

# Molecular profile of *Streptococcus pneumoniae* from Nasopharyngeal swabs of patients with pneumonia using RAPD and PCR detection of *lytA*, *PsaA2*, and *HtrA* virulence genes

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## Abstract

The present work does underline the molecular profiling of ten *Streptococcus pneumoniae* clinical strains isolated from nasopharyngeal swabs of patients suffering from pneumonia by the aid of RAPD technique and molecular detection of three virulence genes namely *lytA*, *PsaA2*, and *HtrA*. Nasopharyngeal swabs taken from patients with pneumonia, consulted a pulmonologist at Nasiriya Hospital in Iraq, were streaked on blood agar plates. Ten clinical strains showed typical *Streptococcus pneumoniae* colonial morphology: Gram-positive diplococci, small, grey, moist, and mucoid with typical green zone of  $\alpha$ -hemolysis on blood agar plates. All tested clinical strains were sensitive to optochin on Møller Hinton agar plates. Confirmatory tests with VITEK2 system, bile solubility, and catalase test did verify the affiliation of the ten tested clinical strains as *Streptococcus pneumoniae*. The frequency of occurrence of *lytA*, *PsaA2*, and *HtrA* genes was 100% (n= 10 /10), 100% (n= 10/10), and 100% (n= 10 /10), respectively among the ten *S. pneumoniae* clinical strains. The RAPD-640 PCR did succeed to categorize the ten *S. pneumoniae* clinical strains under study into 9 groups strains (9 clades) according to the resultant DNA banding profile for each strain. The present data would greatly suggest that the *S. pneumoniae* is the most prevailing pathogen among patients with pneumonia within the taken sample size.

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## Keywords

*Streptococcus pneumoniae*; Nasopharyngeal swabs; of patients with pneumonia; RAPD technique; PCR detection of *lytA*, *PsaA2*, and *HtrA*.

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Invasive pneumococcal diseases (IPDs) do include pneumonia, septicemia, otitis media, and meningitis. IPDs are judged one of the main causes of mortality globally. *Streptococcus pneumoniae*, the causative agent of IPDs, is the primary etiology of community acquired pneumonia (CAP) in the adults and the young children (1). Invasive pneumococcal disease (IPDs) does show an incidence rate of 3.8 per 100,000 populations. Notably, developing countries does exhibit high mortality rates 10 to 40% due to poor health care,

lack of health education, comorbidities with HIV infection, and malnutrition (2-3).

The colonization of the nasopharynx with *S. pneumoniae* is judged a prerequisite to create a reservoir of lower respiratory tract (LRT) infection (4). Consequently, this would facilitate the evolution of IPD among human carriers (5- 6). The nature of the LRT is usually not sterile. The nasopharyngeal microbes could disseminate in healthy individuals with a high prevalence during the respiratory diseases seasons (7- 8).

N-acetylmuramoyl-L-alanine-amidase (LytA) is a major autolytic cell wall degrading enzyme that is produced by pneumococcus. The major autolytic enzyme of the pneumococcus is LytA (N-acetylmuramoyl-L-alanine-amidase). This would in turn help facilitate the cell lysis which is induced by the deoxycholate- and penicillin in the stationary phase. The lytA-encoded major autolysin of *S. pneumoniae* located in the cell envelope and hypothesized to perform a multiplicity of physiological functions (9-10).

Serine proteases, virulence factors for *S. pneumoniae*, were discovered for the first time ever in 1991 (11). After that, the serine protease orthologs from a variety of bacterial species were described (12). The high temperature requirement A (HtrA) was recognized as a virulence factor in a diverse bacterial member (13-14). HtrA in *S. pneumoniae* does perform a multiplicity of functions like bacterial stress response and competence. Additionally, HtrA is a posttranslational regulator; participated in binary fission and bacteriocin activity. It was reported that HtrA is a key determinant in virulence for pneumococci since HtrA deficient pneumococci demonstrated a remarkable diminished virulence in models of bacteremia and pneumonia (15-16).

