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Isolation, Identification and Biochemical profile of *Streptococcus pyogenes* from Sore throat

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ABSTRACT

Throat infection includes tonsillitis and pharyngitis may be caused by a wide variety of microbial agents, but the most common bacterial cause is group A β hemolytic *Streptococci*. However, prescription of antibiotics due to clinical diagnosis only could disturb the microbiota and result in antibiotic resistant. So that, the aim of this study is to investigate the presence of *Streptococcus pyogenes* as common cause of throat infection.**:** 591 throat swabs samples from tonsils and auropharynx were obtained from 308 participants most of them were healthy. Throat swabs were cultured followed by isolation of *S. pyogenes* and other types of bacteria, which were subjected to morphological, microscopic and biochemical analysis include the biochemical tests and sensitivity tests. PCR confirmation was performed for commonly isolated *S. pyogenes* using spy1258 primer.
Among all isolated bacteria (556 isolates), *S. pyogenes* represented the most common (65%), 13% of them were positive by PCR using spy 1258 primer, while other *Streptococci* represented 21%. Other bacteria formed 3.7% of all isolated bacteria. 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 and extraeosaphageal reflux (laryngeopharyngeal 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.

Introduction:

Regardless of the causative agent, most of the general physicians prescribe antibiotics for patient with sore throat (Kumar *et al*, 2003), 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. This study aimed to investigate the presence of *Streptococcus pyogenes* as a common causative agents of the bacterial sore throat, and to clarify whether there is other types of bacteria suspected to cause throat infections.

Material and Method:

There were 308 participants only 30 of them with respiratory signs, they provided formal and verbal consent. Additional data collections with questionnaire were performed. A questionnaire was 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 was performed according to Barrow and Feltham, (2003).

Cultural and microscopic technique:

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 done 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, 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 Barrow and Feltham identification manual (2003) including cultural, morphological, microscopic, and biochemical features.

DNA Extraction: This was done using GF-1 Bacterial DNA Extraction Kit from Vivantis™ as described by Boonyayatra *et al*, 2018.

PCR detection for *S. pyogenes*:

DNA Extraction:

This was performed using GF-1 Bacterial DNA Extraction Kit from Vivantis™ as described by Boonyayatra *et al*, 2018

PCR:

In the course of experiment PCR played an important role for confirmation of *S. pyogenes* a spy 1258 was 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) was used, and, the protocol of Al-Saadi *et al*, (2015) and Dunne *et al*, (2013) was adopted for PCR and gel documentation system (UV SoloTS Biometra). The PCR product was 407 bp in length.

Table(1) 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 were 396 isolates (Percentage of *Streptococcus pyogenes* isolates among other isolates was 71.2%)

All these isolates were tested by PCR (396) and 72 were positive: Number of confirmed *S. pyogenes* with ideal biochemical result was **27** isolates (6.8%), the number of PCR confirmed *S. pyogenes* with uncommon 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** isolated *S. pyogenes* (44%).

2. Other *Streptococcus sp.:*

Among isolated microorganism there were 4.8% isolates of *Streptococci* group C (*Streptococcus equi and S. disagalactiae)*, 2.3% of all isolated bacteria were *Streptococcus spp. Group L,* 1.9% among isolated bacteria are *Streptococcus suis,* and 1.1% were *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* 0.2%.

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.

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

Biochemical tests:

Catalase enzyme test was not detected in all *Streptococcus sp.* Isolates except 6 isolated *Streptococci* which were further detected as *S. pneumococci*.

Many biochemical tests were unique and tested different from Barrow and Feltham, (2003); after confirmation of the *S. pyogenes* isolates identity by PCR, four of these isolates were positive to V.P. test, while *S. pyogenes* was negative according to Barrow and Feltham, (2003). Also 17 (23.6%) isolates was Argenine hydrolysis test negative unlike what mentioned in Barrow and Feltham, (2003). In addition 8 (11.1%) isolates were negative to Starch fermentation test opposite to Barrow and Feltham, (2003). Sorbitol fermentation test was negative for *S. pyogenes* according to Barrow and Feltham, (2003), but it was unexpectedly positive in 9 (12.5%) of the confirmed isolates. Lactose and Trehalose fermentation tests also were negative in 7 (9.7%) and 12 (16.6%) isolates respectively, while they should be positive as in Barrow and Feltham, (2003).

