

DETECTION OF AUTOLYSIN, PNEUMOLYSIN AND PNEUMOCOCCAL SURFACE ADHESION A GENES AMONG *STREPTOCOCCUS PNEUMONIAE* CAUSING BACTERIAL MENINGITIS IN CHILDREN

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ABSTRACT

The aim of the present study is to assess the presence of autolysin, pneumolysin and pneumococcal surface adhesion A genes as virulence factors that may contribute in pathogenicity of *Streptococcus pneumoniae* causing bacterial meningitis in children. Non-culture tests such latex agglutination test and PCR for *S.pneumoniae* diagnosis should be considered for patients who need early identification of pathogens or have previously received antibiotics, or whose initial cerebrospinal fluid Gram's stain is negative with negative culture at (72)hrs incubation. There were (303) cases delivered their cerebrospinal fluids samples to the central public health laboratory distributed by (183) male (60.39%) and (120) female (39.60%). Seventy seven cases (25.41%) were diagnosed as bacterial meningitis; (36) cases (11.88%) as viral meningitis. *S. pneumoniae* was isolated from (16) cases (20.7%) of bacterial meningitis. DNA from *S. pneumoniae* was extracted then subjected to amplification by PCR leading to detection of autolysin and pneumolysin genes in (9) out from (16) isolate and presence of pneumococcal surface adhesion A gene in (8) out from (16) isolate.

Key words: *S. pneumoniae*, Bacterial meningitis, *lyt A*, *pl*, *psa A*.

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التحري عن جينات
AUTOLYSIN, PNEUMOLYSIN, PNEUMOCOCCAL
STREPTOCOCCUS SURFACE ADHESION A **في بكتريا**
PNEUMONIAE **المسببة لإلتهاب السحايا البكتيري في الأطفال**

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الخلاصة

هدفت الدراسة الحالية الى التحري عن جينات Autolysin, Pneumolysin, Pneumococcal surface adhesion A كعوامل ضراوة يمكن أن تساهم في إمرضية بكتريا *Streptococcus pneumoniae* المسببة لإلتهاب السحايا البكتيري في الاطفال. الاختبارات غير المعتمدة على الزرع مثل إختبار التلازن وإختبار تضخيم الدنا التضاعفي المتسلسل لتشخيص بكتريا *S. pneumoniae* ذو أهمية خاصة بالنسبة للمرضى الذين يحتاجون الى تشخيص مبكر للممرضات المسببة أو المرضى الذين تناولوا علاجاً من المضادات الحيوية مسبقاً أو المرضى سالبى تصبغ كرام لنماذجهم من سائل النخاع الشوكي مع نتيجة زرع سلبية بعد (72) ساعة من الحضان. تم تسليم (303) عينة من سائل النخاع الشوكي الى مختبر الصحة المركزي. توزعت العينات الى (183) ذكراً (60,39%) و(120) أنثى (39,60%). (77) حالة (25,41%) شخصت كحالات التهاب سحايا بكتيري و(36) حالة (11,88%) كحالات التهاب سحايا فايروسي. بكتريا *S. pneumoniae* عزلت من (16) حالة (20,77%) من حالات التهاب السحايا البكتيري. تم إستخلاص دنا بكتريا *S. pneumoniae* ومن ثم تم تضخيمه بتقنية تضخيم الدنا التضاعفي المتسلسل ليتم إيجاد جيني Autolysin, Pneumolysin في (9) من أصل (16) عزلة وإيجاد جين Pneumococcal Surface Adhesion A في (8) من أصل (16) عزلة.

