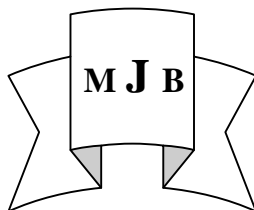


Prevalence of β -Hemolytic Groups C and F Streptococci in Patients with Acute Pharyngitis in Hilla City/Iraq

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Abstract

Background: The roles of group C and F streptococci in causing endemic pharyngitis are still controversial, although group C streptococci are implicated in the outbreaks of pharyngitis and associated disorders.

Aim: The aim of this study was to determine the prevalence and the role of these groups of β -hemolytic streptococci in acute pharyngitis with emphasis on *Streptococcus anginosus* group. The antimicrobial susceptibility profile of these bacterial isolates and their ability to produce some virulence factors was also determined.

Materials and Methods: Throat swab specimens were collected from 177 patients suffering from acute pharyngitis who had been admitted to Hilla teaching hospital (Hilla, Iraq) during October 2009 to January 2010. The necessary biochemical tests were conducted and the organisms identified using standard procedures. Susceptibility of isolates pathogens to several antibiotics was examined using standard susceptibility testing. Virulence factors of these isolates were also determined using standard methods. **Results:** Results revealed that a total of 67 isolates were belonged to β -hemolytic streptococci. Of which, 11(16.4%) isolates belonged to anginosus group streptococci, which possess Lancefield group C and F antigens. Most of these bacterial isolates have the ability to produce more than one virulence factor such as capsule, hemolysin, CFA III, and lipase enzyme. The bacterial isolates were highly resistant to ampicillin, cefotaxime, and cefepime while they exhibited moderate resistance to tetracycline, ceftriaxone, and ciprofloxacin. On the other hand they showed high sensitivity to vancomycin, ofloxacin, and clindamycin.

Conclusion: This study concluded that group C and F Streptococci were implicated as a cause of acute pharyngitis in 6.2% of the specimens among other groups of streptococci. Most of these isolates have the ability to produce more than one virulence factor and there was a high rate of resistance among isolates for β -lactam antibiotics, but they were highly susceptible to vancomycin, ofloxacin, and clindamycin.

Introduction

The *Streptococcus milleri* group (SMG) is a highly diverse group which includes three species: *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus*. The group also includes hemolytic streptococci belonging to Lancefield group A, C, F and G as well as non-groupable and non-hemolytic streptococci [1].

The nomenclature, identification and classification of *Streptococcus milleri* have been confusing. In 1989, it was proposed in the United States to rename this different group under one species name, *Streptococcus anginosus*, while in Great Britain the designation *Strep. milleri* was preferred [2].

Group C streptococci is a common cause of infection in animals but there is little information about their overall

importance as a cause of human infection. Group C and G streptococci were first recognized as human pathogens by Lancefield and Hare in 1935 [3].

The role of Lancefield group C beta-hemolytic streptococci in causing endemic pharyngitis are still controversial [4,5], although Lancefield group C streptococci are implicated in the outbreaks of purulent pharyngitis and associated disorders [6, 7]. It is well known that group C streptococci are often isolated from clinical specimens. is the most common beta-hemolytic group C streptococcus isolated from the human throat [8]. *S. anginosus* group (SAG), is the most common beta-hemolytic group C streptococcus isolated from the human throat [8, 9]. Other group C streptococcal species are generally isolated only from patients with zoonotic infections [7].

Strains belonging to the '*Streptococcus milleri*' group are distinct among the viridans

streptococci because of their tendency to cause suppurative infections and bacteremia [10]. The frequent presence of polysaccharide capsule may help these pathogens to escape from being phagocytosed before adhering to the site of tissue damage. The production of extracellular enzymes including hyaluronidase, deoxyribonuclease, ribonuclease, gelatinase and collagenase by these organisms may contribute to its pathogenicity by degradation of connective tissues. SMG have also been observed to release extracellular products with immunosuppressive effects which may allow the organism to survive within an abscess [11]. Strains belonging to the '*S. miller*' group are considered uniformly susceptible to the antibiotics usually employed for streptococcal infections [10]. However, few reports and studies

have studied the antimicrobial susceptibility of the "*Streptococcus milleri*" group and have demonstrated the presence of resistant strains [12, 13].

