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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Journal of Microbiology and Antimicrobials

Full Length Research Paper

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

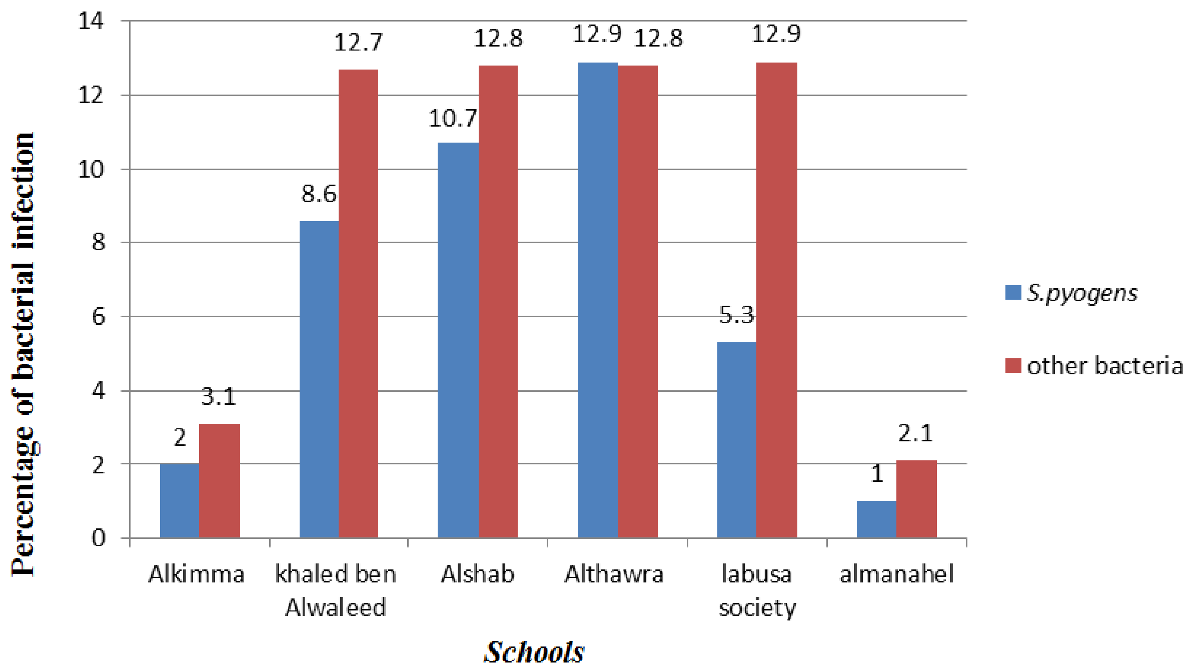

Figure 1. Presence of bacterial infection among school-children in the AL-Mashana district.

