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ORIGINAL ARTICLE

Analysis of Autolysin-Encoding Gene (*lytA*) Sequencing of *Streptococcus pneumoniae* Isolated from Iraqi Patients with Bacterial Meningitis

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ABSTRACT

Streptococcus pneumoniae is an important human pathogen associated with a variety of diseases, from mild infections to invasive pneumococcal disease and meningitis. Autolysin (N-acetylmurayl-L-alanine amidase) (LytA) is its most important virulence factor, regulating autolysis, promoting inflammatory processes and biofilm formation, and inhibiting bacterial clearance. In this research we tried to study the *lytA* gene enclosed autolysin in *S. pneumoniae* isolates that were detected in Iraqi patients suffering from bacterial meningitis. One hundred CSF samples were collected, and viable bacterial isolates were identified using classical and molecular microbiological methods. All isolates underwent an antibiotic susceptibility test. Out of 100 samples the result showed that 32 isolate of *S. pneumoniae* was identified by VITEK2 system. The majority of isolates exhibited high resistance to ciprofloxacin and chloramphenicol while higher sensitivity was recorded for Vancomycin and Ceftazidime. All the isolates were also positive for specific genes (*lytA* and *ply*) of *S. pneumoniae* using PCR amplification. Sanger sequencing of the *lytA* gene indicated that all strains showed high homology with reference strains and no significant mutations were detected (a sign of genetic stability). The autolysin enzyme also possesses conserved domains suggesting the likely functional significance of autolysin in virulence and pathogenesis, notably in meningitis. These findings underscore the potential of *lytA* and *ply* as diagnostic biomarkers and therapeutic targets. The study reveals important epidemiological relevance of *S. pneumoniae* in Iraqi hospitals and confirms the previously described global data on conservation of virulence determinants at the molecular level.

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1- INTRODUCTION

Streptococcus pneumoniae remains a leading cause of childhood illness and death, particularly in low-income countries. Pneumococcal infection is the most common cause of vaccine-preventable childhood death, at least in part due to community-acquired pneumonia (CAP), which accounts for approximately 11%, of childhood

deaths [1]. *Streptococcus pneumoniae* is also frequently associated with meningitis, bacteremia, and CAP in adults. The incidence of pneumococcal disease is significantly higher in developing countries than in developed countries, which can be attributed to significant differences in economic and healthcare conditions between the two groups [2].

Streptococcus pneumoniae is also frequently associated with meningitis, bacteremia, and CAP in adults. The major autolytic enzyme in *Streptococcus pneumoniae*, LytA (N-acetylmurayl-L-alanine amidase), belongs to a large class of cell wall degrading enzymes. These enzymes are located on the cell membrane and are believed to play a role in various physiological processes, including microbial structure, life cycle, and cell division. During its stationary phase, *Streptococcus pneumoniae* undergoes autolysis, leading to excessive lysis of in vitro cultures [3]. The secretion of cytolytic toxins is often crucial in this process. For example, the cytolytic pore-forming toxin pneumolysin (Ply) produced by Streptococcus pneumoniae binds to cholesterol in the host cell membrane and inserts into the lipid bilayer to form transmembrane pores, thereby lysing the host cell. Ply has been shown to possess immunomodulatory activity, including pro-inflammatory and anti-inflammatory effects on the host. Although the data presented in this paper may seem contradictory, previous studies have shown that Ply can promote colonization [4]. Currently, quantitative PCR detection of the *lytA* gene is the preferred method for culture-free studies. LytA, the major autolysin of *Streptococcus pneumoniae*, has been found to be both species-specific and commensal-specific. 67S real-time PCR detection was initially tested on 67S *Streptococcus pneumoniae* cultures and 104 non-*Streptococcus pneumoniae* isolates. Many laboratories frequently use this technique for studies in patients and carriers [5].

The aim of this study was to isolate and identify *Streptococcus pneumoniae* from meningitis patients in several Baghdad hospitals, to detect genes (*lytA* and *ply*) in the isolates using PCR, and to assess the sequences of these genes to examine for any variations among local isolates.

2- MATERIALS AND METHODS

2.1 Clinical Sample Collection

Cerebrospinal fluid (CSF) samples were collected within 24 hours of admission from clinically diagnosed bacterial meningitis patients in some of Baghdad hospitals (total number of samples =100). Patients were aged between 1 and 75 years of age. 41 of 41 (41%) were female; 59 of 59 (59%) were male. Study ethical approval was obtained and informed consent was given by the study participants or their guardians prior to sample collection.

