefotaxime, while cured isolates of Proteus mirabilis lost their resistance to Chloramphenicol, and same for Citrobacter freundii to amoxicillin after curing. Conjugation experiments were done for six isolates. Their resistance to antibiotics and production of virulence factor were changed after curing as donor isolates with the standard strain E.coli HB 101 prepared in university labs. That not harbor any plasmid which have a chromosomal resistance to Tetracycline and not able to produce all studied virulence factors as recipient strain. The results showed that just one isolate of Proteus mirabilis was able to transfer its resistance to Chloramphenicol to the recipient bacteria.

Conclusion: This proved that Chloramphenicol resistance gene was carried on conjugative plasmid and proved the results of curing experiment. Other isolates have failed to transfer their resistance for any antibiotics in conjugation experiments, because their genes may be carried on non-conjugative plasmids.

Keywords: Acute pharyngitis, Buccal cavity, Bacteria, Curing, Conjugation.

Iraqi Medicals Journal Vol. 64, No. 2, July 2018; p.144-151.

The upper respiratory tract infections and pharyngitis are the most common illnesses in the world[1]. Most patients have benign viral infections, and only 20% have bacterial pharyngitis (organisms detected by way of throat cultures are S. pyogenes, S. aureus, M. catarrhalis, Meningococci, Gonococci, C. diphtheriae, B. perturssis, and H. influenzae) and usually require treatment with appropriate antibiotics[2]. However, the pharyngeal mucosa exhibits a brisk inflammatory response to any opportunistic bacteria, therefore, treatment should ideally be based on the result of bacteriological examination. Bacteriological diagnosis of pharyngitis is complicated by the fact that the mouth and pharynx

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contains a heavy, mixed, normal flora of aerobic and anaerobic bacteria. The normal flora generally outnumbers the pathogens and the role of the bacteriologist is to distinguish between the commensals and the pathogens. Most drug resistance is due to a genetic change in the organism, either a chromosomal mutation or the acquisition of a plasmid or transposon. Plasmid-mediated resistance is very important from a clinical point of view for three reasons: (1) It occurs in many different species, especially gram-negative rods, (2) Plasmids frequently mediate resistance to multiple drugs, (3) Plasmids have a high rate of transfer from one cell to another, usually by conjugation. Mediate conjugation is the main process by which resistance genes are transferred from one bacterium to another[3].Plasmids can be eliminated from host cells in a process known as curing, that facilitates to detect the location of antibiotics resistance genes(chromosomal or plasmid)[4].

The aim of the present study is to isolate and identify the aerobic bacterial causes of pharyngitis and that colonize the buccal cavity, and determine the antibiotic resistance and virulence factors' genes whether related to plasmid(s) or chromosome by curing experiments, also, to assess whether the plasmids related to antibiotic resistance and virulence factors are conjugative or not.

Methods

Strain of Escherichia coli HB101 free of plasmid was used for bacterial conjugation experiments. It was taken from Biotechnology department, College of Science, Al-Nahrian University.

The study was performed from 12/12/2008 to 22/2/2009 in Tikrit Teaching Hospital in Tikrit city. One hundred twenty two(122) patients were collected, who were infected by bacteria only according to the clinical diagnosis and the bacterial cultures. From each patient two samples, (throat swabs and buccal cavity swabs) were collected by a physician using sterile cotton swabs. Throat and buccal cavity swabs were rolled across one of the following media: Blood agar, Chocolate agar, and MacConkey's agar. After overnight incubation at 37°, the primary inoculated plates were examined and the predominant of the isolated colonies were selected to be isolated and identified using conventional methods and analytical profile index (API) system[5].

Curing experiments were performed on seven bacterial isolates, four from throat (Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, and Citrobacter freundii) and three from oral cavity (Staphylococcus saprophyticus, Pseudomonas aeruginosa, Proteus mirabilis), using chemical factor as sodium dodecyl sulphate (SDS)[6].