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

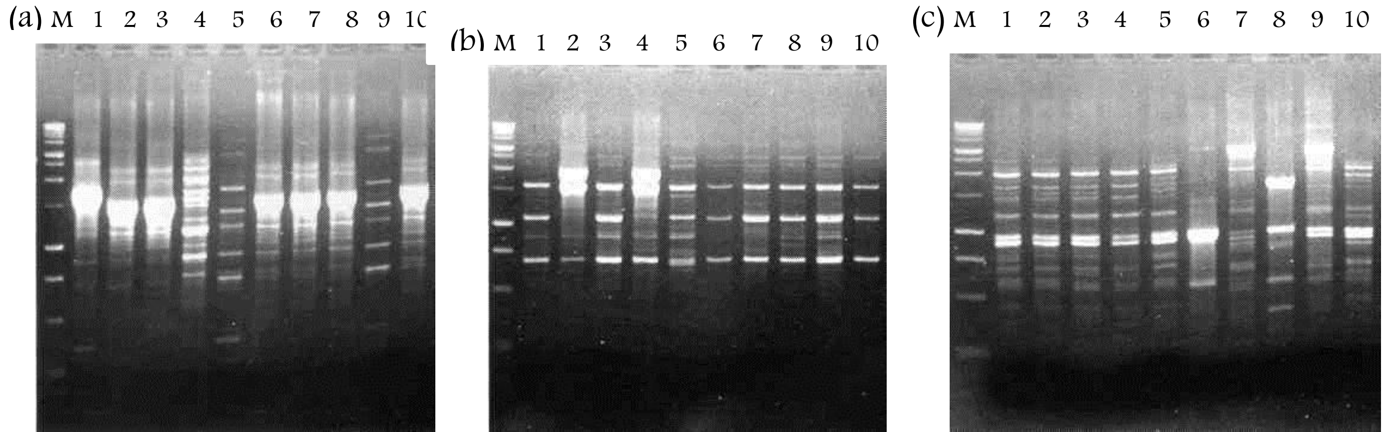

Figure 2. RAPD-PCR amplification pattern of different subtypes of GAS isolates with (a) OPA13 primer, (b) OPA14 primer, (c) EZ primer. Lane M: 1 kb DNA molecular weight marker (Promega, USA), Lanes 1 to 10 : representative clinical isolates of GAS.

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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Full Length Research Paper

In vitro evaluation of virulence factors of *Candida* species isolated from oral cavity

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The yeast *Candida* is a normal flora of the skin and the mucous membrane and it then becomes pathogen in immunocompromised people. Various virulence factors are contributing to establishment of the infection in the host. Adherence of the pathogen to host tissues, yeast-hyphal transition and extracellular hydrolytic enzymes secretion are important virulence factors of *Candida* species. These hydrolytic enzymes play important roles in pathogenicity of *Candida* infection. The present study was conducted with an aim to determine in *vitro* phospholipase, proteinase, haemolysin, esterase activities and biofilm formation in oral *Candida* isolates. A total of 38 *Candida* species were isolated from oral cavity of patients with symptoms of oral candidiasis. The specimens were identified by standard mycological techniques up to species level and were investigated for production of hydrolytic enzymes and biofilm formation. Phospholipase activity was in 52.6% of isolates, 86.8% produced proteinase and haemolysin activity was seen in 63.1%, esterase activity was demonstrated in 50% of isolates, 78.9% of *Candida* isolates showed biofilm formation. *Candida albicans* showed more extracellular hydrolytic enzyme activity, whereas, *Candida tropicalis* showed more biofilm formation. Both the C. *albicans* and Non-*albicans Candida* (NAC) species are capable of producing extracellular hydrolytic enzymes and biofilm formation.

Key words: *Candida* species, virulence factors, extracellular hydrolytic enzymes, biofilm formation.

INTRODUCTION

The dimorphic fungus *Candida* sp. can respond rapidly to environmental changes, and this flexibility could allow this organism to take advantage of impaired immunity and facilitate establishment of disease. Although *Candida* is normal flora of skin and mucous membranes of healthy people, they cause infections that range from superficial infections to life-threatening systemic infections in immuno-compromised and immunosuppresed people. Various virulence factors are contributing to the colonization and pathogenicity of *Candida* infection, including the expression of adhesins and invasins on the cell surface, yeast-hyphal morphogenetic transformation, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes (Francois et al., 2013).

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Among the various factors, extracellular hydrolytic enzymes of which SAPs (secreted aspartyl proteinases) are considered to be one of the major virulence factors play a major role in over growth of the *Candida*, since these enzymes pave way to adhere, penetrate and for tissue invasion (Schaller et al., 2005).