INTRODUCTION

Acute bacterial meningitis is responsible for most infections affecting the central nervous system. Close to two-thirds of all cases occur among children *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* are the pathogens responsible for 80 to 90 % of the cases (1,2). A rapid and precise etiologic diagnosis of bacterial meningitis is essential to determine adequate treatment and the risk for long-term sequelae (3). Among the routine tests used for the etiologic diagnosis of bacterial meningitis, bacterial culture is considered the gold standard. However, this is a time-consuming technique, which requires viable microorganisms for cultivation, and its sensitivity is directly affected by the start of antibiotic treatment before spinal tap (4). Other faster tests such as latex agglutination, have a lower sensitivity and present reliable results only with samples containing more than 10^5 CFU/ml (5). Currently, molecular biology techniques, such as Polymerase Chain Reaction (PCR), are being widely used for the etiologic diagnosis of central nervous system infections (6). The greatest problem in quantifying the burden of disease is diagnosis. PCR is a highly sensitive diagnostic technique for identifying microorganisms in human body specimens. The specificity of the technique depends upon a combination of two factors: the extent to which a gene fragment of similar size can be amplified from an alternative species with the PCR primers and the probability that the target organism could be found in the selected body specimen without necessarily being the cause of the disease (7). Autolysins are enzymes that degrade different bonds in the peptidoglycan and eventually cause the lysis and death of the cell. The autolysin *lyt A* is responsible for release of lipoteichoic and teichoic acids that are mediators of host inflammatory response. Cell wall autolysin is likely to have a function in pathogenesis of *S. pneumoniae* through lysing a proportion of the invading pneumococci leading to the release of potentially lethal toxins. Autolysin releases highly inflammatory cell wall break down products, which ultimately contribute to pathogenesis (8). Pneumolysin is a (471) amino acid toxin with both cytolytic and complement activation properties which appear to be major virulence determinant in *S. pneumoniae*. *ply* is the gene that codes for the pneumolysin and its detection is used in the diagnosis of *S. pneumoniae* infection in many studies. Pneumolysin was found to be present in almost all invasive isolates studied (8). Pneumococcal surface adhesin A *psa A* is a 37-kDa lipidated protein expressed on the cell surface of *Streptococcus pneumoniae* (9). In a murine model, *psa A* has been shown to stimulate antibodies that are protective against fatal pneumococcal disease (10). Monoclonal antibody studies indicate that Psa A is present at the surface of all 90 serotypes of *S. pneumoniae* (11). *psa A* PCR analysis therefore has the species specificity required for use in diagnosis of pneumococcal infection.

MATERIALS AND METHODS

1- CSF samples

The study included a total of (303) samples of CerebroSpinal Fluid (CSF) collected from: Al-Kadhymia Pediatric Hospital, Central Child Hospital, Children Welfare Teaching hospital, Elwiyah Pediatric Hospital, Basrah General Hospital and Babil Pediatric Hospital) from April 2010 to May 2011.

2- Laboratory examination

Once the CSF has arrived at the microbiology laboratory, it was centrifuged for 20 minutes at 2000 rpm. The supernatant was drew off with a Pasteur pipette. One or two drops of sediment were used to prepare the Gram stain and another one drop from the same sediment was used to streak the primary culture media (Blood agar and Chocolate agar plates) (12). The agar plates were incubated at 37°C in (5%)CO₂ for(18-24)hours. The α -haemolytic colonies with morphology indicative of *S. pneumoniae* in Gram staining were subcultured to a fresh plate with optochin disk (5mg) (13).

Detection of Bile solubility was done by preparing each isolate as a suspension of bacterial cells from fresh growth on agar plate which were suspended in 1 ml of saline to make heavy suspension (McFarland 2.0 or greater). The suspension was divided in equally in two glass tubes, and 0.5 ml of saline was added to one tube and 0.5 ml of (10%) deoxycholate was added to the other. The tubes were incubated up to (30) minutes at 35°C. Lysis of the cells in the tube with deoxycholate indicates a positive test (13). Isolates that exhibited an inhibition zone of (14) mm or more around the optochin disk and those showing bile-solubility were identified as *S. pneumoniae*.

3- DNA extraction

DNA isolation from *S.pneumoniae* isolates was performed as follow (14):

- 1) Isolates from which DNA was to be extracted were grown for an overnight at 37°C on blood agar plates. Bacteria were resuspended in 500 μ l SET Buffer.
- 2) 10 μ l of lysozyme solution was added to bacteria suspension, mixed in vortex, then incubated for (30-60) min at 37°C.
- 3) 14 μ l of Protease solution was added to the mixture mixed in a vortex then 60 μ l of (10 %) SDS was added and mixed by inversion and incubated for 2 hr at 55°C in incubator or 55 °C overnight in water bath.
- 4) 200 μ l of 5 M NaCl was added and mixed thoroughly by inversion and let cool to 37 °C.
- 5) 500 μ l of Chloroform was added and mixed by inversion for 30 min at 20°C. Then it was centrifuged for 15 min at 4500 rpm at 20°C. Supernatant was transferred to fresh tube.
- 6) 600 μ l of Isopropanol was added to the supernatant mixed by inversion and then centrifuged at 4500 rpm at room temperature for 15 min.
- 7) DNA was rinsed in 200 μ l 70 % Ethanol, centrifuged 14000 rpm for 2 min, air dried and dissolved in 50 μ l of TE Buffer.

4- PCR assays

After DNA extraction from the isolates of *S.pneumoniae* , presence of genes was detected by using primers with sequence as mentioned in Table (1), by using PCR programs mentioned in table (2).

