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Multiplex and Real Time PCR for Serotyping of Important Epidemiologic Pneumococcus

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Pneumococcal diseases caused by the bacterium Streptococcus pneumoniawere a worldwide public health problem.. According to the World Health Organization, community-acquired pneumonia was the main cause of pediatric death. This study was curriedatKing Saud University during 2008- 2010 in Saudi Arabia.Four hundred eighty eight isolates of *Streptococcal pneumonia* were kindly supplied by Al Kharj Military Hospital and these isolates represented a part of a large collection maintained at -70 ^{0}C. Their sources of isolation were infected patients of different ages. The viability of the isolates was tested on blood agar. Unfortunately only 266 isolates were viable. The viable isolates were serogrouped by using latex agglutination method and were found to fall into 20 serogroups that matched the same serogroups listed in the source culture (488isolates).Out of these 20 serogroups, 170 isolates were chosen for serotyping because they were common in all serogroups.Multiplex polymerase chain reaction (PCR) with specific primer was used for serotyping.Real time PCR were also used applying DNA belonging to 19F serotypes using the specific primer 19F produced five distinct beaks and illustrated variable amount of DNA concentration and similar melting point. The process was repeated using with known serotypes 19A and specific primer 19A. The products were four peaks and showed variable concentration of DNA and similar melting point.All serotypes detected in this study included in the available vaccine PCV-7 (4, 6B, 9V, 14,18C, 19F, 23F) which is currently being used in Saudi Arabia. But in this study we found two new serotypes (19A, 6A) which do not include in this PCV-7 vaccine.

Key words: Real time PCR, Multiplex PCR, *S. pneumonia*, Serogouping, Serotyping.

Streptococcus is a genus of spherical gram positive bacteria belonging to the phylum firimicutes and the lactic acid bacteria group. Pneumococcal diseases caused by the bacterium *Streptococcus pneumonia* are worldwide public health problem[1].According to the World Health Organization, community-acquired pneumonia is the main cause of pediatric death, accounting for 20 % of deaths in children younger than 5 years old, and 90 % of these deaths occur in non-industrialized countries[2].In addition to streptococcal pharyngitis (or strep throat) certain Streptococcus species are responsible for many cases of meningitis, bacterial pneumonia, endocarditic, erysipelas and necrotizing. Most human especially young children (0–5 years of age) are likely to be colonized at least once during their lifetime with pneumococci that are spread by droplet infection. Pneumococcal-related disease is usually preceded by colonization of the nasopharynx. Therefore, pneumococcal carriage is believed to play an important role in horizontal spread of *S. pneumonia* in the community[3]. However, many

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streptococcal species are nonpathogenic as a rule; individual species of streptococcus are classified based on their hemolytic properties. The hemolytic reactions of streptococci on blood agar plates can be used for their preliminary identification and classification. For instance, Streptococcus pneumonia forms alpha hemolysis, Streptococcus pyogenes forms beta hemolysis, and *Enterococcus faecalis* displays gamma hemolysis on blood agar plates. A definitive diagnosis of pneumococcal *pneumoniae*is difficult to establish using conventional diagnostic tests isolation of *streptococcus pneumoniae* from blood lacks sensitivity; isolation of *S. pneumoniae* from sputum may represent colonization and invasive tests are rarely performed. The increasing difficulty in the management of pneumococcal disease and of preventative measures against infection by S. pnumoniae has led to renewed interest in pneumococcal capsular typing techniques. Multiplex PCR method accurately detects the majority of serotypes and serogroups frequently isolated from young children, adults and elderly patients, allowing the characterization of the colonization patterns before and after vaccination. In medical setting, the most important groups are the alpha- hemolytic streptococci, *S. pneumonia* and *streptococcus viridians* group and the beta hemolytic streptococci of Lancefield groups A and B. Beta hemolytic streptococci are further characterized via the Lancefield serotyping based on specific carbohydrates in the bacterial cell wall. These are named field lance groups A to V. They have come to public attention recently as antibiotic resistant strains have appeared causing serious infections.Thepneumolysin PCR assay adds little to existing diagnostic tests for S. pneumoniae and is unable to distinguish colonization from infection when respiratory samples are tested[4].

