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Genotypic characterization of Malaysian human isolates of *Streptococcus pneumoniae* from carriage and clinical sources

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ABSTRACT

This study characterized carriage and clinical pneumococcal isolates for serotypes, penicillin susceptibility, virulence genes and restriction fragment length polymorphism (RFLP) pattern of penicillin binding protein (PBP) genes. DNA fingerprint of isolates was generated by BOX-PCR. Majority of serotypes were 23F followed by 19F, 19A and 6A. Twenty-four percent of isolates were penicillin non-susceptible (PNSP). All of the targeted virulence genes were detected in all isolates with the exception of pili; 20.6% ($n = 22$) for PI-1 and 14.0% ($n = 15$) for PI-2. Of the 13 isolates which carried both PI-1 and PI-2, 10 were of clinical origin. Digested *pbp*-DNA produced three PBP-RFLP profiles for *pbp1a* (A1 to A3), six profiles for *pbp2b* (B1 to B6) and seven for *pbp2x* (X1 to X7) mostly in PNSPs. Based on BOX-PCR analysis, the majority of isolates were genetically diverse with a small number of potentially related isolates carrying pili genes. No obvious genotypic association was observed pertaining to carriage and clinical origin of isolates.

1. Introduction

Streptococcus pneumoniae (pneumococcus) causes invasive pneumococcal diseases (IPD) and non-invasive pneumococcal diseases (non-IPD) with significant morbidity and mortality worldwide [1]. There are more than 90 serotypes of *S. pneumoniae* which differ in virulence, geographical distribution and extent of drug resistance. Current pneumococcal vaccines such as pneumococcal conjugate vaccines (PCV) provide a limited coverage due to serotype-specific protection. Currently there are PCV7, PCV10 and PCV13 where the number represents the number of included serotype, respectively [1,2].

Penicillin has been the drug of choice for pneumococcal disease treatment since the 1940s. However, treatment for pneumococcal diseases is complicated by the increasing rate of resistance to penicillin and other β-lactams antibiotics [2,3]. Resistance to penicillin and β-lactams in *S. pneumoniae* arises from alterations in penicillin-binding proteins (PBPs) which are located in the bacterial cell wall. Among the PBPs, modifications of *pbp1a, pbp2b* and *pbp2x* contribute to their decreased affinity to those antibiotics, which leads to development of penicillin and β-lactams resistance in pneumococci [4].

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S. pneumoniae possesses several virulence properties such as autolysin A (LytA), pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumolysin (Ply), choline binding protein A (CbpA), choline binding protein G (CbpG), pneumococcal adherence and virulence factor A (PavA) and the multimetric filaments; the pili [5–7]. These proteins and enzymes have been proposed to have distinct roles in pneumococcal pathogenesis in different host niches [8]. There are two different pilus islets (PIs) that encode the structural and biosynthetic genes for two antigenitically different types of pili, PI-1 [5] and PI-2 [9]. The PI-1 was reported to play a role in virulence in murine models of pneumococcal infection [5] and PI-2 was proposed to play a role in pneumococcal adhesion to human respiratory cells [10].

BOX-PCR typing method targets a dispersed repetitive motif in the genome of *S. pneumoniae*. There are approximately 25 BOX repetitive elements in non-coding regions dispersed throughout the pneumococcal genome with three discriminate regions; *boxA* (59 bp), *boxB* (45 bp) and *boxC* (50 bp) [11]. The multicopy BOX elements have been recognized to be useful for rapid molecular discrimination of pneumococcal strains and have been successfully employed elsewhere [12–16].