To our knowledge, the prevalence and role of groups C and F in patients with acute Pharyngitis have not been documented in our locality. Therefore, this study aims to determine the prevalence and the role of these groups of β -hemolytic streptococci in acute pharyngitis with emphasis on *Strep. anginosus* group in Hilla City, Iraq. The antimicrobial susceptibility profile of these bacterial isolates and their ability to produce some virulence factors will also be determined.

Materials and Methods

Patients and specimen collection

One hundred seventy seven throat swab specimens were collected from patients suffering from acute pharyngitis who were submitted to Hilla general teaching hospital during a period of three months (from October 2009 to January 2010). The patients age ranged from (4-75) years. These specimens were collected with the help of physician to avoid any possible contamination. Each swab was inserted into the oropharynx and rotated there then carefully withdrawn avoiding contamination from the mouth. Swabs for culture were placed in tubes containing readymade media to maintain the swab wet until taken to laboratory. Each specimen was inoculated on a blood agar plates. All plates were incubated anaerobically in a Candle Jar at 37 C° for 24-48 hrs. Throat swabs were collected aseptically and transported immediately to laboratory.

Bacterial isolates

The isolation and identification of *Strep. anginosus* group bacteria in patient's pharynx were performed

through colony morphology of bacterial isolates, microscopic gram stain investigation and biochemical tests [14]. Isolates identification was also confirmed using API 20 test system (Biomérieux/France).

Streptex agglutination test

Streptex agglutination test (provided by Remel/USA) for Lancefield grouping was used to identify streptococci groups (A, B, C, D, F, and G) according to manufacturer instructions.

Detection of Virulence factors

virulence factors were detected in bacterial isolates according to standard procedures. Capsule production was detected by Hiss's Method of capsule staining [14], Hemolytic reaction was detected on Blood agar medium by streaking a pure culture of bacterial isolate and incubated at 37°C for 24-48 hrs. The appearance of a clear zone surrounding the colony is an indicator of β - hemolysis while the greenish zone is an indicator of α - hemolysis [14].

Extracellular protease production was tested and carried out by using M9 medium [15]. Colonization Factor antigens (CFA) I and III production test was performed to detect the ability of bacterial isolates to produce colonization factors antigen [16]. Bacteriocin production was performed using cup assay method on Brain Heart Infusion agar (BHI) supplemented with 5% glycerol to enhance their growth and bacteriocin production [17]. Lipase production was carried out in egg-yolk agar medium [14]. Gliding motility of bacterial isolates was detected by swarm assay method to measure spreading of bacteria over agar surface [18].

Antimicrobial susceptibility testing

The susceptibility of the bacterial isolates to antimicrobial agents was determined using disk diffusion

method [19] and interpreted according to Clinical and Laboratory Standards Institute (CLSI) documents [20]. The following antimicrobial agents were obtained (from Oxoid, U.K.) as standard reference disks as known potency for laboratory use: Penicillin (P) , Ampicillin (Am) 10 μ g, Cefotaxime (CTX), 30 μ g, Ceftriaxone (CTR) 30 μ g, Cefepime (FEP) 30 μ g, Vancomycin (VA) 30 μ g, Erythromycin (E), 15 μ g, Clarithromycin (AZM) 15 μ g, Chloramphenicol (C) 30 μ g, Tetracycline (TE), 30 μ g, Clindamycin (DA) 2 μ g, and Ofloxacin (OFX) 5 μ g.

All these tests were performed on plates of Muller- Hinton agar (Oxoid, U.K.).

