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Molecular Characterization of Some Virulence Factors of *Streptococcus pneumoniae* Isolated from Children with Acute Otitis Media in Hilla, Iraq

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Isolation and identification of *Streptococcus pneumoniae* from children with acute otitis media. Molecular detection of some virulence genes of *S. pneumoniae* such as pneumolysin (*ply*), autolysin A (*LytA*), Neuraminidase (*NanA*), Luminescence *luxS*, capsular polysaccharide synthesis (*cpsA*), pneumococcal surface antigen *A* (*psaA*), α- Enolase (*Eno*) by using PCR primer.
Place and Duration of Study: Hilla Teaching Hospital (ENT unit) and privacy during a period from November (2014) to March (2015), Hilla, Iraq.
Methodology: A total 0f 120 Ear swabs obtained from children suffering from acute otitis media ranged from (1 months - 12 years). Only eight isolates of *S. pneumoniae* were isolates. Seven virulence genes were investigated in eight clinical isolates of *S. pneumoniae* by using PCR techniques.
Results: Pneumolysin and autolysin were seen to be present in only 4 isolates which were isolated from otitis media samples, whereas Luminescence and α - Enolase gene were present in five

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isolates, Besides, Neuraminidase and pneumococcal capsule synthesis A were detected in all isolates. It was found that Pneumococcal Surface antigen A is present in 2 isolates.

Keywords: Isolation S. pneumoniae; DNA extracts; detection virulence gene by polymerase chain reaction (PCR).

1. INTRODUCTION

Streptococcus pneumoniae is an vital pathogen that causes both serious invasive infections, such as septicemia, otitis media, pneumonia, meningitis, and upper respiratory infections, it is fits to the normal nasopharyngeal microbial flora that involves of bacteria with physiologic and genetic properties correct for colonization and multiplication under sure conditions [1].

Infection with *S. pneumoniae* occurs through respiratory droplets from person to person in most cases; initially leads to asymptomatic carriage of pneumococci in the upper respiratory tract, development of disease can happen by local spread from the nasopharyngeal mucosa leading to sinusitis and otitis media [2].

Pneumococcus virulence factors can contribute to pneumococcus disease in different way: By adhere and colonize in mucosal barrier [3].

S. pneumoniae have many virulence factors have been recognized, such as pneumococcal surface protein (*PspA)* that prevent the activation of complement, and pneumococcal enzymes such a Neuraminidases A (*nanA*), autolysin A (*LytA*), and pili that enable *S. pneumoniae* to attach to epithelial cells in the upper respiratory tract. These pili contribute to adherence and virulence, as well as increase the inflammatory response of the host [4]. Pneumolysin *(Ply)* it is a member of the household of cholesterol-dependent cytolysins that are manufactured by gram-positive bacteria. In height concentrations enzyme is toxic to bronchial epithelial cells, decreasing the ciliary movement, ending the integrity of joints, cellular bronchial epithelial monolayer, *ply* also correlates with the epithelial cells of the alveoli and pulmonary endothelial cells, causing alveolar edema, hemorrhage during pneumococcal pneumonia, facilitation the diffusion from the epithelium to the pulmonary interstitium and ultimately into the blood stream [5].

Autolysin A be in the right place to extra-cellular choline binding proteins, and its size is 36 kDa. It is a group of enzymes that destroy bacterial peptidoglycan. Their action leads to cell lysis. The careful role of *LytA* in the virulence of pneumococcus is unclear [6]. *lytA* plays a role in virulence through the release of highly inflammatory cell wall degradation products and also release of *ply* from the cytoplasm [7].

However, the LuminescenceS *(luxS)* gene plays an important role in quorum sensing for many bacterial species. Quorum-sensing systems improve bacterial access to nutrients and environmental niches, and enhance production of virulence factors and other defense capabilities against the host and other microorganisms [8].

Neuraminidases, also known as sialidases, cleave terminal sialic acid residues from glycoproteins, glycolipids and cell-surface oligosaccharides. Studies have shown that neuraminidases can remove sialic acid from soluble proteins, such as lactoferrin, IgA2 and secretory component [9], also is able to directly bind epithelial cells via a lectin domain, eliminate sialic acid to expose receptors to aid pneumococcal adherence, help in formation of biofilms [10].

