Pneumococcal surface protein C group 4 of Streptococcus pneumoniae is a significant factor to human invasive pneumococcal diseases

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Abstract

Background

Streptococcus pneumoniae is a major causative pathogen of non-invasive and invasive disease worldwide. Although many virulence factors of S. pneumoniae have been reported, the knowledge of the relationship between these factors and clinical features of pneumococcal infection is limited. To clarify factors leading from non-invasive to invasive disease, we analyzed virulence factor genes of S. pneumoniae isolates from patients and evaluated relationship between presence or absence of the genes and clinical features.

Methods

Pneumococcal surface protein (Psp)A families (pspA F1 and pspA F2), PspC group 4 (pspC.4), Pilus-1 (rrgC), and Pilus-2 (sipA) virulence factor genes were measured by PCR using 13 isolates from patients with invasive pneumococcal disease (IPD) and 111 from patients with non-invasive pneumococcal diseases (NIPD) in a community hospital in Japan during 2016. We also tested serotype of isolates. Statistical analysis was performed using multivariable logistic regression analysis for calculating adjusted odds ratio (AOR).

Results

Isolates from IPD carried pspC.4 at significantly higher rate (69.2%) than from NIPD (AOR 10.58 95% CI, 2.67-41.87). Proportions of pspC.4 positive isolates were varied depending on serotype. There found 13 serotypes (including non-typeable) that had pspC.4, and 7 of the 13 pspC.4-carrying serotypes caused invasive diseases while 2 of 12 non-pspC.4 serotypes did. Relationships between IPD and pspA F1, pspA F2, rrgC, or sipA were not significant.

Conclusions

It is suggested that positive pspC.4 gene in S. pneumoniae is related with invasive pneumococcal diseases in this study. Further studies of virulence factors are required to
elucidate S. pneumoniae pathogenicity.

Background

*Streptococcus pneumoniae*, one of the most prevalent human pathogens worldwide, constitutes a major causative agent of both non-invasive (e.g., otitis media and pneumonia) and invasive disease (e.g., bacteremia and meningitis), leading to the death of a large number of young children and the elderly in particular [1-3]. In Japan, pneumococcal conjugate vaccine (PCV13) and pneumococcal polysaccharide vaccine (PPSV23) have been approved for routine vaccination of children and the elderly, respectively.

*S. pneumoniae* colonises the nasopharynx of many healthy young children asymptotically [4]. However, despite *S. pneumoniae* being one of the most extensively studied microorganisms and the identification of many virulence factors, little is known regarding the factors that contribute to cause of disease and invasion. The pneumococcal surface protein A (PspA), a cell-wall-associated protein, interferes with complement deposition on the bacterial surface [5] and binds human lactoferrin [6].

PspA is variable at the DNA level, and can be classified into three families by polymerase chain reaction (PCR) [7] with PspA family 1 and 2 as the major alleles. PspC, also termed choline binding protein A (CbpA), constitutes a major pneumococcal adhesin. PspC binds to the polymeric immunoglobulin receptor (pIgR) in human nasopharyngeal cells and promotes translocation across a mucosal barrier. PspC also binds secretory IgA (sIgA), platelet activating factor receptor and more specifically complement proteins such as C3 [8].

PspC can inhibit C3b deposition but also binds the host complement inhibitor
factor H, leading to inhibition of alternative pathway activation. Dieudonné-Vatran et al. demonstrated that the pneumococcus exploits host C4b-binding protein (C4BP) for complement evasion in a PspC group 4-dependent manner [9].

Pilli have been identified on several gram-positive bacteria; two different pilus islets have been described in S. pneumoniae that encode for two different types of pilli, Pilus-1 and Pilus-2 [10].

Pilus-1 is encoded by a pathogenicity islet including genes for three structural proteins, rrgABC, three sortases, srtBCD, and a regulator, rlrA. Pilus-1 was reported to mediate host-bacterial interactions as an adhesin, and a proinflammatory stimulus [10].

In particular, an isolate is defined as pilus-1 positive if a PCR for the rrgC gene is positive [11].

In turn, Pilus-2, detected via primers for the signal peptidase-like protein (SipA) gene [12], mediates adhesion of S. pneumoniae to eukaryotic cells [11].