Aspartyl proteinases are secreted by pathogenic species of *Candida in vivo* during infection. The enzymes are secreted *in vitro* when the organism is cultured in the presence of exogenous protein (usually bovine serum albumin) as the nitrogen source. Phospholipase enzymes, another important virulence factor, are associated with the function related to host cell damage, adherence and penetration (Kabir et al., 2012). They destroy phosphorlipids in the host cell, hence results in the damage to the cell membrane, cell lysis and facilitating tissue invasion (Bhat et al., 2011). There are four secreted phosphorlipase, A to D (PLA, PLB, PLC and PLD). Their activity is very high during tissue damage because these enzymes carry out hydrolysis of one or more ester linkages of glycerophospholipids on the host cell membrane. Furthermore, *Candida albicans* is able to acquire elemental iron from host tissues through haemolysin production, iron chelators (siderophores) and iron-transport proteins, which then is used by the fungus for metabolism, growth and establishment of infection in humans (Weinberg, 1978; Almeida et al., 2009). The ability of *C. albicans* to utilize hemoglobin as an iron source was first described by Moors et al. (1992). In humans, iron is found in some proteins, including hemoglobin (a component of erythrocytes). The first step of *C. albicans* infection *in vivo* involves binding to erythrocytes through receptors of the complement system. Next, *C. albicans* produces a hemolysis factor that induces lysis of the erythrocyte. This factor most likely corresponds to a mannoprotein bound to the cell surface of the fungus (Almeida et al., 2009; Watanabe et al., 1999). Almeida et al. (2008) observed that *C. albicans* caused greater damage to oral epithelial cells containing elevated concentrations of ferritin as compared to cells with lower iron levels.

Traditionally, antifungal drugs were developed either to inhibit or to kill the pathogenic organism. Because of the development of anti fungal resistance to various anti fungal drugs by the pathogen, there is a need to develop new antifungal strategy which specifically targets the virulence factors. The study of virulence factors provides a way to specifically target virulence of *Candida* sp. Therefore, the present study was conducted with an aim to determine *in vitro* phospholipase, proteinase, haemolysin, esterase activities and biofilm formation in oral *Candida* isolates.

MATERIALS AND METHODS

Collection of samples

A total of 38 clinical samples were obtained from patients attending Tertiary Care Hospitals, Coimbatore, TamilNadu, India, with symptoms of oral candidiasis. Oral swabs were collected with all aseptic precautions using sterile swabs from tongue and buccal mucosa by gently rubbing over the lesional tissue. The swabs were then dispensed in a test tube containing sterile SDA broth. Then, they were identified by Gram staining, lactophenol cotton blue test, germ tube test, carbohydrate fermentation test, urease test, morphology on HiCHROM agar and corn-meal agar with Tween-80. Culture on *Candida* HiCHROM agar was for the species identification whereas corn-meal agar was for demonstration of chlamydospores.

Preparation of the yeast suspension

Yeast suspension was prepared from the isolates. A small amount of stock culture was inoculated on Sabouraud dextrose agar (SDA) containing chloramphenicol by using a sterile loop and incubated at 37°C for 24-48 h. Then, the yeasts were harvested and suspended in sterile phosphate buffered solution (PBS) at turbidity equal to optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain 1×10^7 yeast cells/ml.

Determination of phospholipase activity

The extracellular phospholipase activity of *Candida* sp. was determined by growing them on egg yolk agar and measuring the size of zone of precipitation by the method prescribed by Samaranayake et al. (1984). The egg yolk medium was prepared according to Tsang et al. (2007) and Mohandas (2011). A 10 ml suspension of yeast cells per ml saline was placed on the egg yolk medium and left to dry at room temperature. The culture was then incubated at 37°C for 48 h, after which the diameter of the precipitation zone around the colony was determined. Phospholipase activity was measured by dividing colony diameter by the diameter of the precipitation zone (pz) around the colony formed on the plate. The pz was scored as follows: pz = 1, negative phospholipase activity; pz = 0.64-0.99, positive phospholipase activity; and pz ≤ 0.63, very strong phospholipase activity (Price et al., 1982). The lower the pz value, the higher the enzymatic activity.