2.2 Bacterial Isolation and Identification

The CSF samples were cultured on blood agar and then incubated at 37°C in 5% CO_2 for 24 h. Colonies with typical morphology of *Streptococcus pneumoniae* (α-hemolytic, mucoid colonies) were subjected to Gram staining, optochin sensitivity, and bile solubility tests for confirmation. The specimen were identified by VITEK2 system too.

2.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined using the Mueller-Hinton disk diffusion method on agar plates. The Kirby-Bauer disk diffusion method was used to detect resistance patterns of isolated *Staphylococcus aureus* strains to nine antibiotics. Antibiotic disks used included ciprofloxacin (CIP), tetracycline (TE), and vancomycin (VA). Antibiotic disks used in this study included ceftriaxone (CRO), ceftazidime (CAZ), erythromycin (ERY), amoxicillin (AMX), chloramphenicol (CMP), and cefepime (FEP). After 24 hours of incubation at 37°C, the size of inhibition zones on the agar plates was assessed, and the percentage of strains transitioning to susceptible, intermediate, or resistant was determined according to the CLSI breakpoint interpretation guidelines.

2.4 DNA Extraction

The confirmed isolates genomic DNA was extracted from those isolates with a commercial bacterial DNA extraction kit (e.g., Qiagen DNeasy Blood &Tissue Kit) according to manufacturer instructions. The DNA was assessed for quality and quantity via spectrophotometry and 1% agarose gel electrophoresis.

2.5 Amplification of *lytA* and ply genes by PCR

The PCR assays were performed for specific genes (*lytA* and *ply* genes) for *S. pneumoniae*. The amplification primers were summarized in table 1.

Table (1): Oligonucleotide primers sequences of *Streptococcus pneumoniae* virulence genes used in this study

gene	Primer name	Sequence	Product size (bp)	Reference
lytA	***lytA* (F)**	'5-CAACCGTACAGAATGAAGCGG-3'	319	[6]
	***lytA* (R)**	'5-TTATTCGTGCAATACTCGTGCG-3'		
ply	***ply* (F)**	'5-ATTTCTGTAACAGCTACCAACGA-3'	348	[7]
	***ply* (R)**	'5-GAATTCCCTGTCTTTTCAAAGTC-3'		

The PCR reaction conditions were as follows: pre-denaturation at 94℃ for 5 minutes; then 35 cycles, each cycle consisting of denaturation at 94℃ for 30 seconds, annealing at 55℃ for 30 seconds, and extension at 72℃ for 45 seconds; and a final extension at 72℃ for 5 minutes. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and observed under ultraviolet light.

2.6 Sequencing and Bioinformatic Analysis

DNA Purification and Sequencing DNA PCR products associated with the *lytA* gene were purified using a spin column and sent for Sanger sequencing (Macrogen, Geumcheon-gu, South Korea). Sequences were aligned using NCBI BLAST and compared to reference strains using MEGA11 software. We examined conserved domains to evaluate the genetic stability and potential mutations.

3- RESULTS AND DISCUSSION

One hundred cerebrospinal fluid clinical samples were collected from meningitis patients in various Baghdad hospitals. Thirty strains of *Streptococcus pneumoniae* were identified using the VITEK2 system. Most isolates were sensitive to all tested antibiotics, except for ciprofloxacin and chloramphenicol. The resistance rate to ciprofloxacin was 68%, and to chloramphenicol was 74%. High sensitivity was observed to vancomycin (93%) and ceftazidime (89%). Moderate resistance to erythromycin and tetracycline was also observed.

Table (2): Percentage of susceptibility of 32 *Streptococcus pneumoniae* isolates to 9 antimicrobial agents

Antibiotic	Resistance (%)	Sensitive (%)
Ciprofloxacin	**68**	**32**
Chloramphenicol	**74**	**26**
Cefepime	**24**	**76**
Ceftriaxone	**21**	**79**
Ceftazidime	**11**	**89**
Amoxicillin	**27**	**73**
Erythromycin	**35**	**65**
Vancomycin	**7**	**93**
Tetracycline	**38**	**62**

Ciprofloxacin (CIP), Tetracycline (TE), Vancomycin (VA). Ceftriaxone (CRO), Ceftazidime (CAZ), Erythromycin (ERY), Amoxicillin (AMX), Chloramphenicol (CMP), Cefepime (FEP)

This finding is consistent with the global tracking of increasing resistance trends in *S. pneumoniae* [8]. Past overuse of chloramphenicol in much of the developing world, where it remains available and inexpensive, may account for the high resistance observed. Chloramphenicol resistance is usually mediated by cat genes, which encode chloramphenicol acetyltransferase, resulting in inactivation of drug [9]. Under the study conditions, this finding restricts its practical use as a probable empirical remedy for pneumococcal illness.

