

# The Dominance of Pilus Islet 1 in Pneumococcal Isolates Collected From Patients and Healthy Individuals

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## Abstract

**Background:** Pili in *Streptococcus pneumoniae* have been shown to be one of the adherence factors for epithelial cells in the human upper respiratory tract. Two types of pilus-like structures (pilus islet-1 and pilus islet-2) have been distinguished in *S. pneumoniae*.

**Objectives:** To investigate the presence of pilus islet-1 (PI-1) in *S. pneumoniae* and the correlation between our isolates.

**Materials and Methods:** In this study, 162 *S. pneumoniae* isolates were collected from clinical specimens, and normal flora were also examined for the distribution of PI-1 using the presence of the *rlrA* and *rrgC* genes as markers for this islet and *sipA* as an indicator of pilus islet-2 (PI-2). BOX-PCR analyses were performed to determine the genetic relationship between isolates.

**Results:** The results confirmed the presence of *rlrA* and *rrgC* genes in both clinical (n = 39) and normal flora (n = 26) isolates. The minimal inhibitory concentration results revealed that the rate of resistance of these isolates to the three antibiotics tested ranged from 26% for penicillin to 46% for erythromycin and tetracycline. Furthermore, 12% of the isolates were resistant to all three antibiotics. Strain typing using repetitive element BOX-PCR analysis among the 65 isolates identified 8 different band patterns.

**Conclusions:** Our results indicated that the dissemination of PI-1 was widespread in *S. pneumoniae* isolates, although no PI-2 isolates were detected. Furthermore, the frequency of *rlrA* and *rrgC* of clinical isolates was significantly more than that of normal flora isolates.

**Keywords:** Pilus, BOX-PCR, *Streptococcus pneumoniae*

## 1. Background

*Streptococcus pneumoniae* colonizes the nasopharynx of approximately 50% of adults as a part of their normal flora (1). This process is a primary step for the interaction of bacteria with host cells (2). Afterward, these bacteria can migrate to the bloodstream and establish disease in the host, causing respiratory tract infections and leading to invasive diseases, such as septicemia and meningitis (3). Recent studies have shown that the presence of pili enhances the ability of the initial bacteria to adhere to epithelial cells (4). Two different pilus islets (PI-1 and PI-2) encoded by *S. pneumoniae* play a role in the initial host cell's contact with the respiratory tract (5). A chromosomal *rlrA* pathogenicity islet with a size of 14.2 kb encodes the PI-1 (6) and contains seven genes: *rlrA*, a positive transcriptional regulator of the gene cluster for pilus expression, three pilus subunits (*rrgA*, *rrgB*, and *rrgC*), and three sortase enzymes (*srtB*, *srtC*, and *srtD*) (7).

PI-2 is a 7-kb region that consists of five genes that encode two surface proteins (PitA and PitB), a signal peptidase-like protein (SipA), and two sortases (SrtG1 and

SrtG2) (5). The PI-2 is composed of polymers of the major pilus protein PitB that contain accessory pilus proteins that serve as adhesins, while both the signal peptidase-related protein SipA and sortase SrtG1 are necessary for the assembly and polymerization of the pilus (8). As previously mentioned, the presence of pili is not only associated with serotype but is related to clonal property as well (9). The molecular typing of *S. pneumoniae* can be performed using different techniques, such as ribotyping, pulsed-field gel electrophoresis, and DNA analysis using polymerase chain reaction (PCR) (10). DNA analysis based on the BOX element has some of the best discriminatory potential. BOX-PCR is DNA typing procedure for amplifying human genome-like tandem repeat DNA elements (10). This method is a quick and very reproducible technique for quantitating the genotypic variation among pneumococcal isolates. Typing of microbial pathogens in a quick and effective manner is of great importance for microbiology and epidemiology (11). Furthermore, this method enables the analysis of precise band sizes without requiring software (12).

## 2. Objectives

In view of the importance of pili in pneumococcal infections and introducing new protein conjugated pneumococcal vaccine-like pili in recent studies, this investigation was undertaken in Tehran, Iran, to study the prevalence of pili in pneumococcal strains isolated from patients and normal flora and also to determine their clonal types using BOX PCR.