A 0.5 MacFarland suspension (provided by Biomérieux/ France) of tested bacterial isolates was applied to the plates , which were dried in an incubator at 35 °C for 15 minutes . Antimicrobial disks were placed on the agar with sterile forceps .The agar plates were incubated inverted at 35 °C for 18 hours. Results were recorded by measuring the inhibition zone (in millimeters) and interpreted according to Clinical and Laboratory Standards Institute (CLSI) documents [20].

Detection of β -Lactamase production

This test was performed for all isolates that were resistant to β -lactam antibiotics, using Rapid Iodometric Method [21].

Determination of minimum inhibitory concentration (MIC)

Two methods were used for determination of MICs of isolates against seven different antibiotics: The MICs for Cefotaxime and Ciprofloxacin were determined by HiComb MIC Test using HiComb strips (Himedia/India). This test was

carried out according to procedure recommended by the manufacturer. The MICs of the other antibiotics (Ampicillin, Ceftriaxone, Vancomycin, Erythromycin, and Tetracycline) were determined by the two-fold agar dilution susceptibility method [22]. The MIC values were based on break points recommended by Clinical and Laboratory Standards Institute (CLSI) documents [20] for estimation of the response. The break point represents the optimum concentration of the drug that can reach the serum and provide high level of therapy. The microorganism was considered sensitive if the estimated MICs were less than the break point. Standard strain (*E.coli* ATCC 25922) was used as a negative control.

Results and Discussion

Morphological and biochemical characterization revealed that 137 isolates belonged to the genus *Streptococcus*. Of which 67 (37.8%) β-hemolytic isolates, The results showed that out of 67 β-hemolytic streptococcal isolates, 11(16.4%) isolates belonged to the

Strep.anginosus group. This represented about (6.2%) of all the positive culture throat swabs collected in this study. The percentage of the positive isolates (6.2%) in the present study is higher than that recorded by other studies [23] which reported that (4.4%) throat swabs were positive for group C streptococci. This could be attributed to many factors like cultural, ecological, and others like the habit of using antibiotics without medical roles. There was a difference in this study in the isolation rates of BHS between males and females. Of the 11 isolates which belonged to the anginosus group, 4 isolates (36.3%) were recovered from males and 7 isolates (63.6%) from females. This result is inconsistent with other investigators who found a higher incidence of infections among males by this group of bacteria [24].

Results found that used for identification of *Streptococcus anginosus* group was not enough for identification of β-hemolytic streptococci, so the following tests were used in addition to tests in API 20 strep system:

Test	Result
Colony characteristics	Small, grey, and large colonies on blood agar
Cell morphology	Gram positive , Cocci
Hemolysis	Beta-hemolytic
<u>Biochemical reactions</u>	
Catalase	Negative
Mannitol	Negative
Salicin	Positive
Raffinose	Positive
Lancefield group reaction	Group F and C

The use of biochemical profiling tests in API 20 strep system test was not sufficient for final identification of species of this group of streptococci. This result is in accordance with other researchers who reported that

identification may be difficult, and many laboratories reported the identification of these bacteria to the group level but not the species level [2].

Results in (Figure 1) showed that the isolates in our study possess Lancefield group antigens C ,2 (18.1%) and F, 8 (72.7%) with one isolate posses both C and F antigens that found in β-hemolytic streptococci. This result is near to the results of [25] who found

that *Strep. milleri* represented 56% of the group C, 100% of group F, isolated in their clinical laboratory, whereas the incidence of *Strep. milleri* among group A and G streptococci was estimated to be low.