Bacterial conjugation experiment was performed for six bacterial isolates, four from throat (S. aureus, P. aeruginosa, P. mirabilis, and C. freundii), and two from oral cavity (P. aeruginosa, P. mirabilis), which were resistant for most tested antibiotics, and this antibiotic resistance proves by curing experiment. The six bacterial isolates chosen as donor isolates (to prove whether the plasmids responsible for antibiotic resistance and production of some virulence factors conjugative or non-conjugative), and the standard strain E.coli HB 101 which is free of plasmid and has a chromosomally resistance tetracycline and not able to produce any of virulence factors as a recipient. This experiment was performed according to Prescott, (2003)[5].

Results

Of 122 throat specimens, 46(37.7%) specimens were caused by GAS, while 76(62.3%) were caused by other bacteria, (Figure 1).

From 122 throat isolates, 46 (37.7%) were GAS, 41 (33.6%) were Staphylococcus aureus, 16 (13%) were Citrobacter freundii, 7 (5.7%) were Moraxella catarrhalis, 5 (4%) were Pseudomonas aeruginosa, 4 (3.2%) were Proteus mirabilis, 2 (1.6%) were Citrobacter

diversus, and 1 (1.2%) was Neisseria meningitidis, (Figure 2).

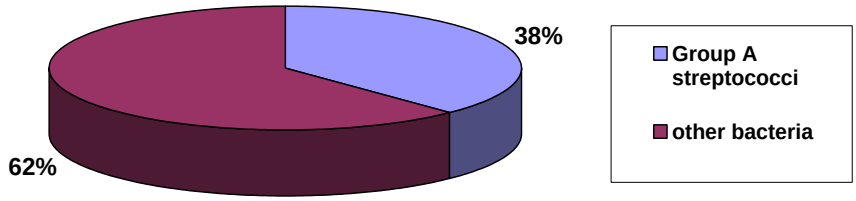

Figure 1: Percentage of group A Streptococci compared with total number of isolated bacteria (n = 122).

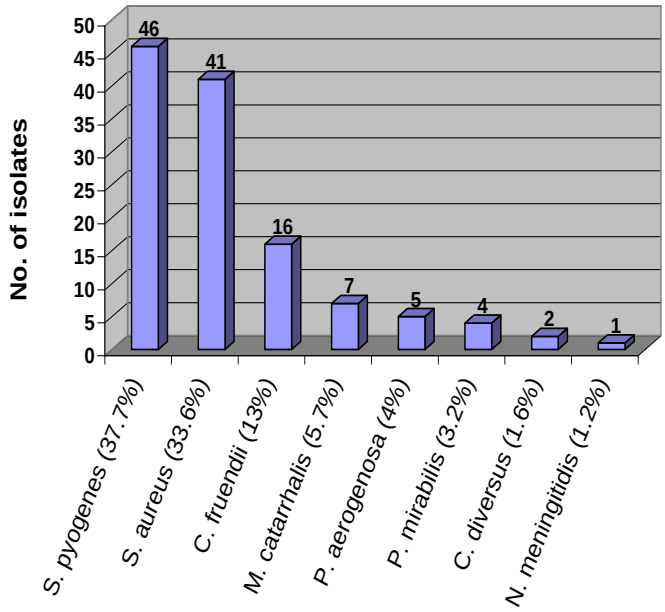

Figure 2: Number and percentage of bacterial isolates from pharyngitis cases.

From 122 buccal cavity specimens collected from 122 pharyngitis patients, 87 (71.3%) were Viridans Streptococci, while, 26 (21.3%) were Coagulase-negative Staphylococci, as the following: 9 (34.6%) were Staphylococcus epidermidis,5 (19.3%) were Staphylococcus saprophyticus, 4 (15.4%) were Staphylococcus hominis, 4(15.4%) were Staphylococcus hysicus, 3 (11.5%) were Staphylococcus warneri, 1(3.8%) was Staphylococcus gallinarum. Also, 5 (4.1%) were Pseudomonas aeruginosa, and 4 (3.3%) were Proteus mirabilis, (Figure 3).

Many concentrations of SDS solutions were used in this study. For Gram-positive bacterial isolates, the concentrations of SDS were from 0.5% to 22%, while for Gram-negative bacterial isolates they were from 8% to 22%.