Determination of proteinase activity

Extracellular proteinase activity of *Candida* sp. was analyzed in terms of bovine serum albumin (BSA) degradation by the technique described by Staib et al. (1965). To determine proteinase activity, bovine-serum albumin agar (0.1% KH_2PO_4, 0.05% $MgSO_4$, 4% agar and 1% bovine serum albumin) was employed (Tsang et al., 2007). The final pH was adjusted to 4.5. Ten microliters of previously prepared yeast suspension was inoculated into the wells punched onto the surface of the medium onto the plates; these were then incubated at 37°C for 10 days in both aerobic and anaerobic conditions. After incubation, the plates were fixed with 20% trichloracetic acid and stained with 1.25% amidoblack. Decolourization was performed with 15% acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that could not be stained with amidoblack, indicated degradation of the protein. The presence of proteinase activity was determined by the formation of a transparent halo around the yeast colonies. The diameter of unstained zones around the well was considered as a measure of proteinase production. The proteinase activity (Prz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. Proteinase activity (Prz) was determined by the method described by Price et al. (1982). Prz was scored as follows: Prz = 1, negative proteinase activity, Prz =

Table 1. Various virulence factors exhibited by *Candida* sp.

Organism	No. of isolates	Phospholipase (P_z) activity No. (%)	Proteinase (Pr_z) activity No. (%)	Haemolytic (H_z) activity No. (%)	Esterase activity No. (%)	Biofilm formation No. (%)
C. albicans	22	22 (100)	20 (90.9)	14 (63.6)	13 (59)	19 (86.3)
C. tropicalis	9	4 (44.4)	9 (100)	7 (77.7)	3 (33.3)	8 (88.8)
C. krusei	6	2 (33.3)	3 (50)	2 (33.3)	2 (33.3)	2 (33.3)
C. glabrata	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Total	38	20 (52.6)	33 (86.8)	24 (63.1)	19 (50)	30 (78.9)

0.64-0.99, positive proteinase activity; and Prz ≤ 0.63, very strong proteinase activity. Thus, a low Prz indicated high production of the enzyme.

Determination of haemolysin activity

To determine hemolytic activity, SDA (Oxoid) containing 7% sheep blood and 3% glucose with a final pH adjusted to 5.6 ± 0.2 was employed. Ten microliters of yeast suspension was inoculated onto plates; these were then incubated at 37°C for 48 h in aerobic condition. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive hemolytic activity (Manns et al., 1994). The ratio of the diameter of the colony to that of the translucent zone of haemolysis (mm) was used as the haemolytic index (Hz value).

Determination of esterase activity

To determine esterase activity, Tween-80 opacity test medium was used. The test medium with a pH adjusted to 6.8 consisted of 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$ and 1.5% agar. After cooling the medium (50°C), 0.5% of Tween-80 was added. Ten microliters of previously prepared suspension was carefully deposited on the Tween-80 opacity test medium. This was then incubated at 30°C for 10 days in aerobic conditions. Esterase activity was considered as positive in the presence of a halo pervious to light around the inoculation site (Slifkin, 2000).

Determination of biofilm formation

Candida sp. was evaluated for biofilm formation using the method described by Melek et al. (2012). Sterile 96-well microplates were used to evaluate biofilm formation. Yeast culture was inoculated using a loop into a tube containing 2 ml of brain heart infusion broth (BHIB) medium with glucose (0.25%) and incubated at 37°C for 24 h. Then, all tubes were diluted at a ratio of 1:20 by using freshly prepared BHIB. From this final solution, 200 µL was placed into the microplate, which was then incubated at 37°C for 24 h. After incubation, the microplate was rinsed with PBS 3 times and then inverted to blot. Then 200 µL of 1% crystal violet was added to each well, followed by incubation for 15 min. After incubation, the microplate was again rinsed with PBS 3 times. Then 200 µL of ethanol : acetone mixture (80:20 w/v) was added to each well. They were read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader and the OD was recorded for each well. Three wells were used for biofilm formation and the arithmetical mean of 3 readings was used in analysis. *Enterococcus faecalis* ATCC 29212 was employed as the control strain. Sterile BHIB without microorganism was employed as the negative control. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative.