MATERIALS AND METHODS

Two hundred sixty six viable out of 488 isolates of invasive specimen *streptococcus pneumonia which* were penicillin resistant were from the collection of (October 2008 to March 2010) they include sixty three of sputum, 37 blood culture, 25 tracheal aspirates, 5 spinal fluid,1 23 nasopharyngeal aspirate, 2 broncheallevеal lavage, 2 tissues, 1 peritoneal fluid, 1 tongue , 3 sternum and 4 plural fluid.

Control strains *Streptococcus faecalis* (ATCC 29212) as negative control. *Streptococcus-pneumoniae* (ATCC 6303) as positive control

Streptococcus pneumoniae (ATCC 49619) as positive control

Streptococcus viridians (ATCC 13419 as negative control were bought.

Preparation of blood agar medium

The medium was prepared as described in Oxoid manual (17) as follows:

Peptone complex 10.0 grm/L , peptone bacteriological 10.0 gm/L , sodium chloride 5.0 gm/ L, agar bios LL 12.0 gm/L and maize starch 1gm/L. The medium was sterilized by autoclaving at 121°C for 15 minutes, cooled down at 50°C added horse blood5% (SBML) mixed gently and poured into petri dishes dried and stored at 4^0C.

The controls and the 266 isolates were cultured on 5% horse blood agar in a humidified atmosphere supplemented with 5% CO_2. The colonies of *streptococcus pneumoniae* were picked and stored in three different media egg yolk media at R/T for six months, in skimmilktryptone glucose-glycerol (STGG) transport media at -70C and 1.2ml of Todd Hewitt Broth at -70°C.

Confirmation of the isolates

To confirm the isolates we did morphological identification of *Streptococcus pneumonia***.**

The isolates were suspended in McFarland standard tubes containing 5ml of sterile distilled water equivalently to 0.5 (108cfu/ml).The 0.5 McFarland colony suspensions were inoculated on the blood agar plates then incubated at 35°C plus 5% CO_2 for 18 hours.

Mueller Hinton Blood Agar preparation

The medium was prepared accordingly to Oxoid manual as follows:

Beef dehydrated infusion 300.0 gm /L, Casein hydrolysate 17.5 gm/L , starch 1.5 gm/ L and agar 17.0 gm /L, were dissolved in distilled water and sterilized by autoclaving as 121°C for 15 minutes , Cooled down to 50°C and a 5% lysed horse blood (SBML), was added mixed gentlely, poured into petri dishes and stored at 4^0C.

Optochin disk diffusion test

All strains were identified by using optochin disk on blood agar andMueller Hinton

lysed horse blood agar mediaas follows:

The bacterial suspension was smeared onbloodagar plates dried, placed the disks and incubated at 35 ^{0}C suplmented with 5% CO_2.

Likewise bacterial suspension was smeared on Mueller Hinton lysed horse blood agar plates dried and incubated at 35 ^{0}C supplemented with 5% CO as in the case of blood agar.

Oxacillin and penicillin disk diffusion test by (Kirby Bauer)

Sterile cotton swab was deeped into the tube that contained the suspended isolate and strocke on the Mueller Hinton lysed horse blood agar plates, dried, placed the disks and incubated at 35 ^{0}C supplemented with 5% CO2.Oxacillin and penicillin disk diffusion test by[18]. Sterile cotton swab was deeped into the tube that contained the suspended isolates and inoculated on Mueller Hinton lysed horse blood agar plates by spreading the inoculum from the center rotating on the rotory plate to cover two third of theplate agar. The suspended control*streptococcuspneuminae* was also inoculated on Mueller Hinton lysed horse blood agar plates spreading the inoculum at the edges of the plates by using rotory plate covering the whole edge of theplate. And then the Oxacillin and penicillin disks were placed in between the test isolate and the control. Dried, incubated at 35 °C supplemented with 5% CO_2.

Agglutination test

Using the Pneumo test latex kit, one drop of each pool antisera reagent from each vail was placed on the circle reaction card. One colony of overnight pneumococcal sub culture was picked from blood agar plate and mixed with the antisera on the circle card for 5-10 secands then immediately agglutination was observed indicating positive reaction.

Bile solubility (plate test method)

In this method three blood agar plates were used, namely *Streptococcus pneumonia* (ATCC-63 03) blood agar for positive control and Streptococcus viridans (ATCC-13419) for negative control as well as the bacterial isolates to be identified, Using plastic pipette 4drops of 10% sodium dodecyl sulfate (SDS) were placed on each colony of the controls and bacterial isolates on the blood agar then incubated at 35°C supplemented with 5% CO_2 for 2hours leaving the plates slightly opened, colony lyses of bacterial isolates and the positive control were observed.