## 3. Materials and Methods

### 3.1. Sample Collection and Identification of the Isolates

We analyzed 162 isolates (92 collected from patients in 15 medical centers in Tehran hospitals and 70 from normal flora samples) for the presence of PI-1 and PI-2 from 2010–2013. Participants who had not completed any antibiotic course of treatment at least six months prior to the sampling were analyzed for the carriage of nasopharyngeal *S. pneumoniae*. The sources of the clinical isolates were found to be cerebrospinal fluid (CSF) (n = 10), blood (n = 20), eye infections (n = 24), the trachea (n = 5), lung secretions (n = 16), ear infections (n = 6), pleural fluid (n = 3), urine (n = 1), the sinuses (n = 5), ascites (n = 1), and abscesses (n = 1); these results are shown in Table 1.

All clinical samples were initially assessed for the presence of *S. pneumoniae* using blood agar plates that were incubated at 37°C. These samples were also sub-cultured for purity before further testing. First, pneumococcal identification was confirmed by colony morphology,  $\alpha$ -hemolysis,

optochin susceptibility, and bile solubility assays. All pneumococcal isolates were isolated and confirmed using specific primers for the amplification of the *lytA* gene (13).

### 3.2. Antimicrobial Susceptibility

In this study, the minimum inhibitory concentrations (MICs) of penicillin, erythromycin, and tetracycline for the 162 isolates were determined using the E test (Liofilchem, Via Scozia, Roseto d. A., Italy) method on Mueller-Hinton agar. All plates were incubated at 37°C for 24 hours and were evaluated according to the guidelines of the clinical laboratory standards international (CLSI) (14).

### 3.3. DNA Extraction and PCR Protocol

Extraction of bacterial DNA was performed using a DNA purification kit (Roche, Manhiem, Germany) according to the manufacturer's instructions. Primers were used on the principle of the primary structure of PI-1 and PI-2 for *rlrA*, *rrgC*, and *sipA*, as listed in Table 2 (8, 15, 16). The PCR program was as follows: *rlrA*, *rrgC*, and *sipA*, 30 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by 5 minutes at 72°C. Each PCR product was purified and directly sequenced for confirmation.

### 3.4. BOX-PCR

BOX-PCR was carried out using the primer of *boxA* 5'-CTA CGG CAA GGC GAC GCT GAC G-3', an interspersed, repetitive DNA sequence disseminated widely among bacterial genomes. The PCR program was designed as follows: pre-denaturation at 95°C for 7 minutes, 30 cycles each of denaturation at 90°C for 30 seconds, primer annealing at 48°C for 1 minute, chain extension at 65°C for 8 minutes, and a single cycle extension at 65°C for 16 minutes (17).

### 3.5. Gel Electrophoresis and Pattern Analysis

The amplified products were separated by size on a 1% agarose gel run in 0.5 X Tris-borate-ethylenediaminetetraacetic acid at 100 V for 3 hours. The gel was stained with ethidium bromide (1 mg/mL) and viewed with ultraviolet light. The details were evaluated using a visual examination of the banding patterns.

### 3.6. Statistical Analysis

All statistical analyses of the links were carried out using the IBM statistical package for the social sciences (SPSS) for the analysis of categorical data. All information was calculated with the fisher's exact test.

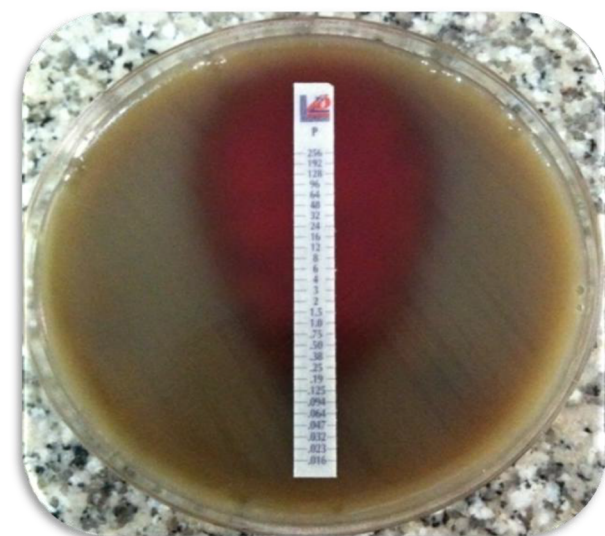


Figure 1. The E Test Image of Penicillin

**Table 1.** Properties of the PI-1 Positive Isolates

BOX Type	Normal Flora No.	Clinical Sample No.	Sources of Clinical Isolates
1	7	17	CSF (2), ear (1), blood (3), eye (9), ascites (1), trachea (1)
2	-	1	Eye (1)
3	2	2	Blood (1), pleura (1)
4	-	3	Lung secretions (3)
5	-	1	Pleura (1)
6	1	1	Sinuses (1)
7	12	5	Abscess (1), eye (2), blood (1), lung secretions (1)
8	4	9	Blood (3), eye (2), lung secretions (1), CSF (2), trachea (1)