RESULTS AND DISCUSSION

Of the 38 isolates, 22 isolates (58%) were identified as *C. albicans*, while 9 (24%) were identified as *Candida tropicalis*, 6 (16%) as *Candida krusei*, 1 (2%) as *Candida glabrata* (Table 1). These isolates were studied for the production of hydrolytic enzymes such as phospholipase, proteinase, esterase, haemolytic activity and for the biofilm formation (Figure 1).

Phospholipase activity was found in 20 (52.6%) isolates and positivity for proteinase activity was found in 33 (86.8%) *Candida* isolates. Hemolysin activity was seen in 24 (63.1%) isolates and esterase activity was found in 19 (50%). About 30 (78.9%) isolates gave positive result for biofilm formation. Maximum phospholipase (100%) activity and esterase activity (59%) was seen in *C. albicans* whereas maximum proteinase (100%) activity, haemolysin (77.7%) production and biofilm formation (88.8%) was seen in *C. tropicalis*. *C. krusei* and *C. glabrata* also showed positive results for all the activities (Table 1).

C. albicans is an opportunistic pathogenic micro-organism that has developed several virulence factors facilitating the invasion of host tissues (Schaller et al., 2005). The ability of *Candida* species to persist on mucosal surfaces of healthy individuals is an important factor contributing to its virulence. This is particularly important in the oral cavity, where the organism has to resist the mechanical washing action of a relatively constant flow of saliva toward the esophagus (Sitheeque and Samaranayake, 2003). Various virulence factors contribute to the colonization and pathogenicity of *C. albicans* infection, including the expression of adhesins and invasins on the cell surface (Cannon and Chaffin, 1999), yeast-hyphal morphogenetic transformation, phenotypic switching (Francois et al., 2013), the secretion of hydrolytic enzymes (Schaller et al., 2005), iron acquisition from the environment (Manns et al., 1994), the ability to form biofilm on various surfaces (Williams and Lewis, 2011).

Many different hydrolytic enzymes are identified in

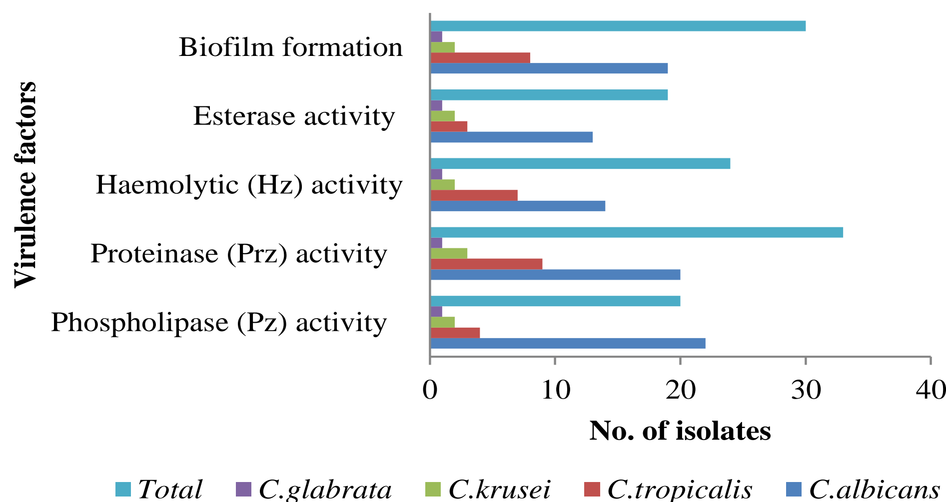

Figure 1. Virulence factors produced by different *Candida* sp. isolated from oral cavity.

Candida sp. including secreted aspartyl proteinase, phospholipase, lipase and esterase. The production of hydrolytic enzymes helps in colonization of host surfaces, increase adhesion by degrading host surface molecules, allow penetration into host tissues by digesting host cell membranes or evasion of host defense mechanism by digesting cells and molecules of the host immune system hence modulate host immune responses (Calderone et al., 2002).