Sub culturing of the Isolates

Ten µl plastic loop was used to pick up 10 µl of the bacterial suspension and smeared on agar plate sublimated with 5% horse blood and then dried, incubated at35^{o}C sublimated with 5% CO_2 for 24 hours.

Microscopic examination

Procedure

Ten µl of distilled water were placed on clean glass slide, bacterial colony were mixed with distilled water, smeared and air dried. One drop of crystal violet was covered on the bacteria, left for 10 seconds, washed with water, a drop of iodine was added and after tenseconds, washed with water and flooded with acetonethen immediately washed with water, flooded with carbolfuchsin for 60 seconds, washed with water, left to dry, droped an oil andexamined under microscopy.

Catalase activity

Procedure

A loobfull of Hydrogen peroxide powder was placed in a sterile test tube, 5ml of sterile distilled water was added, mixed to dissolve, and fresh colony was deeped in the Hydrogen peroxide solution then observed for gas production.

DNA Extraction

For DNA extraction Magna pure LC DNA Isolation Kit III (Bacteria, Fungi), Roche protocol was used.

Electrophoresis of Isolated DNA

A liter TBE of 0.5 X buffer was prepared, poured in the tank, deeped the solidified 2% gel then removed the comb, two µl of SYBER green was mixed with two µl of phenol blue and 10µl of DNA sample, the whole mixture was loaded in each well, Electrophoretic machine was adjusted at 120volt and operated, then results were observed under ultra violet light

DNA amplification

For DNA amplification, the primers used see (table3).

Multiplex PCR

One µl of each reverse and forward specific primer of (6A, 19A, 6B, 19F, 23F) were added into Ready to go PCR beads, added 2.5µl of $MgCl_2$, 7 µl of DNA, and completed the volume to 25µl with 10.5µl of sterile distilled water mixed well and thermal cycled with Initial Denaturation temperature 95^{0}C for 5 minutes followed by

annealing 60°C for 45 seconds/ final extension 72°C for 2 minutes, 30 cycles. PCR reaction using six primers (6A, 6B, 19F, 19A, 23F and 9V)

One µl of each reverse and forward specific primer of (6A, 6B, 19F, 19A 23F and 9V)were added into Ready to go PCR beads, added 2.5µl of $MgCl_2$, 7 µl of DNA, and completed the volume to 25µl with 9. 5µl of sterile distilled water mixed well and thermal cycled with Initial Denaturation temperature 95°C for 5 minutes followed by annealing 60°C for 45 seconds/ final extension 72°C for 2 minutes, 30 cycles

Sample and Marker Loading

Two µl of SYBER green was mixed with two µl of phenol blue and 10µl of sample in separate tubes and two µl of DNA marker was mixed two µl of SYBER green, 2µl phenol blue, 8µl of sterile distilled water, the whole mixture of each tube was loaded in the well of the agarose gel. Electrophoretic machine was adjusted at 120 volt and operated, and then results were observed under ultra violet light.

Real time PCR

Materials

Primers (19A & 19F), Light Cycler, Light Cycler Fast Start DNA Master SYBR Green1 (Roche, Germany) kit, Pipettes, tips, microphage tubes, precooked light cycler capillary tubes and sample holder carousel centrifuge.

Preparation of master mixtur

Ten µl from thawed vial (1A) colorless cap was taken and added tovial (1B) green cap and mixed well, two µl from this mixture was taken and placed in small centrifuge tube, added 1.2µl from vial (2 blue cap), added 2µl of primers 19F and 2µl 19A in separate tubes, 3µl DNA template in each tube, the volume was completed to 20µlwith water from vial (3 colorless cap).DNA templates of five isolates were used with primer 19F and four DNA isolates with primer 19A in two separate reaction mixture. The whole volume was transferred to capillary tube and placed in carousel centrifuge, capillary tube holder and was centrifuged at 3000rpm for 5 seconds, placed the carousel holding the capillaries in light cycler machine following the parameters for programming PCR light cycler protocol mention in Roche Applied Science Light cycler fast start DNA Master SYBR Green1 (Roche Germany) kit, version September 2005.

