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Full Length Research Paper

A combination of two molecular typing methods for characterization of Pnemococci isolates from Saudi

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Streptococcus pneumonia **is a major worldwide causative agent of morbidity and mortality among young children, adults and old patients. The ability of pneumococci to cause disease is directly related to its capsule which is a major virulence factor of pneumococcal pneumonia. The aim of the present study was to characterize epidemiologically important Pneumococcus by using two molecular typing. A total of 266 pneumococci isolates from cerebral spinal fluid, nosopharengile aspirate, pleural fluid from children, adults and old patients were donated by Al-Karj hospital, Riyadh Saudi Arabia were analyzed by polymerase chain reaction (PCR) and light cycler with specific primers. Our study indicated the presence of frequent strains (6A and 19A). These new strains are not included in (11-Valent conjugate vaccine) component which is being used as a vaccine in Saudi Arabia. Since the capsular components of these new serotypes do not include this vaccine, we propose the addition of these invasive serotype capsular components to the current (11-Valent conjugate Vaccine) which is being used in Saudi Arabia.**

Key words: Pneumococci, capsular typing, invasive serogroups, multiplex PCR.

INTRODUCTION

Streptococcus is a genus of spherical gram positive bacteria belonging to the phylum firimicutes and the lactic acid bacteria group. Precise etiologic diagnosis in pediatric community-acquired pneumonia (CAP) remains challenging (Nuorti et al., 2010). *Streptococcus pneumoniae* being a normal habitant of the human respiratory tract, it is also responsible for many cases of meningitis, bacterial pneumonia, endocarditic; erysipelas. The bacterium can cause pneumoniae, usually of the lobar type, paranasal sinusitis and otitis media. In medical setting, the most important groups are the alpha-hemolytic streptococci, streptococcus viridians- group and the beta hemolytic streptococci of lance field groups A and B. Beta hemolytic streptococci can be further characterized via the lancefield serotyping - based on specific carbohydrates in the bacterial cell wall. These are named lance field groups A to V. *S. pneumoniae* have come to public attention recently as antibiotic-resistant stains have started appearing and causing epidemics. In an effort to battle the evolution of these pathogens, researchers have sequenced the genomes of 9 different strains in 4 different species of *Streptococcus*. Research for new antibiotics to treat the diseases and new vaccines to prevent them has escalated in recent years. *S. pneumoniae* access to alveolar sites by aspiration or inhalation may eventually cause a lobar pneumoniae, with consolidation of bacteremia which may predispose adults to pneumococcal disease including broncho-pulmonary disease, compromised humoral immunity, example are myeloma, lymphoma, chronic lymphocytic, leukemia and squamous cel.l Carcinoma of the lungs emphasis the urgent need for vaccines to control pneumococcal disease (Washington et al., 2010). *Pneumococci* carry numerous surfaces proteins of which several have been studied in detail. Pneumococcal surface protein (PSPA) shows some similarities to M proteins of *S. pyogenes*

