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Identification and genotypic analysis of *Streptococcus pyogenes* isolated from pharyngitis and tonsillitis infected children in IBB city in Yemen

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Streptococcus pyogenes **is beta-hemolytic bacterium that belongs to Lancefield serogroup A, also known as group A streptococci (GAS). GAS causes a wide variety of life threatening diseases including pharyngitis and tonsillitis disease. A total of 93 throat swab and 93 blood specimens were collected from pharyngitis and tonsillitis infected children in different schools in Almashana, IBB city in Yemen. All isolates were diagnosis by using two methods: throat swab culture and serological test (ASO test). The result shows difference between throat swab culture method and blood specimens serology method (ASO). 38 isolates (40.8%) for throat swab culture and 60 isolates (64.5%) for blood specimens serology method (ASO) were positive in 93 total isolates. All isolates were characterized by their antimicrobial susceptibility test to different antibiotics including penicillin G, chloramphenicol, erythromycin, clindamycin and streptomycin. All isolates were sensitive to penicillin G and chloramphenicol. Most isolates (61.3%) showed a high degree of resistance to erythromycin. Resistance to clindamycin and streptomycin were observed in 34.4 and 46.2% of isolates, respectively. The 93 isolates were subjected to fingerprinting by random amplified polymorphic DNA (RAPD) analysis. Amplification of genomic DNA of GAS was performed with three primers. The results reveal that approximately 36 different amplified DNA fragments (rapdemes) were observed in all, of which 21 (58.3%) were shared and 15 (41.7%) unshared or unique rapdemes. RAPD analysis provides a practical alternative for genomic typing of GAS. It can be recommended for the typing of GAS, especially if used in parallel with serotyping.**

Key words: Group A streptococci (GAS), IBB city, antistreptolysin-O (ASO) test, pharyngitis, random amplified polymorphic DNA (RAPD).

INTRODUCTION

The Gram positive bacterium *Streptococcus pyogenes* has the ability to cause disease, both relatively mild local diseases as well as life-threatening invasive or systemic disease. *S. pyogenes* is beta-hemolytic bacterium that

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belongs to Lancefield serogroup A, also known as group A streptococci. They are classified based on their hemolytic capacity (α-, β-, γ-hemolysis) and the antigenicity of a carbohydrate occurring in their cell walls (Lancefield antigen) (Murray et al., 2002).

Group A streptococcus (GAS) elaborate several extracellular products include Streptolysin O (SLO) which derives its name from its oxygen lability. It is a member of a family of highly conserved pore-forming cytolysins. In addition to its effect on erythrocytes, it is toxic to a variety of cells and cell fractions, including PML, platelets, tissue-culture cells, lysosomes (Madden et al., 2001). The antistreptolysin-O (ASO) test is used to determine recent streptococcal infection and post streptococcal complications including rheumatic fever and glomerulonephritis. The presence and level of ASO antibodies in human serum directly reflects the extent and degree of infection. Elevated levels of ASO may also be present in other conditions including scarlet fever, acute rheumatoid arthritis, tonsillitis and various other streptococcal infections as well as in health carriers (Johnson et al., 1996).

S. pyogenes is the cause of many human diseases, ranging from mild superficial skin infections to life-threatening systemic diseases. Pharyngitis is the most common clinical manifestation of *S. pyogenes* infection.

The diagnosis of GAS is necessary to demonstrate evidence of recent infection. This can be established in one of the three ways: (a) positive throat culture, (b) positive group A streptococcal antigen test from throat swab, or (c) elevated or rising serum antistreptococcal antibody titer. The antistreptolysin-O (ASO) titer is a commonly used streptococcal antibody test in establishing the diagnosis of these bacteria (Kaplan et al., 1992; Seppala et al., 1994).

Therefore, a number of genotyping techniques like ribotyping (Shundi et al., 2000), pulse field gel electrophoresis (Gonzalez–Rey et al., 2003), random amplified polymorphic DNA (RAPD) analysis (Gonzalez–Rey et al., 2003; Fica et al., 2003) are being applied worldwide for typing of GAS isolates. RAPD typing was found to be a simple, rapid, effective method for the epidemiological investigation of any outbreak for the study of *S. pyogenes* infections (Cleary et al., 1988).

The present work aimed to: 1) Determine the prevalence of Streptococcus group (A) among pharyngitis and tonsillitis infected children in different schools in Almashana region in IBB city in Yemen by using two methods; throat swab culture and serological test (ASO test); 2) Determine the susceptibility of all bacteria isolates to various antibiotics; 3) Fingerprint the genetic variation among all bacteria isolates by using the RAPD.