Seven bacterial isolates were used in this experiment, which were multi-drug resistant and have the ability to produce most of the studied virulence factors from throat and oral cavity. Two isolates were Pseudomonas aeruginosa, one isolate from throat and another from oral cavity. Two isolates were Proteus mirabilis, one isolate from throat and other from oral cavity. One isolate of each Staphylococcus aureus, and Citrobacter freundii from throat, and one isolate was Staphylococcus epidermidis from oral cavity.

The results of the curing experiment showed that the minimal inhibition concentration of SDS for S. aureus isolate was 6%.

The results also showed that the minimal inhibition concentration of SDS for Gram-negative bacteria were 16% for P. mirabilis and C. freundii isolates and 18% for P. aeruginosa isolates.

After cultured on antibiotics plates, an antibiotics sensitivity test was done to ensure the antibiotics resistance loss and to determine the loss of resistance for other antibiotics. The results showed that (30%) of P. aeruginosa colonies lost their resistance to Cefotaxime. Also, (36%) of S. aureus colonies lost their resistance to Cefotaxime. One- hundred percent (100%) of P. mirabilis colonies lost their resistance to Chloramphenicol, and (9%) of C. freundii colonies lost their resistance to Amoxicillin, (Table 2).

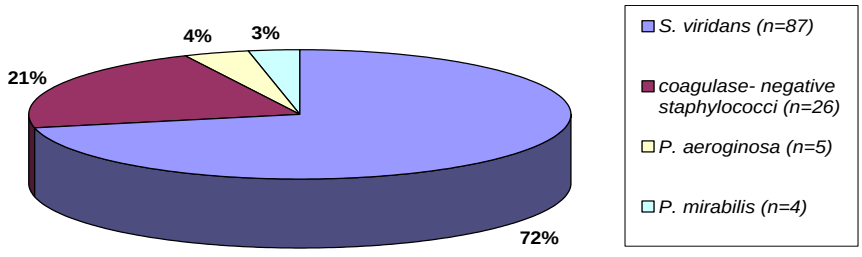

Figure 3: Number and percentage of bacterial isolates from oral cavity.

Table 1: The antibiotic resistance of bacteria isolated from pharyngitis and buccal cavity cases.

Bacterial isolates	Total No.	P 10		Ax 10		Cl 10		CTX 30		Ox 10		CN 10		Ak 10		TOB 2		E 30		Azm 15		Tmp 5		Te 30		C 30		CIP 5		VA 30	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
S. pyogenes	46	33	72	0	0	30	65	0	0	0	0	20	44	16	36	14	31	4	9	5	10	44	96	11	24	5	11	2	4	24	52
S. aureus	44	44	40	98	29	20	49	41	100	21	51	40	98	41	100	40	98	7	17	7	17	6	15	9	22	6	15	7	17	0	0
Citrobacter Spp.	16	16	100	16	100	16	100	16	100	16	100	0	0	0	0	0	0	16	100	0	0	0	0	0	0	0	0	0	0	16	100
P. aeroginosa	10	10	100	10	100	10	100	10	100	10	100	0	0	0	0	0	0	10	100	0	0	10	100	0	0	10	100	0	0	10	100
P. mirabilis	8	8	100	8	100	8	100	0	0	8	100	0	0	0	0	0	0	8	100	0	0	8	100	0	0	8	100	0	0	8	100
Coagulase negative Staphylococci	26	20	77	18	69	19	73	19	73	26	100	19	73	19	73	19	73	24	92	24	92	14	54	9	35	0	0	3	12	0	0

P: Penicillin, **Ax**: Amoxicillin, **Cl**: Cephalexin, **CTX**: Cefotaxime, **Ox**: Oxacillin, **CN**: Gentamicin, **Ak**: Amikacin, **TOB**: Tobramycin, **E**: Erythromycin, **Azm**: Azithromycin, **Tmp**: Trimethoprim, **Te**: Tetracyclin, **C**: Chloramphenicol, **CIP**: Ciprofloxacin, **VA**: Vancomycin

Table 2: The antibiotics resistance of bacterial isolates after and before curing.