On the other hand Pneumococcal Surface Protein A *(PsaA)* is surface protein found on the cell wall of *Streptococcus pneumoniae*. *PsaA* is a virulence factor of pneumococci; it has a molecular weight of 34,539 KDa and is composed of 309 residues, it is suggested to play a crucial role in pneumococcal survival in response to oxidative stress, as mutants lacking *PsaA* exhibited increased cell death in presence of H2O2 compared to wild-type [11].

Streptococcus pneumoniae capsular polysaccharides (*CPS*) are of interest both as virulence factors and as a protective immunogens for prevention of invasive disease [12].

In addition, the *cps* gene cluster encodes the capsule formation. It comprises several genes, and is located in the pneumococcal chromosome, which are not involved in CPS synthesis, particularly within the first four genes, *cpsA*-*cpsD*, are common to all serotypes [13].

Table 1. Primers of virulence gene used in PCR

Genes	Primer sequence (5'-3')	Size of produt bp	PCR condition			References
			94ºC		3 min	[17] Sakai et al.
nanA F	ATAGACGTGCGCAAAATACAGAATCA			1x		
			94ºC		1 min	
		550	52ºC		1 min	
			72ºC		1.30 min	
nanA R	GTCGAACTCCAAGCCAATAACTCCT			30X		
			72ºC		7 min	
				1 X		
			94ºC		3 min	[18] Brito.
CpsA F	GGTGTTCTCTATCCTTGTCAGCTCTGTGTCGCTC			1x		
		157	94ºC		1 min	
			52ºC		1 min	
CpsA R	GTGTGAATGGTCGAATCAACTCTATAAATGCC		72ºC		1.30 min	
				30 X		
			72ºC		7 min	
				1x		
			95ºC		5 min	[17] Sakai et al.
LuxS F	ACATCATCTCCAATTATGATATTC			1x		
			95ºC		20 sec	
		254	55ºC		30 sec	
LuxS R	GACATCTTCCCAAGTAGTAGTTTC		68ºC		1 min	
				35 X		
			68ºC		7 min	
				1x		
			95ºC		5 min	[17] Sakai et al.
Eno F	GACGGTACTCCTAACAAAGGTAAA			1x		
		110	95ºC		20 sec	
			55ºC		30 sec	
Eno R	ATAGCTGTAAAGTGGGATTTCAAG		68ºC		1 min	
				35 X		
			68ºC		5 min	
				1x		

Genes	Primer sequence (5'-3')	Size of produt bp	PCR condition			References
			94ºC		3 min	[19] Anthony et al.
PsaA F	CTTTCTGCAATCATTCTTG			1x		
			95ºC		30 sec	
		838	52ºC		30 sec	
psaA R	GCCTTCTTTACCTTGTTCTGC		72ºC		2 min	
				35x		
			72ºC		7 min	
				1x		
			94ºC		3 min	[20] Garnier et al.
Ply F	ATTTCTGTAACAGCTACCAACGA			1x		
		329	94ºC		1 min	
			52ºC		1 min	
Ply R	GAATTCCCTGTCTTTTCAAAGTC		72ºC		1.30 min	
				28x		
			72ºC		7 min	
				1x		
			94ºC		3 min	[21] Stralin
LytA F	CGGACTACCGCCTTTATATCG			1x		and Korsgaard
		229	94ºC		1 min	
			52ºC		1 min	
lytA R	GTTTCAATCGTCAAGCCGTT		72ºC		1.30 min	
				28x		
			72ºC		7 min	
				1x		

Finally α- Enolase has a molecular weight of 82-100 K Daltons depending on the isoform, also it is found in the cytoplasm and is a glycolytic enzyme that catalyses the conversion of phosphoglycerate to phosphoenolpyruvate [14].

The activity of α-enolase was found in the cytoplasm and in whole cells. Activity was also demonstrated in cell wall fractions, which confirmed that α-enolase is a cytoplasmic antigen also expressed on the surface of *S. pneumoniae*. The plasminogen-binding activity of α-enolase was examined by Western blot, which showed that purified α-enolase was able to bind human plasminogen [15].