To clarify bacterial factors which foresee invasive diseases, we analysed five virulence factor genes, pspA F1, pspA F2, pspC group 4 (pspC.4), rrgC, and sipA, of clinical isolates and evaluated the relationship between gene possession and clinical features in this study.

Methods

Clinical isolates

A total of 511 clinical S. pneumoniae isolates were collected at Saiseikai Yokohamashi
Tobu Hospital, a regional core hospital in Kanagawa prefecture, Japan, during January–December 2016. Those patients from whom multiple pneumococcal isolates were obtained within a 30-day period, they were regarded as a single episode. In the 331 episodes (187 males and 144 females), 124 episodes were confirmed pneumococcal diseases based on both bacteriological isolation and consistent clinical symptom, signs and laboratory findings with *S. pneumoniae* infection.

Table 1 shows the distribution of patient’s age, type of specimen, clinical diagnosis, and serotype. Thirteen episodes were diagnosed as invasive pneumococcal diseases (IPD) according to that *S. pneumoniae* had been isolated from normally sterile body sites in patients. Patients who had been diagnosed with pneumonia as well as bacteremia were designated as having IPD. Thirteen isolates from normally sterile body sites were defined as IPD isolates; 11 isolates from blood, and 2 from cerebrospinal fluid. We defined the other 111 episodes as non-invasive pneumococcal diseases (NIPD), although NIPD might have contained IPD because all episodes did not have blood culture test. The first isolate per NIPD episode subjected to genetic analysis; 97 isolates were from sputum, 10 from bronchial wash, 3 from nasopharyngeal swab, and 1 from otorrhea.

**DNA extraction and serotyping**

Isolates were cultured on 5% blood agar plates at 37 °C with 5% CO₂. DNA was extracted using the Cica Geneus DNA Extraction Reagent (Kanto Chemical Co, Tokyo, Japan) according to manufacturer instruction. Capsular serotypes were determined via sequential multiplex PCR analysis [15] using QIAGEN Multiplex PCR Kits (QIAGEN, Hilden, Germany) or pneumococcal capsule-specific antisera (Statens Serum Institut, Copenhagen, Denmark) per manufacturer instruction. Strains whose serotypes could not be determined by PCR or the
Quellung reaction were defined as non-typeable.

**Analysis of PspA family 1 and 2**

PspA family classification was performed by PCR using DNA extracted from isolates with primers reported by Hollingshead, et al. [16] as follows: LSM12 and SKH63 for PspA family 1 (pspA F1), and LSM12 and SKH52 for PspA family 2 (pspA F2) (Table 2). PCR reactions were carried out using Quick Taq HS DyeMix (TOYOBO, Osaka, Japan). The PCR conditions were 95 °C for 3 min; then 30 cycles of 95 °C for 1 min, 62 °C for 1 min, 72 °C for 3 min, and finally 72 °C for 10 min. PCR products were loaded onto 1% agarose gels, electrophoresed at 100 V for 30 min, and stained with 0.5 μg/ml ethidium bromide. The isolates that were not initially amplified were further processed with the same cycling pattern at an annealing temperature of 58 °C, or, if that also failed, of 55 °C.

**Analysis of pspC.4, rrgC, and sipA genes**

The presence of pspC.4 was investigated by PCR using primers LU9 and LU10 (Table 2), which were designed to specifically amplify the pspC group 4 locus [9]. The PCR conditions were 96 °C for 5 min; then 30 cycles of 96 °C for 45 sec, 50 °C for 45 sec, 72 °C for 3 min, and finally 72 °C for 10 min. PCR products were electrophoresed at 100 V for 30 min, then stained with ethidium bromide.

For detection of the presence of the Pilus-1 operon, an isolate was defined as Pilus-1 positive if PCR for the rrgC gene was positive. Primers to evaluate the presence or absence of rrgC were c5 and c3 (Table 2) [13]. The PCR conditions were 94 °C for 3 min; then 35 cycles of 94 °C for 15 sec, 60 °C for 15 sec, 72°C for 1 min, and finally 72°C for 5 min. PCR products were electrophoresed in 2% agarose gels and stained.
To determine whether Pilus-2 was present, primers *sipA*-up and *sipA*-dn (Table 2), designed against the Pilus-2-specific gene *sipA*, were used [1]. The PCR conditions were 95 °C for 15 min; then 35 cycles of 95 °C for 20 sec, 55 °C for 30 sec, 68 °C for 1 min, and finally 68 °C for 10 min.