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(Borriello et al., 1992). The capsule of *S. pneumoniae* is a critical virulence factor. It enables the bacteria to resist phagocytosis. The sizes of pneumococcal capsules vary depending on the strain; the capsular polysaccharides can be isolated by alcohol precipitation but most preparations, including commercial vaccine preparations, contain contaminates of cell wall polysaccharides (C polysaccharides) which cannot be eliminated owing to covalent lining through the cell wall peptidoglycan (Borriello et al., 1992). Pneumococci also produce protease which cleaves IgA1, antibody and exotoxin pneumolysin which inhabit neurtophils chemotaxis phagocytosis and immunoglobulin synthesis (Lawrence et al., 2003). Alcohol or drug intoxication cause's depresses of phagocytic activity, depresses the cough reflex, and facilitates aspiration of foreign material. Abnormal Circulatory Dynamics: causes pulmonary congestion, heart failure. The capsule of *S. pneumoniae* is critical virulence factor. It enables the bacteria to resist phagocytosis. The sizes of pneumococcal capsules vary depending on the strain; the capsular polysaccharides can be isolated by alcohol precipitation but most preparations, including commercial vaccine preparations, contain contaminates of cell wall polysaccharides (C polysaccharides) which cannot be eliminated owing to covalent lining through the cell wall peptidoglycan (Borriello et al., 1992). The production of hydrogen peroxide by *S. pneumoniae* can also lead to tissue damage caused by reactive oxygen intermediates. Finally phosphoryLcholine present in the bacterial cell wall can bind to the receptors of the platelet – activating factory that are expressed on the surface of endothelial cells, leukocytes, platelets, and tissue cells such as those in the lungs. By binding these receptors, the bacteria can enter the cells, where they are protected form phagocytosis, and pass into areas such as blood and central nervous system. This activity facilitates the spread of disease (Murray et al., 2002). Penicillin – resistant strains has now emerged and is particular problem in South Africa and parts of Europe. In some areas of U.K., up to 5% of strains are resistant to penicillin. A vaccine containing 14 of the most commonly isolated pneumococcal serotypes can be given to patients at particular risk of associated infections, including those with sickle cell disease, asplenia, renal, heart, lung or liver disease (Elliott et al., 2007). Pneumococci can produce at least 90 immunologically distinct capsules that differ in chemical structure. An even more limited number of serotypes and serogroups are most often associated with pediatric disease although the rank of serotypes may vary somewhat with the geographic region and time period (Brito et al., 2003). Significant decline in invasive pneumococcal disease caused by the vaccine targeted serotypes has been observed in young children (Pai et al., 2006). However, little is known about the long-term impact of the vaccine, with early reports indicating some replacement of vaccine serotypes (VT) by non-vaccine types (NVT) both among the nasopharyngeal colonizers and among invasive isolates. Such changes in the sero epidemiology of the organisms need to be constantly monitored to evaluate the effect and appropriate-ness of newer vaccines.

MATERIALS AND METHODS

A total number of 266 isolates of invasive resistant for penicillin *S. pneumoniae* were obtained from Al- Karj hospitals, Riyadh Saudi Arabia. These samples were collected (October 2004 to March 2009) from different sites, 63 of sputum, 38 of blood culture, 25 tracheal aspirates, 5 spinal fluid, 123 nasophyrangeal aspirates, 2 broncheal leveal lavage, 2 tissues 2 peritoneal fluid, 2 tongue, 2 sternum and 2 plural fluid. Serotyping was performed by latex agglutination test (Statens Serum Institut). Control strains for serotyping were *Strepto faecalis* (ATCC 29212), *S. pneumoniae* (ATCC 6303), *S. pneumoniae* (ATCC 49619), and S. viridians (ATCC 13419). Bacteria were propagated on 5% horse blood agar in a humidified atmosphere supplemented with 5% CO_2. The colonies of *S. pneumoniae* were stored in egg yolk media, at -700°C, in skim milk tryptone glucose-glycerol (STGG) transport media at -700°C and in 1.2 ml of Todd Hewitt Broth at -70°C and the serogroups found were included 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 17, 18, 19, 22, 23, 29.

Preparation of blood agar

The medium was prepared as described in Oxoid manual, was sterilized by autoclaving at 121°C for 15 min, Cooled at 50°C, added 5% horse blood (SBML) mixed gently, poured into Petri dishes, dried and stored at 40°C.

Preparation of meuller hinton blood agar

The medium was prepared as Oxoid manual (1995) used for an anti microbial suspceptibility testing (AST). The media was dissolved in water bath and sterilized by autoclaving 121°C for 15 min, cooled at 50°C, added 5% horse blood (SBML) 100 ml, mixed gently, poured in Petri dishes, dried and stored at 40°C.

Inoculation and incubation

Suspensions of micro organisms were prepared equivalent to 0.5 MacFarland standard tubes (108 cfu/ml) MacFarland equivalence turbidity standard (Remal) for all strains. All suspensions were inoculated on to blood agar and place optichin disk 5 µg (Oxoid) and were incubated overnight in 5% CO_2 at 35°C.

