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Original Article

Quantitative detection of periodontopathic bacteria in lower respiratory tract specimens by real-time PCR

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ABSTRACT

The presence of common periodontopathic bacteria, the *Fusobacterium nucleatum-periodonticum-simiae* group, *Prevotella intermedia*, and *Porphyromonas gingivalis* was determined from respiratory tract specimens of bacterial pneumonia by real-time PCR using universal and species-specific TaqMan probe/primer sets. 42 patients with infectious pneumonia and 45 patients without infectious pneumonia were retrospectively enrolled in clinical studies. Periodontopathic bacterial DNA was found in 57.1% cases of infectious pneumonia and 31.1% cases of noninfectious pulmonary disease. However, the proportion of periodontopathic bacterial DNA did not differ between the two groups, and the presence or proportion of periodontopathic bacterial DNA was not related to any clinical index of pneumonia. Only two pneumonia cases consisted of >30% *Fusobacterium* DNA, suggesting that *Fusobacterium* was the causal pathogen in these cases. Our findings suggest that the periodontopathic bacteria rarely proliferate and be etiological pathogen in lower airway tract. However, further study is necessary, focusing on the pathogenicity of *F. nucleatum* in pulmonary infectious disease.

1. Introduction

Periodontitis is a highly prevalent worldwide infectious disease that leads to bone and connective tissue attachment loss. Recently, there has been increasing interest in potential associations between periodontal disease and various chronic systemic diseases and conditions. These conditions include atherosclerosis (stroke and coronary heart disease) [1,2], adverse pregnancy outcomes [3], and diabetes [4]. Although several studies indicate that oral care reduces the incidence and mortality of pneumonia [5–7], there are few studies that describe the relationship between pneumonia and periodontal disease.

Among more than 500 different species of common oral bacteria, only a small fraction of these bacterial species are thought to relate to periodontal disease [8]. Interestingly, several periodontopathic bacterial species have been reported as the etiologic pathogen of pulmonary infections. In a Bulgarian study, *Fusobacterium nucleatum* and *Prevotella intermedia* were detected in 27.2% and 2.7% of pleural fluid specimens in 147 cases of thoracic empyema, respectively [9]. In another study of empyema patients, which used 16S ribosomal RNA gene amplification followed by clone library methods, the phylotypes *F. nucleatum* and *Porphyromonas gingivalis* were predominantly detected from pleural fluid specimens in 2 of 16 cases (12.5%) and 1 of 16 cases (6.3%), respectively [10]. In a Japanese study that evaluated BAL specimens from 64 community-acquired pneumonia (CAP) patients by 16S rDNA analysis, the phylotype *F. nucleatum* was predominantly detected in 3 cases (4.7%) [11]. On the basis of these data, we expected that certain periodontopathic bacteria have higher potential as etiologic pathogens of pulmonary infection than previously known. Moreover, there is possibility that the presence of periodontopathic bacteria in oral cavity or lower respiratory tract may also affect severity of pneumonia via its products. As we recently

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reported, the culture supernatant of *P. intermedia* exhibits synergic effects on *Streptococcus pneumoniae*-lower respiratory tract infections in mice by enhancing pneumococcal adhesion to lower airway cells [12]. However, there are few clinical data which examined the presence of periodontopathic bacteria in lower respiratory tract, and its frequency or influence on pneumonia is unclear.

Herein, we examined the presence of common periodontopathic bacteria in respiratory tract specimens from patients with/without infectious pneumonia. Because periodontopathic bacteria are facultative anaerobes and difficult to culture [13,14], we conducted real-time PCR using universal and species-specific TaqMan probe/primer sets, which method widely used in surveillance of periodontopathic bacteria [15]. In this study, we focused on three periodontopathic bacteria, the *F. nucleatum-periodonticum-simiae* group, *P. intermedia*, and *P. gingivalis*, which had been already reported as lower respiratory tract pathogens. We then examined and compared the presence or proportion of periodontopathic bacterial DNA of these two groups.

The main aims of this study were to determine whether the lower respiratory tract infection by the periodontopathic bacteria exist, and to clarify whether the prevalence of the periodontopathic bacteria has a relationship with clinical features of infectious pneumonia.