Bacterial isolates		Antibiotics with percentage of curing		
		CTX	AX	C
Staphylococcus aureus	Before curing	+	+	+
	After curing	-	36%+	+
Pseudomonas aeroginosa	Before curing	+	+	+
	After curing	-	30%+	+
Proteus mirabilis	Before curing	+	+	+
	After curing	+	100%+	-
Citrobacter freundii	Before curing	+	+	+
	After curing	+	-	9%+

+ = Resistant, - = Sensitive, CTX= Cefotaxim, AX= Amoxicillin, C= Chloramphenicol

Discussion

Current results showed that GAS represent about 37.7% of cases, which disagree with Chreitahet al[7] study, who collected 91 throat bacterial specimens and their results showed that 85(93.4%) were Beta-hemolytic streptococci and 6(6.6%) were other bacterial causes. While other studies were in agreement with our results, since Jeffrey and Rosita[8] collected 325 throat bacterial specimens and their results showed that 145(44.6%) were GAS and 180(55.4%) were by other bacterial causes. Brook and Alan[9] also collected 548 throat specimens and reported that 112(20.4%) were GAS and 436(79.6%) were by other bacterial causes. The most prevalent bacterial pharyngitis is due to GAS, however, microbiologic data regarding bacterial pharyngitis show the role of other bacteria as a cause of infection[10]. Also, the causes of pharyngitis may share (viral, viral), (viral, bacterial), and (bacterial, bacterial); a mutual symbiotic enhancement of growth of GAS in the presence of other aerobic and anaerobic bacteria has been demonstrated in an animal model[11].

Colonization by potential pathogens and complication infection play an important role in secondary respiratory infections, for example in pharyngitis infections, viral infection facilitates invasion by colonizing bacteria, which in turn facilitates superinfection by normal flora. Also, viral

infections caused low immunity, increased adherence, cilial paralysis, and with time (duration of infection), and random use of antibiotics, leads to the aerobic bacterial infections from colonizing bacteria[12]. The present results indicate that other bacteria play an important role as a cause of pharyngitis.

Current results agree with Radosz et al[13] who studied 158 patients with pharyngitis GAS were isolated from 47(30%) of patients, while the majority of isolated bacteria 111(70%) belong to potential pathogenic flora of other bacteria.

A very common situation during winter is the high prevalence of bacterial pharyngitis. The diagnosis of the bacteria that cause pharyngitis through classical microbiological methods is a difficult, but usually very efficient task[14]. Many bacterial organisms are capable of inducing pharyngitis, either as a single manifestation or as part of a more generalized illness[15]. Furthermore, aerobes and facultative anaerobes are the most numerous components of the normal human oropharyngeal bacterial flora (Enterobacteriaceae, Pseudomonas aeruginosa, Neisseria meningitidis), so that causes of bacterial infections of the upper respiratory tract (sinusitis, otitis, pharyngitis) have an endogenous origin. This means that they play an important role as co-pathogens and not as primary pathogens[13,16,17].

Individuals who are previously hospitalized for several days may become colonized in the upper respiratory tract by gram-negative rods, particularly members of the Enterobacteriaceae[18]. Saphylococcus aureus, Neisseria meningitidis, Haemophilus influenza, Escherichia coli, Proteus spp., Pseudomonas aeruginosa, and Bacteriods spp. are potential pathogens commonly found in the pharynx and the mouth with exception of Escherichia coli, Proteus spp., and Pseudomonas aeruginosa are common about 5-25%[19].

Fifteen different antimicrobial antibiotic discs were used to performances this test, along with all bacterial isolates, which were from the throat with acute pharyngitis, and buccal cavity of each patient. To determine whether the isolates were resistant or not, (Table 1), it was confirmed according to the comparison between the diameter of inhibition zones obtained and the values determined by NCCL[20].

This study showed that most of bacterial isolates were multi-drug resistant. Antibiotic resistance was widely spread in both hospital and community pathogens. Recent data from large programs has illustrated the growing issue of antibiotic resistance, especially among bacteria from upper respiratory tract infections[21].