Table(1):Primers of PCR

Genes	Sequence of primers (5'→ 3')	Gene size (bp)	Reference
<i>lyt A</i>	Forward primer CAA CCG TAC AGA ATG AAG CGG Reverse primer TTA TTC GTG CAA TAC TCG TGC G	319	(15)
<i>ply</i>	Forward primer TGC AGA GCG TCC TTT GGT CTA T Reverse primer CTC TTA CTC GTG GTT TCC AAC TTG A	80	(16)
<i>psa A</i>	Forward primer CTT TCT GCA ATC ATT CTT G Reverse primer GCC TTC TTT ACC TTG TTC TGC	838	(7)

Table(2):PCR Programs

Genes	Initial denaturation	No. of cycles	Final extention
<i>lyt A</i>	5 min at 94 °C	30 cycles , each cycle consisting of : 30 seconds at 94 °C 30 seconds at 53 °C 30 seconds at 72 °C	10 min at 72 °C
<i>ply</i>	3 min at 94 °C	35 cycles , each cycle consisting of : 40 seconds at 92 °C 30 seconds at 55 °C 20 seconds at 72 °C	10 min at 72 °C
<i>psa A</i>	1 min at 95 °C	35 cycles, each cycle consisting of : 30 seconds at 95 °C 30 seconds at 52 °C 2 min at 72 °C	8 min at 72 °C

Optimization of PCR for each gene was accomplished after several trials, thus the following mixture were adopted table (3).

Table(3):The mixture of PCR working solution

Working Solution for Simplex PCR			
	<i>lyt A</i>	<i>ply</i>	<i>psa A</i>
Distilled Water	4	2.4	4.8
Gotaq master mix	10	10	10
Forward Primer	2	1.2	0.6
Reverse Primer	2	1.2	0.6
MgCl ₂	-	1.2	-
DNA	2	4	4
Final volume 20 µl			

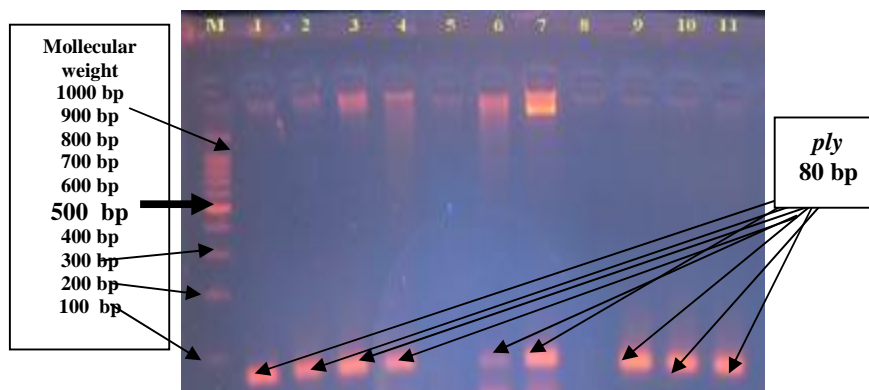
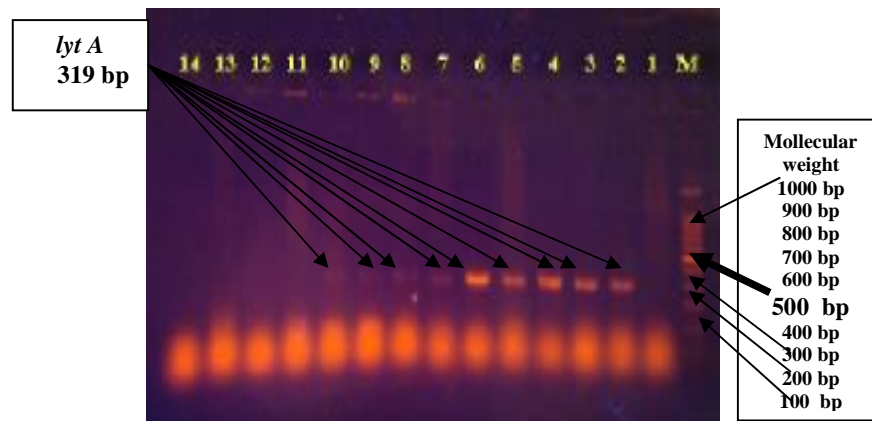
PCR end products were analyzed on 1 % agarose gel for *lyt A* and *ply*, and at 1.5 % for *psa A* stained with ethidium bromide and the bands were visualized under UV illumination (260 nm).