Identification

The *s. pneumonia* (ATCC-6303), *Streptococcus faecalis* (ATCC-19212) as positive control and *Streptococcus viridians* (ATCC-13419) as negative control were used along with the 266 isolates of *S. pneumonia* which were mentioned above. After 18 h of incubation on blood agar at 35°C, each isolate was identified by:

Colonial morphology

In this study, their shape, diameter, alpha haemolysis on blood

chocolate agar, color, greenish discoloration (alpha haemolysis) produced underneath narrow zone around the colonies, and their sizes were all observed.

Microscopic examination

Smears of the suspected colonies were stained with gram stain and examined for appearance, length, diameter, ends and arrangements of the cell.

Catalase activity

The colony was picked up using a clean sterile loop and placed into hydrogen peroxide solution in a small clean tube. The production of gas bubbles indicated a positive reaction.

Optochin disk

All strains were identified on different media, blood agar and Mueller Hinton lysed horse blood agar in two different conditions at 35°C with 5% CO_2 and 35°C without any percentage of CO_2 for 24 h incubation in the two different methods. We have examined all plates for any diameter zone of inhibition including the zone of inhibition surrounding the optochin disk.

Serotyping was performed by slide agglutination with capsular typing sera and factor sera (Statens Serum Institut) SSI Diagnostica.

Methods

An overnight pneumo culture growth on blood agar plate was used. Bottles of latex reagents were brought to room temperature and shaken before use. The pneumococci were tested with latex pool sera A to I and preceded by testing latex pool sera P to T. For each reaction one drop of 10 µl of latex suspension was added by squeezing the bottle gently in a circle on the reaction card, mixed and spread over the area of the circle. Rocked the card slowly and observed for agglutination within 5 to 10 seconds

DNA extraction

Total genomic DNA was recovered by using the product of Roche. T (Magna pure LC DNA Isolation Kit III (Bacteria, Fungi).

PCR amplification

For PCR amplification, the (IllustraTM pure Taq Ready-to-Go PCR beads) containing Taq, $MgCl_2$ and dNTPs were used. Final reaction volume 25 µl. 1 µl, reverse primer, 1 µl forward primer and 7 µl template DNA were added to Ready-to-Go PCR beads and the volume was completed to 25 µl with sterile distilled water. Vortexed gently and then centrifuged the tube for a few seconds and placed the reaction mixtures on ice until ready for cycling.

Thermal cycling

Denaturation: 95°C 5 min), 94°C for 45 s, 60°C (45 s) for annealing, 72°C (30 s) and 72°C (5 min) for extension for 30 cycles. PCR product yield can be improved by increasing the number of cycles to 40.

Molecular capsular typing (1) oligonucleotide primers

The sequence specific oligonucleotide Primers for Molecular Capsular Typing was obtained from data base and synthesized by a company (Pharmacia). Class one primers were intended to serve as internal control and are represented by the primer pair CPSA-F and CPS A-r which targets CPSA, a highly conserved gene that exists in all capsular loci. The specific primers in the second class were intended d to target genes specific for most common serotypes in Riyadh region 3, 6 A, 6 B, 9 V, 19 A, 19 F, 23 F comprising seven of the components of the 11 valent vaccine 9V also present in this vaccine. The primers in this second class were designed to target different genes as follows: gene creative problem solving group (CPSB) for serotype 3, gene WYZ for serotype 6B, Carbamoyl Phosphate Synthetase I (CPSI) for serotype 19 F, Coherent Phase-Shift Keying (CPSK) for serotype 19A, Chesterfield Psychic Study Group (CPSG) for serotype 23F and CPSO for serotype 19F and CPSB for 19 F. The third class of primers was designed to identify genes common to certain sets of serotypes. CPSB is a highly conserved pneumococcal gene, whereas CPSC can be divided into two distinct classes. One forward (CPSB-F) and two reverse primers (cpscr1 and cpscr2) were designed to target genes CPSB and CPSC.