More than 98% of respiratory tract infections prescriptions were written empirically (so they would not know if the patient had a resistant pathogen initially or not researchers soon discovered that pathogens develop resistance to antimicrobials through a process known as natural selection[22].

Most drug resistance is the result of a genetic change in the organism, caused either by a chromosomal mutation or the acquisition of a plasmid or transposon. Plasmid-mediated resistance occurs at a higher frequency than chromosomal-resistance, because of three reasons: It occurs in many different species, especially gram-negative rods, and plasmids frequently mediate resistance to multiple drugs, and plasmids have a high rate of transfer from one cell to another, usually by conjugation[3].

Antibiotic resistance and production of virulence factors genes in bacteria may be located on chromosomes or located on plasmids or both, thus, elimination of plasmids results in loss of antibiotics resistance and production of virulence factors that are located on plasmids. Curing refers to the ability to spontaneously or under chemical and physical effect loss of plasmids. In this study sodium dodecyl sulfate is used as a curing agent

Salisbury[23] mentioned that SDS have strong activity in bacterial curing more than other chemical curing factors like Acridine Orange and Rifampicin but not mutagenic.

Curing experiments' results were nearly agreed with Sonstein[24] who mentioned that the minimal inhibition concentration of SDS for Gram-positive bacteria was 8%. Thus, we cannot get a cured cell of S. epidermidsis isolate using SDS. There were many reasons that may be related to this result; (1) the culturing conditions were not good, because of continuous electrical cease, (2) production of slime and biofilm as a extracellular barrier, consists from complex material; chemical analysis of slime indicate that it is a glycoconjugate composed of glycerol phosphate, D-alanine, N-acetylglucosamine and glucose[25], whereby, the production of slime may prevent SDS to penetrate the bacteria.

The results of minimal inhibition concentration of SDS for Gram-negative bacteria agreed with Tomoeda et al[26], who mentioned that the optimal minimal inhibition concentration of SDS for Gram-negative bacteria was 19%, and Raoof[27] showed that the minimal inhibition concentration of SDS for P. aeruginosa isolates was 18%. This resistance result agrees with Shahcheraghietal[28] who found that the genes responsible for resistance to Cefotaxime are located on plasmid.

The results of curing experiments also agreed with Harod[29] who mentioned that the Cefotaximase enzyme gene was located on plasmid in S. aureus, and the results were not agreed with Yah et al[30] who found that all isolates of P. mirabilis lost the resistance to Chloramphenicol after curing by SDS, and the genes responsible for resistance to Chloramphenicol are located on plasmid. Other results disagrees with Oliva[31] who found that the resistance to β-lactam antibiotics were not plasmid encoded after curing experiment, unlike others who showed that the genes coded of β-lactamase in C. freundii were located on plasmid and have transposable activity.

In addition, Harod[29] mentioned that the β-lactamase genes in Enterobacteriaceae were located either on chromosomes or plasmid. These can move more or less randomly around bacterial genome.

The aim of bacterial conjugation in the current study was to determine whether the plasmids of cured bacterial isolates were conjugative or not. The conjugation method was carried out according to Prescott[5], using Escherichia coli HB 101 as a recipient, which was chromosomally resistance to tetracycline only. The donor bacterial isolates selected from the cured bacterial isolates (six isolates) under study have plasmid resistance to Cefotaxime, Amoxicillin, and Chloramphenicol and are sensitive to Tetracycline.

This experiment lasted for a long time and was repeated many times during our study, and only one isolate of Proteus mirabilis (isolated from throat) succeeded in conjugation, by transconjugating of Chloramphenicol resistance character into the recipient strain. This result agreed with Yah et al[30] who showed that all bacterial isolates of Proteus mirabilis from patients with wounds succeeded in transconjugate with the recipient strain, and the resistance gene coded for Chloramphenicol was located on conjugative plasmids.

In conclusion; Most of isolated bacteria of throat and oral cavity were resistant to many antibiotics under study, with ability of isolated bacteria of each throat and oral cavity to produce some important virulence factors, with the possession of conjugative plasmid which may help to propagate the drug resistance between the different genera of bacteria present in the oral cavity.

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IMJ 2018;64(2):144-151.