MATERIALS AND METHODS

Bacterial isolates

A total of 93 throat swab and blood specimens were collected from same children patients (aged 6 to 15 years) from Almashana in IBB city during April 2012 to December 2012 (during the winter). The isolates were identified as *S. pyogenes* by colony morphology, β-haemolysis on sheep blood agar, and Lancefield grouping by using a commercially available agglutination technique (Seroiden Strepto kit) and bacitracin disc test (0.04 U, Taxo A, BBL Microbiology system) (Bisno, 1996; Gerber, 1984).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by Kirby-Bauer disk diffusion according to the guidelines recommended by the CLSI (1990); the examined antibiotic disks contained penicillin, chloramphenicol, erythromycin, clindamycin and streptomycin (HiMedia) (Snow et al., 2001).

Molecular fingerprinting for the *S. pyogenes* 93 bacterial isolates

Genomic DNA was extracted from overnight cultures using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. Random amplified polymorphic DNA was performed on all the isolates using three random primers in three separate RAPD-PCR tests in an effort to type *S. pyogenes* isolates: Primers EZ (5'-GCATCACAGACCTGTTATTGCCTC3'), OPA14 (5'-GACCGCTTGT-3') and OPA13 (5'-CAGCACCCAC-3') [Operon Technologies, Inc., Atlanta, USA] (Martin and Single, 1993; Micheli et al., 1993). RAPD-PCR was carried out in a 25 µl reaction mixture containing 2.5 µl 10x buffer, 0.2 mM dNT'Ps, 0.5-1 µl (100 pmol) primer, 2 U Taq DNA polymerase, 3.0 mM $MgCl_2$, 50 ng DNA template and nuclease-free water. Amplification conditions consisted of denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 1 min, annealing at 43°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were detected in 2% agarose gel. A 100-bp ladder was used in each gel as a DNA fragment size marker. After staining with ethidium bromide, PCR products were photographed with UV light. The arbitrarily primed PCR patterns were examined by direct visual comparison of the patterns and the 100-bp ladder. In repeated analyses of selected GAS isolates, the results obtained with RAPD analysis were found to be reproducible (Martin and Single, 1993; Micheli et al., 1993; Penner et al., 1993).

RESULTS

Identification of throat swab by culture

In this study, 93 throat swab were collected from children to investigation the GAS from different school of AL-Mashana district in IBB city (Table 1). These specimens were collected according to the recommendation of the public health official in IBB Province. Table 1 shows the distribution of GAS at Almashana district. These specimens were collected in six schools of Almashana district as follows: 1) AL-thawrh, 2) AL-shaab, 3) Khaled ben alwaleed, 4) Labusa society, 5) Al-kimma and 6) Kl-manahel.

The result of throat swab culture was reported from a total of 93 specimens, 38 (40.8%) only represent *S. pyogenes*. This result is based on β-haemolytic and bacitracin disc sensitivity. Whereas 55 (59.2) was

Table 1. Disc sensitivity test against the isolated specimens. The results of throat specimens are shown for culture in blood agar.

Result	Diagnosis by culture In B.A	Ratio (%)
Positive	38	40.8
Negative	55	59.2
Total	93	100

Table 2. Blood agar culture results among different schools

S/N	Name of school	Number of specimens	Diagnosis by culture			
			Positive	Ratio (%)	Negative	Ratio (%)
1	al-Kimma	5	2	40	3	60
2	Khaled ben alwaleed	20	8	40	12	60
3	AL-shab	24	10	41.6	14	58.3
4	AL-thawrh	24	12	50	12	50
5	Labusa society	17	5	29.4	12	70.5
6	al-Manahel	3	1	33.3	2	77
Total	6	93	38	40.8	55	59.2

