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High prevalence of multidrug-resistant Pneumococcal molecular epidemiology network clones among *Streptococcus pneumoniae* isolates from adult patients with community-acquired pneumonia in Japan

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Abstract

A total of 141 *Streptococcus pneumoniae* isolates from patients with community-acquired pneumonia were collected from May 2003 through October 2004. The strains were tested for antimicrobial agent susceptibility, serotype and genotype by multilocus sequence typing (MLST) and the presence of the pilus *rlrA* islet. MLST analysis identified 49 sequence types (STs), of which 19 were novel. eBURST analysis using the MLST database (3773 STs) grouped the isolates into 27 clonal complexes and three singletons. A total of 92 (65.2%) isolates were related to ten of the 43 international Pneumococcal Molecular Epidemiology Network (PMEN) clones; major clones found were multidrug-resistant Netherlands^{3}-31 [clonal complex (CC) 180], Taiwan^{19F}-14 (CC271), Taiwan^{23F}-15 (CC242), and Colombia^{23F}-26 (CC138) (the latter new to Asia). We adopted univariate and multiple logistic regression models to identify factors associated with PMEN CCs. Multivariate analysis showed that multidrug resistance (OR 6.3; 95% CI 2.0–22.9), carriage serogroups (OR 7.2; 95% CI 2.5–23.7), prevalence of *rlrA* (OR 12.6; 95% CI 3.6–59.7) and central nervous system-related disorders (OR 7.7; 95% CI 1.8–48.4) were independently associated with PMEN CCs. Our data indicate that multidrug-resistant PMEN clones are highly prevalent, contributing to the high frequency of resistance to antimicrobial agents in Japan, and suggest that certain predisposing factors in patients contribute to the high frequency of these clones.

Keywords: Carriage, host factor, multidrug resistance, multilocus sequencing typing, *rlrA*, serotype, *Streptococcus pneumoniae*
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Introduction

Several clinical and epidemiological studies of *Streptococcus pneumoniae* have revealed that a small number of dominant resistant clones is responsible for the spread of *S. pneumoniae* resistance to different classes of antimicrobials [1]. Although increased antibiotic use has been the most important selective force driving the appearance and circulation of resistant strains [2], other, as yet unidentified, mechanisms of dissemination and successful establishment of such clones are likely.

Multilocus sequencing typing (MLST) is a nucleotide sequence-based approach for characterizing isolates of bacteria and other organisms via the internet (http://www.mlst.net/), having the advantage over pulsed-field gel electrophoresis of ease of manipulation and unambiguous and convenient comparison [3]. The Pneumococcal Molecular Epidemiology Network (PMEN) includes 26 multidrug-resistant and 17 susceptible *S. pneumoniae* clones found worldwide using MLST (http://www.sph.emory.edu/PMEN/) [4]. In Asian countries, Spain^{23F}-1, Spain^{6B}-2, Taiwan^{19F}-14, Taiwan^{23F}-15 and Netherlands^{3}-31 have been spreading, which could be a major reason for the rapid increases in penicillin- and macrolide-resistant *S. pneumoniae* strains [5–8].

Because the ecological niche of *S. pneumoniae* is the human nasopharynx, horizontal transfer of antimicrobial resistance genes occurs easily via commensal organisms that reside in the nasopharynx [9]. Various pneumococcal virulence factors have a role to play in bacterial colonization and disease [10,11].

Although at least 90 different capsular serotypes have been described, their carriage potentials differ [12]. Bruegg-

eman *et al.* have performed a meta-analysis to calculate the ORs for different serogroups/serotypes causing invasive diseases. Using serotype 14 as the reference for serogroups/serotypes, it was concluded that 1, 5 and 7 cause more invasive disease, whereas 18, 9, 8, 33, 38, 19, 6, 23, 3 and 15 are responsible for carriage, irrespective of any temporal change or major geographical differences [13]. Recent studies have revealed that pneumococcal pili encoded by the *rlrA* islet also enhance adherence to lung epithelial cells [14] and that the presence of the *rlrA* gene was correlated more with MLST genotypes than with serotypes; the *rlrA* gene was also found to be carried in several PMEN clones [15–17].