*S. pneumoniae* does carry the Psa transporter, an ATP-binding cassette (ABC) transporter for  $Mn^{2+}$  (17). The Psa transporter consists of the products of three genes, *psaB* (ATP-binding protein), *psaC* (integral membrane protein), and *psaA* (solute-binding lipoprotein) (18). *PsaA* was paid attention for the first time upon identifying it as a potential adhesion and virulence factor as well (19). *psaA* mutants were found to be characterized by pleiotropic phenotype (diminished virulence in multiple infection models, lowered devotion to A549 pneumocytes, escalated sensitivity to oxidative stress, and a necessity for extra  $Mn^{2+}$  for competence and growth (17- 18- 20). *PsaA* does assign to a binding proteins designated *LraI*. This class of binding proteins, designated *LraI* (21)

The aim of the present study is to determine the molecular profile of *Streptococcus pneumoniae* clinical strains isolated from patients suffered from pneumonia by discovering the distribution of the three virulence genes *lytA*, *PsaA2*, and *HtrA* among the tested clinical strains and unraveling the RAPD profile of these strains as well.

## Patients and methods

### Patients

A total of ten patients suffering from pneumonia, collected from Nasiriya Hospital, Iraq from

February 2019 to May 2020, was enrolled in the present study. Demographic profile such as age, gender and symptoms for each patient enrolled in this study was performed.

### Samples collection

A total of ten nasopharyngeal swabs were taken from patients suffering from pneumonia the causative agent is unknown. All nasopharyngeal swabs were transferred immediately to the transport Amies medium (22).

### Media

Amies medium was utilized in the course of transport the nasopharyngeal swabs carrying the suspect causative agent (22). For isolation purposes, sheep blood agar was utilized. M $\ddot{u}$ ller–Hinton agar plates were used in the course of optochin antibiotic susceptibility. The long term preservation of the bacterial strains were carried out in nutrient broth at -80 °C using glycerol stock (23). All media were purchased from HiMedia Laboratories, India.

### *Streptococcus pneumoniae*: isolation and identification

The ten clinical samples in the form of nasopharyngeal swabs were plated on sheep blood agar plates. Then, all plates were incubated for 24 hrs at 37 °C in a candle jar. Next day, Gram-staining was performed for all appeared colonies on the sheep blood agar plates. The catalase test was carried out for all colonies showing Gram-positive diplococci appearance under the light microscope. Then, the catalase negative colonies were subjected to bile solubility test (24- 25) in order to discriminate the suspect pathogen *S. pneumoniae* from viridians streptococci. Briefly, 10 g of sodium deoxycholate was added to 100 mL sterile distilled water until complete dissolution. A fine drop 10% deoxycholate solution was plotted on the surface of an overnight grown *S. pneumoniae* suspect colony on sheep blood agar plates. The intactness of the colony of the suspect *S. pneumoniae* was monitored until five minutes. The suspect *S. pneumoniae* colonies with positive bile solubility was subjected further to VITEK2 identification. The interpretation of the VITEK2 results was done according to the instructions of the manufacturer.

### Optochin sensitivity test

The colonies with the following profile: Gram-positive diplococci,  $\alpha$ -hemolytic zone of hydrolysis on blood agar, catalase negative, bile solubility test

positive, and confirmed VITEK2 profile for *S. pneumoniae* were subjected to optochin sensitivity test. The optochin susceptibility was carried out using the disk diffusion method according to the guidelines of CLSI (Clinical & Laboratory Standards Institute) (26).

All suspect strains of *S. pneumoniae* were activated on sheep blood agar plates overnight at 37°C for 18–24 hrs in a candle jar. A fine touch of an overnight colony from each strain was streaked on the surface of sheep blood agar plates. After that, an optochin disc (5µg) was overlaid on the surface of each sheep blood agar plate. All plates were incubated at 37°C in a candle jar. The growth nearby the optochin disc was observed thoroughly, however, the zone of inhibition around the disc was measured in every sheep blood agar plate. A zone of inhibition with diameter of 14 mm or more was indicative of optochin sensitivity with confirmation of *S. pneumoniae*.