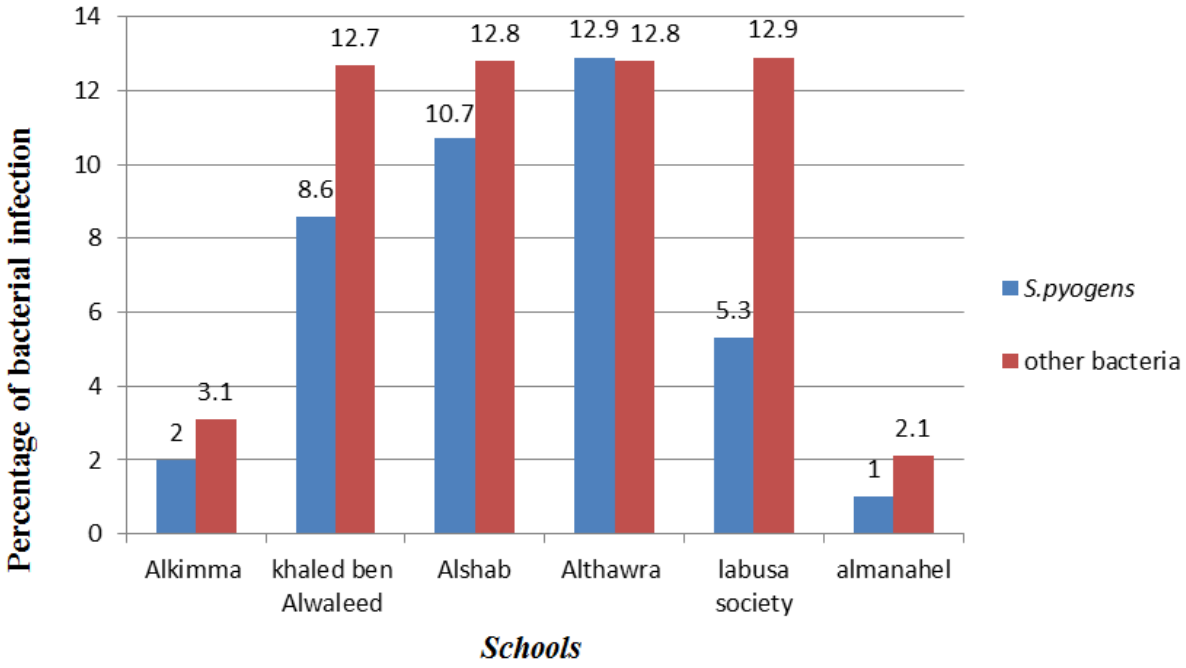

Figure 1. Presence of bacterial infection among school-children in the AL-Mashana district.

negative and represent other bacteria.

The result reported high prevalence of GAS in AL-thawrh school, twelve cases from 24 specimens (50%), then AL-shab showed 10 cases from 24 speci-mens (41.6%), followed by Khaled ben Alwaleed where 8 from 20 in Labusa society, five from 17 was observed as shown in Table 2 and Figure 1.

Identification of results by ASO test

ASO test was performed for 93 blood specimens and the result show only 60 (64.5) were positive and 33 (35.5)

Table 3. Results of ASO test for blood specimens.

Result	Diagnosis by ASO	Ratio (%)
Positive	60	64.5
Negative	33	35.5
Total	93	100

Table 4. ASO test results among different schools.

S/N	Name of school	Number of specimens	Diagnosis by ASO(Serology)			
			Positive	Ratio (%)	Negative	Ratio (%)
1	Al-kimma	5	2	40	3	60
2	Khaled ben alwaleed	20	14	70	6	30
3	AL-shab	24	16	66.6	8	33.3
4	AL-thawrh	24	16	66.6	8	33.3
5	Labusa society.	17	11	64.7	6	35.2
6	Al-manahel	3	1	33.3	2	66.6
Total	6	93	60	64.5	33	35.5

Table 5. Comparison of two methods.

Result	Diagnosis by culture	Percent (%)	Diagnosis by ASO	Percent (%)
Positive	38	40.8	60	64.5
Negative	55	59.2	33	35.5
Total	93	100	93	100

Table 6. Susceptibility pattern of *S. pyogenes* isolates.

Antibiotics type*	P	C	E	Cl	S
No. of *S. pyogenes* isolates resistance (%)	Nil (0)	Nil (0)	57 (61.3)	32 (34.4)	43(46.2)

*P, penicillin; C, chloramphenicol; E, erythromycin; Cl, clindamycin; S, streptomycin.

negative as show in Table 3. The result reports high prevalence of GAS in Khaled ben Alwaleed, fourteen specimens out of the 20 specimens (70%), then AL-thawrh and Al-Shab schools with 16 specimens out of twenty four specimens (66.6%), followed by Labusa society with 11 out of 17 specimens (64.7%) and Al-kimma with two out of five specimens (40%); finally, Al-manahel with one out of three specimens (33.3%) as show in Table 4.