**Statistical analysis**

Statistical analysis was performed using multivariable logistic regression model to calculate adjusted odds ratios (AORs) of developing IPD by existence of virulence factors. In the model IPD status was used as dependent variables, and five virulence factor genes (i.e. *pspA* F1, *pspA* F2, *pspC* 4, *rrgC*, and *sipA*) were used simultaneously as independent variables. Findings of $p < 0.01$ were considered significant.

**Results**

**Serotype distribution of isolates**

Serotype distribution of the isolates showed that serotype 11A/11E was the most common serotype, followed by 15A, among isolates from total pneumococcal diseases including IPD (Table 1). IPD distribution did not follow the total serotype distribution. Among IPD isolates, serotype 7F was the most common, and serotype 24B and 38 were the only one isolates in total studied (Table 1). It was noted that three of five 7F isolates caused IPD (bacteremia), and two of the three 7F isolates that caused bacteremia were isolated from patients with no underlying diseases in age group of 5–64 years.

**Virulence factor gene distribution in clinical pneumococcal isolates**

Figure 1 shows the proportions of the *pspA* F1, *pspA* F2, *pspC* 4, *rrgC*, and *sipA* presence in IPD and NIPD. Among the 124 strains tested, 96.0% had *pspA* F1 or *pspA* F2; 46.8% were
identified as belonging to PspA family 1, whereas 50.8% belonged to family 2. Two isolates of serotype 22 belonged to both families 1 and 2. All IPDs were included in PspA family 1 or family 2 groups. However, the relationship between IPD and pspA F1 (adjusted odds ratio [AOR] 4.09 [95% CI 0.13-125.70]) or pspA F2 (AOR 1.77 [95% CI 0.06-55.25]) were not statistically significant. Isolates from the IPD group showed significant relation with pspC.4 carriage than the NIPD groups (AOR 10.58 [95% CI 2.67-41.87], p <0.001). rrgC did not show difference between IPD isolates and NIPD isolates (AOR 0.25 [95%CI 0.02-2.62]). Although sipA was more frequently detected in the IPD group (23.1%) than in the NIPD group (8.1%), the differences were not significant statistically (AOR 2.65 [95% CI 0.41-17.00]).

Proportions of pspC.4 presence varied depending on serotype (Figure 2). For example, all isolates of 7F, 19F, 24B, 33F, and more than half of 6C, 20 were pspC.4 positive, whereas, no isolate had pspC.4 in serotype 11A/11E which was the most common serotype in this study. Neither of the second common serotype 15A had pspC.4 even in 2 IPD isolates. Some serotypes such as 24B and 33F had pspC.4 and caused IPD although they were not frequent serotypes. There found 13 serotypes (including non-typeable) that had pspC.4, and 7 of the 13 pspC.4-carrying serotypes caused invasive diseases while 2 of 12 non-pspC.4 serotypes did (Figure 2).

Discussion

Pneumococci express multiple virulence factors, which include, for example, the polysaccharide capsule, pneumolysin, pneumococcal surface proteins, and pili. It is considered that they can cause diseases because they possess efficient complement evasion strategies and resist opsonophagocytosis. Complement resistance constitutes a major contributor to pneumococcal virulence and pathogenesis. In particular, the capsule reduces the amount of bound C3b and restricts the access of phagocytes to cell-bound
C3b, which hampers opsonophagocytosis [18]. Pneumolysin quenches complement away from the pneumococcal surface [19].

PspA is present on almost all strains of S. pneumoniae [16]. PspA families 1 and 2 (fusion PspA) are considered as promising candidate antigens for pneumococcal vaccines [7, 20].

Our data showed that all isolates from patients with IPD and 96.0% of total isolates in this study contained PspA families 1 or 2 and that relationship between clinical invasiveness and each of them were not significant in this study.

PspC proteins contribute to virulence in colonisation and systemic mouse models [22].

PspC binds to the secretory component of plgR to promote the adherence and invasion of epithelial cells [23]. It also binds soluble host factors such as sIgA and IgM as well as C3 and complement inhibitor factor H ([8, 25]).

PspC exhibit high variability at the sequence level, with allelic variants of PspC being divided into 11 groups. The presence of PspC group 4 was found to be correlated with the ability to bind C4BP [9].

