Phenotypic and Gene-technological Methods for the Identification of Clinically Isolated *Streptococcus pneumoniae* from Egyptian Children

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Abstract: *Streptococcus pneumoniae* is an important human pathogen that causes both serious invasive infections, such as septicemia, meningitis and pneumonia, as well as mild upper respiratory infections. The purpose of the study was to identify the *Streptococcus pneumoniae* using the conventional phenotypic methods and the PCR assay; especially, to evaluate their usefulness in the identification of the suspected pneumococcal isolates lacking one or more of their typical phenotypic characteristics. A total of 123 nasopharyngeal specimens obtained from children under five years of age, with acute upper respiratory tract infection were subcultured and identified by conventional and gene-technological methods. Forty-one isolates were identified as *Streptococcus pneumoniae*. Approximately (7.31%) were found to be atypical optochin-resistant, while, (4.87%) were bile insoluble. A 209-bp fragment indicative the pneumolysin (ply) gene was obtained from all typical and atypical isolates. The bile solubility test is more specific than the optochin test for identification of *Streptococcus pneumoniae*. Genetic test (PCR) for *ply* could be used to evaluate any isolates giving questionable results by any of the other phenotypic methods.

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1. Introduction:

Streptococcus pneumoniae (pneumococcus) is a major bacterial infection worldwide, ranging from common infections such as otitis media to life threatening invasive infections such as sepsis, meningitis and pneumonia. Pneumococcus is the sixth most frequently isolated organism from human patients (Harakeh *et al.*, 2006). It has one of the largest public health and economic impacts of any bacterial infectious disease agent in both developing and industrialized countries (O'Brien *et al*., 2003). Pneumococcal disease kills over 1.6 million people each year. The vast majority of its victims come from the world's poorest countries (All-Party Parliamentary Group (APPG) on Pneumococcal Disease Prevention in the Developing World, 2008). It affects people of all ages, but its incidence is especially high in children less than 2 years and in adults more than 65 years (Domínguez *et al*., 2002). World Health Organization estimates that between 700 000 and 1 million children under five die from pneumococcal diseases each year (World Health Organization, 2005), and at least one child dies of pneumococcal disease every minute (All-Party Parliamentary Group (APPG) on Pneumococcal Disease Prevention in the Developing World, 2008).

Streptococcus pneumoniae is a member of the *Streptococcus mitis-Streptococcus oralis* group (the Smit group) of viridans group streptococci, which includes *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus cristatus*, *Streptococcus infantis*, *and Streptococcus peroris* (Arbique *et al*., 2004). There are now 90 recognised serotypes of *Streptococcus pneumoniae*, and the involvement of different serotypes in invasive disease varies between countries and between different age groups within the same country (Mckenzie *et al*., 2000). Most of the ninety pneumococcal serotypes immunologically distinguishable by their polysaccharide capsules are potentially pathogenic (García-Suárez *et al*., 2006).

Rapid and accurate diagnosis of pneumococcus infections plays an important role in treatment, effective management and control of outbreaks. The laboratory identification of

Streptococcus pneumoniae is based on the hemolysis pattern when it is cultured on blood agar plates and by confirmatory tests that include optochin (ethylhydrocupreine hydrochloride) sensitivity, bile solubility, miniaturized manual systems such as the API 20 Strep system, reaction with specific antisera, and PCR assays (Rudolph *et al*., 1993; Gardman & Miller,1998; Kellogg *et al*., 2001; Scott *et al*., 2003; Verhelst *et al*., 2003; Arbique *et al*., 2004; Messmer *et al*., 2004; Saukkoriipi et al., 2004; Slotved *et al*., 2004).

The purpose of the study was to identify the *Streptococcus pneumoniae* using the conventional phenotypic methods and the PCR assay; especially, to evaluate their usefulness in the identification of suspected pneumococcal isolates lacking one or more of their typical phenotypic characteristics.

2. Materials and methods

Bacterial strains:

A total of 123 nasopharyngeal specimens were obtained from children under five years of age suffering from acute upper respiratory tract infections defined as an illness having a sudden onset with rhinorrhea, pharyngitis, or cough, indicating mucosal involvement of the nose, throat, or bronchus. The cases were visiting the ENT Department of Ismailia General Hospital and the outpatient Department of Ismailia Fever Hospital. Samples were collected between September and November 2007. All specimens were immediately submerged into test tubes containing 2 ml of Skim-milk tryptone glucose glycerol (STGG) transport medium and cultured within 3-4 hours of collection.

