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Difference of Type 3 secretion system (T3SS) effector gene genotypes (*exoU* and *exoS*) and its implication to antibiotics resistances in isolates of *Pseudomonas aeruginosa* from chronic otitis media

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ABSTRACT

Objective: Type 3 secretion system (T3SS) is the most important virulence factor in *Pseudomonas aeruginosa* infection. Of the various T3SS effector genes, *exoS* and *exoU* showed mutually exclusive distributions, and these two genes showed varied virulence. In many pseudomonal infections, the distribution of these genes showed different pattern and it influenced severity of infection. This study was aimed to evaluate differences of virulence factors and antibiotics resistance between chronic otitis media and other body infection caused by *P. aeruginosa.*

Methods: To estimate the prevalence of effector genes of T3SS, especially the distributions of *exoS* and *exoU* genes and their association with antibiotic resistance in COM, we compared the prevalence of T3SS genes in isolates from COM with those from lower respiratory infection and bacteremia. Other virulence genes, including *groEL*, *pilA*, *ndvB*, *lasB*, *rhlI*, and *apr*, were also studied to evaluate prevalence. These isolates were tested for antibiotic susceptibility, and we examined the association between antibiotic susceptibility and the prevalence of T3SS effector genes.

Results: The COM group showed a significantly higher e*xoU*-positive rate than the control group (70.6% vs. 6.7%; $P < 0.01$). Furthermore, COM patients with *exoU* showed significant antibiotic resistance to ciprofloxacin and tobramycin ($P = 0.035$), whereas there was no significant difference in the control group.

Conclusions: The high incidence of *exoU*-positive *P. aeruginosa* and ciprofloxacin resistance can explain the chronicity and intractability of infection in COM. Elucidation of this pathogenicity will facilitate the development of new treatment options for COM patients.

1. Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that causes infection in humans. The manner and severity of *Pseudomonas* infections vary according to the age of the host, mode of transmission, and site of infection.

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These infections cause tissue damage via several pathways that are mediated by a range of virulence factors.

P. aeruginosa is a common pathogen isolated from patients with chronic otitis media (COM), which is a common chronic hygiene-related infection. Although prevalence estimates vary widely due to differences in disease definition, sampling methods, and methodological quality, the incidence of COM has declined over the years, and community-based epidemiological studies have found a global estimated COM incidence rate of 4.76 per thousand people for a total of 31 million cases. Oceania had the highest incidence, 9.37 per thousand people, whereas the Latin America Andean region had the lowest incidence, 1.70 per thousand [1].

Pseudomonas was considered to be the most common pathogen associated with chronic otitis media [2]. However, a recent study identified *P. aeruginosa* in one-quarter of chronic otitis media infections [3]. In another study, the percentage of *Pseudomonas*-associated COM was 31.8% [4]. Chronic otitis media is typically a long-standing infection, which may sometimes cause life-threatening intracranial complications, such as otic meningitis, brain abscess, and lateral sinus thrombosis [5].

There are two main treatment methods for chronic otitis media. The first involves use of suitable antibiotics for infection control and prevention of complications, whereas the other involves surgical treatment by tympanomastoidectomy to remove pathological lesions and reconstruct the middle ear cavity. However, even with surgical intervention, the use of appropriate antibiotics is crucial. Identification of the pathogen and examination of its antibiotic sensitivity are necessary for proper treatment of COM.

Drug-resistant strains have made infection control more difficult in recent years. Quinolones, anti-pseudomonal penicillins, and cephalosporins were the major drugs used for the treatment of pseudomonal infection. Unfortunately, strains of clinical isolates were reported to have become resistant to quinolone and/or anti-pseudomonal cephalosporins, and the prevalence of these drug-resistant strains has increased. Among patients presenting with COM, ciprofloxacin resistance was reported in 51.9% of adults and 57.1% of children, whereas ceftazidime resistance was reported in 5.7% of adults and 13.3% of children [6].

The toxicity caused by pseudomonal infection is mediated by several virulence factors, including bacterial cell-surface factors and secreted factors. The type 3 secretion system (T3SS), which is the most important of the secreted virulence factors, consists of four secreted exotoxins: ExoS, ExoT, ExoU, and ExoY [7]. There have been no previous studies regarding the prevalence of *exoU* and *exoS* genes expressed in *P. aeruginosa* isolated from COM samples. As *P. aeruginosa* is often isolated from COM and previous studies suggested its association with the chronicity of infection, this study was performed to determine whether certain virulence genes differ in COM compared with infections at other clinical sites. To explore this hypothesis, the prevalence of T3SS effector genes and other virulence-associated genes in isolates from COM were compared with those isolated from pneumonia and bacteremia. All isolates were tested for antibiotic susceptibility.

2. Methods

2.1. Ethics statements

This study has been approved by the institutional review board at Boramae Medical Center (06-2011-123/109). Informed consent was waived for all participants. The data from hospitalized patients with respiratory tract infections and bacteremia were identified retrospectively from microbiology records. All data were analyzed anonymously.

2.2. Bacterial strains and culture conditions

Clinical isolates of *P. aeruginosa* were collected from outpatient clinics at Boramae Medical Center (Seoul, Korea). To eliminate selection bias, bacterial isolates were collected from consecutive cases between September 2011 and August 2012. Seventeen isolates of *P. aeruginosa* from COM and 30 isolates from blood and respiratory tract sites as a control were collected during this period. All of the isolates were acquired from the symptomatic patients to identify causative pathogen of infectious disease, and they were treated with the antibiotics in accordance with the results of the antibiotic susceptibility testing. There were no statistically significant differences in age or sex between the two groups of patients.

A well-characterized laboratory *P. aeruginosa* strain, PAO1 (A^+B^+ O-antigen serotype), was used as a positive control [8]. *P. aeruginosa* strains were routinely grown in Luria–Bertani (LB) broth (Sigma–Aldrich, St. Louis, MO) at 37 °C with shaking at 250 rpm for 18 h.

2.3. DNA isolation and detection of target virulence factor genes

P. aeruginosa isolates from otorrhea, blood, and bronchial lavage fluid were transferred to a medium and cultured. DNA extraction was performed using a G-spin Genomic DNA extraction kit for Bacteria (iNtRON Biotechnology, Seongnam, Korea). DNA extraction was performed as follows: cultured cells were suspended in the medium and precipitated by centrifugation; lysis buffer was added to the cell precipitate and dissolved at 65 °C (149 °F) for 15 min. The extract was mixed with binding buffer and centrifuged at 13,000 rpm for 1 min, after which the supernatant was discarded. The solution was mixed with washing buffer and centrifuged at 13,000 rpm for 1 min. The extract was placed in a DNA separation column, to which elution buffer was added. The column was kept at room temperature for 1 min and then centrifuged to extract DNA. The optical density (OD) of the extracted solution was measured to confirm that absorbance at (A260/A280) was between 1.8 and 2.0. Ten genes associated with the virulence of *P. aeruginosa* were assessed: the molecular chaperone *groEL* [9], two genes encoding biofilm-forming factors (*pilA* and *ndvB*) [10], three quorum-sensing genes (*lasB*, *rhlI*, and *apr*) [11,12], and four type III secretion toxin-encoding genes (*exoY*, *exoT*, *exoS*, and *exoU*) [13–15]. To determine the presence of these genes, bidirectional nucleotide sequence analysis was performed on the PCR amplification products. Two sequences of

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