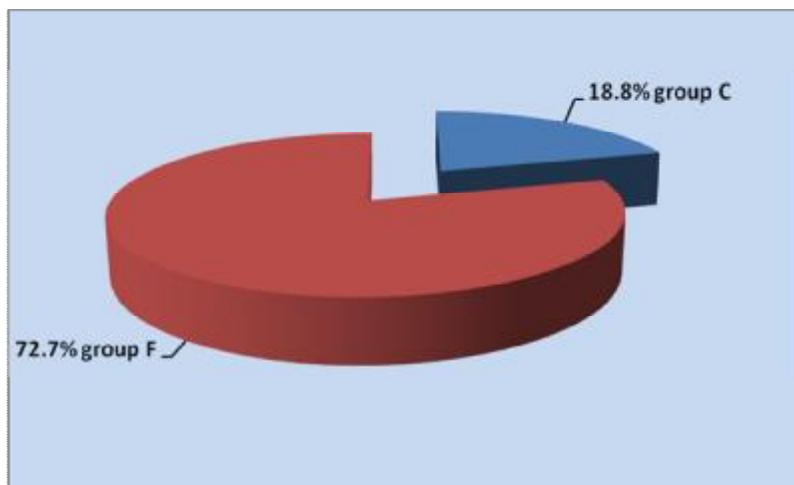


Figure 1 Percentages of group C and F antigens in the β-hemolytic streptococci recovered in this study.

Virulence Factors of the bacterial isolates:

The virulence factors of anginosus group streptococcal isolates in the

present study were detected and the results are shown in (Table -1).

Table 1 Virulence Factors detected in isolates of anginosus group streptococci

No. of isolates	Virulence Factors							
	Capsule	β-Hemolysiss	Protease	Lipase	Colonization factors		Bacteriocin	Gliding motility
					I	III		
17	-	+	-	+	-	-	-	-
46	+	+	-	+	-	-	-	-
91	+	+	-	+	-	+	-	-
93	+	+	-	+	-	+	-	-
108	+	+	-	+	-	+	-	-
113	-	+	-	+	-	+	-	-
114	-	+	-	+	-	+	-	-
116	-	+	-	+	-	+	-	-
137	+	+	-	+	-	+	-	-
147	+	+	-	+	-	+	-	-
151	+	+	-	+	-	-	-	+

All isolates in the present study showed β -hemolysis, (Table 1). This result is similar to that of [26] who reported that strains of *Strep. constellatus* and *Strep. anginosus* (13 of 15) which belong to Lancefield serological group F, were beta-hemolytic. However, this result is not matched with the results of other studies that reported that most strains of *Strep. milleri* are non-hemolytic, and only 25% of them are beta-hemolytic and may possess Lancefield group A, C, F, or G antigen [27].

The inability of our isolates to produce the enzyme protease when tested on M9 medium containing gelatin (Table 1) was also confirmed by another study which reported that all *Strep. milleri* isolates tested were unable to show gelatinase activity [28].

All isolates in the present study produced the enzyme lipase after incubation for 48 hrs. on egg yolk agar medium (Table 1), this result is inconsistent with [29] who found that all isolates examined in their study gave negative reaction in a test for lipase production. While [28] reported that lipase have been observed in some strains of *Strep. milleri* group.

Most isolates in the present study (72.7%) possessed the colonization factor antigens III (Table 1). This result is consistent with [30] who have demonstrated fine fimbrial structures on the surface of oral streptococci, including *Strep. milleri* which may be important in adherence to surfaces, and

in inter-species interactions common in the mixed flora of mucosal surfaces, dental plaque, and purulent lesions.

No isolate in the present study produce bacteriocin when tested with sensitive Gram positive indicator isolates (Table 1). This result disagreed with [31] who found that three of 30 human strains of group C Streptococci produced inhibitors that had bacteriocin-like properties.

In this study only one isolate showed a positive result regarding gliding motility test (Table 1), this result is consistent with [32] who described isolates belonging to the *Strep. milleri* species group that appear to exhibit a gliding type of motility, which is expressed as spreading growth on certain types of chocolate agar medium.

Antibiotic Susceptibility of the isolates

Results of disk diffusion method revealed that most isolates were found to be resistant to at least 4 antibiotic tested, (Table 2). The results also revealed that there was highly bacterial resistance to β -lactam antibiotics ampicillin (90%), third generation cephalosporins (cefotaxime and ceftriaxone) (100%), and fourth generation cephalosporin (cefepime) (81.8%). These results are comparable with the data reviewed by [33] who reported that resistance to penicillin G was associated with resistance to the other beta-lactam antibiotics.

