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Isolation, identification and biochemical profile of pathogenic and opportunistic bacteria from sore throat

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ABSTRACT

Background: *Streptococcus pyogenes* is the most common bacterial agent of sore throat. However, prescription of antibiotics due to clinical diagnosis only could disturb the microbiota and result in antibiotic resistant.

Method: 591 throat swabs samples were obtained from 308 participants. Swabs culture followed by isolation of all types of bacterial growth, which were subjected to morphological, microscopic and biochemical analysis. PCR confirmation performed for *S. pyogenes* isolates.

Results: Among all isolated bacteria (556 isolates), *S. pyogenes* represents the most common (65%), 13% of them were positive by PCR using spy 1258 primer. While other streptococci represent 21% and other bacteria formed 3.7%. Isolation of 12 isolates of Group L Streptococci as well as *Staphylococcus chromogenes* from throat were a remarkable finding in this study. The results also revealed a significant correlation between sore throat caused by Group A Streptococci (GAS) and extraesophageal reflux (laryngopharyngeal reflux, LPR). The low sensitivity of Spy 1258 primer and the variability in S. pyogenes genome sequence necessitate developing new primers according to the environmental and geographical distribution of *S. pyogenes* isolates.

Keywords: Sore throat, *Streptococcus pharyngitis*, Group A Streptococci (GAS) and spy 1258.

Introduction

Regardless of the causative agent, most of the General physicians prescribe antibiotics for patient with sore throat [1], however this will affect the composition of throat flora and will aggravate the status of antibiotics resistance of subclinical *S. pyogenes*, in order to rectify this problem is to investigate and report a full biochemical profile and antibiotic resistance test of all bacterial isolates (specially *S. pyogenes*) present in patient with sore throat and the deviations of those bacteria in any biochemical test.

Acute pharyngeal infection caused by *Streptococcus pyogenes* may lead to myriad illness, a very serious health condition as it may leads to autoimmune rheumatic carditis (rheumatic fever), acute glomerulonephritis and/or skin infections.

Materials and Methods

There were 308 participants in this study. They provided formal and verbal consent. So, additional data collections with questionnaire were performed.
A questionnaire had been designed to investigate the correlation between isolated bacteria and the health condition of patients with sore throat.

Sampling technique:

The samples of respondents included 591 from palatine tonsils and superior oropharynx, swab samples were obtained from patients were attending infirmary and others from healthy people. Collected swaps were directly transferred to the lab for culturing.

Media preparation and sterilization technique: Prior to each experiment Different sterilization techniques were held, autoclaving, dry heat oven, sanitation and UV light in addition to chemical disinfection.

Media description and preparation were performed according to Cowan & Steel laboratory manual.

Cultural and microscopic technique:

Culturing in Blood agar (incubation conditions):

The specimens were inoculated on 5% sheep blood agar and incubated in 10% carbon dioxide enriched atmosphere at 37°C for 24 hours. Subculture technique was required for pure culture.

For *Streptococcus* sp.: The identification of different types of *Streptococcus* sp. was established with the colony morphology and the haemolysis pattern. Then gram's stain was applied for microscopic examination.

Further examinations of biochemical tests including most of differentiating tests (catalase, arginine, bile Esculin, Voges-Proskauer (VP), and sugar fermentation tests) and other sensitivity tests including Bacitracin and Optichin.

For other bacteria: Different primary and secondary identification keys were followed in (Cowan and steel identification manual) including cultural, morphological, microscopic, and biochemical features.

DNA Extraction: This has been done using GF-1 Bacterial DNA Extraction Kit from Vivantis™ as described [2].

PCR detection for S. pyogenes:

PCR:
In the course of experiment PCR played an important role for confirmation of *S. pyogenes* a spy 1258 had been used as primer, although this primer can be found in 13 potential *S. pyogenes* strains (out of more than 200 strains discovered so far), it considered as the most ubiquitous and common primer for molecular detection of *S. pyogenes*. F (AAAGACCGCCTTAACCACCT) and R (TGGCAAGGTAAACTTCTAAAGCA).