It has been reported that the enzymatic activity of *Candida* sp. may vary depending on the species and source of isolates (Mohandas and Ballal, 2002). In this study, out of 38 isolates, phospholipase activity was detected in 100% of the *C. albicans*. Tsang et al. (2007) also reported the same positivity rate of phospholipase activity, in samples from patients with oral *Candida* infection. Previous studies have reported phospholipase activity in 30 to 100% of Candidal isolates from various groups of patients and from various sites (Price et al., 1982; Wu et al., 1996). As shown in Table 1, 100% of *C. albicans* produced phospholipase, among the NAC species, *C. tropicalis* followed by *C. krusei* showed maximum phospholipase production. Phospholipase enzyme digests the host cell membrane phospholipid causing cell lysis and changes in the surface features that enhance adherence and consequent infection and hence phospholipase production may be used as one of the parameters to distinguish virulent invasive strains from non-invasive colonisers. About 90.9% of *C. albicans* showed proteolytic activity in the present study. *C. tropicalis* showed 100% proteolytic activity followed by *C. krusei.* This observation was similar to the reports given by previous workers (Marcos-arias et al., 2011).

It was noted that haemolysin activity was higher in *C. tropicalis* (77.7%) followed by *C. albicans* (63.63%). Manns et al. (1994) defined the condition under which *C. albicans* can display haemolytic activity and found that haemolysis is non-existent when no glucose is available in the culture medium. Rossoni et al. (2013) found that non-*Candida* species also produced same haemolytic activity as *C. albicans. C. albicans* secretes a haemolytic factor that causes the release of haemoglobin, which is then used as an iron source by the organisms. Watanabe et al. (1999) demonstrated that mannoprotein released from *C. albicans* bound to the band 3 protein on RBCs, thereby promoting their disruption. They detected the haemolytic activity in the culture supernatant of C. *albicans in vitro*. In the oral cavity, extracellular iron is bound mainly to lactoferrin, a protein present in saliva, while intracellular iron is stored as ferritin. Although, this element is bound to proteins and/or is present in the cytoplasm of cells, oral infections with *C. albicans* are frequent, suggesting that this yeast is able to take up different forms of iron from the oral cavity (Almeida et al., 2008).

About 59% of *C. albicans* expressed esterase activity (Pakshir et al., 2013; Aktas et al., 2002). It has been reported that both *C. albicans* and non-*albicans Candida* sp. express esterase activity. Rudek et al. (1978) demonstrated that esterase activity would appear to be a common feature of *Candida* species that are frequently isolated from clinical specimens. Kumar et al. (2006) reported that Tween 80 opacity test cannot be used as the sole phenotypic trait in the differentiation of *C. albicans* and *C. dubiliancis* though it appears to be simple, economical and easy method to perform for use in small clinical laboratories. Melak et al. (2012) detected that C. *albicans* showed esterase activity in aerobic conditions but not in anaerobic conditions.

Biofilm formation is one of the most important virulence factors of *Candida* sp. (Figure 1). *Candida* biofilms occur on tissue surfaces as well as the biomaterials of medical devices. As reported by Gultekin et al. (2011), no biofilm formation was detected in any *C. albicans* strains by

microplate method, while it was found in 50% of non-*albicans Candida* sp. Demirbilek et al. (2007) also detected that the biofilm formation rate was higher in non-*albicans Candida* sp. than in *C. albicans* strains by the microplate method. In our study, the biofilm formation rates were found to be higher in *C. tropicalis* (88.8%) than *C. albicans* (86.3%). According to the above mentioned studies, the biofilm formation rate was higher in non-*albicans Candida* species as compared to *C. albicans* isolates.

Conclusion

It is necessary to understand the pathogenicity mechanisms of the *Candida* sp. for the development of new antifungal strategy. Developing anti-fungal therapies against selective target virulence factor is very crucial nowadays because of the multi-drug resistance developed by *Candida* sp. Hence, our study on virulence factors of *Candida* sp. pave way for the better understanding of the various virulence factors exhibited by *Candida* sp.

Conflict of interests

The authors did not declare any conflict of interest.

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