Optochin sensitivity test: The suspected α-hemolytic colonies were touched with a sterile loop and streaked onto a tryptic soy agar plate with 5% defibrinated sheep blood in a straight line. Then, aseptically place an optochin disk (Oxoid) with a diameter of 6 mm containing 5 µg of ethylhydrocupreine HCl on the streak of inoculum. The plates were incubated in 5% CO_2 in a candle-jar at 35°C for 18–24 hours. Zone of inhibition of growth ≥14 mm in diameter indicated positive result.

Tube bile solubility test: Cells from fresh growth on agar plate were suspended in 2ml of sterile saline similar to that of a 2.0 McFarland or greater turbidity standard. The suspension was divided into two equal amounts (1ml per tube), 1ml of 0.9% saline was added to one tube (control), and 1ml of 10% sodium deoxycholate was added to the other. The tubes were shaked gently and incubated up to 30 minutes at 35°C. The tubes were visually compared; if clearing of turbidity occurred in the tube containing bile reagent, the tube was considered positive, indicating *Streptococcus pneumonia*e. Partial clearing was not accepted as a positive result (Messmer *et al*., 2004).

API 20 Strep system: Biochemical test was carried out according to the instructions of the manufacturer.

DNA extraction: Five to ten colonies were suspended in 100µl sterile distill H_2O and incubate at 100°C for 10 min. Then, a centrifugation at 12000g for 1 min was carried out. The supernatant was collected and stored at -20°C until used (Morrison *et al*., 2000; Mayoral *et al*., 2005).

Ply gene PCR assay: The oligonucleotide primers used for the amplification are IIa, (5'-CCC ACT CTT CTT GCG GTT GA-3') and IIb, (5'-TGA GCC GTT ATT TTT TCA TAC TG-3') amplify a 209-bp region of the *ply* gene. This target DNA sequence was used in developing the PCR assay according to Verhelst et al., 2003. The PCR mixture (20µl) contained 10µl of 2x TaqMix complete (Alliance Bio), 50 pmoles for each primer, 4µl Sterile distill H_2O, and finally 5µl of the extracted DNA was added. Amplification was performed using an automated thermal cycler (BIO-RAD) with the following parameters: Predenaturation 94°C for 10 min, followed by 30 cycles of (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C for denaturing, annealing, and extension, respectively) with a final post-extension at 72°C for 6 min. Approximately 10 µl of each PCR amplicon was electrophoresed using a 2.0% agarose/1XTAE buffer gels and subsequently stained with ethidium bromide and visualized with a UV transilluminator. Amplified product size was determined by comparison with a 100bp ladder DNA marker (Axygen biosciences).

Control strain: A positive control (*Streptococcus pneumoniae* ATCC 49619) was included in all assays.

3. Results

Out of 123 isolates**,** 41(33.3%) isolates surrounded by a greenish zone of α- hemolysis after incubation on tryptic soy blood agar medium plates in a 5% CO_2 atmosphere for 18-24hours. Thirty-eight of them had typical pneumococcal colonial morphology and showed optochin inhibition zones: range, 18 to 22 mm with a clear zone, and they were bile soluble. Three isolates (7.31%) were found to be atypical optochin-resistant with no inhibition zone: Two isolates of them (4.87%) were small, dry colonies and bile insoluble, while, one isolate was typical colonial morphology and bile soluble. The API 20 Strep system (bioMérieux, Marcy L'Etoile, France) failed to definitively identify any of the isolates. Applying conventional PCR technique for *ply* gene, a 209-bp fragment was obtained from all isolates (Fig; 1).