Maxime™ PCR PreMix (i-Taq) had been used, and, the protocol of Al-Saadi [3] and Dunne et al. [4] was adopted for PCR and gel documentation system (UV SoloTS Biometra). The PCR product was 407 bp in length.

The thermal cycler program was as follows:

Cycle		Program temperature	Time
Initial Denatutation		95°C	5min.
35 cycle	Denaturation	95°C	30sec.
	Annealing	64°C	30sec.
	Extension	72°C	45sec.
Final extension		72°C	2min.

Results

Data analysis:

Frequency of each microorganism among 556 individual bacterial isolate:

1. Streptococcus pyogenes
S. pyogenes was the most isolated bacteria. There are 396 isolates (Percentage of *Streptococcus pyogenes* isolates among other isolates is 71.2%).

All these isolates were tested by PCR (396) and 72 were positive: Number of confirmed *S. pyogenes* with ideal biochemical result is **27** isolates (6.8%), the number of PCR confirmed *S. pyogenes* with unusual biochemical results is **45** isolates (11.3%), the number of unconfirmed *S. pyogenes* with ideal biochemical result is **146** isolates (37%) and number of unconfirmed *S. pyogenes* with some deviations in biochemical tests is **178** isolates (45%).

2. Other Streptococcus sp.:
Among isolated microorganism there are 4.8% isolates of *Streptococci* group C (*Streptococcus equi and S. disagalactiae*), 2.3% of all isolated bacteria are *Streptococcus spp. Group L*, 1.9% among isolated bacteria are *Streptococcus suis*, and 1.1% are *Streptococcus pneumonia*.

3. Staphylococcus sp:
0.4% of isolates were coagulase negative *Staphylococcus aureus* and 0.9 % was coagulase positive *Staphylococcus aureus*. There was also 0.5% *Staphylococcus chromogenes*.

4. Gram negative rods:
Bacillus mycoides: 0.5%, *Bacillus licheniformis* 0.2%, *Bacillus pantothenicus* 0.4% and 0.2% *Clostridium* sp.

5. Gram negative rods:
0.4% Shwanella putrefaction and 0.2% Pseudomonas alkaligenes.
The first step of recognition was the β-haemolysis on the blood agar, and the colony morphology showed tiny white to colorless, smooth and mucoid characteristic colonies.
S. pyogenes showed a characteristic microscopic shape which is long chain of round to ovoid gram-positive cocci (Figure 1).

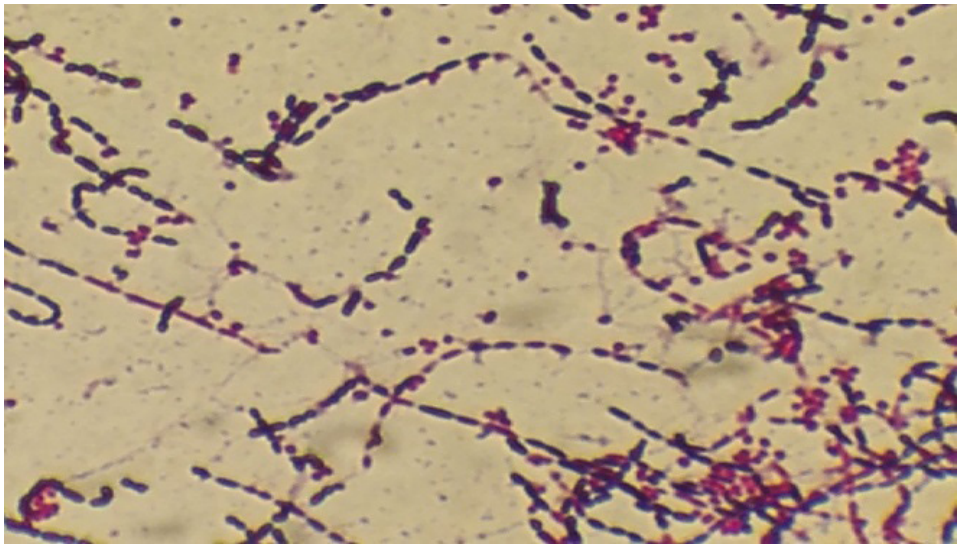

***Figure 1:* *S. pyogenes* in gram's staining method**

The bacitracin sensitivity test result in sensitivity clear zone, on the contrary the optichin exhibited resistance of *S. pyogenes*.