C4BP retains its inhibitory function when bound to the bacteria, although the pneumococcus binds via the same site as C4b. Our study showed that isolates from IPD exhibited significantly higher rate of pspC.4, suggesting that pspC.4 is related to invasiveness. It is also suggested that the serotype distribution of IPD isolates did not
follow that of total isolates because proportions of \textit{pspC.4} positive isolates were serotype dependent. Seven of isolates from IPD were serotypes, over half of which shows \textit{pspC.4} positive regardless of serotype frequency. Whereas, the serotype 3 from a patient with pneumococcal bacteremia was the only \textit{pspC.4}-positive isolate among the 22 serotype 3 isolates. However, no serotype 15A isolate including two from IPD carried \textit{pspC.4}, further supporting that \textit{pspC.4} constitutes one of the factors related to invasiveness but not related with invasiveness in certain serotypes.

The presence of \textit{rrgC} had no appreciable difference in frequency between IPD and NIPD, as reported previously [26]. In comparison, \textit{rrgC} was significantly associated with penicillin susceptibility (data not shown).

All \textit{sipA} positive isolates in IPD were serotype 7F. Notably, 7F was the most frequent serotype in IPD in this study and all of them carried both \textit{pspC.4} and \textit{sipA}. Although we studied just five among many potential virulence factors, the results may relate to the observation that 7F is among the serotypes with highest invasive disease potential [27].

The pathogenicity of \textit{S. pneumoniae} has been reported to differ between clones and even isolates of the same clone [28].

Our study suggested, although factors responsible for pathogenicity vary depending on serotypes and isolates, bacterial virulence factors are associated with clinical invasive aspects.

A limitation of this study is that it was performed in a single center for one year and sample number is limited. There are concerns regarding geographical and time variation, and it is not known whether our data are representative of that in the world.

Conclusions
Our results suggested that the presence of genes for PspC group 4 is associated with clinical invasiveness and the results will promote the understanding of virulence factors in pneumococcal diseases. Further studies including more virulence factors are required to elucidate the pathogenic mechanisms of S. pneumoniae.

List Of Abbreviations

PspA: pneumococcal surface protein A, PspC: pneumococcal surface protein C, PCR: polymerase chain reaction, IPD: invasive pneumococcal disease, NIPD: non-invasive pneumococcal disease, AOR: adjusted odds ratio, plgR: polymeric immunoglobulin receptor, slgA: secretory immunoglobulin A, PCV: pneumococcal conjugate vaccine, PPSV: pneumococcal polysaccharide vaccine, C4BP: C4b-binding protein

Declarations

Ethics approval and consent to participate

This study was approved by the Saiseikai Yokohamashi Tobu Hospital ethics committee (approval number 2016002) and by the Tokyo Medical University ethics committee (approval number 2016-218). Patients’ data were anonymised for analysis. The committees permitted to omit informed consents by providing a means to opt out, according to Ethical Guideline for Medical and Health Research Involving Human Subjects.

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

HM received a grant for education from Pfizer (grant no. 30049557). Other authors declare
no conflicts of interest associated with this study.

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Authors’ contributions

HM designed the study, collected data, performed the experiments, analyzed results and wrote the manuscript. BC performed the laboratory examinations. HK and NF carried out the statistical analysis. RS participated in the data collection and laboratory examinations. YM and TM involved in assessment and reviewed the manuscript. All authors approved the manuscript.

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Tables
Due to technical limitations, all Tables are only available as a download in the supplemental files section.

Figures
Proportions of the presence of the pspA F1, pspA F2, pspC.4, rrgC, and sipA, in isolates from each diagnosis group. IPD: isolates from invasive pneumococcal diseases; NIPD: isolates from non-invasive pneumococcal diseases. *: statistically significant difference (p < 0.001). Black: gene positive isolates; grey: gene negative isolates.
Figure 2

Serotype distributions and pspC.4 gene presence of isolates from patients with pneumococcal diseases and invasive pneumococcal disease (IPD). a. Serotype distribution and pspC.4 gene presence of total isolates (n=124). b. Serotype distribution and pspC.4 gene presence of isolates from patients with IPD (n=13).

Black: pspC.4 gene positive; grey: pspC.4 gene negative.

Supplementary Files

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5-15-19 Table 2.xlsx
5-16-19 Table 1.xlsx