**Table 2.** List of Primers Used in This Study

Gene	Sequence (5' - 3')	Product Size	Reference
<i>rtrA</i>	CTTCCACGAAGTCTTTCAATGG	850	(8)
	GTCTTAGAATATCATGGTTTACGTGC		
<i>rrgC</i>	GCCTGTGTTTTCTCTGTATGG	1030	(15)
	ATCAATCCGIGGTCGCTGTATTTTTA		
<i>sipA</i>	CTCTAGGAGGGATCTCTTTATCATC	500	(16)
	CTACAGCCGTGTTCGGATTGTC		

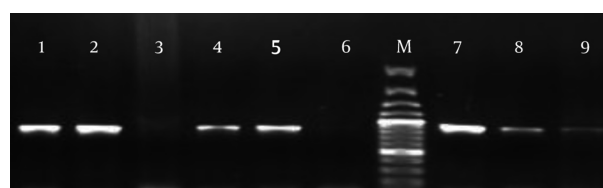
## 4. Results

### 4.1. Prevalence of PI-1 and PI-2

Among the *S. pneumoniae* isolates, 65 (40%) isolates (39 (60%) clinical and 26 (40%) normal flora isolates) were positive for *rtrA* and *rrgC* (Figure 2). The most common sources of clinical isolates harboring *rtrA* and *rrgC* were found to be eye infections in 36% (n = 14), followed by the blood (n = 8), which were significantly (P < 0.05) higher than any other sites. Other sources included CSF (n = 4), ear infections (n = 1), the pleura (n = 2), the trachea (n = 2), the sinuses (n = 1), abscesses (n = 1), lung secretions (n = 5), and ascites (n = 1) (Table 1). The presence of PI-2 was detected by PCR analysis that used the *sipA* gene as a marker for this islet; no positive isolate was found in the 162 *S. pneumoniae* isolates.

### 4.2. Antibiotic Resistance

The MIC results for penicillin showed that 25% (n = 40) of our isolates were penicillin nonsusceptible (MIC > 2). Seventeen (26%) isolates that contained PI-1 were penicillin nonsusceptible (MIC > 2). Amongst the remaining isolates



**Figure 2.** The Representative image shows amplicons of 850 bp for the *rtrA* gene. The negative control is located in lane 6.

without PI-1, 23 (23%) were penicillin nonsusceptible. In addition, the MIC for erythromycin and tetracycline revealed that 30 (46%) isolates were resistant to these antibiotics. Eight (12%) isolates with PI-1 were resistant to all three antibiotics, while 8% (8/97) of the isolates without PI-1 were resistant to all three antibiotics (Table 3). Furthermore, the MIC results indicated that 46% of the clinical isolates were resistant to at least one antibiotic.

### 4.3. Clonality of Pilus-Harboring Isolates

The BOX-PCR analysis of 65 isolates carrying PI-1 provided a total of 8 different patterns with two to five bands (Figure 3 and Table 1). Six types (with > 2 isolates) accounted for 98% of the total isolates. Types 1, 7, and 8 contained the highest number with 24, 17, and 13 isolates, respectively. Types 1, 3, 7, and 8 included isolates from the blood and CSF. Type 1 constituted 9/14 (64%) of all strains isolated from eye infections. Some of the types (e.g., 2, 4, and 5) contained only clinical isolates. Other types were found both in clinical and normal flora samples.

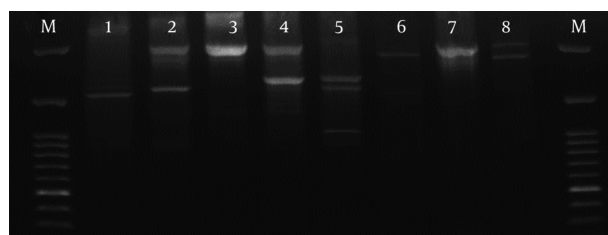
## 5. Discussion

Because they play a major role in adhesion to host cells and are also associated with a number of different virulence mechanisms, the pili of Gram-positive bacteria have

**Table 3.** Antimicrobial Susceptibility of PI-1 Positive and Negative Isolates

Antibiotic Resistance	No. (%) MIC Penicillin > 2	No. (%) MIC Erythromycin > 16	No. (%) MIC Tetracycline > 16
Pilus islet 1 (+)	17 (26)	30 (46)	30 (46)
Pilus islet 1 (-)	23 (23)	58 (59)	58 (59)

Abbreviation: MIC, minimum inhibitory concentration.