RESULTS

Pneumo test latex kit, one drop of each pool antisera reagent from each vial was placed on the circle reaction card. One colony of overnight pneumococcal sub culture was picked from blood agar plate and mixed with the antisera on the circle card for 5-10 seconds then immediately

Table 1. Pneumococcal serogoup infection related to age

Sero group	No=5	Percentange	No. 6-17	Percentange	No. =18	Percentange
19	63	22.1 %	1	1.8 %	36	24.3 %
23	49	17.2 %	9	16.3 %	27	18.2 %
6	32	11.2 %	12	21.8 %	4	2.7 %
9	19	6.7 %	14	25.4 %	19	12.8 %
3	9	3.1 %	2	3.6 %	12	8.1 %
14	23	8.1 %	1	1.8 %	3	2 %
15	17	6 %	0	0 %	10	6.7%

Table 2. Percentage Resistance of 266 isolates to (Penicillin, Erythromycin and Ceftriaxone)

Antibiotic	Numberof Isolates						Total of percentage
	S	%	I	%	R	%	resistance
Penicillin	109	14	134	50.4	23	8.6	59%
Erythormycin	165	62	0	0	101	38	38%
Ceftriaxone	265	99.6	1	0.37	0	0	0.97

agglutination was observed indicating positive reaction. In the bile solubility test, the isolates were lysed by the bile's activity and clear area was seen on the agar plate pointing that they were Streptococcus pneumonia. A loobfull of Hydrogen peroxide powder that was placed in a sterile test tube, 5ml of sterile distilled water was added, mixed to dissolve, and fresh colony that was inserted in the Hydrogen peroxide solution showed gas production indicating catalase activity of the isolates, whilemicroscopic examination indicated gram positivity of the strains. The isolates were confirmed also morphologically that they were *Streptococcus pneumonia*and were serogrouped (table 4). The viable isolates were serogrouped by using latex agglutination method and were found to fall into 20 serogroups that matched the same serogroups listed in the source culture (488isolates) see (table5).The 0.5 McFarland colony containing suspension were inoculated on the blood agar plates incubated at 35°C plus 5% CO_2 for 18 hours showed 266 viable isolates out of 488 isolates that were donated. Multiplex PCR using 5 serotypes with 5 primers (6A, 19A, 6B, 19F, 23F) see (plate2).

Table 3. Primers used in this study

19F-cpslr	CATAGGCTATCAGAATTTTAATAATATCTTGC (32)
19A cpsK-f	GTTAGTCCTGTTTTAGATTTATTTGGTGATGT (32)
19 A cpsK-r	GAGCAGTCAATAAGATGAGACGATAGTTAG (30)
23 FcpsG-f	GTAACAGTTGCTGTACAGGGAATTGGCTTTTC (32)
23 FcpsG-r	CACAACACCTAACACACGATGGCTATATGATTC (33)
19FcpsB-f	CGAACCATTGTCTCTACCTCTCAC (24)
19FcpsB-r	CAATTACTGGCGTGATTC (20)
19FcpsO-f	TAGAGATGATTTTAATTACAGGGCAAATG (30)
19FcpsO-r	CAAGGTTGGAATAACAAATCCAGTACTTG (30)
CAPSc-f	CGGAAGAGAAGATAGCAGAAAATTTCTTC (30)

Table 4. Serogroups and frequencies of each of the viable 266 isolates

Serogroups	Number of Isolates	Percentage 96
1	3	1.1 %
3	20	7.5 %
4	1	0.4 %
5	4	1.5 %
6	31	11.6 %
7	4	1.5 %
8	4	1.5 %
9	27	10.1 %
10	4	1.5 %
11	8	3 %
12	4	1.5 %
14	11	4.1 %
15	11	4.1 %
17	5	1.9 %
18	3	1.1 %
19	54	20.3 %
22	5	1.9 %
23	38	14.2 %
33	1	0.4 %
G	13	4.95 %

M: is marker, 1 is 6B, 2 is 19F, 3 is 19F, 4 is 6A, 5 is 19F , 6 is 19A, 7 is 6A 8 is 23F, 9 is 6A . Another Multiplex PCR using 6 isolates with 6 primers (6A, 19A, 6B, 19F, 23F, 9V1 is 9V) (Plate3). from left to right M: is marker, 1 is 9V, 2 is 23F, 3 is 19F, 4 is 6B, 5 is 19A, and 6 is 6A.In Light Cycler method using five isolates with one primer 19F, the melting temperature of all isolates were 77.5 °C showing homogeneity in melting peaks but differed in peaks due to the difference in molecular weight (Fig2). However, Light Cycler usingfour isolates with primer (19A) showed melting temperature 81 °C for all the isolates but differed also in DNA concentration (Fig3). We observed in Light Cycler PCR, when primers were combined by two, three, four and five and the peaks were separated and readable but it was observed a change of melting temperature and mixed peaks unclear when the combination was exceeded five primers. Therefore, it is not recommended using a combination of more than five primers in one reaction with one serotype template when applying light cycler technique for serotyping as it is very difficult to distinguish