PCR ANALYSIS

The total volume of the PCR mixture was analyzed by electrophoresis on 5% agrose gel (Agarose NA, Healthcare GE, Sweden) in 10 × TBE buffer (0.89M Tris, 0.89 M Boric acid, 0.02 M EDTA: usb, OH, USA) at volume 250 ml). The TBE buffer was prepared by 3800 distal water with 200 ml of distal water mixed with the TBE buffer. Gel was stained with SYBR Green1 nucleic acid gel stain (Roche Molecular Biochemicals, Germany) and blue orange loading dye, 6 × (Promega, USA). The sizes of the PCR products were estimated by comparison with a molecular size standard (100 bp Ladder, *Invirtrogen*, United Kingdom).

RESULTS

All 266 isolates of streptococcus pneumonia were saved in three different media such as (STGG) skim milk trypton soya glucose and glycerin at –70°C, EGG yolk at 25°C and Todd Hewit broth at -70°C subcultured them on blood agar. The isolates which were stored in (STGG) media were the best subcutureed. Genomic DNA of all the isolates was extracted by using MAGNA PURE machine. The source of all isolates was from different sterile body sites (Table 1). The susceptibility of penicillin, ceftriaxone and erythromycin against all isolates were determined by disc diffusion and E-test method. The percentage of penicillin resistant was 59%, ceftriaxone 0.37% and erythromycin 38% (Table 2) and comparison of serotypes in pneumococcal vaccines from the hospital data base were collected (Table 3). Young cultures on blood agar plates of pneumococci produced circular, glistening colonies about 1 mm diameter. Colonies produced by type 3 showed largeness and more mucoidiness than those colonies produced by other types. Un-capsulated strains produced small rough colonies. Colonies incubated aerobically were observed and surrounded by a zone alpha hemolytic greenish with

Table 1. Categories of specimens collected from 2004 to 2010.

Specimens type	Number	Percentage (%)
Nasopharyngeal aspirate (NPA)	123	46.4
Blood Culture (BC)	37	13.9
Sputum (SP)	63	23.7
Trachial aspirate (TA)	25	9.4
Cerebral spinal fluid (CSF)	5	1.9
Bronchial evol lavage (BAL)	2	0.8
Tissue	2	0.8
Peritoneal fluid (PTFL)	1	0.4
Tongue	1	0.4
Sternum	3	1.1
Pleural fluid	4	1.5

Table 2. Isolates resistant to Penicillin, Erythromycin and Ceftriaxone.

Antibiotic	Number of isolates						Total percentage resistance
	S	%	I	%	R	%	
Penicillin	109	41	134	50.4	23	8.6	59
Erythromycin	165	62	0	0	101	38	38
Ceftriaxone	265	99.6	1	0.37	0	0	0.37

Table 3. Comparison of serotypes in pneumococcal vaccines.

Conjugate vaccines					Polysaccharide vaccine		
PCV7	PCV9	PCV10	PCV11	PCV13	PCIV15	PPSV23	
4	4	4	4	4	4	4	2
6B	6B	6B	6B	6B	6B	6B	8
9V	9V	9V	9V	9V	9V	9V	9V
14	14	14	14	14	14	14	9 N
18C	18C	18C	18C	18C	18C	18C	11A
19F	19F	19F	19F	19F	19F	19F	12f
23F	23F	23F	23F	23F	23F	23F	15B
	1	1	1	1	1	1	17F
	5	5	5	5	5	5	20
			3	3	3	3	22F
			7F	7F	7F	7F	33F
			19A	19A	19A		
			6A	6A			
				22F			
				33F			

*PCV 7: 7-Valent pneumococcal conjugate vaccine; PCV 9:9 Valent pneumococcal conjugate vaccine; PCV11: 11-valent pneumococcal conjugate vaccine; PCIV13: 13-Valent pneumoccoccal conjugate vaccine; PCV 15:15-valent pneumooccal conjugate vaccine; PPSV23: 23-Valent pneumococcal polysaccharide vaccine.

viridians streptococci. Serological agglutination test revealed five most common serogroups, 3, 6, 9, 19 and 23 {3 (7.5%), 6 (11.6%), 9 (10.1%), 19 (20.3%), 23 (14.2%)}. The four common sero groups were 11 (3%), 14 (4.1%), 15 (4.1%) G (4.9%) and the rare serogroups were 4 (0.4%), 5 (1.5%), 7 (1.5%), 8 (1.5%), 10 (1.5%) 12

Table 4. Percentage of serogroups detected from specimens by serological latex agglutination.

