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Prevalence of SARS-CoV-2 co-infection with β-haemolytic streptococci in a sample of Iraqi patients

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ABSTRACT

Bacterial co-infections with respiratory viral pathogens are very common. This study was designed to investigate the incidence of β-haemolytic streptococci in patients with COVID-19 and study their antimicrobial susceptibility patterns. A total of 388 clinical samples were collected from Covid-19 patients who suffering from acute pharyngitis. β-hemolytic isolates were diagnosed using the Vitek 2 system. Streptex agglutination test was used to classify streptococci into different groups. These beta-hemolytic isolates were tested for their production of virulence factors and study their antimicrobial susceptibility patterns. Results of PCR showed that (260) patients had Covid-19 positive results, and (128) patients had negative results, who have been excluded. The isolation rate of β-hemolytic streptococci bacteria was (113; 43%). By using Streptex agglutination test, β-hemolytic isolates were divided into (59;52.2%) isolates belonged to group A, 7 (6.1) belong to group C, (3; 2.6%) belong to group F, and 44 (38.9) belong to group G. Five (4.4%) isolates were diagnosed as *S. anginosus* and 39 (34.5%) as *S. dysgalactiae equisimilis*. All five isolates were lipase and protease producers and had a capsule but they were negative for Nitrocefin disk method. Resistance to antibiotics showed that all isolates were resistant to most of the antibiotic classes tested. This study is considered one of the few studies in Iraq that accomplished for isolation and characterization of bacteria isolated from Covid-19 patients with infected acute pharyngitis. The study concluded that a high rate of *Streptococcus dysgalactiae equisimilis* (SDE) strains was isolated than *Streptococcus anginosus* group (SAG) in acute pharyngitis.

1. INTRODUCTION

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has presented a formidable medical challenge to health systems and clinicians [1], [2]. Bacterial co-infections with respiratory viral pathogens are very common, often through synergistic interaction among viruses such as influenza virus, and bacterial pathogens and the host immune system of the human being; nevertheless, the interaction between viruses and unusual bacteria is not yet fully understood [3]. The Streptococcus anginosus group (SAG), commonly referred to as the Streptococcus milleri group, is among five subgroup within the viridans-group streptococci that includes three distinct species streptococcal species: Streptococcus anginosus, Streptococcus intermedius, and Streptococcus constellatus [4]. SAG bacteria are considered commensals of

mucosal membranes, of the oropharyngeal gastrointestinal, and genitourinary tract [5]. These bacteria are now increasingly being found to cause infection at any anatomical site in the body, including the lungs, liver, brain, intra-abdominal areas, as well as the skin and soft tissues [6]. The Streptococcus anginosus group (SAG) play important roles in respiratory infections. It is ordinarily difficult to distinguish them from contaminations as the causative pathogens of respiratory infections because they are often cultured in respiratory specimens [7]. The Streptococcus milleri group (SMG) is a highly diverse group which includes hemolytic streptococci belonging to Lancefield groups A, C, F and G as well as non-groupable and non-hemolytic streptococci. Group A beta-hemolytic streptococci (GAS) is the most frequently isolated pathogen in acute pharyngitis. However, the role of Group C (GCS) and Group G (GGS) streptococci in disease burden is under recognized [8].

Penicillin remains the treatment of choice for beta-hemolytic streptococci (BHS) pharyngitis, and macrolide are recommended only for patients who are allergic to penicillin. Erythromycin resistance in BHS ranges from as low to high reported from outbreaks in Finland, Sweden, and Japan. High rates of resistance have also been reported sporadically from Australia, United Kingdom, Taiwan, and Italy. Resistance to tetracycline and co-trimoxazol has been reported to be high, making them virtually unusable as an alternative for the treatment of streptococcal infection. Antimicrobial drugs for BHS are used to avoid suppurative complication, prevent rheumatic fever, abort person-to-person transmission, and reduce the signs and symptoms associated with these infections.