Biochemical tests:

Catalase enzyme test has not been detected in all *Streptococcus* sp. Isolates except few of them, which were further detected as *S. pneumococci*.

Many biochemical tests were unique and tested different from Cowan and Steel, 2005; after confirmation of the *S. pyogenes* isolates identity by PCR, four of these isolates were positive to V.P. test, while *S. pyogenes* is negative according to Cowan and Steel [5]. Also, 17 (23.6%) isolates was Argenine hydrolysis test negative unlike what mentioned in Cowan and Steel, 2005. In addition, 8 (11.1%) isolates was negative to Starch fermentation test opposite to Cowan and Steel [5]. Sorbitol fermentation test is negative for *S. pyogenes* according to Cowan and Steel [5], but it was unexpectedly positive in 9 (12.5%) of the confirmed isolates. Lactose and Trehalose fermentation tests were also negative in 7 (9.7%) and 12 (16.6%) isolates respectively, while they should be positive as in Cowan and Steel, 2005.

The other biochemical tested typical to Cowan and Steel, 2005, like Catalase, Bile Esculin hydrolysis test, Mannitol fermentation test and sensitivity testing for Optochin and Bacitracin.

The result of some of other biochemical tests such as (Arginine, VP and sugar fermentation tests) reveals variations arrange between typical biochemical profile of the reference Cowan and Steel [5] and unusual results that are confirmed by PCR detection.

Statistical analysis:

Among all isolated bacteria from pharynx, *S. pyogenes* identified by biochemical test but was not detected by spy 1258 primer in PCR constitutes 65.1%, other streptococci constitute 21.6% and the PCR confirmed *S. pyogenes* was 13.3%.

Among all isolated bacteria from tonsils, *S. pyogenes* identified by biochemical test but was not detected by spy 1258 primer in PCR constitutes 64.9%, other streptococci constitute 21.1% and the PCR confirmed *S. pyogenes* was 14%.

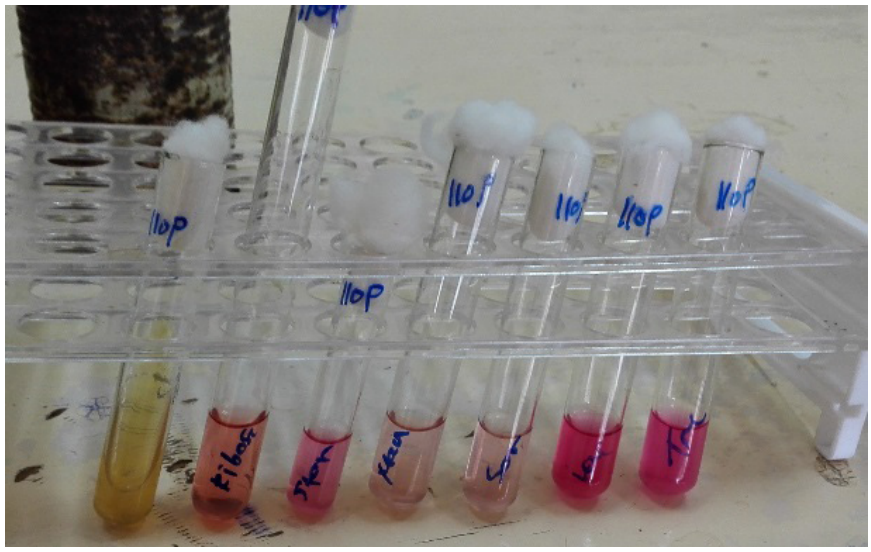

Figure 2: Typical biochemical results of *S. pyogenes*.