### Genomic DNA isolation

All clinical strains under study was subjected to DNA isolation using the Genomic Bacterial DNA isolation kit (Anatolia, Turkey) according to the instructions of the manufacturer. The genomic DNA of strains under study was evaluated regarding its quality by running aliquots of it on 1% agarose gel electrophoresis. The visualization of agarose gels was carried out under ultra violet illumination using UV-Transilluminator (27). However, the quantity of the DNA was determined spectrophotometrically.

### PCR detection of *lytA*, *PsaA2*, and *HtrA* virulence genes

The distribution of the three genes *lytA*, *PsaA2*, and *HtrA* were determined in the bacterial strains under study by PCR partial amplification of each gene using three specific primers sets. The three gene specific primer sets F- *lytA*: 5'- CCATTATC AACAGGTCC TACC -3'/ R- *lytA*: 5'- TAAGAAC AGATTTG CCTCAAG -3' [xxxxxx], F- *PsaA2*: 5'- GCCC TAATA AATTGGAGG TCTAATGA -3'/R- *PsaA2*:5'- GACC AGAA GTTG TATCT TTTT TTCCG -3', F- *HtrA*:5'- ATGAA ACATCTAA AAACATT -3'/R- *HtrA*:5'- AGATTCT AAATCAC CTGAAC -3' (28). The three primer sets was carried out in Integrated DNA Technology, USA. For each clinical strain under study, three polymerase chain reactions were carried out separately using the aforementioned primer sets. Each PCR reaction mixture included genomic DNA (30 ng), forward primer (0.3µM), reverse primer (0.3µM), 25µL of PCR Master mix (2X) (iNTRON, Korea), and nuclease free water to

a final volume of reaction mixture 50 µL. The thermocycler (Biometra, Germany) was programmed as follow: 95 °C, 5 min for initial denaturation, 30 cycles each cycle: 94 °C, 45 sec for denaturation, 57 °C (for *lytA*), 50 °C (for *PsaA2*), 54 °C (for *HtrA*), 45 sec for annealing, 72 °C, 15 sec (for *lytA* and *HtrA*), 25 sec (for *PsaA2*) for extension, and 72 °C, 10 min for final extension. After the PCR termination, the existence of the expected PCR products in each PCR reaction was tested on 1% agarose gel electrophoresis alongside with 100 bp DNA ladder (abm, Canada). The agarose gels were visualized under UV-Transilluminator (Cleaver Scientific, UK). The expected lengths of PCR products were 326, 464, and 226 bp, for *lytA*, *PsaA2* and *HtrA*, respectively.

### RAPD-PCR technique

RAPD640-PCR was performed in the present study work for DNA profiling of the suspect clinical strains using the RAPD-640: 5'- CGTGGGGCCT-3' (4). The RAPD primer was synthesized by Integrated DNA Technology, USA. Shortly, the PCR mixture for RAPD-640-PCR encompassed 12.5 µL of PCR master mix (iNtRON, Korea), 3 µL (0.3 µM) of RAPD-640-primer, 9.5 µL of distilled water, and 2.0 µL (100 ng) of DNA template. All reaction mixtures were performed in the thermocycler (Biometra, Germany). The cycling conditions were programmed to be as follow: 94 °C for 2 min (an initial denaturation step), 45 cycles each cycle 94 °C for 1 min (denaturation), 25 °C for 1 min (annealing), 72 °C for 5 min (extension), and a final extension at 72 °C for 8 min. RAPD640 DNA fragments was run on 1.5% agarose gel alongside with 100 bp DNA ladder (abm, Canada) at 100 Volt for 60 min. The raised DNA bands were visualized under ultra-violet using UV-Transilluminator (Cleaver Scientific, UK). The optical analysis of the resultant RAPD 640 DNA fragments was conducted and the molecular weights were measured. DNA standard curve was established using 100 bp DNA ladder (abm, Canada).