In Iraq, There are no information are available on prevalence of β-haemolytic streptococci in patients with COVID-19 suffering from acute pharyngitis, in addition there is no information regarding their virulence and antibiotic resistance patterns. So the aim of this study is to investigate the incidence of β-haemolytic streptococci in patients with coronavirus disease 2019 (COVID-19) and study their antimicrobial susceptibility patterns.

2. MATERIALS AND METHODS

2.1 Patients and Samples collection

This study cross-sectional study was designed to investigate the incidence of Beta-haemolytic streptococci in patients with coronavirus disease 2019 (COVID-19) and study their antimicrobial susceptibility patterns.

A total of (388) clinical samples were collected from Covid-19 patients who suffering by acute pharyngitis for both the sexes (aged 15 years and above). These patients were admitted to the two main hospitals in Hilla city, Iraq (Merjan Teaching Hospital, Hilla Teaching Hospital) during the period extending from October 2020 to February 2021.

2.2 Bacterial Isolates

All beta-hemolytic streptococci isolates were recovered and identified based on their morphology, Gram-staining, and catalase test [9]. All isolates were identified by VITEK 2 automated system (bioMerieux, France) for Gram positive identification test (GPI). To confirm the phenotypic diagnosis, beta-hemolytic isolates were diagnosed using the Vitek 2 system. β-lactamase production was determined by Nitrocefin disk method. These beta-hemolytic isolates were subjected to a preliminary screening for their production of the virulence factors and study their antimicrobial susceptibility patterns. Lancefield grouping of streptococci isolates was carried out using Streptex agglutination test (bioMerieux, France).

2.3 Screening of β-Lactam Resistant Isolates

All beta-hemolytic streptococci were subjected to β-lactam resistance screening test as a phenotypic selection test. Using Kirby Bauer disk diffusion test method with Ampicillin on Muller-Hinton agar plates [10].

2.4 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility patterns of isolates to different antibiotics were determined using disk diffusion test and interpreted according to CLSI guidelines [10]. The following antibiotics were obtained (from Oxiod, UK, and Himedia, India) as standard reference disks as known potency for laboratory use: Ampicillin (10 μg), Oxacillin (5 μg), Rifampin (5 μg), Cefoxitin (30 μg), Clindamycin (10 μg), Penicillin G (10 μg), Ciprofloxacin (10 μg), Gentamycin (10 μg), Teicoplanin (10 μg), Erythromycin (10 μg), Trimethoprim-Sulphamethoxazol (25μg), Linezolid (30 μg) and Tetracycline (10 μg). The susceptibility to penicillin, oxacillin, cefoxitin, and vancomycin was also determined using MIC method according to the breakpoints recommended by CLSI [10].

2.5 Detection of β-Lactamase Production

Nitrocefin diagnostic disk (Fluka, Switzerland) was used to detect the ability of beta-hemolytic streptococci isolates to produce β-lactamase. A number of required Nitrocefin disks were placed into sterile empty Petri dish and moistened with one drop of sterile D.W.; then the disk was holed by sterile forceps and wiped across a young colony on agar plate. The development of a red color in the area of the disk where the culture was applied indicated a positive result [11].

2.6 Determination of minimum inhibitory concentration (MIC).

In our study MIC of beta-hemolytic streptococci isolates were determined with Vitek 2 compact Gram positive identification test (GPI) card and AST-P580 card (bioMerieux, France). MIC was recorded according to the Clinical Laboratory Standards Institute guidelines [10].

2.7 Statistical analysis

All isolates were performed with the Statistical Package for the Social Sciences version 26 (SPSS Inc., Chicago, IL, USA). Numerical data were using the chi-squared and Fisher's exact test and compared of antibiotic resistance with among streptococci isolates in acute pharyngitis infections, P values (< 0.01) was considered significant.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Bacterial Isolations

The present study included collection of (388) throat swab samples from Covid-19 patients suffering from pharyngitis for both the sexes. Depending on PCR results for covid-19 test showed that (128) patients have Negative results it has been deprecated, and (260) patients had positive results. Streptococcal identification was initially determined by colonial morphology, microscopic and biochemical tests. The positive culture was found in the clinical specimens; only (53.8%) isolates belonged to the genus streptococci, of which (9) (6.4%) α-hemolytic, (113) (80.7%) β-hemolytic, and 18 (12.8%) non-hemolytic, while 120 (46.1%) isolates to other bacterial genera (Table 1).