In a similar manner, 68% ciprofloxacin resistance is important as well. Despite being non-first-line agents for pneumococcal disease penicillin, resistance implies selective pressure, possibly from inappropriate use for the treatment of respiratory or urinary infections. Mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* genes have been implicated to cause fluoroquinolone resistance [10]. Tetracycline resistance (38%) typically mediated by tet(M), protecting bacterial ribosomes from tetracycline action. This level of moderate resistance reflects typical usage for community-acquired infections, and especially for outpatient and pediatric use.

The relatively low resistance rates for ceftazidime and ceftriaxone support the continued use of both agents for the treatment of more serious pneumococcal infections such as pneumonia and meningitis. While alterations in penicillin-binding proteins (PBPs) are common mechanisms of β-lactam resistance in *S. pneumoniae*, high-level resistance is rare in a number of geographies [11]. This finding (35%) rises on concern on the efficacy of macrolides due to erythromycin resistance rate. The resistance is often due to the presence of the erm(B) or mef(A/E) genes, which are responsible for target modification and efflux, respectively [12]. This high level of resistance clearly highlights the need for local susceptibility testing prior to use, as the increased use of macrolides for respiratory infections.

3.1 Detection of *S. pneumoniae* by *lytA* and *ply* Genes

Molecular characterization of *Streptococcus pneumoniae* clinical isolates included in this study isolated from Iraqi patients with bacterial meningitis was confirmed with PCR targeting the *lytA* and *ply* genes which are regarded as highly specific markers for *S. pneumoniae*. LytA is one of the major autolysins and ply encodes the pneumolysin, a pore-forming cytotoxin. The *lytA* gene was amplified (approximately 319 bp) from all tested isolates (n = 10) (Figure 1). Likewise, a ~348 bp PCR product of the ply gene was identified in all isolates (Figure 2).

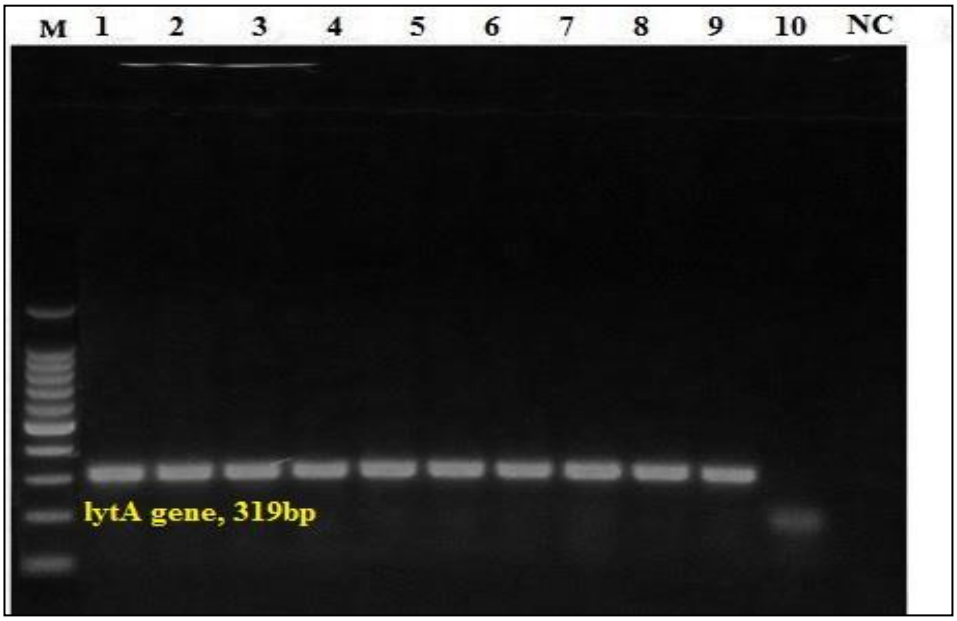

Fig (1): Gel electrophoresis amplification of the *lytA* gene from *Streptococcus pneumoniae* isolates. M: 100 bp DNA molecular weight standard. Lanes 1-10 are similar to 319 bp PCR products. Lane 11: Negative control. (1.5% agarose gel electrophoresis, ethidium bromide staining, 100 volts, 60 minutes)