2. MATERIALS AND METHODS

2.1 Patients

A total 120 samples, only eight isolates of *Streptococcus pneumoniae* were obtained from children suffering from acute otitis media by standard bacteriological methods. All samples were obtained from patients or individuals who admitted to Al-Hilla Surgical Teaching Hospital in Babylon Governorate.

2.2 Bacterial Identification

The samples were processed on blood agar and chocolate plate agar were incubated at $37c_o$ with ~5% CO2 (or in a candle-jar). The identification of gram positive bacteria was performed by standard biochemical methods (catalase test, oxidase test, optochin sensitivity, bile solubility, present of capsule) [16].

2.3 DNA Extraction for Gram Positive Bacteria

DNA extraction was carried out according to the genomic DNA purification kit supplemented by manufactured company (Viogene, Taiwan).

2.4 Detection of Some Virulence Gene Markers by PCR

The primers and PCR conditions used to amplify genes encoding virulence factors with PCR are listed in Table (1 above). The primers includes *ply* gene, *lytA, cpsA, Eno, nanA, psa A* and *luxS*. Each 25µl of PCR reaction contained 2.5µl of each upstream and downstream primer, 2.5µl of free nuclease water, 5µl of DNA extraction and 12.5 µl of master mix. The PCR amplification product were visualized by electrophoresis on 1% agarose gels for 45 min at 70v. The size of the amplicons were determined by comparison to the 100 bp allelic ladder (promega, USA).

3. RESULTS AND DISCUSSION

Seven primers were used to investigate the presence of seven important genes these are *ply, lytA, nanA ,luxS, cpsA, eno and psaA* genes for eight *S. pneumoniae* isolated from acute otitis media samples.

It was found that *ply* is present only in 4 isolates, were shown in Fig. (1).

This results is agreement with results obtained by [22], who found our isolates were PCR positive for pneumolysin (17 out of 24 isolates). and this result disagrees with the result obtained by [23]who found that (85%) rate for ply, during the screening of *S. pneumoniae* isolates. These differences may be due to the difference in the sequence of this marker. However, phenotypically all *S. pneumoniae* isolates were found to be able to produce pneumolysin extracellulary in blood agar that will give an interpretation that pneumolysin may be encoded by more than one genetic loci may have a role in pneumolysin production and may be other bacterial exotoxins have the ability to direct lysis of cells and ultimately help with microbial spread through tissues by causing momentous damage to the extracellular matrix or the plasma membrane of eukaryotic cells. Perhaps, these toxins result in this cellular injury by dint of enzymatic hydrolysis or pore development, such as these protein are hyaluronidases, protein kinase, collagenases, and phospholipases are capable of decaying cellular membrane or matrices [24]. Ply gene was investigated for identification of *S. pneumoniae* in clinical specimens from infected patients with otitis media, pneumonia or meningitis [25]. It was found that lyt A is present only in 4 isolates, were shown in Fig. (2).

However, the result of this study was found that *lytA* gene was observed only in four isolates (50%) of *S. pneumoniae* strains. These results is identical with results obtained by [23] who found the prevalence of *lytA* is (51%) of isolates by PCR, but the results is not identical to the results obtained by [26] who found *lytA* gene detected in (23%) from Middle ear effusion samples. The major autolysin of pneumococci is *LytA* which responsible for the cleavage of peptidoglycan, it may be play important role, both directly and indirectly in pathogenic process in two ways:

(i) by generating inflammatory cell wall degradation products but under certain conditions such as in the stationary phase of growth in vitro or on antibiotic or detergent treatment it induces cell lysis and (ii) by releasing the pneumococcal cytoplasmic contents, including virulence factors such as pneumolysin [27].

However, it was found that psa A is present only in 2 isolates, were shown in Fig. (3).

Psa A is present in two isolates (25%). This result is agreement with result obtained by [19] who was found the ability of *S. pneumoniae* to produce *psaA* gene are (30%), but this result is disagreement to the result obtained by [28] who found *psaA* gene detected in all isolates of *S. pneumoniae* from clinical samples.