Serogroup	Number	Percentage (%)
1	3	1.1
3	20	7.5
4	1	1.5
5	4	1.5
6	31	11.6
7	4	1.5
8	4	1.5
9	27	10.1
10	4	1.51
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	4.9%

(1.5%), 17 (1.9), 18 (1.1%), 22 (1.9 %), 33(0.4%) (Table 4). The total numbers of the most common invasive serogroups that was found from 170 patient were: 19, 6, 9, 23, 3. Most common serogroup or serogtypes causing invasive disease for children less than 5 years in Saudi Arabia matched with those of developing and developed countries which are (19, 23 6, 9, 3, 14, 15). Serogroup 19 was more frequently isolated from children less than 5 years old than adults between 6 to 17 years older but the serogroup was frequently found in persons who were ≥ 18 years older. Serogroup 9 was isolated exclusively in adult 6 to 17 years (19) and 5 years older. Serogroup 23 is a second highest in age group of 5 and 18 years older while the second highest serogroup was serpgroup 6 in adult 6 to 17 years old. We screened the serotypes of serogourp 19 from 54 patients with two separate primers 19 A and F (Figure 1) and Serotypes of serogroup 6 were also screened from 31 patients with two separate primers; 6 A and B (Figure 2).

DISCUSSION

Accurate serotyping is essential to monitor the changes in the seroepidemiology of *S. pneumoniae*. Pai et al. (2006) devised a simple and schematic sequence-based system of seven multiplex PCRs, in a sequence order based upon Active Bacterial Core surveillance to reliably deduce specific pneumococcal serotypes. A polymerase chain reaction assay using taxon-specific oligonucleotide primers was used for microbial detection. Amplification using ubiquitous 16S rDNA primers detected *Enterococcus faecalis*, *Streptococcus* spp and *Tannerella forsythensis* (Rôças et al., 2004). The molecular diagnosis by PCR method could improve the etiologic diagnosis and might help to guide the treatment of parapneumonic effusion in children (Menezes-Martins et al., 2005). Bacterial infections are considered to be a major cause of sudden deaths. The recognition of infections caused by *Neisseria meningitidis* is an essential duty of medicolegal offices due to the risk of secondary cases. Since other microorganisms, such as *Haemophilus Influenzae* and *S. pneumoniae*, are also involved in infectious sudden deaths, the identification of the pathogen responsible for death is essential in order to establish a positive diagnosis while also preventing secondary meningococcal cases (Fernández-Rodríguez et al., 2005). Bovine mastitis is the most important source of loss for the dairy industry. A rapid and specific test for the detection of the main pathogens of bovine mastitis is not actually available. Molecular probes reacting in PCR with bacterial DNA from bovine milk, providing direct and rapid detection of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis* (Riffon et al., 2001). *Streptococcus*