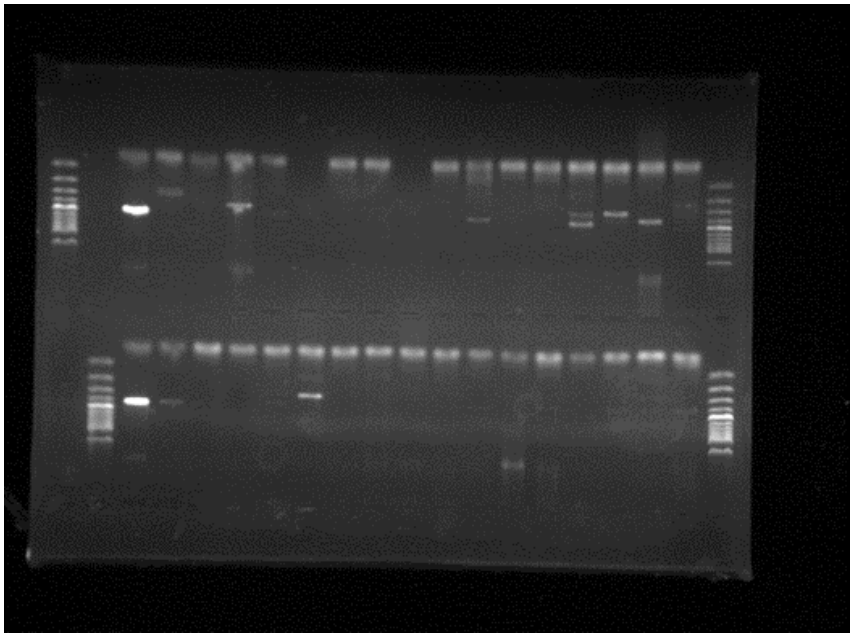

Figure 3: Detection of *S. pyogenes* by using conventional PCR.

Among our patients, 69% were youth between 14 and 40 years old, 11.9% were children under 14 years old, while 13.5% were between 40 and 60 years old and only 5.6% were elderly over 60 years old. The majority was males (58.5%) and the females were 41.5%.

From questionnaire data, patients suffering upper digestive tract symptoms were 61%, while 9.7% had respiratory signs, 5.2% had lower digestive tract illness symptoms, only 4.5% had fever, up to 24.3% ware complaining of signs of general body fatigues. There was 15.3% among all had headache and dizziness. Only 2.6% were diabetic, those with history of smoking were 3% and those with blood hypertension were 2.3%.

Result analysis showed very significant correlation between isolation of Group A Streptococci (*S. pyogenes*) and signs of upper digestive tract (Correlation is significant at the 0.01 level "2-tailed". There was also significant correlation at the 0.05 level (2-tailed) between isolation of Group A Streptococci (*S. pyogenes*) and the presence of respiratory signs.