β- hemolytic isolates, those results showed that of (113) β-hemolytic streptococcal isolates, have (59) (52.2) isolates belonged to the group A, 7 (6.1%) isolates belong to group C, 3 (2.6%) isolates belong to group F, and 44 (38.9%) isolates belong to group G. (Table 2). The morphological and biochemical characterization showed that group G belonged to two difference strains of bacteria, *S. anginosus* 5 (4.4%) (small colony) and 39 (34.5%) *S. dysgalactiae* equisimilis (large colony).

This result was similar to that of [12], but it was inconsistent with several authors who found that this group has a higher incidence of infections among throat culture [13]. Of the isolates of SAG, we found a predominance of males over females with ratio of 3 to 2, and this result was compatible with [14] who reached to the same isolation rate, which was identical to the original data in adults. This result was incompatible with other studies, which generally reported almost equal sex distribution for SAG infections among adults [15]. The percentage of SDE strains in our study (13.0%) was higher than that recorded by other studies worldwide [16-18] who isolated *Streptococcus dysgalactiae* subsp. *equisimilis* strains from invasive and non-invasive patients. This result may be due to the increased infections with pharyngitis caused by SDE streptococci strains in the area of the study (Hilla city, Iraq).

Streptococci expressing group G antigen have clearly been linked to outbreaks of pharyngitis. Many of those outbreaks were related to a common source, usually a food product [19]. Epidemiologic and clinical features of acute pharyngitis associated with SDSE are indistinguishable from those with SP with the exception of age and seasonal variation [17]. It is difficult to determine whether they are the causative pathogens of respiratory tract infections when the SAG bacteria are obtained in sputum cultivation because the SAG bacteria are resident members of the flora of both the oral and/or upper respiratory tract. In addition to the culture results, it is therefore important to understand the clinical characteristics and the laboratory findings related to thoracic SAG infection [7].

Table (1) an (2) showing the distribution of β-hemolytic streptococci and others bacterial isolated from throat swab samples and anginosus Lancefield grouping respectively.

3.2 Biochemical and Physiological tests

Results in tables (2) and (3) showed that the isolates belonging to the anginosus group characteristic by gram-positive, catalase-negative, cocci, nonmotile, and have typically small colonies ≤0.5 mm of diameter with a characteristic caramel odor and beta-hemolytic colonies [4]. They were resistant to bacitracin and optochin [20], hydrolyzed arginine and esculine and produced acetoin from glucose (Voges-Proskauer positive), some of the anginosus group isolates can be VP negative. These results were compatible with Mascini and Holm [21].

The anginosus group possessed Lancefield group G and negative CAMP test, produce acid from lactose and raffinose but not manitol, sorbitol, and ribose. [5] reported that *S. anginosus* strains had fermented raffinose or mannitol or both or fermented just either raffinose or mannitol.

Catalase test was used to exclude *Staphylococcus spp*. (positive) from isolates (negative) in this study. CAMP test was used to differentiate between β-hemolytic group B *Strep. agalactiae* (positive) and *Strep. anginosus* group. Also the results of bacitracin and optochin sensitivity test were used to exclude streptococci group A *Strep. pyogenes* and *Strep. pneumoniae* respectively [20]. In the past, classification of Strep¬tococci was based mostly on phenotypic traits such as carbohydrate fermentation and later on 16S rDNA sequencing. Unfortunately, even with the use of 16S rDNA sequencing, some of the strains cannot be clearly classified to previously described streptococcal species [22].

3.3 Serological identifications

Streptex agglutination test was used for identification of Lancefield groups A, B, C, F, and G for tested isolates and the results are shown in (Table 4). The results showed that the isolates possessed Lancefield group antigens G 5 (4.4%) that found in β-hemolytic streptococci. This result was incompatible with the result of [13], who found that SMG represented 1.7% of the group C, 6.9% of the group A, 41.2% of the group F,

and 34.5 for the group G.