Table 5. Characterization of (488) isolates donated by the military Al- Kharj Hospital, Saudi Arabia

Serogropus	Number of infected patient = 5 year	Number of infected patient 6-17 year	Number of infected patient = 18 year	Total of infected patients per serogropus
1	18	4	2	24
2	-	-	-	-
3	9	2	12	23
4	-	1	1	2
5	-	1	-	1
6	32	12	4	48
7	-	1	5	6
8	2	-	5	7
9	19	14	19	52
10	4	-	-	4
11	11	1	1	13
12	3	-	1	4
13	-	-	-	-
14	23	1	3	27
15	17	-	10	27
16	-	-	-	-
17	8	1	1	10
18	1	3	4	8
19	63	1	36	100
20	3	3	2	8
22	-	-	5	5
23	49	9	27	85
33	-	-	1	1
G	18	1	4	23
Non grouping	5	0	5	10
Total Isolates				488

between the melting temperatures for each serotype. Pneumococcal serogoup infection related to age is indicated in (table1 & Fig1). Percentage Resistance of 266 isolates to (Penicillin, Erythromycin and Ceftriaxone) was tested (table 2)

DISCUSSION

In a study of mortality rate and its associated variables in community-acquired pneumoniae (CAP) due to Streptococcus pneumoniae indicated 19.3% due to septic shock and antibiotic delay and the most frequent serotype was 7F(5). Study of the clinical value of the expression of neutrophil surface CD64 in the diagnosis of community acquired pneumonia in children were investigated in ninety-eight children with community acquired pneumonia.The expression of neutrophil surface CD64 in the diagnosis of community acquired pneumonia in children was studied and the levels of peripheral blood neutrophil CD64 were measured using flow cytometry. Dynamic changes of C-reactive protein were also detected for each patient. CD64 index and CRP levels in the bacterial pneumonia group were significantly higher ($P<0.05$) (6). During 2007-2008, in northeast London suburb anoutbreak of pediatric pneumonia caused by serotype 5 pneumococci was identified by using tandem repeat analyses. This highlighted the importance of this discriminative typing method in supporting epidemiological investigations[7]. *Streptococcus-pneumoniae* is a Gram-positive bacterial pathogen that colonizes the mucosal surfaces of the host nasopharynx and upper airway. Through a combination of virulence-factor activity and an ability to evade the early components of the host immune response, this organism can spread from the upper respiratory tract to the sterile regions of