The other biochemical tests tested typical to Barrow and Feltham, (2003), 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 (Barrow and Feltham, 2005) and unusual results that are confirmed by PCR detection.

Statistical analysis: Among all isolated bacteria from pharynx, *S. pyogenes* that identified by biochemical test but wasn't detected by spy 1258 primer in PCR constituted 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 wasn't detected by spy 1258 primer in PCR constituted 64.9%, other *Streptococci* constituted 21.1% and the PCR confirmed *S. pyogenes* was 14%.

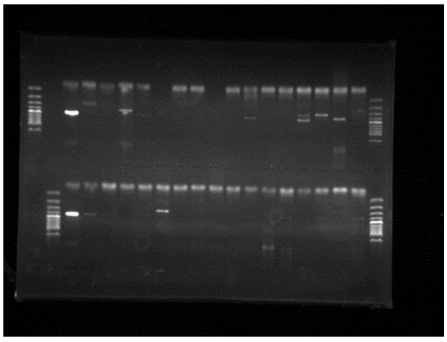

Figure: detection of *S. pyogenes* by using conventional PCR.

Most of our patients were youth between 14 and 40 years old 69%, 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% were complaining of signs of general body fatigues. There were 15.3% among all had head ache 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 respiratory signs.

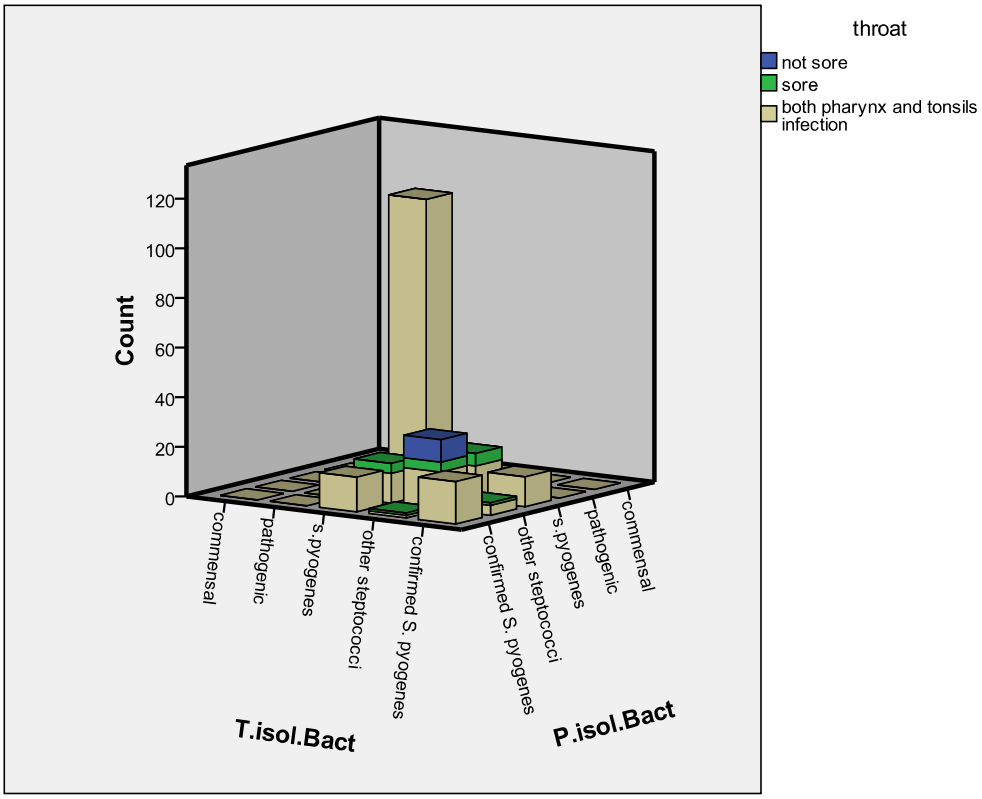

Figure 4: The high frequency of isolation *Streptococcus pyogenes* among all other isolated bacteria. *S. pyogenes* represent about 71.2% among about 520 bacterial isolate, obtained and identified from throat in this study.