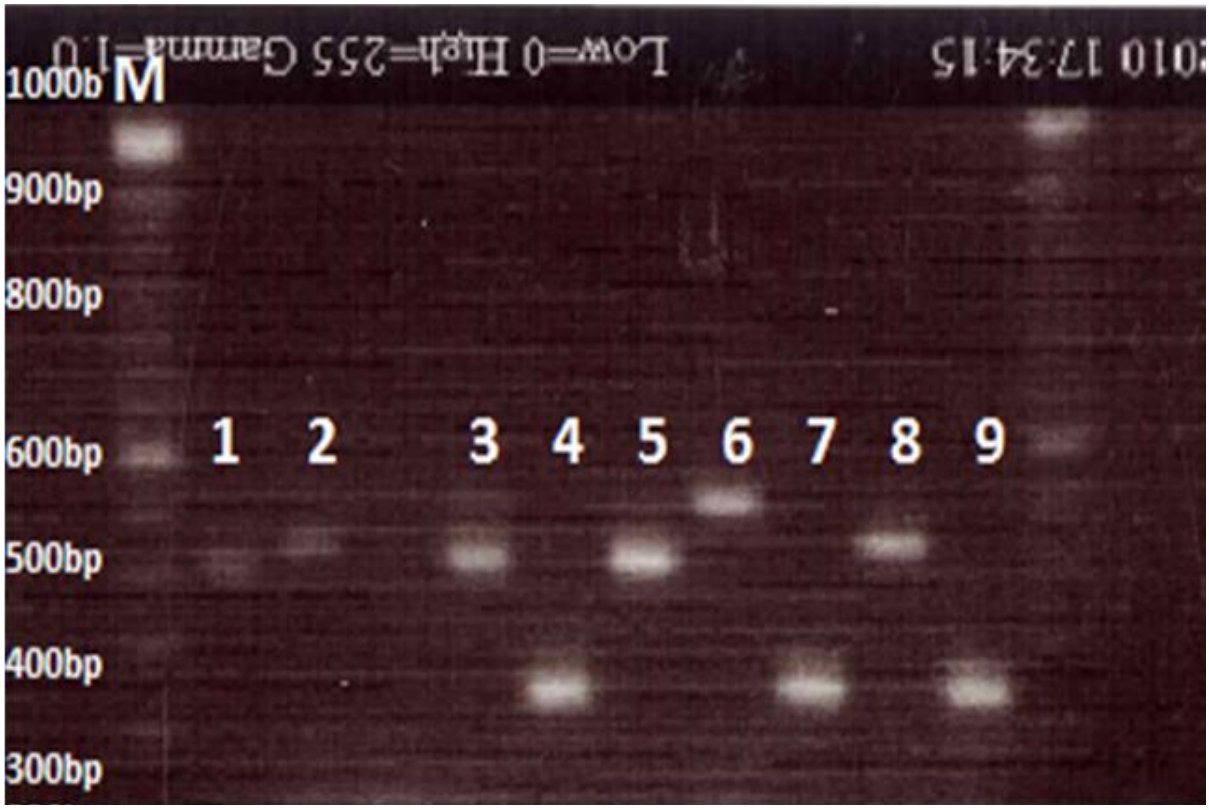

Figure 1. Multiplex PCR results of the serotypes of serogourp 19 from 54 patients with two separate primers 19A and 19F. From left to right: M is marker, 4, 7, and 9 are 19 A serotypes.

Figure 2. From left to right: M is markers, 1, 2, 3, 4, are 6A serotype.

suis serotype 2 is an emerging zoonotic pathogen and is the main cause of acute bacterial meningitis in adult patients in Vietnam. an internally controlled real-time PCR for detection of *S. suis* serotype 2 in cerebrospinal fluid (CSF) samples targeting at the cps 2J gene has been used (Nga et al., 2011). Microbial biofilms are communities of sessile microorganisms, formed by cells that are attached irreversibly to a substratum or interface or to each other and embedded in a hydrated matrix of extracellular polymeric substances. Microbial biofilms have been implicated in > 80% of human infections such as periodontitis, urethritis, endocarditis, and device-associated infections (Romero et al., 2008). All infants carried *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* at several time points during infancy. *S. pneumoniae* co-colonized the infant nasopharynx with at least one other pathogen nine out of ten times. There was early colonization of the newborns and neonates, the average times