**Figure 3.** Representative BOX-PCR Patterns for Isolates with PI-1 of *S. pneumoniae* Performed with Box A Primer

recently received considerable attention (4). In particular, the *S. pneumoniae* pilus was found to be associated with the initial adherence of bacteria to epithelial cells (3) and is regarded as a potential vaccine candidate (18).

As already mentioned in other studies, PI-1 was present in 21% - 27% of clinical isolates depending on the geographic region analyzed (9), but PI-2 is rarely detected in pneumococcal isolates (19). We found that 40% of isolates contained PI-1, which was in agreement with previous findings. A comparison of our results with those of other studies suggested a higher incidence of PI-1 (5, 9). On the other hand, our study demonstrated that none of the isolates contained PI-2. In contrast, Zahner and colleagues identified 21% PI-2 in their isolates (8). Another study also indicated the presence of PI-2 (16%) in global collections (1). This result confirms that the prevalence of PI-2 is related to particular geographic regions and populations; published data have shown PI-2 isolates in global collections are included in the 13-valent pneumococcal conjugate vaccine now being used in an increasing number of countries (8).

PI-1 (*rtrA*, *rrgC*) and PI-2 (*sipA*) genes were used to detect these structures between our isolates. The *rtrA* gene has been identified as an essential virulence gene in the murine infection model (7). Moreover, other studies have shown that these genes are also essential for expression of the pili (1, 9). Although Basset et al. discussed the limitations of using the detection of *rrgC* by PCR as a marker for the presence of a pilus islet (6), this may have been too broad a criterion because strains that carry the *rrgC* gene may lack other genes essential for pilus expression (9). The present study also had the same limitation. The PCR per-

formed to identify the *rrgC* and *rtrA* genes indicated that the *rtrA* islet was present in all isolates. Regarding PI-2, the signal peptidase-related protein SipA is necessary for the assembly and polymerization of the pilus (8). Our isolates lacked this gene, which explains their absence of PI-2.

Our results revealed that the prevalence of PI-1 in clinical isolates was significantly ( $P < 0.05$ ) higher than normal flora isolates. As suggested in previous studies (4, 20), our results indicated that the pili are important factors in bacterial pathogenesis and disease. Conversely, it seems that strains that are overrepresented in the normal flora would be more likely to acquire the pilus from other strains (6). However, Basset and colleagues demonstrated that the similar frequency of *rrgC*-positive strains in both normal flora and bacteremic isolates denotes that the pilus does not play an important role in virulence (6). In addition, they suggested that the pilus does not represent an important virulence factor for invasive diseases in humans (6).

However, our results showed that the prevalence of PI-1 was significantly higher in blood samples as well as in pneumococcal isolates from eye infections. We also proved a connection between the presence of a pilus islet and resistance to antimicrobials in clinical collections. Most of the clinical isolates were resistant to at least one antibiotic. However, we did not observe this same association between the pilus islet and normal flora collections of *S. pneumoniae*. In addition, our results indicated that the prevalence of resistance to the three antibiotics we tested was significantly higher in pilus-harboring isolates than it was in the isolates that lacked it. Other studies (8, 9) have also concluded a similar relationship between the *rtrA* and antimicrobial resistance.

Our BOX PCR result showed that the isolates harboring a pilus had four main types. This result is inconsistent with a previous study, which showed that the presence of a pilus locus is a clonal property among invasive pneumococcal isolates (6). In all, the data obtained in this study revealed that the rate of PI-1 in our society is high. The fact that the majority of isolates with PI-1 were collected from clinical samples (especially in invasive isolates) suggests the role of this structure in infection. According to the BOX PCR typing, the strains were also arranged in clonal groups.

## Footnotes

**Authors' Contribution:** Study concept and design, Malihe Talebi, Mohammad Reza Pourshafi; acquisition of data, Malihe Talebi, Farzaneh Khodaei; analysis and interpretation of data, Malihe Talebi, Ali Ahmadi, Shirin Sayahfar, Gholamreza Irajian; drafting of the manuscript, Malihe Talebi, Farzaneh Khodaei, Gholamreza Irajian; critical revision of the manuscript for important intellectual content, Mohammad Reza Pourshafi; administrative technical and material support, Mohammad Reza Pourshafi, Shirin Sayahfar; study supervision, Mohammad Reza Pourshafi, Malihe Talebi.