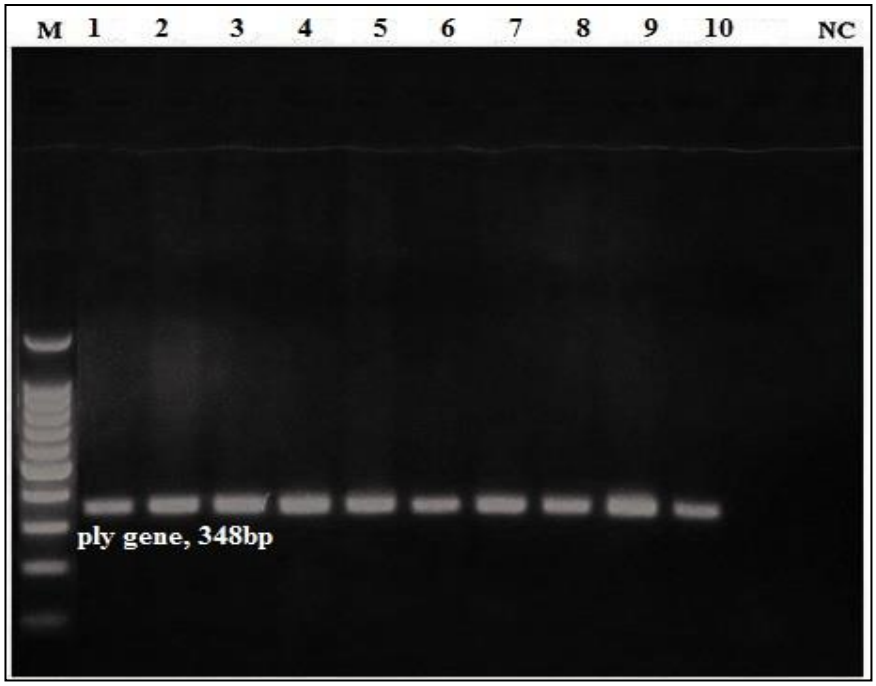

Fig (2): Gel electrophoresis amplification of *ply* gene of *Streptococcus pneumoniae* isolates, M: 100bp ladder marker. Lanes 1-10 resemble 348bp PCR products. Lane11: Negative Control. (1.5% agarose gel electrophoresis stained with Ethidium Bromide, 100volt, 60 min.)

Taken together, these findings support that these markers are specific for the identification of *S. pneumoniae*, as previously demonstrated globally [13, 14].

3.2 Molecular Study of Autolysin (*lytA*) Gene (Sequencing and Alignment)

All the empirical high-confidence *lytA* genes identified in the study were tested by sequencing analysis against the reference sequences in GenBank, comparable to that of NC_003028.3. The alignment showed some conserved regions important to autolysin function (amidase catalytic domains). The genetic conservation highlights the evolutionary significance of lytA in *S. pneumoniae* pathogenesis [15].

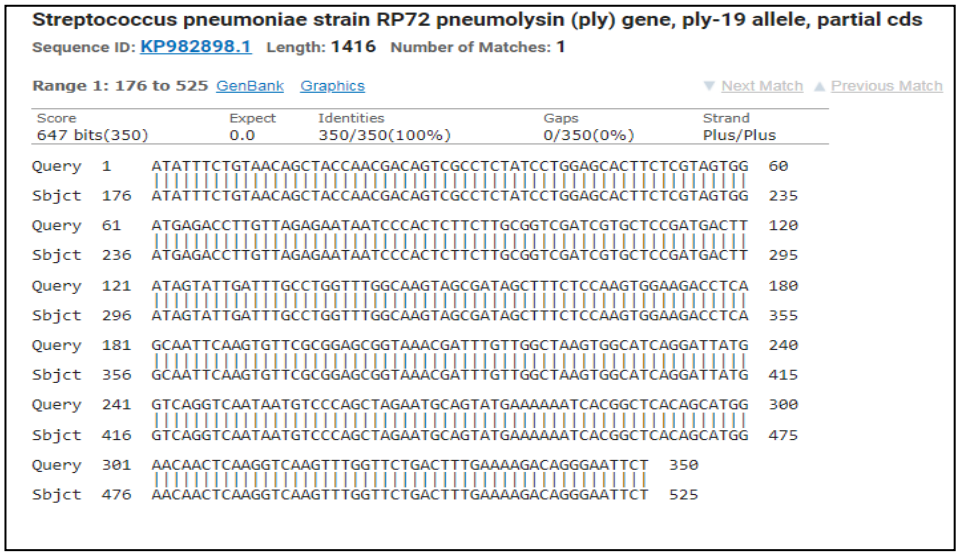

Fig (3): Alignment of *S. pneumoniae ply* gene sequence from this study with gene *plyA* from *S. pneumoniae* (Accession no. KP982898.1) available in GenBank