PsaA are major group lipoproteins of bacterial surface proteins that have diverse functions, and often have important effects on pathogen host interactions during the development of infection. The majority of bacterial pneumococcal surface antigen A are substrate-binding proteins for ATP binding cassette transporters involved in the transport of a wide range of substrates including cations, sugars, amino acids, oligopeptides, polyamines, and minerals and which individually can be vital for full virulence [29].

Also, It was found that *cpsA* is present in all isolates, were shown in Fig. (4).

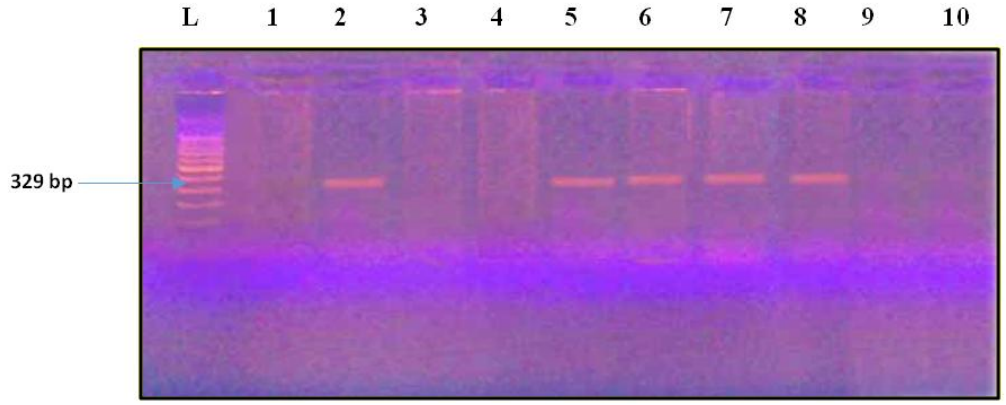

Fig. 1. Gel electrophoresis of PCR of ply amplicon; L (ladder) molecular weight marker of ladder (100 bp), 1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

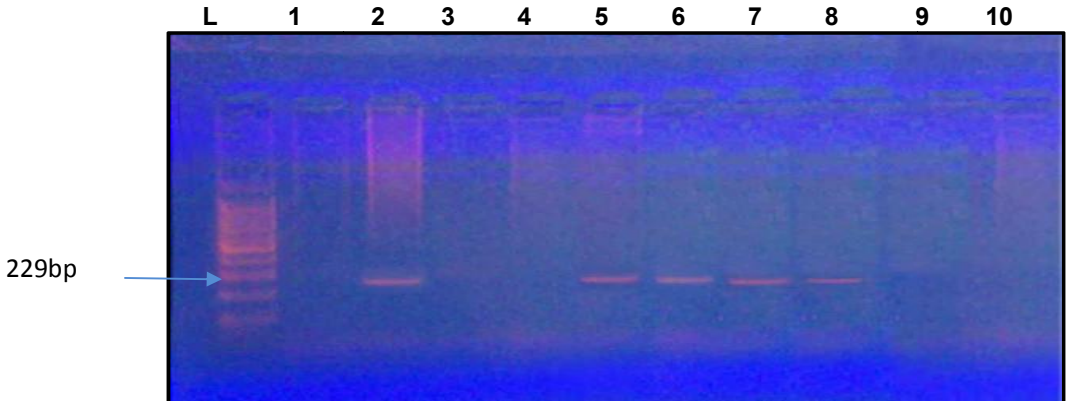

Fig. 2. Gel electrophoresis of PCR of lytA amplicon; L(ladder) molecular weight marker of ladder (100 bp), 1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