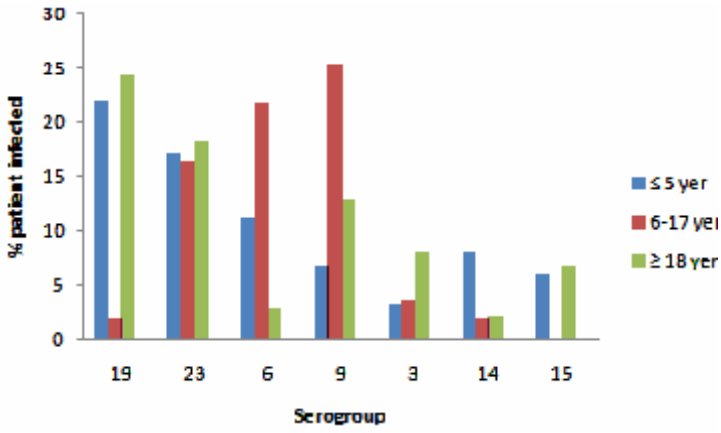

Fig. 1. Pneumococcal serogoup infection related to age

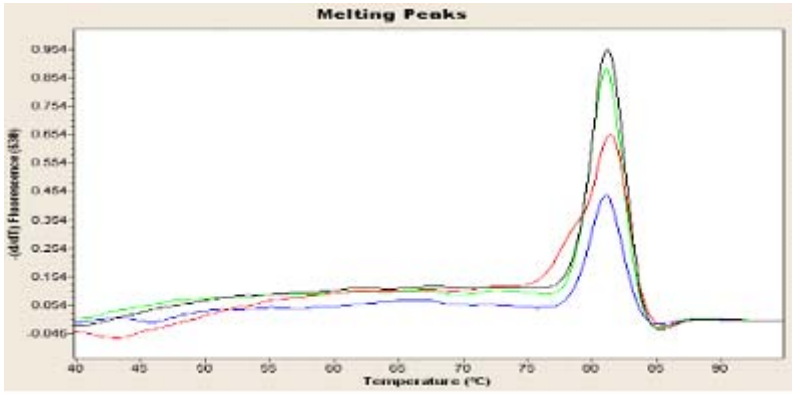

Fig. 2. Light Cycler using four isolates with primer (19A) Melting temperature (MT) of four isolates with specific 19A primer 1, 2, 3, 4 showed same malting peaks 81^0C and differing in DNA concentration

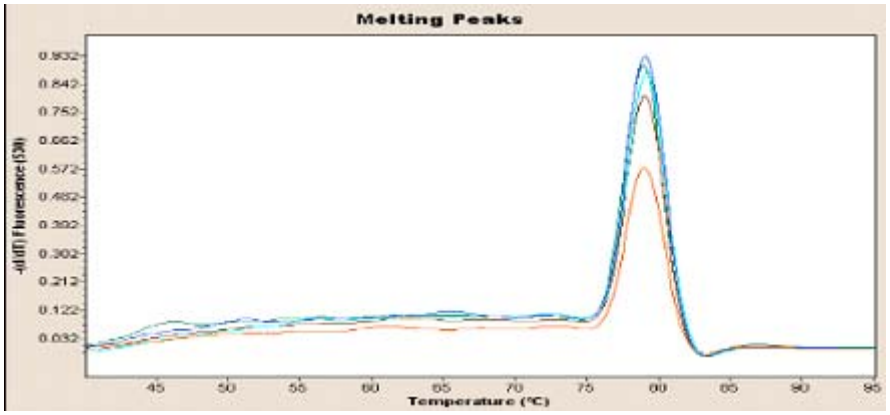

Fig. 3. Light Cycler using five isolates with primer (19F) Melting temperature (MT) of four isolates with specific 19F primer 1, 2, 3, 4 and 5 showed same malting peaks 77.5 ^{0}C and differing in DNA concentration

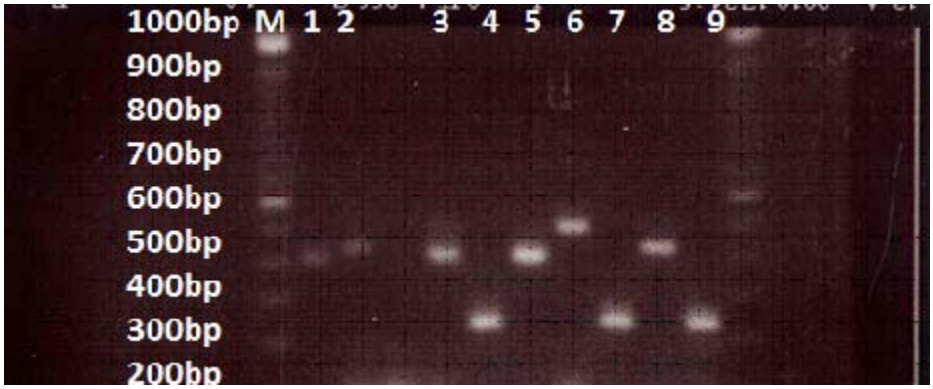

Plate 1. Multiplex PCR using 5 serotypes with 5 primers (6A, 19A, 6B, 19F, 23F) from left to right: M is marker, 1 is 6B, 2 , 3 and 5 are 19A, 6 is 19F, 4, 7 and 9 are 6A, 5 is 23F