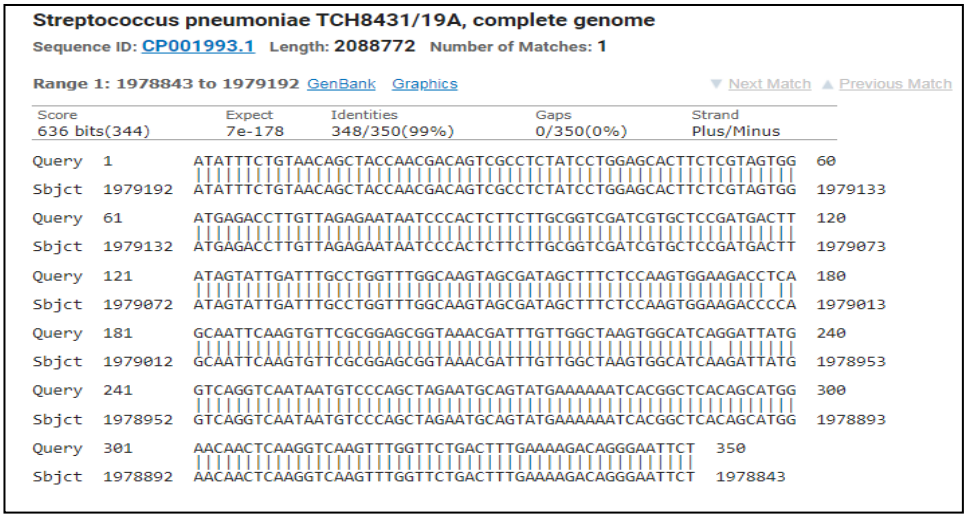

Fig (4): Alignment of *S. pneumoniae ply* gene sequence from this study with complete genome from *S. pneumoniae* (Accession no. CP001993.1) available in GenBank

In particular, the virulence factor does not seem to be unstable in Iraqi clinical isolates because no significant mutations nor deletions which change the autolysoin protein function were observed [16, 17].

The recurrent persistence of the *lytA* and *plyA* genes observed in the *Streptococcus pneumoniae* population observed in this study suggests that the products of these two genes may interact in the form of a (patho) physiological protein network. Co-transcription of bacterial genes leads to gene aggregation, resulting in high concentrations of local proteins near the coding genes. This aggregation can promote selective interactions even without co-transcription. As previously reported; plyA and *lytA* are essential for in vitro biofilm formation. However, we found that double mutants carrying the ply-out gene did not exhibit a further decrease in biofilm-forming ability compared to single mutants [18, 19].

Autolysin (lytA) promotes invasion of host tissues by degrading the bacterial peptidoglycan cell wall and liberating pro-inflammatory factors. It plays the second most critical role after pneumolysin (ply) in the pathophysiology of pneumococcal infections, such as meningitis. All isolates carried some of these virulence genes suggesting that they have a high level of pathogenicity. For instance, in meningitis, lytA-mediated autolysis may contribute to blood-brain barrier traversal and exacerbating neuroinflammation [20]. As such virulence genes represent valuable molecular diagnostic markers but may also be vaccine or antimicrobial targets.