Relationship between throat swab culture and serology diagnostic test

The result shows difference between the diagnosis by throat swab culture method and blood specimens serology method. ASO positivity ratios are very high. Thirty eight (38) specimens were positive from 93 total specimens (40.8%) for culture method and 60 specimens were positive from 93 total specimens (64.5%) for serology method (ASO) as show in Table 5.

Susceptibility of *S. pyogenes* to antibiotics

The susceptibility of the all bacteria isolates (93) to antibiotics were tested by disk diffusion methods (Table 6). All clinical *S. pyogenes* were sensitive to penicillin G and chloramphenicol, 61.3% (57) were resistant to erythromycin.

Resistance to clindamycin and streptomycin were

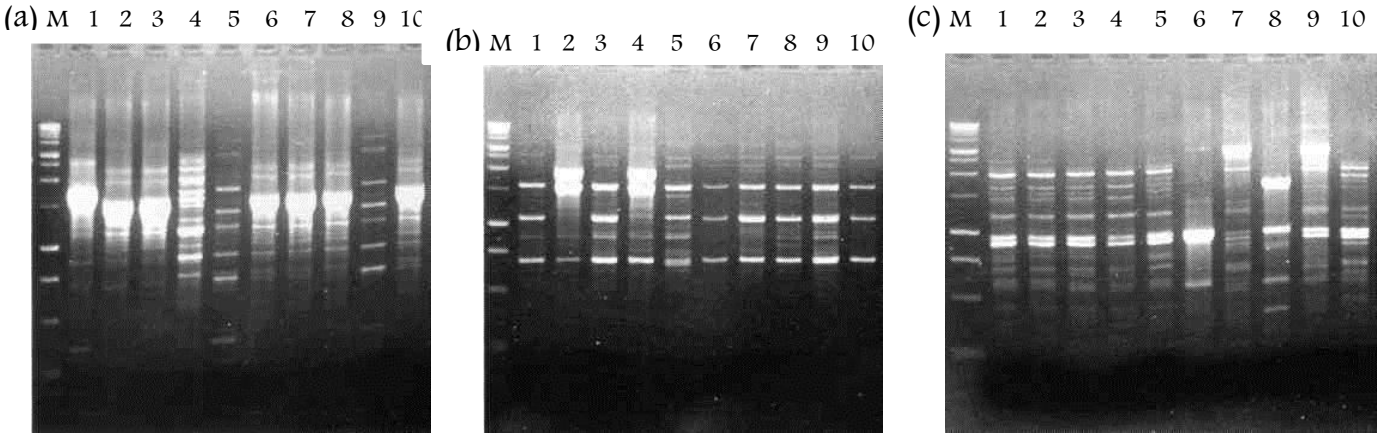

Figure 2. RAPD-PCR amplification pattern of different subtypes of GAS isolates with (a) OPA13 primer, (b) OPA14 primer, (c) EZ primer. Lane M: 1 kb DNA molecular weight marker (Promega, USA), Lanes 1 to 10 : representative clinical isolates of GAS.

observed in 34.4 and 46.2% of isolates, respectively.

RAPD analysis

RAPD analysis of 93 isolates was carried out using 3 different primers. RAPD fingerprinting showed highly polymorphic nature of the isolates. The results revealed that approximately 36 different amplified DNA fragments (rapdemes) were observed in all, of which 21 (58.3%) were shared and 15 (41.7%) unshared or unique rapdemes representing a 41.7% overall genetic heterogeneity among the isolates. The primer OPA14 and OPA13 revealed more discrimination as compared to EZ primer. As shown in Figure 2a-c, OPA14 and OPA13 resulted in 12 to 13 patterns, and EZ9 patterns. Hence, the primer OPA14 and OPA13 provided the higher level of discrimination.

DISCUSSION

GAS has continued to be a major health problem in developing and industrialized countries, especially since the outbreaks that emphasized the need for practitioners to remain vigilant and to maintain prevention efforts (David, 1998), In contrast to the developed countries, this disease has remained a significant problem for years in developing countries (Zangwill et al., 1991); furthermore, the overall quality of epidemiological data from developing countries is poor, particularly with respect to research documenting the incidence of GAS (Carapetis, 2004; Cunningham, 2000).