Table 2 Antibiotic resistance of anginosus group Streptococci

Isolates designation	ANTIBIOTICS											
	AM	CTX	CRO	VA	E	AZ M	CLR	TE	OFX	C	DA	FEP
<i>Strep.anginosus</i> T 17	R	R	R	S	S	S	S	R	S	S	S	S
<i>Strep.anginosus</i> T 46	R	R	R	S	S	S	S	S	S	S	S	R
<i>Strep.anginosus</i> T 91	R	R	R	S	S	S	S	S	S	S	S	R
<i>Strep.anginosus</i> T 93	R	R	R	S	R	R	R	R	S	S	S	R
<i>Strep.anginosus</i> T 108	S	R	R	S	S	S	S	S	S	S	S	S
<i>Strep.anginosus</i> T 113	R	R	R	S	S	S	S	S	S	S	S	R
<i>Strep.anginosus</i> T 114	R	R	R	S	R	R	S	S	S	R	S	R
<i>Strep.anginosus</i> T 116	R	R	R	S	S	S	S	S	S	S	S	R
<i>Strep.anginosus</i> T 137	R	R	R	S	S	S	S	S	S	S	S	R
<i>Strep.anginosus</i> T 147	R	R	R	S	R	R	R	R	S	R	S	R
<i>Strep.anginosus</i> T 151	R	R	R	R	S	S	S	S	S	S	R	R
% of Resistance	90	100	100	9.0	27.2	27.2	18.1	36.3	0	18	9.0	81.8

AMP=ampicillin, FEP=cefepime, CTX=cefotaxim, CRO=ceftriaxon, A=vancomycin, E=erythromycin, AZM=azithromycin, CLR=clarithromycin, TE=tetracycline, OFX= ofloxacin C=chloramphenicol, DA=clindamycin.

The result of beta-lactamase detection test in this study revealed that there was no beta-lactamase activity could be detected in isolates of this group. This results may be attributed to the fact that the mechanism of beta-lactam resistance in this group of bacteria is be due to altered target (penicillin binding proteins, PBPs). Penicillin-resistance of viridans streptococci is not due to beta-lactamase production, hence no benefit from using agents such as ampicillin-sulbactam as bacterial therapy [34].

Bacterial Susceptibility to Macrolide group (erythromycin, azithromycin, and clarithromycin), has also been investigated (Table 2), and it was found that most isolates were sensitive to these antibiotics with sensitivity rate (72.7%) for erythromycin, (72.7%) for azithromycin, and (81.8%) for clarithromycin. However, the erythromycin resistance rate seen in this study was significantly higher than that of SAG isolates from Netherlands (2.6%) and Spain (17.1%) [12, 35]. Because of the high frequency of

genetic exchange between different streptococcal species, it is likely that the increased macrolide resistance among viridans streptococci has contributed to an increased macrolide resistance among SAG isolates [13].

Most isolates were sensitive to tetracycline (Table 2) with resistance rate (36.3%). This result is comparable with [36] who found a resistance rate of for tetracycline of 70 clinically significant isolates of *Streptococcus milleri* group was (37.1%).

Results also showed that most isolates (10 of 11) were highly sensitive to vancomycin, clindamycin and chloramphenicol with very low rate of resistance (9.0%), (18%), (9.0%) respectively. (Table 2) showed that one isolate of SAG was resistant to vancomycin.

According to CLSI documents [20], disk diffusion test for vancomycin sensitivity is ≥ 17 mm with no intermediate value regarding to *Streptococcus* spp. The disk diffusion procedure cannot differentiate isolates

with reduced susceptibility to vancomycin from susceptible isolates (MIC range $\leq 1 \mu\text{g/mL}$) even when incubated for 24 hours.