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## References

- Hava DL, Hemsley CJ, Camilli A. Transcriptional regulation in the *Streptococcus pneumoniae* rlrA pathogenicity islet by RlrA. *J Bacteriol*. 2003;**185**(2):413-21. [PubMed: 12511486].
- Nallapareddy SR, Singh KV, Sillanpaa J, Garsin DA, Hook M, Erlandsen SL, et al. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J Clin Invest*. 2006;**116**(10):2799-807. doi: 10.1172/JCI29021. [PubMed: 17016560].
- Abbot EL, Smith WD, Siou GP, Chiriboga C, Smith RJ, Wilson JA, et al. Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. *Cell Microbiol*. 2007;**9**(7):1822-33. doi: 10.1111/j.1462-5822.2007.00918.x. [PubMed: 17359232].
- Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci U S A*. 2006;**103**(8):2857-62. doi: 10.1073/pnas.0511017103. [PubMed: 16481624].
- Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, et al. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J Bacteriol*. 2008;**190**(15):5480-92. doi: 10.1128/JB.00384-08. [PubMed: 18515415].
- Basset A, Trzcinski K, Hermos C, O'Brien KL, Reid R, Santosham M, et al. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. *J Clin Microbiol*. 2007;**45**(6):1684-9. doi: 10.1128/JCM.00265-07. [PubMed: 17392439].
- Hava DL, Camilli A. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol Microbiol*. 2002;**45**(5):1389-406. [PubMed: 12207705].
- Zahner D, Gudlavalleti A, Stephens DS. Increase in pilus islet 2-encoded pili among *Streptococcus pneumoniae* isolates, Atlanta, Georgia, USA. *Emerg Infect Dis*. 2010;**16**(6):955-62. doi: 10.3201/eid1606.091820. [PubMed: 20507746].
- Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. The Presence of the Pilus Locus Is a Clonal Property Among Pneumococcal Invasive Isolates. *BMC Microbiol*. 2008;**8**(1):1. [PubMed: 18307767].
- Hermans PW, Sluijter M, Hoogenboezem T, Heersma H, van Belkum A, de Groot R. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. *J Clin Microbiol*. 1995;**33**(6):1606-12. [PubMed: 7650196].
- Maslow J, Arbeit R. Molecular Epidemiology for the Clinician: I. Selected Techniques. *Infect Dis Clin Pract*. 1995;**4**(2):128-33.
- Currie BJ, Gal D, Mayo M, Ward L, Godoy D, Spratt BG, et al. Using BOX-PCR to exclude a clonal outbreak of melioidosis. *BMC Infect Dis*. 2007;**7**:68. doi: 10.1186/1471-2334-7-68. [PubMed: 17603903].
- Stralin K, Korsgaard J, Olcen P. Evaluation of a multiplex PCR for bacterial pathogens applied to bronchoalveolar lavage. *Eur Respir J*. 2006;**28**(3):568-75. doi: 10.1183/09031936.06.00006106. [PubMed: 16737990].
- Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement. Wayne, PA: CLSI; 2010.
- Regev-Yochay G, Lipsitch M, Basset A, Rubinstein E, Dagan R, Raz M, et al. The pneumococcal pilus predicts the absence of *Staphylococcus aureus* co-colonization in pneumococcal carriers. *Clin Infect Dis*. 2009;**48**(6):760-3. doi: 10.1086/597040. [PubMed: 19207082].
- Zahner D, Gandhi AR, Yi H, Stephens DS. Mitis group streptococci express variable pilus islet 2 pili. *PLoS One*. 2011;**6**(9):e25124. doi: 10.1371/journal.pone.0025124. [PubMed: 21966432].
- Payne DB, Sun A, Butler JC, Singh SP, Hollingshead SK, Briles DE. PspA family typing and PCR-based DNA fingerprinting with BOX A1R primer of pneumococci from the blood of patients in the USA with and without sickle cell disease. *Epidemiol Infect*. 2005;**133**(1):173-8. [PubMed: 15724724].
- Moschioni M, Donati C, Muzzi A, Masignani V, Censini S, Hanage WP, et al. *Streptococcus pneumoniae* contains 3 rlrA pilus variants that are clonally related. *J Infect Dis*. 2008;**197**(6):888-96. doi: 10.1086/528375. [PubMed: 18269316].
- Basset A, Turner KH, Boush E, Sayeed S, Dove SL, Malley R. Expression of the type 1 pneumococcal pilus is bistable and negatively regulated by the structural component RrgA. *Infect Immun*. 2011;**79**(8):2974-83. doi: 10.1128/IAI.05117-11. [PubMed: 21576325].
- Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. *Nat Rev Microbiol*. 2006;**4**(7):509-19. doi: 10.1038/nrmicro1443. [PubMed: 16778837].