3.4 Virulence factors of the bacterial isolates

The virulence factors such as capsule, Lipase, and Protease of streptococcal group *anginosus* isolates were tested in the present study and the results revealed that all *anginosus* group streptococcal isolates were positive foe these tests (Table 5).

One of the factors for delay of organism clearance in vivo is the bacteria capsule [23]. The presence of capsules in *Streptococcus milleri* strains has rarely been reported. However, all isolate in this study were found to be capsule-producers. The presence of a polysaccharide capsule might be a necessary virulence factor in suppurative infections, but not in lethal ones. Brook and his colleagues reported that uncapsulated anaerobic gram-positive streptococci had a stronger ability to produce an abscess than un-encapsulated ones. This result suggests that the capsular material itself might play a key role for bacteria to escape from host defenses [23]. Parts of some studies results showed few un-encapsulated strains produced abscesses, and suggest that the capsule might not always responsible for the pathogenicity to cause the abscess [23].

Regarding Lipase production test, our results were compatible with [24] who reported that lipase in some Strep. milleri group strains was observed, whilst [25] found that all streptococci isolates examined had a negative reaction with this test.

Regarding protease production, our findings were compatible with [26] who found that that Strep. anginosus were protease producers. Protease enzyme secreted outside the cell by a growth process as it builds up in a significantly stable phase in bacteria, and it is one of the most important virulence factors of streptococci [27].

Extracellular protease plays a significant role in the survival and the communication of cells. Several authors reported that clinical isolates are highly pathogenic and responsible for human diseases, as various toxins are secreted [28]. Many species of Streptococcus have the ability to produce disease and virulence. However, within Streptococcus anginosus virulence mechanisms have been identified that permit host cell invasion, the evasion of host immune activities, propagation and colonization of host tissues [29].

3.5 Detection of β-Lactamase Production

Results of Beta-lactamase production in anginosus group isolates revealed that all beta-hemolytic streptococci isolates tested were negative for Nitrocefin disk method. This result was compatible with the results of several authors worldwide [30], who found that all anginosus group isolates were negative for beta-lactamase production tests.

Hence, β-Lactam resistance in isolated viridans streptococci is likely to mediated by altered PBPs which have decreased affinity to antibiotics and then higher concentrations of drugs are thus both required to inhibit the altered PBPs [30]. Considering β-lactamases, they weren't known to occur in Streptococcus species. However, in 1986, streptococci producing β-lactamase were isolated from the sub-gingival plaque of adults with periodontitis [31].

Production of β-lactamases is the most important mechanism of resistance against b-lactam antibiotics. These enzymes constitute a family of proteins that degrade or modify the b β-lactam drugs before they can reach the penicillin-binding protein target sites. Enzymatic degradation of antibiotic by the production of beta-lactamases, or by decreasing the affinity and susceptibility of their target site, the penicillin-binding protein (PBP), by either acquisition of exogenous DNA or by changes in the native PBP genes [32].

Streptococcus viridans are an upper respiratory tract commensal bacterial that developed resistance to penicillin and other β-lactam antibiotics due to alteration in the penicillin-binding protein. In addition, other reports demonstrated that Streptococcus viridans can serve as reservoirs for resistance genes such as mef (E) and mel genes which develop resistant to the macrolide-lincosamide-streptogramin B (MLS(B)) antibiotics [33].

3.6 Antibiotic Resistance of Strep. anginosus isolates

For determination of the antibiotic resistance of Strep. anginosus isolates, 14 various clinically important antibiotics were tested using disk diffusion method. Results of the present study found that most Strep. anginosus isolates were resistant to most of antibiotics used, in particular, β-lactams (100%), like Penicillin, Ampicillin, cefotaxime, and ceftriaxone. This result was compatible with results obtained by several authors [34]. Although no susceptibility to penicillin was found in our isolates, it has been reported by others as in [35].

Antimicrobial resistance is one of the biggest challenges facing modern medicine. Because the management of COVID-19 is increasingly becoming dependent on pharmacological interventions, there is greater risk for accelerating the evolution and spread of antimicrobial resistance [36].