2. Material and methods

2.1. Study location and patients

During a 2-year period (July 2011 to March 2013), 42 consecutive pneumonia patients treated at our university hospital were enrolled in this study. Because our laboratory division routinely stores all respiratory tract specimens until 7 days after submission, we could retrospectively examine the respiratory tract specimens of patients with infectious pneumonia. To exclude contamination with oral flora, we only examined the respiratory tract specimens collected directly from the lower respiratory tract, sputum from intubated patients, bronchoalveolar lavage fluid (BALF), or transtracheal aspirations. As representative samples of noninfectious pulmonary disease, BALF specimens obtained from 45 patients with chronic respiratory disease were also evaluated. This study was conducted as a retrospective study, approved by the Nagasaki University School of Medicine Research Ethics Committee. Informed consent was waived.

2.2. Inclusion and exclusion criteria

Inclusion criteria for infectious pneumonia were defined as follows: patients >20 years old with a body temperature of >38 °C or <36 °C and a newly developed lesion detected by chest radiography consistent with the diagnosis of pneumonia. CT scans were admissible as supporting evidence. Signs and symptoms included two or more of the following: cough, purulent sputum, abnormal auscultatory findings, signs of respiratory failure, signs of dyspnea, and worsening of tracheal aspirate fluid in mechanically ventilated patients. One of the following was required: leukocytosis or leukopenia (WBC count > 10,000/mm^3 or <4500/mm^3, respectively), band neutrophils > 15%, pulse rate >120 beats/min, or systolic hypotension. Pneumonia was classified into CAP, healthcare-associated pneumonia (HCAP), hospital-acquired pneumonia (HAP), or ventilator-associated pneumonia (VAP) according to American Thoracic Society guidelines [16,17].

Patients who received BAL examination for the diagnosis of chronic respiratory disease, such as pulmonary sarcoidosis, lung cancer, or idiopathic interstitial pneumonia, and also lacked any sign of infectious pneumonia as described above were included in the noninfectious pulmonary disease group. Patients with another simultaneous infection or mixed fungal/viral infection were excluded from evaluation.

Assessment of the severity of pneumonia in each patient was conducted using the pneumonia severity index (PSI). Mortality 30 days after admission was also evaluated.

2.3. DNA extraction

DNA samples were extracted from respiratory tract specimens using Qiagen DNA extract kits, according to the manufacturer's instructions.

2.4. Quantitative analysis by real-time-PCR

We conducted real-time PCR as described previously [15]. Primer/probe sets used are shown in Table 1. Real-time PCR assays were carried out using a Light Cycler 480 (Roche). For amplification reactions, duplicate samples were routinely used, and assays were performed in a total volume of 25 μL containing 12.5 μL of 2× TaqMan Universal Master Mix (Applied Biosystems), 0.2 μL each of forward and reverse primer (final concentration 200 nM each), 0.1 μL TaqMan probe (final concentration 100 nM), 2 μL template DNA solution, and an appropriate volume of sterilized DNase- and RNase-free water. Amplification reactions were performed in a thermocycler programmed as follows. An initial denaturation at 95 °C for 10 min was followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The negative control was PCR TaqMan Master Mix without DNA. The total number of bacterial cells was also determined using the TaqMan PCR procedure with a universal primer/probe set. In order to establish a quantitative assay, we cloned plasmids containing the amplified region of each target bacteria by using PCR 2.1 TOPO TA cloning procedures (Invitrogen). Each of the PCR amplicons for *P. gingivalis*, *P. intermedia*, and *F. nucleatum* were individually inserted into separate plasmid vectors. The recombinant vectors were transformed into chemically competent *Escherichia coli*. Insertion was confirmed by restriction enzyme digestion and agarose gel analysis. Plasmids were purified using MaxiPrep (QIAGEN). The purified plasmids of multiple dilutions were quantified by spectrophotometry. Quantification of target DNA was achieved by using serial 10-fold dilutions from 10^2 to 10^7 plasmid copies of the previously quantified plasmid standards. Plasmid standards and clinical samples were run in duplicate and the average values were used for calculation of bacterial load. A sample was considered positive for a target microorganism when the fluorescence emitted by the sample indicated higher than 10^2 plasmid copies.

2.5. Statistical analysis

Mean values ± SD were calculated for each bacterial species and for total eubacteria. The X^2 test was used for univariate comparison of categorical data. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Clinical characteristics and microbiological findings of patients

Of 42 patients included to the infectious pneumonia group, 33 were nosocomial pneumonia patients (83.3%), 35 patients were intubated (83.3%), and 5 patients underwent bronchoalveolar lavage (BAL) (11.9%). The mean PSI score was 119.8 ± 48.8, and 16 patients were scored as Pneumonia Patient Outcomes Research