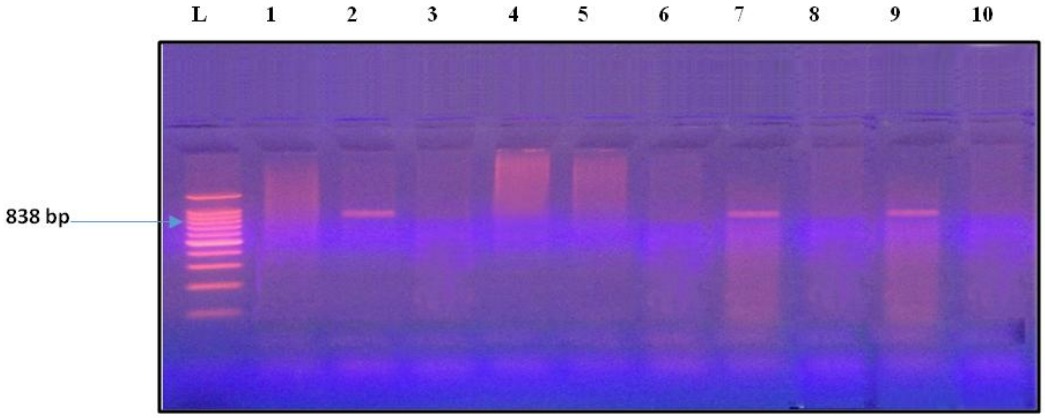

Fig. 3. Gel electrophoresis of PCR of psaA amplicon; L(ladder) molecular weight marker of ladder (100 bp), 1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

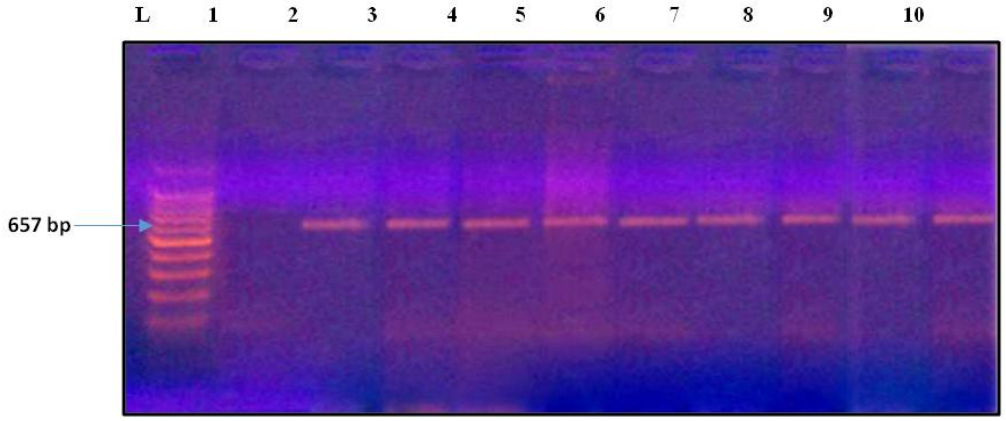

Fig. 4. Gel electrophoresis of PCR of cpsA amplicon; L (ladder) molecular weight marker of ladder (100 bp),1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

Besides, *CpsA* gene is present in all isolates of *S. pneumoniae*. Therefore, this result confirms that *cpsA* is present in this bacteria and it considered as a housekeeping gene and used it for molecular identification. In addition, this positive isolates for confirmation of the PCR were in complete concordance with the nigrosin stain test results, this results correlate with the results obtained by [30] who pointed that all clinical isolates of *S. pnumoniae* contained this gene.

CpsA is the first gene of pneumococcal capsule operon and level of its expression is associated to invasiveness of isolates [31]. The *cpsA* gene as a novel genomic marker specific for *S. pneumoniae*. It is specific primer set discriminated *S. pneumoniae* from other closely related viridans group members. This new primer set may be useful for the routine diagnosis and identification of presumptive *S. pneumoniae* isolates. Capsule is thought to be to protect the bacteria from phagocytosis following invasion, and in the nasopharynx to repel mucus and so aid colonization. It seems that the capsule type either enables a pneumococcus to reside for a long time in the nasopharynx (high colonization prevalence serotype) or causes it to be cleared quickly from the nasopharynx requiring invasion for its survival (low colonization prevalence serotype) [32].

However, It was found that *luxS* is present in all isolates, were shown in Fig. (5).

Fig. 5. Gel electrophoresis of PCR of luxS amplicon; L (ladder) molecular weight marker of ladder (100 bp),1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

Also*, LuxS* gene was present from five isolates (62.5%) of *S. pneumoniae*, this result is agreement with results obtained by [33] who was found the ability of *S. pneumoniae* to produce *luxS* gene are (57%) from clinical sample.