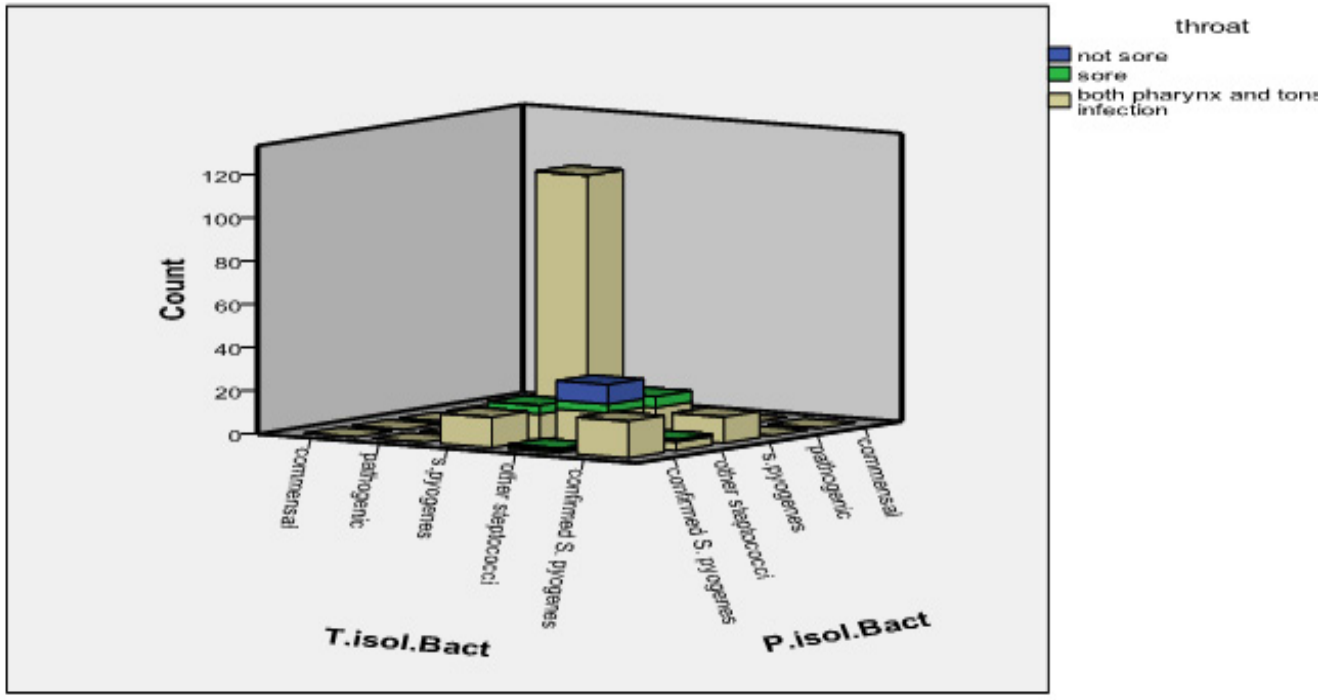

Figure 4: This graph shows the high frequency of isolation *Streptococcus pyogenes* among all other isolated bacteria. *S. pyogenes* represent about 71.2% among about 520 bacterial isolate, obtained and identified from throat in this study.

Discussion

Although the spy 1258 is the most widely common primer used for molecular detection of *S. pyogenes* [6], it can only detect 13 strains out of more than 200 strains and we found so many isolates which are typical for *S. pyogenes* by microscopic characteristics, colony features and biochemical tests results, but couldn't be detected by spy 1258 primer.

Colman and Ball [7] reported that fermentation of lactose was present in 72% and 89% of isolates cultured in Hartley-digest horse and Columbia horse blood agar blood agar respectively. In addition, hydrolysis of arginine was present in 62% and 60% of isolates cultured in Hartley-digest horse and Columbia horse blood agar blood agar respectively. In contrast, all our isolates were cultivated in Sheep blood Agar and 76.4% had arginine hydrolysis activity while 90.3% of them can ferment Lactose.

We had 1 isolated was frankly fermenting Ribose and another 13 isolates tested positive weak to ribose 8 of which was confirmed as *S. pyogenes* by PCR, unlike Colman and Ball, [7] and Cowan and Steel, [5].

Occasionally nonhaemolytic strains of Group A streptococci are isolated [8], but All the isolates of *S. pyogenes* in this study were haemolytic.

M-type 6 and M-type 55 are known to ferment mannitol, and we had many as 38 isolates fermented mannitol weakly (seven of them were confirmed by PCR) and another 64 isolates was strongly positive for Mannitol fermentation test (only three were confirmed by PCR).