to first detection were 5, 7, 3 and 14 weeks for *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus*, respectively. The prevalence of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* increased among the neonates and exceeded 80% by 13, 15 and 23 weeks, respectively (Kwambana et al., 2011). Fifty-five bacteriocinogenic lactic acid bacteria (LAB) isolated from seven different sources. Eight isolates were found to produce pediocin PA-1 like bacteriocin as detected by pedB gene PCR and dot-blot hybridization. The culture filtrate (CF) activity of these isolates exhibited strong antilisterial, antibacterial activity against tested food-borne pathogens and LAB. The identification and genetic diversity Devi SM among the selected LAB was performed by conventional morphological and molecular tools like restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), and 16S Recombinant DNA (rDNA) gene sequencing. The isolates were identified as, 1 each of *Pediococcus acidilactici* Cb1, *L. plantarum* Acr 2, and *Streptococcus equinus* AC1, 2 were of *P. pentosaceus* Cb4 and R38, and other 3 were *Enterococcus faecium* Acr4, BL1, V3 (Devi and Halami, 2011). PCR analyses confirmed the bacteriological results demonstrating the reliability of the 16S rRNA PCR assay for detecting Streptococci, the multiplex PCR for differentiating between *Streptococcus zooepidemicus*, and *Streptococcus equi*, and PCR assays based on streptokinase genes for identification of *Streptococcus equisimilis*. PCRs for genes encoding superantigens revealed seeL and seeM specific amplicons with size of approximately 800 and 810 bp, respectively for *S. pneumonia* is major world wide causative agent of morbidity and mortality among young children and adults and elderly. The ability of pneumococci to cause disease is directly related to its capsule which is a major virulence factor of pneumococcal pneumonia. Our aim was to study a simple, reliable, and economical method for detecting epidemiologically important of invasive *Streptococcus pneumonia* (types 3, 6A, 6B, 9V, 19a, 19F, 23F) which are present in Saudi Arabia and compare the capsular components of these invasive serotypes with the current available vaccines (11-Valent conjugate vaccine). The *S. equi* strains and for 2 *S. zooepidemicus* strains (Casagrande et al., 2011). The aim of this research is to study a simple, reliable and economical method for detecting epidemiologically the importance of invasive streptococcus pneumonia (types 3, 6A, 6B, 9V, 19a, 19F, 23F) which are present in Saudi Arabia and compare the capsular components of these invasive serotypes with the current available vaccines (11-Valent conjugate vaccine).

Conclusion

In our study of *Pneumococcus pneumonia*, we found that all the strains were penicillin resistant pneumococcal serotypes that caused the most invasive disease in the world. Most serotypes found in this study were included in the conjugate vaccine formulation used in the kingdom of Saudi Arabia. Conventional immunological techniques are not enough, as such, alternative method of serotyping was necessary. Therefore, we used single and multiplex typing method which identified serotypes (3, 6A, 6B, 9v, 19A, 19f, 23F) that include in the 11-valent antipneumococal vaccine which is currently being use in Saudi Arabia. For our multiplex PCR typing, we selected the most common serogroup (63.9%) of 170 isolates in Saudi Arabia (3, 6, 9, 19 and 23), obtained by the conventional immunological method in order to facilitate the easy understanding of the frequency of serotyping of each serogroup. The 54 isolates belonging to serogroup 19 by latex agglutination test were further serotyped by using multiplex PCR with two specific primers of 19 F and A and the products were 24 serotypes for 19 F and 5 serotype for 19A. The 31 isolates belonging to serogroup 6 were also serotyped by two specific primers of 6A and B and the PCR product were 6 serotype for 6A and 7 serotype for 6B. Likewise, for the rest of 27, 38 and 20 isolates belonging to serogroup 9, serogroup 23 and serogroup 3, respectively were serotyped by using multiplex PCR with specific primers 9V, 23F and primer 3 giving, 5 serotype, 30 serotype and 8 serotype, respectively. Our study indicated the presence of tow new strains (6 and 19A). These new strains are not included in (11-Valent conjugate vaccine) component which is being used as a vaccine in Saudi Arabia. Since the capsular components of these new serotypes do not include in this vaccine, we propose the addition of these invasive serotype capsular components to the current (11-Valent conjugate Vaccine) which is being used in Saudi Arabia.

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