In *S. pneumoniae*, *luxS* is a virulent gene and a central regulator of competence, fratricide, and biofilm formation. The phenomenon of Quorum-sensing is a cell-to-cell communication mechanism that uses molecules called auto-inducers to regulate gene expression in response to environmental and cell density changes experimental evidence indicates that the *LuxS* Quorum-sensing system is implicated in the persistence, virulence, and dissemination of *S. pneumoniae* [34].

It was found that *α-Eno* is present only in 5 isolates, were shown in Fig. (6).

α- Enolase (*α-Eno*) was observed in five bacterial isolates (62.5%). The results obtained in this study are identical with the results previously mentioned by [35] who detected α-Enolase gene by PCR and they found the ability *S. Pneumoniae* to produce α-Enolase gene at a rate (68.1%) of isolates. However, this result was disagreement with results obtain of [36] who found that the α-Enolase gene is present in rate (75 %).

The pneumococcal α-enolase labeled *Eno*, has been identified and characterized as a surface-displayed protein that binds both plasminogen and plasmin and exhibits glycolytic enzyme activity. The α- enolase gene showed decreased transcription during bacterial growth in blood, infected cerebrospinal fluid and bacteria attached to a pharyngeal epithelial cell line.α-enolase protein expression is influenced by environmental conditions, but with even lower expression in vivo than *in vitro* [36].

Finally, It was found that *nanA* is present in all isolates, were shown in Fig. (7).

NanA was observed in all *S. pneumoniae* isolates**.** This results is corresponding with results obtained by [30] were found that *nanA* is present in all clinical isolates**.** Moreover, *S. pneumoniae neuraminidase* has been detected in 78% of middle ear effusions from patients with acute otitis media and in 96% of *S. pneumoniae* positive middle ear effusions from patients with chronic otitis media [37].

S. pneumoniae neuraminidase may affect middle ear pressure by disrupting the Eustachian tube function, a key determinant in the maintenance of normal middle ear pressure. *NanA* has a highly defined role in pneumococcal colonization, and the presence of *nanA* in commensal relatives probably highlights its role in adherence and colonization of mucosal surfaces as well. *Neuraminidase A* thus appears to play multiple temporal roles in pneumococcal infection, from adherence to host tissues, colonization, and community development, to systemic spread and crossing of the blood-brain barrier [38].

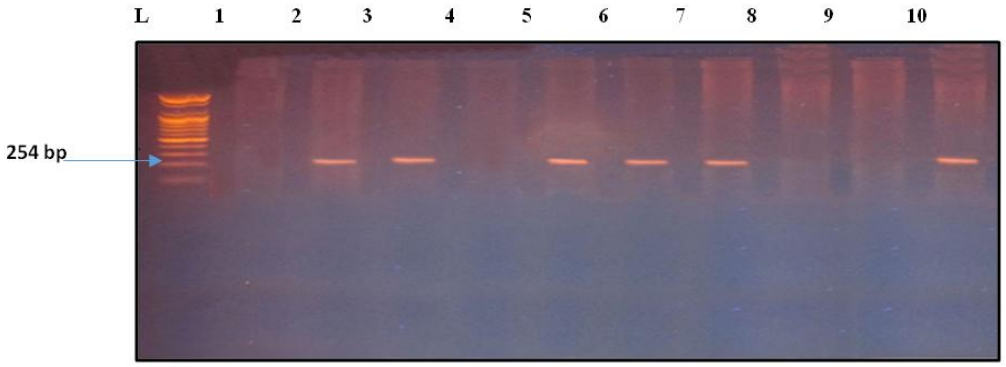

Fig. 5. Gel electrophoresis of PCR of *luxS* amplicon; L (ladder) molecular weight marker of ladder (100 bp), 1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