Statistical analysis of this study showed very significant correlation between isolation of Group A Streptococci (*S. pyogenes*) and signs of upper digestive tract, According to Koufman et al. [9] sore throat is a symptomatic feature of extraesophageal reflux (laryngopharyngeal reflux LPR) and the golden and dominant feature of LPR is the elevated pH of throat, which is the most common upper digestive tract signs we found, so we concluded that is mainly because of *Streptococcus pyogenes* (71.2% among others), because this bacteria can tolerates and resist acidic conditions up to 4 pH, this remarkable properties of *S. pyogenes* is linked to possession of a virulent factor known as Arginine deaminase ADI system, [10]. (We think that the laryngopharyngeal reflux favors the conditions for secondary Group A streptococci pharyngitis).

There is two problems with the PCR primers for group A Streptococci GAS, the first one is that, now a days there is more than 60 different types of strains of Group A Streptococci (GAS), http://lab.rockefeller.edu/fischetti/mstocks, but the most known ubiquitous PCR primer available can only anneal for about 13 strains according to (MFEprimer-2.0 Report), the second problems is the variability of GAS genome, along with rareness of sequenced genomes from developing countries in Africa. This necessitates developing new primer, possible by sequencing new isolates from Africa, Sudan.

Previous studies showed that *S. equi* is associated with a wide variety of diseases in horses and other animals including humans, share over 80% DNA sequence identity with the important human pathogen *Streptococcus pyogenes* [11]. However, *S. equi* rarely cause human infections, several patients with *S. equi* have animal contacts [12]. Thus, our study goes in line with the two mentioned studies [12] and [11]. Clinical presentations are variable, and may include mild upper respiratory tract signs, and pneumonia [13].

Our findings are in accordance with findings reported by Brandt et al., [14] and Jensen *et al* [15]. They found that *S. dysgalactiae* colonizes the human upper respiratory, gastrointestinal, and female genital tracts and was previously considered nonpathogenic [14]. A full list of *Streptococcus dysgalactiae* strains have been provided by Jensen *et al* [15] from human throat as an original habitat.

The original strains of group L Streptococci were isolated by Hare and Fry [16], Topley and Wilson, [17] and from dogs and pigs [18]. Most of the group L streptococci isolates from Blood and cerebrospinal fluid were believed by Broome *et al* [19] to be nonpathogenic, and there have been very few reports of infection in man [20].

β hemolytic Group L Streptococci were first found in the human throat [21] in patients both with [22] and without [23] signs of respiratory infection.

In our knowledge, since 1987 no report of infection by group L or even colonization in man.
Our result ties well with [24] and [25]. Therefore, they reported that *Streptococcus suis* is a pathogen in pigs that can cause severe systemic infection in humans, infections can be complicated by acute respiratory distress. The number of human *S. suis* cases reported in the literature has increased significantly [24]. Human infection that was also associated with cattle [25].

A similar conclusion that *S. pneumoniae* colonizes the upper respiratory tract was reached by [26], [27] and [28]. They yet also have shown that pneumococcal carriage at the individual level plays an important role in pneumococcal disease dissemination in communities.

Recent studies have identified the oropharynx as a potential site of *Staphylococcus aureus* colonization [29], [30]. Caroline *et al* Results showed that higher throat load than nasal carriage of *S. aureus* [31], this also confirm earlier observations that the oropharynx is an important reservoir for *S. aureus* [30], [32] and [33].

Many healthy people may carry *S. aureus* as a part of their normal microflora in the nose, throat, perineum or skin [34]. The coagulase test usually correlates well with staphylococci pathogenicity [35]. A study showed that staphylococci are widely spread among humans and the most common isolates were coagulase-negative staphylococci [36].

We strongly agree with [37] suggestion about the misidentification of atypical coagulase-negative *S. aureus* strains from clinical specimens could be dangerous and lead to failure of treatment. To our knowledge, no study has yielded in isolation of *Staphylococcus chromogenes*. Therefore, as far as we know no previous research has investigated the isolation of *S. chromogenes* in human throat. Our findings on 0.5% *S. chromogenes* of our isolates at least hint that these bacteria could be colonize human throat.

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