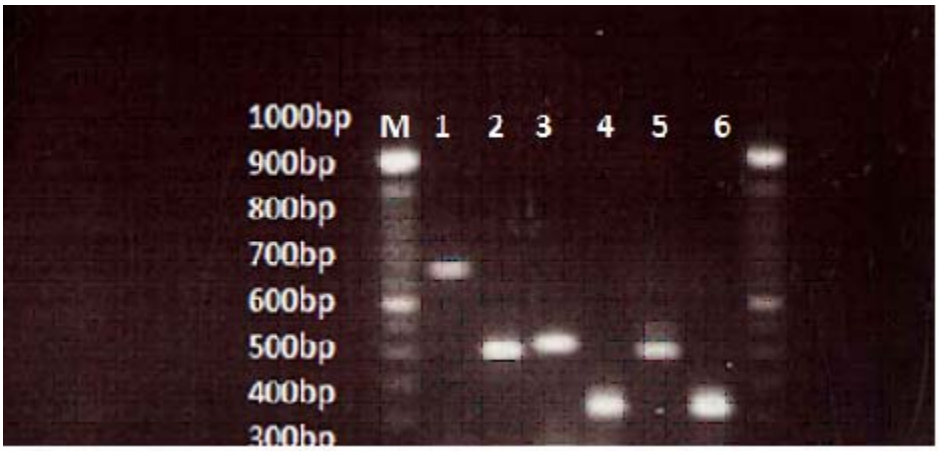

Plate 2. Multiplex PCR using 6 isolates with 6 primers (6A, 19A, 6B, 19F, 23F, 9V) from left to right M: is marker, 1 is 9V, 2 is 23F, 3 is 19F, 4 is 6B, 5 is 19A, and 6 is 6A

the lower respiratory tract, which leads to pneumonia[8]. Laura and his co investigators reported that the use ofheptavalent protein-polysaccharide pneumococcal conjugate vaccine(PCV7) of depressedassociation in -type invasive pneumococcal disease and thatofnasopharyngeal (NP) carriage in children. Vaccine use has also indirectly decreased the rate of invasive disease in adults, presumably through decreased transmission of pneumococci from vaccinated children to adults[9]. S*treptococcus pneumoniae* is the major causative pathogen of community-acquired respiratory tract infections (RTIs) in humans, including community-acquired pneumonia, acute otitis media and acute maxillary sinusitis. Nasopharyngeal carriage of *Streptococcus pneumoniae* is a key factor in the development of invasive disease and the spread of resistant strains within the community[10] .The diagnosis of severe Streptococcus pneumoniae infection relies heavily on insensitive culture techniques[11]. Annually, this organism accounts for _1.2 million deaths in children due to pneumonia and meningitis, mostly in developing countries[12]. In the United States alone in 2000, *S. pneumoniae*caused an estimated 17,000 cases of invasive disease in children _5 years of age, including 700 cases of meningitis[13, 14].Contributing to virulence is a capsular polysaccharide, the immunochemistry of which helps to differentiate pneumococci into 90 distinct serotypes[15]. However, many of these serotypes are rarely recovered from

serious disease, and only about 15 serotypes cause the majority of invasive pneumococcal disease worldwide[16]. The obtained data from this study and those reported by other researchers; supported that age plays a role in Pneumococcal serotype causing infection. Mohammed S. Al-Saggaf and coworkers[18] investigated the genetic relatedness and pathogenicity of three viruses, Bovine Herpesvirus-1, (BHV-1), Bovine Viral Diarrhea virus, (BVDV) and Bovine Respiratory Syncytial Virus (BRSV), based on the nucleotide sequences of the glycoprotein G (gG) gene and by Indirect ELISA. The same techniques can be used in the investigation of streptococcus pneumonia but we found multiplex and real time PCR more specific and less time consuming.

It may be concluded that serotype infection in different age group populations are probably due to geographical, socioeconomically, antibiotic usage and vaccine design. All the serogroups and serotypes detected in this study include in the currently available vaccine PCV-7 (4, 6B, 9V, 14,18C, 19F, 23F) which is currently being used in Saudi Arabia. But in this study we found two new serotypes (19A, 6A) which do not include in this PCV-7 vaccine. Therefore, we highly recommend the addition of these new serotypes in the current PVC-7 which is used as vaccine for pneumococcal infection.