Discussion:

Although the spy 1258 is the most widely common primer used for molecular detection of *S. pyogenes* (Liu *et al*, 2005), it can only detect 13 strains out of more than 200 strains. We found so many isolates which were 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, (1984) 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, also 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 isolate that was frankly fermenting ribose and another 13 isolates showed positive weak reaction to ribose, 8 of which was confirmed as *S. pyogenes* by PCR, unlike Colman and Ball, 1984 and Barrow and Feltham, (2003).

Occasionally nonhaemolytic strains of Group A *Streptococci* are isolated (James and McFarland, 1971), 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 (7 of them were confirmed by PCR) and another 64 isolates were strongly positive for Mannitol fermentation test (only 3 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, (2002) sore throat is a symptomatic feature of extraeosaphageal reflux (laryngeopharyngeal 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, (Cotter and Hill, 2003). We think that the laryngeopharyngeal reflux favors the conditions for secondary Group A *streptococcus* 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, including 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* (Holden *et al.,* 2009). However, *S.equi* rarely cause human infections, several patients with *S. equi* have animal contacts (Krisina *et al,* 2016). Thus our study goes in line with Krisina *et al,* (2016). And Holden *et al*, (2006). Clinical presentations are variable, and may include mild upper respiratory tract signs, and pneumonia (Downar *et al*., 2001).

Our findings are in accordance with findings reported by Brandt *et al*, (2009) and Jensen *et al,* (2011). They found that *S. dysgalactiae* colonizes the human upper respiratory, gastrointestinal, and female genital tracts and was previously considered nonpathogenic (Brandt CM. et al, 2009). A full list of *Streptococcus dysgalactiae* strains have been provided by (Jensen A. *et al*, 2011) from human throat as an original habitat.

The original strains of group L *Streptococci* were isolated by Hare and Fry (Hare *et al*, 1938), (Topley and Wilson, 1964) from dogs and pigs (Laughton, 1948). Most of the group L *Streptococci* isolates from Blood

and cerebrospinal fluid were believed by (Broome *et al*, 1976) to be nonpathogenic, and there have been very few reports of infection in man (Barnham *et al*, 1987).

β hemolytic Group L *Streptococci* were first found in the human throat (White *et al*, 1939) in patients both with (Nordlander *et al*, 1975) and without (Olson, 1957) 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 Lun *et al,* (2007) and Ishigaki *et al,* (2009). 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 acute respiratory distress. The number of human *S. suis* cases reported in the literature has increased significantly (Lun *et al*, 2007). Human infection that was also associated with cattle (Ishigaki *et al*., 2009).

A similar conclusion that *S. pneumoniae* colonizes the upper respiratory tract was reached by (Gwaltney et al, 1975; Bogaert *et al*., 2004; Simell et al., 2012). 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 aureu*s colonization. (Bignardi *et al*, 2009), (Marshall *et al*, 2007). Results were showed by (Caroline *et al*, 2011) that higher throat than nasal carriage of *S. aureus* also confirm earlier observations that the oropharynx is an important reservoir for *S. aureus* (Marshall C. *et al*, 2007), (Nilsson *et al*, 2006) and (Hamdan-Partida *et al*, 2010).

Many healthy people may carry *S. aureus* as a part of their normal microflora in the nose, throat, perineum or skin (Narmeen *et al*. 2009). The coagulase test usually correlates well with *Staphylococci* pathogenicity (Markey *et al*. 2013). A study showed that staphylococci are widely spread among humans and the most common isolates were coagulase-negative *Staphylococci* (Sleiniute *et al*, 2015).

We strongly agree with (Mackay *et al*, 1993) 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 cololnise human throat.

The main conclusion that can be drawn is that, *Streptococcus pyogenes* is the common causative agent of bacterial sore throat. The present findings confirm that, there was considerable percentage in the deviation of biochemical tests results of *S. pyogenes*. Many isolates of *S. pyogenes* in our study were typical for microscopic features, colony characteristics and biochemical tests results but couldn't be detected by spy 1258. This necessitate developing of new primer possible by sequencing new isolates from Africa including Sudan.

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