## Results

### Phenotypic identification of clinical strains from nasopharyngeal swabs

A ten clinical strains was isolated from the ten patients suffered from pneumonia. The ten clinical strains showed typical *Streptococcus pneumoniae* colonial morphology on sheep blood agar: green, mucoid, and α-hemolytic zone of hydrolysis. Moreover, typical gram-positive diplococci were

observed under the light microscope by the ten clinical strains. All the clinical strains were catalase negative with bile solubility profile. The VITEK2 profile did confirm the affiliation of the ten clinical strains as *Streptococcus pneumoniae*. As a result, all clinical strains under study were nominated as *Streptococcus pneumoniae*. The ten *S. pneumoniae* clinical strains were given a nomenclature strain number code starting from SP1 to SP10.

### The susceptibility profile of optochin

The present data did verify that that all tested strains showed sensitivity towards the optochin with a frequency of occurrence 100% (n=10/10). This in turn would confirm that all tested strains assigned as *S. pneumoniae*.

### Molecular profile for *lytA*, *PsaA2*, and *HtrA*

The distribution of the three genes *lytA*, *PsaA2*, and *HtrA* of the ten *S. pneumoniae* clinical strains isolated from patients suffered from pneumonia was monitored by partial amplification of each gene by PCR. For the three virulence genes *lytA*, *PsaA2*, and *HtrA*, all tested ten *S. pneumoniae* clinical strains did carry the three virulence genes (Fig 1, 2, 3). All tested strains did display positive PCR product band pattern with the expected length 326, 464, and 226 bp for *lytA*, *PsaA2*, and *HtrA*, respectively. The frequency of occurrence of *lytA*, *PsaA2*, and *HtrA* genes was 100% (n= 10 /10), 100% (n= 10/10), and 100% (n= 10 /10), respectively among the ten *S. pneumoniae* clinical strains.

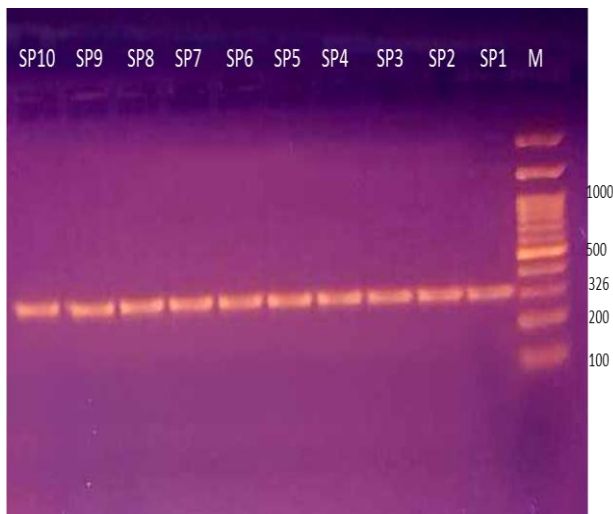


Fig 1: Agarose gel electrophoresis (1.5%) displaying the PCR products of *lytA* gene after partial amplification using gene specific primer. M: 100 bp DNA ladder. Lanes (1-xxxx): PCR products of *lytA* gene partial amplification (326 bp) from ten *S. pneumoniae* clinical strains namely SP1 to SP10.