Analysis on the interrelation of serotypes, antibiotic susceptibility, virulence gene distribution and genomic DNA fingerprint patterns in *S. pneumoniae* from different sources of isolation may provide useful epidemiological perspective and genetic linkage of the studied pneumococcal population. Comparison of such data from various localities may as well indicate the geographical variation of the organism. Subsequently, the findings can be used as guidelines in managing the pneumococcal prophylactic measures either locally or globally [2,3,13,15]. In Malaysia, *S. pneumoniae* is also an important disease-causing pathogen and yet data on local isolates, particularly those at genetic level are still lacking. In this study, we characterized a collection of human pneumococcal isolates, derived from both community and hospital, for their genotypic properties covering serotypes, penicillin susceptibility, virulence genes availability, PBP genes profile (*pbp1a, pbp2b* and *pbp2x*) and BOX-PCR based DNA typing. Comparative analysis was conducted to find potential correlations.

2. Material and methods

2.1. Ethics statement

The Universiti Putra Malaysia (UPM) Medical Research Ethics Committee found no objectionable ethical issues of this study. Study did not involve human subjects except some clinical and demographic data associated with the isolates that are no longer traceable to the sampled individuals.

2.2. Pneumococcal isolates

One hundred and seven viable *S. pneumoniae* isolates were available in our stock culture collection consisting isolates from both carriage (community) and clinical (hospital) sources. Carriage isolates ($n = 50$) were collected from anterior nares of healthy children of five years or younger in 2010 as described previously [17]. Clinical isolates ($n = 57$) were isolated from 1989 to 2012 from patients of five years or younger ($n = 6$), five to twelve years ($n = 5$), 13 to 50 years ($n = 18$) and above 50 years ($n = 12$). Sixteen clinical isolates came from patients of unknown age. The clinical isolates were randomly obtained from various isolation sites from admitted patients at a number of hospitals in Malaysia. Thirty-seven were from sterile anatomical sites (blood, cerebrospinal fluid (CSF) and pleural fluid) and the rest were various including sputum, eye, ear, pus, throat, nasopharyngeal fluid and others (Fig. 1). All 107 isolates were obtained from a different individual.

2.3. Pneumococcal identification and serotyping

All isolates were re-identified as *S. pneumoniae* by standard bacteriological methods consisting of colonial morphology, α-hemolysis on 5% Sheep Blood Agar, Gram stain, catalase reaction, bile solubility and susceptibility to ethylhydrocupreine or optochin. Fourty-one of the carriage isolates were previously serotyped by multiplex PCR in our previous study [18]. The remaining 66 isolates were serotyped using similar PCR-approach in this study with an additional primer set (set F) for detecting serotypes/serogroup 12F, 22F, 15A/F and 8 (Table 1).

2.4. Penicillin susceptibility testing

The minimum Inhibitory Concentration (MIC) value for penicillin was determined by Epsilometer Test (E-Test) method (AB Biodisk, Solna, Sweden) following procedures recommended by the Clinical and Laboratory Standard Institute (CLSI) guidelines [19]. The MIC results obtained were compared to the stated CLSI interpretive criteria; for clinical isolates from CSF, the criterion for meningitis group with parenteral penicillin was referred; MIC $\leq$0.06 μg/ml is susceptible and MIC $\geq$0.12 μg/ml is resistant. As for the rest of clinical isolates, the criterion for non-meningitis with parenteral penicillin was referred; MIC $\leq$2, 4 and $\geq$8 μg/ml for susceptible, intermediate and resistant, respectively. Meanwhile, for carriage isolates, the criterion for oral penicillin was used, with MIC $\leq$0.06, 0.12–1, and $\geq$2 μg/ml for susceptible, intermediate, and resistant, respectively. *S. pneumoniae* ATCC 49619 with known zone of inhibition size and MIC was used as the reference strain throughout the testing.

2.5. Genomic DNA extraction

Pure bacterial cells, grown overnight on Columbia Agar supplemented with 5% sheep blood incubated in the presence of 5% CO_2 at 37 °C were prepared for the DNA extraction. GeneAll® Exgene™ (GeneAll Biotechnology Co. Ltd, Korea) kit was used for the extraction in accordance to the instructions provided by the manufacturer.