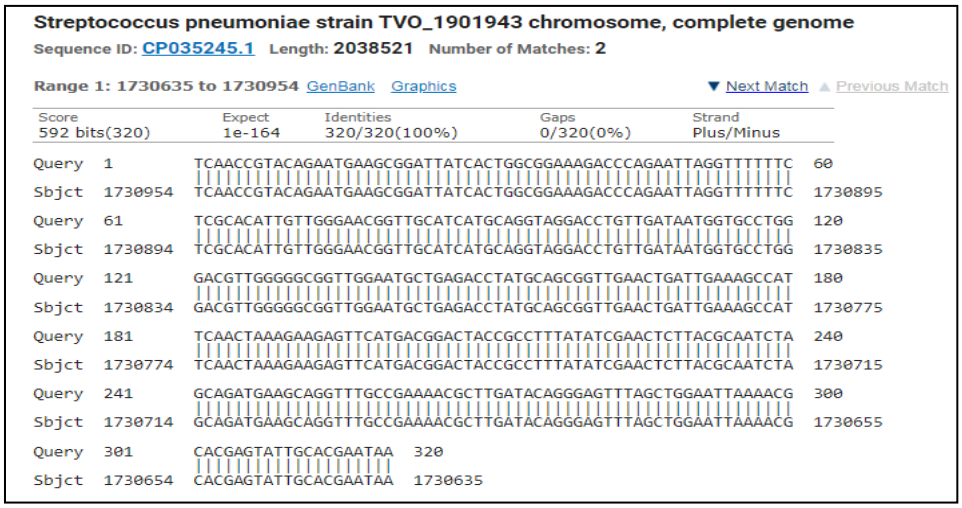

Fig (5): Alignment of *S. pneumoniae lytA* gene sequence from this study with comlete genome from *S. pneumoniae* (Accession no. NC_022082.1) available in GenBank

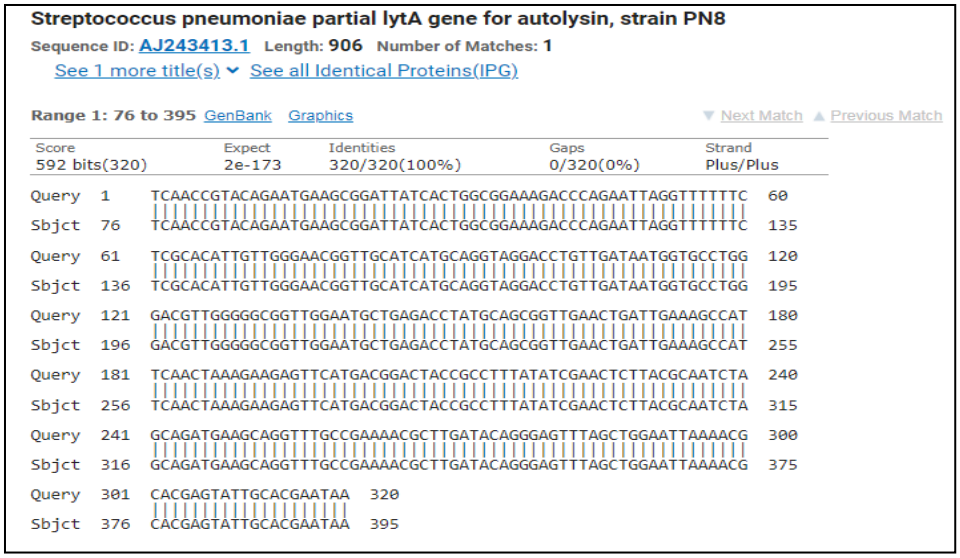

Fig (6): Alignment of *S. pneumoniae lytA* gene sequence from this study with gene *lytA* from *S. pneumoniae* (Accession no. AJ243413.1) available in GenBank

We have analyzed the genetic diversity of the *lytA* gene as part of a systematic study of allelic variations in *Streptococcus pneumoniae* virulence factors, aiming to explore the molecular evolution and vaccine potential of this gene region. Currently, little is known about the allelic diversity of the *lytA* gene in *Streptococcus pneumoniae*; however, the *lytA* gene of an atypical clinical isolate (101/87) shares only 81% homology with the *lytA* gene. In fact, comprehensive sequencing of a series of typical *Streptococcus pneumoniae* clinical isolates has shown that strain 101/87 is genetically significantly different from these clinical isolates [21].

4- CONCLUSION

The PCR detection of *lytA* and *ply* genes among *S. pneumoniae* isolates from Iraqi meningitis patients revealed as reliable markers of susceptibility. Genome analysis indicated that most or all the mutation sites affecting autolysin could not randomly be changed due to high homology with the global strains. Patterns of antibiotic resistance were alarming, especially ciprofloxacin and chloramphenicol. These findings reinforce the utility of molecular diagnostics and implicate *lytA* as a possible therapeutic target. The high resistance rates to commonly used antibiotics observed in this study highlight the need for regular monitoring of antibiotic resistance patterns in Streptococcus pneumoniae. β-lactam antibiotics and vancomycin remain effective.

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