The use of antiparasite, antiviral, antibacterial, and anti-inflammatory drugs for preventing secondary infections in patients with COVID-19 during a prolonged pandemic will inevitably invite future complications, including aggravation of antimicrobial resistance. This is particularly relevant in light of the successive emergence of mutations that increase SARS-CoV-2 fitness, which could be responsible for recurrent COVID-19 waves [37].

Of note, because most of these drugs are used for other target pathogens, we might not only increase resistance in COVID-19 but also face challenges in the treatment of other bacterial and viral infections [36].

With drugs frequently replaced by new therapeutic options, the fear of increased antimicrobial resistance evolution and spread are a reality. There was an increasing demand for and misuse of various drugs in the treatment of COVID-19 irrespective of paucity of scientific evidence [38].

Social media has also played an alarming role in increasing the popularity (both negative and positive) of some drugs, including a number of pharmacological substances with no proven effects. As an additional threat, imperfect drug penetration to patients with COVID-19 might lead to rapid evolution of multidrug resistance [39].

This might be worsened by alterations observed in the gut microbiota of patients hospitalized with COVID-19, which represents a propitious dysbiotic environment for the emergence and dissemination of multidrug resistance [40].

4. CONCLUSION

This study considered a first record in Iraq that accomplished for isolation and characterization of bacteria isolated from Covid-19 patients infected acute pharyngitis. The study concluded that high rate of Streptococcus dysgalactiae equisimilis (SDE) strains were isolated than Streptococcus anginosus group (SAG) in acute pharyngitis.

5. DECLARATIONS

Ethical consideration
The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients (and infants' parents) verbal approval before sample was taken. The study protocol and the patients consent forms were reviewed and approved by the committee on publication ethics at college of Medicine, University of Babylon, Iraq, under the reference No. BMS/0282/016.

Consent for publication
We authorize the journal for publication of any personal or clinical details of participants that compromise anonymity. (Not applicable)

Availability of data and material
All data and material are available upon request

Competing interests
The authors declare that there is no compete or conflict of interests regarding the publication of this article.

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Table 1: Distribution of β-hemolytic streptococci groups and other bacteria isolated from throat swab samples

Bacterial isolates	**No**	**%**	**Total**
Streptococcus Spp.			
β- hemolytic *streptococci*	113	43.4	**140**
α-hemolytic *streptococci*	9	3.4	
Non- hemolytic *streptococci*	18	6.9	
Other genera			
Staphylococcus Spp.	48	18.4	**120**
Fungi Spp.	32	12.3	
Klebsiella Spp.	2	0.7	
Gram negative cocci Spp.	6	2.3	
Gram negative bacilli Spp.	1	0.3	
No growth	31	11.9	

Total	**260**

Table (2) Distribution of S. anginosus Lancefield grouping

β-hemolytic group	No.	%	Total
Group A	59	52.2	**113**
Group B	------	------	
Group C	7	6.1	
Group F	3	2.6	
Group G	44	38.9	

Table (3): Biochemical and physiological tests used for differentiation of Streptococcus anginosus group from other Streptococci:

Isolate	Biochemical and physiological tests			
	β-hemolysis	Bacitracin	Optochin	CAMP test
T 75	+	–	–	–
T 86	+	–	–	–
T 175	+	–	–	–
T 213	+	–	–	–
T 218	+	–	–	–

Table (4): Lancefield grouping of anginosus group by Streptex agglutination test

Isolates	Lancefield group antigens				
	A	B	C	F	G
S. anginosus T	-	-	-	-	+
S. anginosus T	-	-	-	-	+
S. anginosus T	-	-	-	-	+
S. anginosus T	-	-	-	-	+
S. anginosus T	-	-	-	-	+

(Table 5): Virulence factors of anginosus group streptococcal isolates of this study

No. of isolate	Virulence Factors		
	Capsule	Lipase	Protease
T1	+	+	+
T2	+	+	+
T3	+	+	+
T4	+	+	+
T5	+	+	+
T6	+	+	+

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