Furthermore, different host factors, such as male gender, smoking, recent antibiotic use, attendance at day care centres, as well as various underlying diseases, all predispose to pneumococcal colonization [10,18,19].

In the present study, we performed MLST for *S. pneumoniae* isolates collected from patients with community-acquired pneumonia (CAP) in Japan in order to investigate clonal spread of the bacteria, especially the PMEN clones. We then employed univariate and multiple logistic regression models to identify microbiological and clinical risk factors associated with the prevalent PMEN clones compared with the non-PMEN isolates.

Material and Methods

Study design, bacterial strains and clinical data

total of 141 pneumococcal isolates that had been prospectively collected from patients >15 years old diagnosed with CAP between May 2003 and October 2004 in Japan have been previously described [20]. In brief, the sources were sputum (n = 132), blood (n = 6), transtracheal aspiration (n = 2) and bronchoalveolar lavage (n = 1). On the basis of sociodemographic and clinical data, we calculated the Charlson comorbidity index [21] and the Pneumonia Severity Index [22]. The study was approved by the Ethics Committees of each hospital.

MLST

The bacterial strains were analysed by MLST, as described elsewhere [3]. In order to determine nucleotide sequences of new alleles, we used three new primers: xpt2-up, TCGCTCGTAATAGTTTTATC, ddl2-up, AAATGCCTTACGTTGGTTGC, and ddl2-dn, GCGCT TGTCAAAACTTTCCT. The sequence types (STs) were obtained by reference to the MLST database (http://www.mlst.net/). New alleles and new STs were submitted to the curator of the database and were assigned designations. ST180, ST2808 and ST2809 isolates in the present study have already been described in our previous report [6].

eBURST analysis for clonal complexes

To visualize the genetic relationships among the different strains, a dendrogram was generated from the distance matrix between STs by using the unweighted pair group method with arithmetic averages on the PubMLST website (http://pubmlst.org/). eBURST was carried out to estimate the relationship of isolates with software (http://spneumoniae.mlst.net/eburst/). Clonal complexes (CCs) consisted of eBURST sets in which all STs share six of seven identical alleles with at least one other ST within the group [single-locus variants (SLV)]. We ran eBURST with default settings associating each ST with a CC on the entire MLST database (3773STs) and STs newly assigned within our dataset. In this study, we named CCs according to an ST number of the eBURST predicted founder, defined as being the ST with the greatest number of SLVs, a smaller number of STs in a group consisting of two different STs or a singleton itself.

Antimicrobial susceptibility and serotyping

We have already reported the antimicrobial agent susceptibilities and serotypes of 141 isolates [20,23]. In brief, non-susceptibility to penicillin, erythromycin, ceftriaxone, clindamycin, minocycline and trimethoprim-sulfamethoxazole was recorded in 64 (45.4%), 118 (83.7%), five (3.5%), 77 (54.6%), 116 (82.3%) and 41 isolates (29.1%), respectively. Multidrug resistance includes three or more antimicrobial non-susceptibilities. According to a meta-analysis [13], we divided the serotypes into three different groups with different invasive disease potential in our collection. These were high (invasive), serotype 7; medium, 4, 9N, 9V and 14; and low (carriage), 3, 6A, 6B, 15, 19A, 19F, 23A and 23F.

Detection of the *rlrA* islet

The genomic location of the *rlrA* islet was determined by simultaneously assessing five PCR amplifications, as previously reported [15,16]. If the *rlrA* islet or parts of it were absent, the PCR product using these primers would be similar in length to DNA as mainly type A (1310 bp), type B (1912 bp) or type C (2616 bp).

Statistical analysis

Statistical analysis was performed using JMP version 6.03 (SAS Campus Drive, Cary, NC, USA). Related factors and outcomes for PMEN clonal genotypes were identified by comparison with non-PMEN genotypes using univariate analysis.

Associations between categorical variables were tested by the Pearson χ^2 or Fisher's exact test, when appropriate. Means of continuous variables were compared by Student's t-test or Mann–Whitney U-test, as appropriate. ORs and the