The results also showed that all isolates (100%) were sensitive to fluoroquinolone (ofloxacin). Since all isolates were sensitive to ofloxacin, it might serve as the drug of choice for the treatment of infections caused by this group of bacteria.

Results of β -Lactamase production test were negative for all tested isolates in this study. This result showed that resistance to β -lactam antibiotics in this group is not due to β -lactamase production, but may be due to altered penicillin binding proteins. This result is in agreement with other researchers who mentioned that the intrinsic penicillin resistance mechanism in viridans group streptococci involves

alterations of the target enzymes for β -lactams, essential penicillin-binding proteins, with a decreased affinity of all β -lactam antibiotics [37]. This mechanism together with selective antibiotic pressure may play a role in resistance of viridans group streptococci to β -lactam antibiotics.

MICs Determination of anginosus group isolates

Results showed that all 11 anginosus group isolates were highly resistant to ampicillin with a concentrations of one fold of the break point ($\geq 128 \mu\text{g/ml}$) and most isolates were highly to moderate resistant to ceftriaxone (7 isolates) and cefotaxime (8 isolates) with MIC values ranged from (0.25 to $8 \mu\text{g/ml}$) and from (1 to $30 \mu\text{g/ml}$) respectively (Figure 2, Table 3).

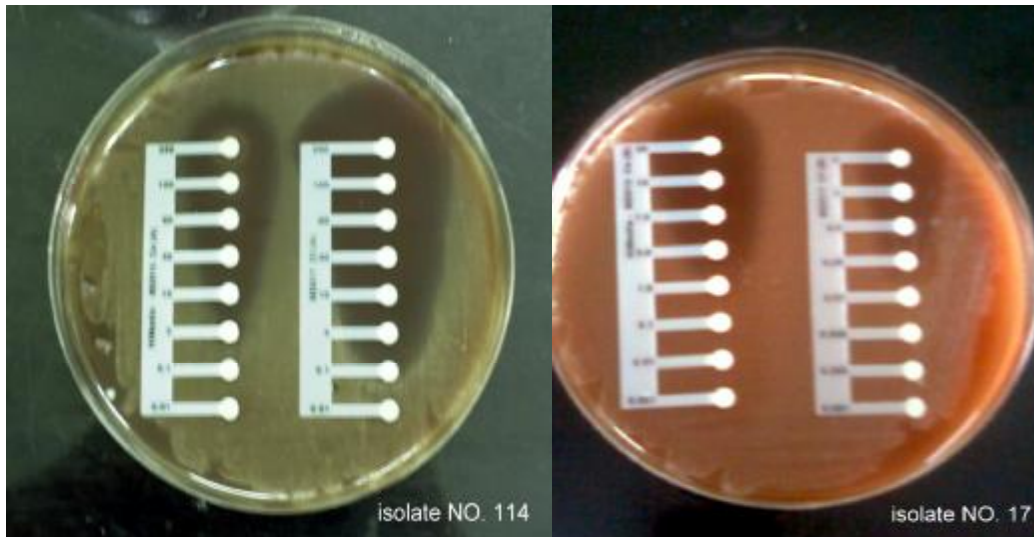


Figure 2 HiComb MIC test used for determination of MIC of cefotaxime against *Streptococcus anginosus* group isolates.

Table 3 MICs of a number of antibiotics against *Strep.anginosus* group isolates.