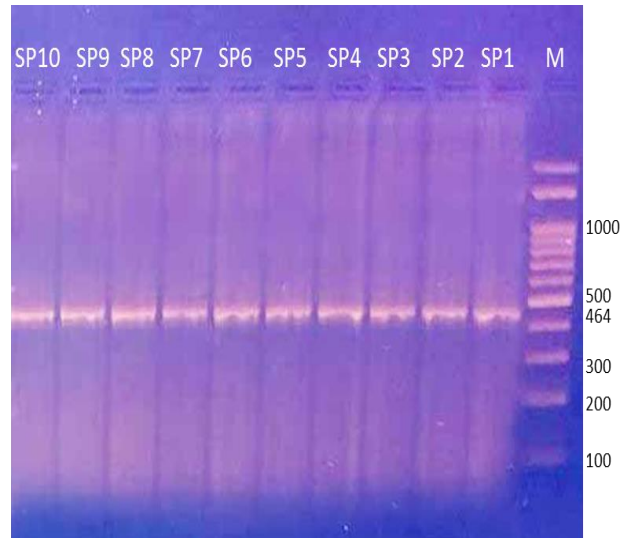


Fig 2: Agarose gel electrophoresis (1.5%) displaying the PCR products of *PsaA2* gene after partial amplification using gene specific primer. M: 100 bp DNA ladder. Lanes (1-xxxx): PCR products of *PsaA2* gene partial amplification (464 bp) from ten *S. pneumoniae* clinical strains namely SP1 to SP10.

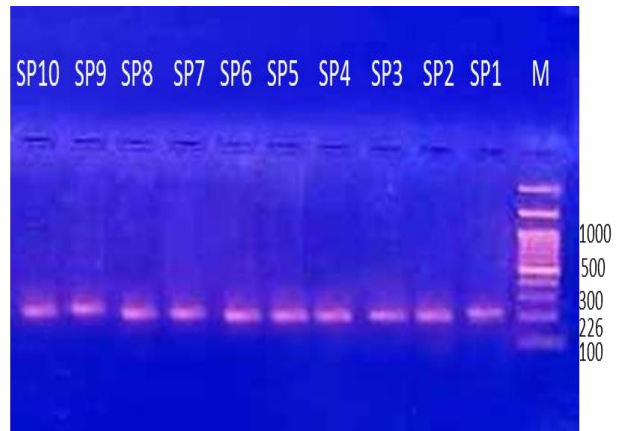


Fig 3: Agarose gel electrophoresis (1.5%) displaying the PCR products of *HtrA* gene after partial amplification using gene specific primer. M: 100 bp DNA ladder. Lanes (1-xxxx): PCR products of *HtrA* gene partial amplification (226 bp) from ten *S. pneumoniae* clinical strains namely SP1 to SP10.

### RAPD-640 profile for ten *S. pneumoniae* clinical strains

The ten *S. pneumoniae* clinical strains were discriminated according to the resultant RAPD640-PCR DNA banding pattern as demonstrated in Fig 4. RAPD640-PCR showed 9 profile of DNA banding patterns (Table 1) among the ten *S. pneumoniae* clinical strains inferred from Fig 4.

The highest and the lowest numbers of banding pattern were 9 and 4 DNA banding pattern of RAPD640-PCR, respectively. The molecular weight of each DNA band demonstrated by



RAPD640-PCR was measured based on DNA standard curve (Fig 5).

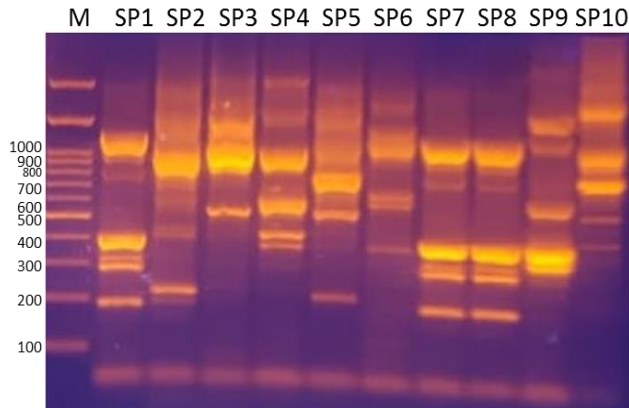


Fig 4: Agarose gel electrophoresis (1.5%) showing the banding pattern of RAPD640-PCR for the ten *S. pneumoniae* clinical strains using RAPD-640 primer.