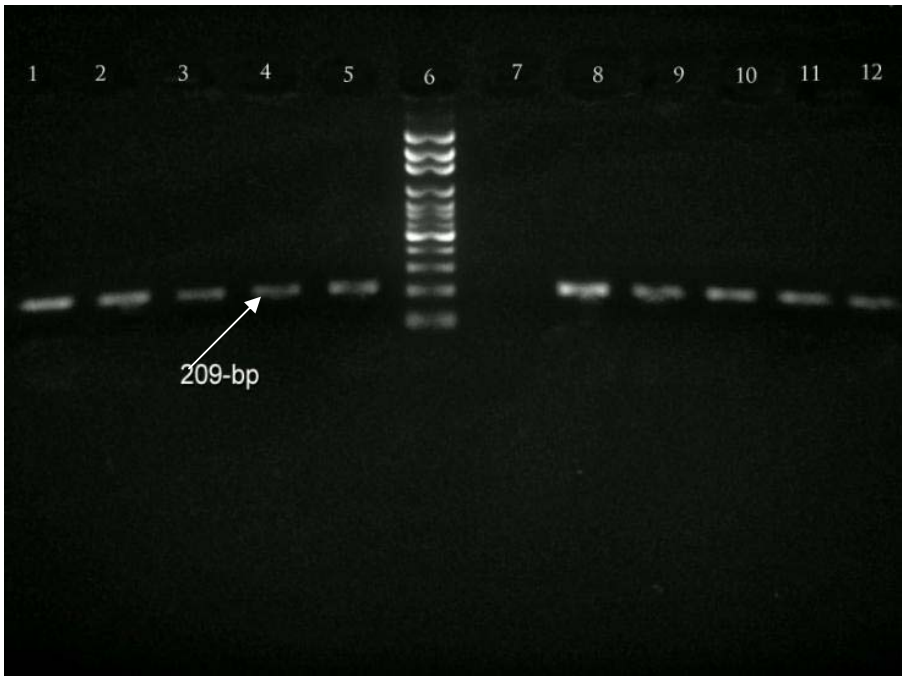

Fig. (1): Agarose gel electrophoresis of PCR-amplified products
Lane 6: Molecular weight marker (Twelve discrete fragments ranging from 100bp to 3000bp);
Lane 7: Negative control; Lanes 1-5, 8- 12: PCR positive

4. Discussion:

The accurate identification of pneumococci isolates has traditionally relied on observations of colony morphology, α-hemolysis on sheep blood agar, optochin susceptibility, and bile solubility tests. Atypical (nontypeable) pneumococci have been previously reported (Kearns *et al*., 2000; Whatmore *et al*., 2000; Obregón *et al*., 2002; Messmer *et al*., 2004). They may produce atypical reactions in one or more of the standard tests, leading to misidentification and thus may influence diagnosis and treatment. PCR for the *ply* gene has been observed to be sensitive and reliable in detecting of pneumococcus. Our result showed that all typical and atypical isolates showed positive band at 209-bp and thus were confirmed to be pneumococci. However, PCR requires special skills and equipment; therefore, at this stage, we suggested that the *ply*-PCR is a basic tool for the identification of "difficult" isolates suspected of being pneumococci.

Bile solubility and optochin sensitivity have shown to have almost complete correlation, but in 10% of cases the interpretation was considered uncertain. In our study, the optochin resistant was observed in 7.31% of isolates that had no inhibition zone. The resistance results from point mutation in the atpC gene, which prevents optochin from disrupting the H^+ transport path, by this way the strains lose their susceptibility to this compound (Pikis *et al*., 2001). However, some studies have presented that the number of colonies and the optochin discs that are used may influence the optochin sensitivity (Wasilauskas & Hampton, 1984; Gardman & Miller, 1998). Kaijalainen *et al.* 2002, reported that the density of colonies has only a small effect on the result of the optochin sensitivity test. However, when heavy inoculums is used, the diameter of the optochin sensitivity test is smaller than when a light inoculums is used, and in borderline cases the result of the optochin sensitivity test should be interpreted as sensitive or the test should be repeated.

On the other hand, the result of the present study suggests that bile solubility test is more sensitive than the optochin sensitivity test as only 4.87% was found to be bile insoluble. This result is consistent with other reported series (Burdash & West, 1982; Wasilauskas & Hampton, 1984; Davis *et al*., 1992; Kellogg *et al*., 2001), on the contrary, Kaijalainen *et al.* 2002 reported that the optochin sensitivity test is still a reliable and practical test for identifying pneumococcus from invasive as well as respiratory infections, and even from nasopharyngeal specimens. A false-positive bile solubility result will occur more often when the test is performed directly

on colonies on the agar surface rather than on those in broth medium (Denys & Carey, 1992).

Biochemical identification of pneumococcus has been proved to be quite difficult. The API 20 STREP system could not identify any of the isolates tested, and in all cases, additional testing was required before identification could be made. Some studies are in agreement with these finding (Fordymacki *et al*., 1998; Verhelst *et al*., 2003; Arbique *et al*., 2004). Other serological tests provide simpler and more rapid serological identification of *Streptococcus pneumonia* from culture (Smith & Washington, 1984; Wasilauskas & Hampton, 1984). These rely on visible detection of an antigen-antibody complex resulting from the reaction between pneumococcal surface antigens and type-specific antibodies. However, pneumococcal strains lacking a polysaccharide capsule cannot be identified by serological tests (Arbique *et al*., 2004).

On the basis of our observations, it is recommended that the genetic test for *ply* could be used to evaluate any isolates giving questionable results by any of the other phenotypic methods. On the other hand, the tube deoxycholate bile solubility test is preferred over the optochin susceptibility assay as a primary means of identification of most routine isolates of *Streptococcus pneumoniae*. Because the latter assay requires overnight incubation and the number of false negative tests were higher.

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