Diagnosis of GAS based on anti-streptolysin O alone is not reasonable unless throat culture is not performed because ASO test represent the former GAS infections, not acute infections and cross infection for ASO (IgG antibody) (Atatoa-Carr et al., 2008; Gerber, 1989). Serological diagnosis of group A streptococcal infection is based on immune responses against the extracellular products streptolysin O which induce strong immune responses in the infected host. Anti-streptolysin O is the antibody response most often examined in serological tests to confirm antecedent streptococcal infection (Gerber, 1989; Gerber and Shulman, 2004). Numerous studies have demonstrated that the currently available rapid streptococcal tests have a sensitivity of 70-90% when compared with standard throat cultures. In contrast to their relatively low sensitivity, the specificity of these rapid tests has consistently been 90-100%. Therefore, if a rapid streptococcal test result is positive, a culture is not necessary, and appropriate antibiotic therapy is immediately initiated. However, when a negative result is encountered, a standard throat culture should always be obtained.

In this study, two methods were used to diagnose *S. pyogenes*: ASO test and throat swab culture. We found the ASO positivity ratios are very high but throat swab culture was more accurate than ASO test because the ASO test represent chronic infections or carrier of *S. pyogenes*, also due to the cross infection for ASO (IgG antibody). Thirty-eight (38) specimens were positive from 93 total specimens (40.8%) for culture method and 60 specimens were positive from 93 total specimens (64.5%) for serology method (ASO).

ASO test currently available were used when compared with blood agar plate cultures (Tanz et al., 2009; Johnson and Kaplan, 2001). False-positive test results are highly unusual, and therefore therapeutic decisions can be made with confidence based on a positive test result (Gerber and Shulman, 2004; Tanz et al., 2009; Johnson and Kaplan, 2001). Unfortunately, the sensitivity of most of these tests is 70-90%, when compared with blood agar plate culture (Gerber and Shulman, 2004; Tanz et al., 2009).

The culture result reported high prevalence of GAS in

AL-thawrh, 12 cases from 24 specimens (50%), then AL-Shab with ten cases out of 24 specimens (41.6%), followed by Khaled ben Alwaleed with eight out of twenty specimens (40%) and Labusa society with five out of seventeen specimen (29%). Whereas, ASO test result reported high prevalence of GAS in Khaled ben Alwaleed school with fourteen out of twenty specimens (70%), then AL-thawrh and Al-Shab schools with sixteen out of twenty four specimens (66.6%), followed by Labusa society with eleven out of seventeen specimens (64.7%) and Al-kimma with two out of five specimens (40%); finally, Al-manahel with one out of three specimens (33.3%) as show in Table 4 and Figure 3. The highest percentage of *S. pyogenes* infection in Althawra and Khaled ben Alwaleed school may be due to crowding of students in these schools.

A number of antibiotics showed to be effective in treating GAS pharyngitis (Table 6). These include penicillin G and chloramphenicol. All isolates were sensitive to penicillin G and chloramphenicol, similar result was reported in USA and other countries (Shulman et al., 2012). Most of the isolates (68.8%) showed a high degree of resistance to tetracycline followed by 61.3% resistant to erythromycin. Resistance to clindamycin and streptomycin were observed in 34.4 and 46.2% of isolates, respectively; a similar study was reported in Korea (Sook et al., 2007). Clindamycin resistance among GAS isolates in the United States is approximately 1%, and this is a reasonable agent for treating penicillin-allergic patients (Tanz et al., 2004).

RAPD method, based in PCR, was used for typing of GAS and it was found to be highly discriminatory. As reported earlier, selection of primers, optimization of PCR condition and combination of different primers play an important role in discriminating the isolates by RAPD (Micheli et al., 1993; Penner et al., 1993; Matthews, 1993). Hence, three arbitrarily selected primers (OPA14, OPA13 and EZ) were tested. The majority of arbitrary primers used, produced distinctly reproducible patterns in all the isolates studied. The results revealed that approximately 36 different amplified DNA fragments (rapdemes) were observed in all, of which 21 (58.3%) were shared and 15 (41.7%) unshared or unique patterns. According to these results, we have found the relationship between genetic diversity and antibiotic susceptibility of *S. pyogenes* isolates.

In conclusion, under carefully controlled spreading of the *S. pyogenes* infection, the results indicate the high prevalence of resistant *S. pyogenes* in patients for tetracycline and erythromycin 68.8 and 61.3% isolates, respectively. Therefore, the choice of the suitable anti-biotic for the treatment of *S. pyogenes* infections should be made carefully to avoid the emergence of resistance isolates. The findings of this study demonstrated the benefit of RAPD fingerprinting (genotype) in comparison with phenotype methods in identifying and characterizing GAS isolates obtained from pharyngitis cases.

Conflict of interests

The authors have not declared any conflict of interests.

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