Lanes (1-10): RAPD640-PCR DNA banding pattern for the ten *S. pneumoniae* clinical strains namely SP1 to SP10. M: 100 bp DNA ladder.

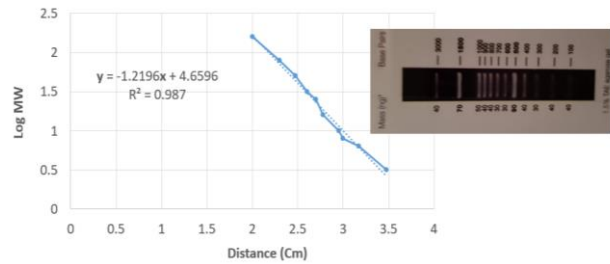


Fig 5: A DNA standard curve using 100 bp DNA ladder (abm, Canada). The R2 value was nearby 1.0 that would refer to the minor difference between the predicted values and the experimental values. MW: molecular weight of DNA ladder bands.

Table 1: RAPD640-PCR profile and molecular weight of DNA bands for the ten *S. pneumoniae* clinical strains namely SP1 to SP10

DNA band size (bp)	<i>S. pneumoniae</i> clinical strains with DNA band size	RAPD640-PCR profile no in relation to the strain
3000, 1500, 1000, 900, 800, 400, 300, 200, 350	SP1	<u>P1</u>
3000, 1500, 1000, 900, 800, 400, 200, 450	SP2	<u>P2</u>
3000, 1500, 1000, 900, 800, 500	SP3	<u>P3</u>
3000, 1500, 1000, 900, 800, 500, 400, 350	SP4	<u>P4</u>
1500, 1000, 800, 500, 200	SP5	<u>P5</u>
1500, 2500, 2000, 1000, 600, 500, 300	SP6	<u>P6</u>
1000, 900, 800, 700, 400, 300, 250	SP7, SP8	<u>P7</u>
1500, 1000, 500, 300	SP9	<u>P8</u>
1800, 2500, 900, 700, 500, 450	SP10	<u>P9</u>

## Discussion

The prevalence of *S. pneumoniae* colonization recorded in various regions of the world did differ broadly. Additionally, the distribution of certain genotypes varies widely among different populations with different ethnicity and socioeconomic conditions. Thus, the information regarding the distribution of certain genotypes of pneumococci as well as their colonization are very important to tailor well controlled strategies for preventive purposes for the IPD diseases. In the present study, the of *S. pneumoniae* colonized in the nasopharynx among patients suffering from pneumonia was tested with regard to three genes of high potential in virulence *lytA*, *PsaA2*, and *HtrA*. Our data did reveal the colonization of nasopharynx with *S. pneumoniae* from ten patients suffered from IPD pneumonia in Iraq. In the context of unraveling the genotype pattern associated with these ten *S. pneumoniae* clinical strains, the frequency of occurrence of the three virulence genes *lytA*, *PsaA2*, and *HtrA* were 100%. Our results are in a good accordance with those of Zhao et al. who stated a high prevalence of the

three virulence genes *lytA*, *PsaA2*, and *HtrA* among their isolates in a retrospective study in Shanghai (29). For *lytA*, there is still a debate about the real role of *lytA* in pathogenesis in *S. pneumoniae* (30) with three postulated theories. One theory states that *LytA* do mediate lysis to induce the release of other virulence factors like pneumolysin (31). Another hypothesis states that *LytA* is secreted to lyse adjacent non-competent pneumococcal cells (32). A third likelihood does state that *LytA* helps mediate lysis in order to liberate proteins included in immune evasion (33).

The RAPD-PCR technique proved to be able to discriminate Group B Streptococci into clusters and it is more quicker and convenient than other alternative techniques like Pulsed Gel Electrophoresis (PGE) (34). Similarly, our data did reveal the potential of RAPD640-PCR technique to discriminate the ten *S. pneumoniae* clinical strains into nine clusters.

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