RESULTS AND DISCUSSION

There were (303) cases distributed by (183) male (60.39 %) and (120) female (39.60 %). Seventy seven cases (25.41 %) were diagnosed as bacterial meningitis, (36) cases (11.88 %) as viral meningitis, (21) cases (6.93 %) as Different medical cases (e.g. febrile convulsion, meningoencephalitis, epilepsy and tonsillitis) and (169) cases (55.77%) were diagnosed as other medical cases. Obviously males were more predominant than females in meningitis infection. This predominance can be resulted from the greater potential contact exposure of men to community cases of meningitis in particular, and gender-based health care attitudes and practices in general (17). The Confirmed bacterial meningitis cases were further divided into the following two groups: *Streptococcus pneumoniae* which isolated from (16) CSF sample (20.77 %) and other bacteria including all other isolated pathogens (e.g *N.meningitidis*, *H.influenzae* as the most important pathogens, in addition to other bacteria like Gram -ve and Gram +ve bacteria which caused meningitis in less numbers) which were isolated from (61) CSF sample (79.22 %). *S.pneumoniae* was isolated from (16) cases of bacterial meningitis (20.7 %). This bacteria caused also bacterial meningitis by (16.7 %) of cases of another study (18), which was close to the present study. Antibiotics taken before lumbar puncture were noticed in (124) case (40.92 %). Administration of antibiotics to children before the performance of diagnostic lumbar punctures increases the number of false-negative CSF bacterial culture results (19). Both genes *lyt A*, *ply* appeared in (9) out of from the (16) isolate, gene *psa A* appeared in (8) out of (16) isolate of *S.pneumoniae* as shown in figure (1,2 and 3). PCR detection of genes ubiquitous in pneumococci, particularly *lyt A* (20), *psa A* (21) and *ply* (22), has been used as an alternative method for species identification.

Culture techniques are effected by the presence of antibiotics and that *psa A* PCR analysis is not , we would predict that among patients whose cultures were negative but whose PCR results were positive there would be a concentration of individuals with evidence of antibiotics (7). To avoid complement immunity and phagocytosis, *S. pneumoniae* has developed a wide arsenal of bacterial virulence factors. Among them, Psp A and pneumolysin, which is released to the medium after cell wall lysis mediated by Lyt A, contribute synergistically to the innate immune diversion by targeting complement immunity at different levels. All these factors increase the ability of *S. pneumoniae* to cause invasive disease (23).

Figure(1): Presence of autolysin gene detected by the amplification of the 319 bp fragment of the *lyt A* gene . Lane M- 100 base pair Molecular marker. Lane 1- Negative control. Lane 2-10 positive *lyt A S.pneumoniae*. Lane 11-14 negative *lyt A S.pneumoniae*.



Figure(2):Presence of pneumolysin gene detected by the amplification of the 80 bp fragment of the *ply* gene. Lane M- 100 base pair Molecular marker. Lane 1-4, 6-7 and 9-11 positive *ply S.pneumoniae*. Lane 5,8 negative *ply S.pneumoniae*.

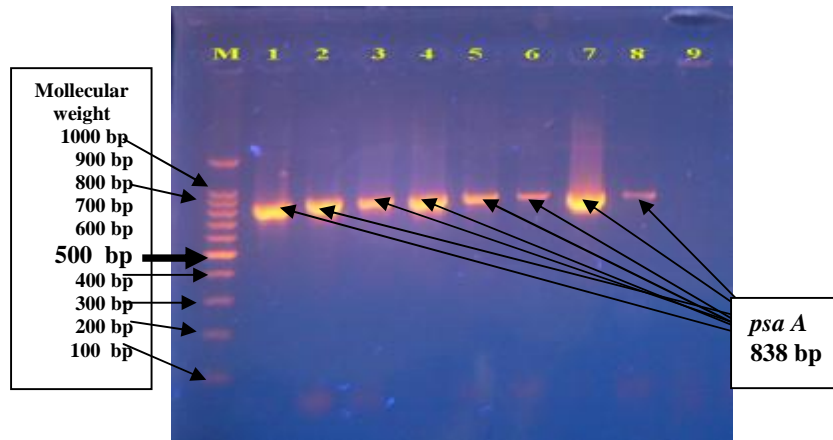


Figure (3): Presence of pneumococcal surface adhesion A gene detected by the amplification of the 838 bp fragment of the *psa A* gene. Lane M- 100 base pair Molecular marker. Lane 1-8 positive *psa A S.pneumoniae*. Lane 9 negative *psa A S.pneumoniae*

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