Isolates Designation	MIC (µg/ml) of:						
	AMP (≤0.25µg/ml)	CRO (≤0.5µg/ml)	CTX (≤1µg/ml)	VA (≤1µg/ml)	E (≤0.25µg/ml)	TE (≤2µg/ml)	CIP (≤1µg/ml)
Strep.anginosus T 17	64	0.5	5	0.48	0.96	0.42	0.1
Strep.anginosus T 46	64	4	30	0.48	0.24	0.03	5
Strep.anginosus T 91	32	1.0	5	0.48	0.96	0.03	0.1
Strep.anginosus T 93	64	0.5	10	0.24	0.96	0.12	5
Strep.anginosus T 108	64	>0.5	0	>0.12	>0.06	>0.03	0
Strep.anginosus T 113	64	4	5	0.24	0.06	>0.03	5
Strep.anginosus T 114	64	2	5	0.24	0.96	>0.03	5
Strep.anginosus T 116	64	2	1	1.92	0.12	0.24	5
Strep.anginosus T 137	64	4	5	0.24	0.12	>0.03	5
Strep.anginosus T 147	64	8	5	0.96	1.92	0.24	5
Strep.anginosus T 151	64	0.25	0	7.68	0.24	0.03	0

*Numbers between the brackets refer to break points recommended by CLSI (2010).
 AMP=ampicillin, CTX=cefotaxim, CRO=ceftriaxon, VA=vancomycin, E=erythromycin, TE=tetracycline, CPI=ceprofloxacin.

These results are not agreed with results of [38] who reported that the β-lactam group provides the antibiotic of choice in infections caused by streptococci, and with [37] who mentioned that many other β-lactam antibiotics have *in vitro* activity similar to that of penicillin against the SMG, but the susceptibilities to different cephalosporins are quite variable.

All isolates in the present study were highly susceptible to vancomycin and tetracycline (Table 3). However, one isolate was found to be less susceptible to vancomycin with MIC reached to (7.68 µg/ml) which is seven times more than the break point recommended by Clinical and Laboratory Standards Institute (≤1 µg/ml) [20].

The percentage of tetracycline resistance (100%) among isolates of this group were higher than that recorded by [36] who found that more variable results are obtained with tetracyclines, where rates of resistance in the range 0-37% have been described.

Result of the single isolate that was less susceptible to vancomycin may be considered as vancomycin tolerant. Up to our knowledge this is the first resistant isolate recorded in Iraq. No vancomycin resistance had been recorded previously locally or worldwide in this group but it was found in *Staphylococcus aureus* and *Strep. pneumoniae*.

This group may be tolerant to vancomycin rather than being resistant.

The result of vancomycin tolerance in this group match with [39] who reported tolerance to vancomycin among pharyngeal isolates of non-group A β -hemolytic streptococci (mostly GCS and GGS) from children, and with [40] who reported that a few clinical isolates exhibit tolerance to vancomycin. The less susceptibility (or resistance) of this isolate to vancomycin may be recorded only when the resistance genes (*VanA*, *VanB*, *VanC*, etc...) could be detected by genetic methods (PCR) which is not determined in the present study. Results in Table (3) showed a resistance rate 45% to erythromycin this result is much higher than that reported by [41] who mentioned that resistance to erythromycin-lincomycin-clindamycin group, although currently rare, is transferable among streptococci, including the SMG, and can be as high as 12-14%.

For ciprofloxacin, seven anginosus group isolates (Table 3) were able to grow in concentrations one fold of the break point ($\geq 4\mu\text{g/ml}$). This result is in accordance with the data of [13] who showed that even in ciprofloxacin-susceptible isolate, the ciprofloxacin MICs are very close to the resistance breakpoints and suggest that ciprofloxacin is inadequate for treatment of SAG infections. In a study of the antimicrobial susceptibility of the *Streptococcus milleri* group, a steady increase in the susceptibility to ciprofloxacin was observed over the study period [35], while some authors reported that ciprofloxacin was noticeably the less active agent than the other agents tested, it was the least active quinolones, especially against all species of streptococci [42]. This study concluded that group C and F Streptococci were implicated as a cause of acute pharyngitis in 6.2% of the specimens among other groups of streptococci in Hilla, Iraq. Most of

these isolates have the ability to produce more than one virulence factor and there was a high rate of resistance among isolates for β -lactam antibiotics, but they were highly susceptible to vancomycin, ofloxacin, and clindamycin.

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