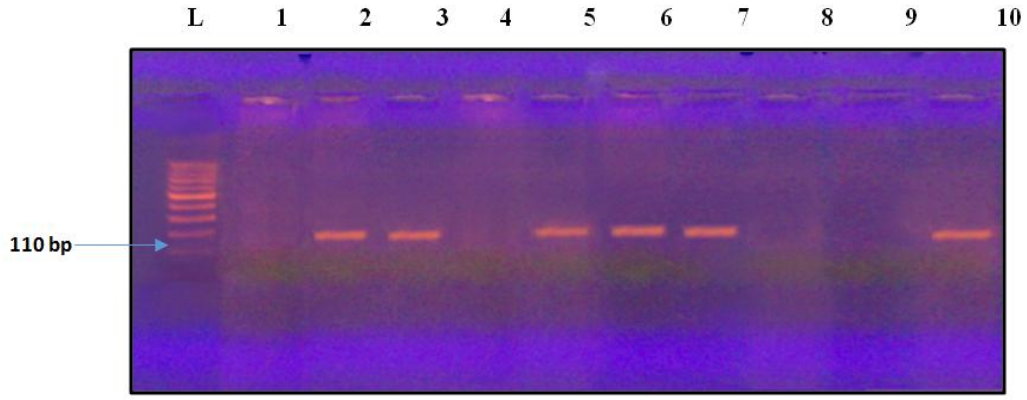

Fig. 6. Gel electrophoresis of PCR of *α-Eno* amplicon; L (ladder) molecular weight marker of ladder (100 bp), 1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

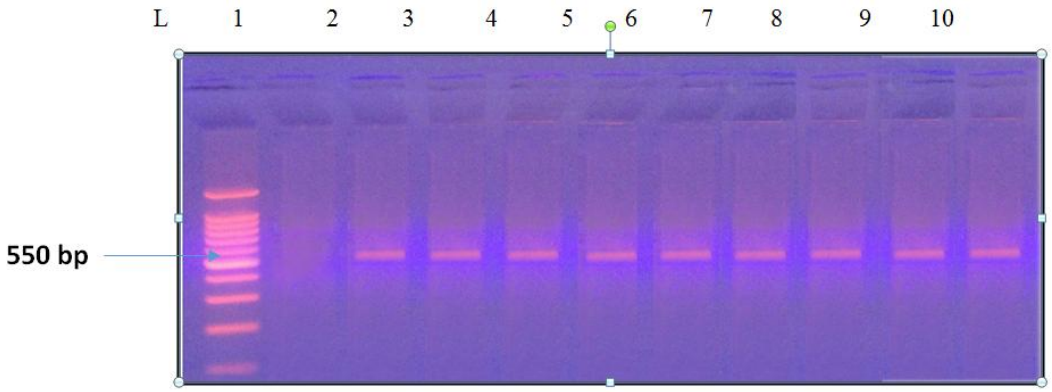

Fig. 7. Gel electrophoresis of PCR of *nanA* amplicon; L (ladder) molecular weight marker of ladder (100 bp), 1 control negative, 2 control positive, 3-10 samples obtained from acute otitis media

These results indicate that each steps in the infection process can be mediated by a number of alternative virulence factors and each strain may have a unique combination of these factors. The absence of virulence genes in some of the isolates suggests that infections by *S. pneumoniae* may require the involvement of multiple virulence factors.

The presence of such virulence genes may promote the ability of these bacteria to cause a disease in human.

4. CONCLUSION

Streptococcus pneumoniae is pathogenic to man if it is isolated from otitis media. The isolates of *Streptococcus pneumoniae* were seen to be able to produce different virulence genes such as *ply, lyt A*, *nan A*, *lux S, Eno*, *Cps A* and *psa A* this makes the bacteria more dangerous. *Cps A* and *nan A* are common in all *Streptococcus pneumoniae* isolates, the isolates that give positive *ply* is the same isolates give the positive results of *lytA* may be attributed to the release of *Ply* is dependent on another *S. pneumoniae* enzyme such as autolysin (*lytA*). The *luxS* is not required during the initial stages of nasopharyngeal colonization; in addition, it is important for survival on mucosal surfaces will significantly contribute to the pneumococcal disease process, *PsaA* plays a major role in pneumococcal attachment to the host cell and virulence, also the present *α-Eno* in five isolates refer to important role of this gene in the pathogenesis of pneumococcal infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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