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REFERENCES

1. Musawi, M., A retrospective epidemiological study of invasive pneumococcal infections in children aged 0-5 years in Bahrain from 1 January 1999 to 31 December 2003.*Vaccine*, 2012; **22**(24): 2-6.
2. Barsam, F. J. //Borges, G. S. //Severino, A. B. de Mello, L. M. da Silva, A. S. //Nunes, A. A. Factors associated with community-acquired pneumonia in hospitalised children and adolescents aged 6 months to 13 years old. *Eur J Pediatr.* 2012; **22**(24): 2.
3. Bogaert D, De Groot R, Hermans PW., *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 2004; **4**: 144-154.
4. Murdoch DR, Anderson TP, Beynon KA, Chua A, Fleming AM, Laing RT, Town GI, Mills GD, Chambers ST, Jennings LC., Evaluation of a PCR assay for detection of *Streptococcus pneumoniae* in respirator andnonrespiratory samples from adults with community-acquired pneumonia. *J Clin. Microbiol*. 2003; **41**: 63-66.
5. Cardinal-Fernandez, P. Garcia Gabarrot, G. Echeverria, PZum, G. Hurtado, J. Rieppi, G., Clinical and microbiological aspects of acute community-acquired pneumonia due to *Streptococcus pneumoniae*. *Rev Clin Esp*. 2012; **22**(24): 25
6. Calvillo-King, L. Arnold, D. Eubank, K. J. Lo, M. Yunyongying, P. Stieglitz, H. Halm, E. A., Impact of Social Factors on Risk of Readmission or Mortality in Pneumonia and Heart Failure: Systematic Review. *J Gen Intern Med*, 2012; **22**(24): 25.
7. Pichon B, Moyce L, Sheppard C, Slack M, Turbitt D, Pebody R, Spencer DA, Edwards J, Krahé D, George R.. Molecular typing of pneumococci for investigation of linked cases of invasive pneumococcal disease. *J Clin Microbiol.* 2010; **48**(5): 1926-8.
8. ArasKadioglu , Jeffrey N. Weiser , James C. Paton & Peter W. Andrew. He role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nature Reviews Microbiology* 2008; **6**: 288-301.
9. Laura L. Hammitt1,2, Dana L. Bruden1, Jay C. Butler1, Henry C. Baggett1,a, Debby A. Hurlburt1, Alisa Reasonover1 and Thomas W. Hennessy1., Indirect Effect of Conjugate Vaccine on Adult Carriage of *Streptococcus pneumoniae*: An Explanation of Trends in Invasive Pneumococcal Disease. *J Infect Dis*. 2006; **193** (11): 1487-1494.
10. B. Laval, A. L. S. S. de Andrade, F. C. Pimenta, J. G. De Andrade, R. M. de Oliveira, S. A. Silva, E. C. de Lima, J. L. Di Fabio, S. T. Casagrande, M. C. C. Brandileone (2006).
11. Michael D. Smith, Carmen L. Sheppard, Angela Hogan, Timothy G. Harrison, David A. B. Dance, Petra Derrington, Robert C. George, Diagnosis of Streptococcus pneumoniae Infections in Adults with Bacteremia and Community-Acquired Pneumonia: Clinical Comparison of Pneumococcal PCR and Urinary Antigen Detection. *J. Clin.Microbiol*. 2009; **47**(4): 1046-1049.

12. RekhaPai, Robert E. Gertz, and Bernard Beall, Sequential Multiplex PCR Approach for Determining Capsular Serotypes of *Streptococcus pneumoniae* Isolate. *J. Clin. Microbiol*. 2006; **44**(1):12.
13. Hausdorff, W. P., J. Bryant, P. R. Paradiso, and G. R. Siber. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin. *Infect. Dis.*2000; **30**:100-121.
14. Advisory Committee on Immunization practices. Prevention of pneumococcal disease: recommendations of the (ACIP). *Morb. Mortal. Wkly. Rep. Recomm. Rep.* 1997; **46**:1-24.
15. Henrichsen, J., Six newly recognized types of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* 1995; **33**: 2759-2762.
16. Robinson, K. A., W. Baughman, G. Rothrock, N. L. Barrett, M. Pass, C. Lexau, B. Lamaske, K.Stefonek, B. Barnes, J. Patterson, E. R. Zell, A. Schuchat, C. G. Whitney *et al.,* Epidemiology of invasive *Streptococcus pneumoniae* infections in the United States 1995–1998. *JAMA* 2001; **285**: 1729-1735
17. E.Y. Bridson., Oxoid Manual 8thEdition. (http://sst-web.tees.ac.uk/external/u0003076/Food_micro/oxoidmanual.pdf). 1998.
18. Mohammed S. Al-Saggaf, Omar H.M. Shair, Rashead M. Al-ssum and William Macasero, Respiratory Viruses in Gazelles at King Khalid WildlifeResearch Centre: Serological Investigation and Surveillance. *J. Pure Appl Microbio*. 2012; **6**(1): p. 53-58.