Abstract

Acute pharyngitis and tonsillitis caused by *Streptococcus pyogenes* are common pediatric infectious diseases. Although the presence of penicillin-resistant *S. pyogenes* has not been confirmed in Japan, the re-isolation rate of *S. pyogenes* after antimicrobial treatment has been increasing. Thus, we attempted to determine whether the presence of *S. pyogenes* in pediatric patients treated with antimicrobial treatment was because of treatment failure or reinfection. We examined 19 patients who visited Daido Hospital between 2013 and 2017. The patient age, drug susceptibility patterns of the isolated bacteria, and random amplified polymorphic DNA (RAPD) analysis results were evaluated. The patient age ranged from 3 months to 9 years, seven patients were 5 years old. Seven patients showed consistent drug susceptible test results, and 12 patients showed inconsistent drug susceptible test results. Among these 12 patients, nine patients showed a greater decrease in drug susceptibility than the other three patients. Genetic mismatch was confirmed by RAPD analysis in all seven patients with consistent drug susceptible results. The paired *S. pyogenes* isolates from the same patient showed the presence of different bacteria. Our results indicate that, in many cases, re-isolation of *S. pyogenes* is not due to treatment failure, but due to reinfection with other clinical isolates.

Keywords: Drug susceptibility; *Streptococcus pyogenes*; RAPD; reinfection; Reisolation

1. Introduction

*Streptococcus pyogenes* is a typical pathogenic bacterium that causes childhood infections resulting in acute pharyngitis and tonsillitis [1]. As it is prone to acquiring drug resistance, more than half of the *S. pyogenes* strains are macrolide resistant in Japan [2]. Thus, in Japan, macrolide antibiotics are not usually used against *S. pyogenes*, and the first choice is penicillin antibiotics [1]. Although no penicillin-resistant strains have been found in Japan, there have been cases of antibacterial treatment failure in which bacteria are detected again from patients despite the use of penicillin in clinical settings [1, 3]. This may be due to the presence of penicillin-tolerant strains or the selection of inappropriate treatment methods, such as antibiotic treatment for an inadequate period [4, 5]. However, it is unlikely that antimicrobial treatment of *S. pyogenes* will fail based on the results of the drug susceptibility test because all *S. pyogenes* are susceptible to penicillin antibiotics. This may only be thought of as a reinfection with a different bacterium, not a failure of antibiotic treatment. Despite the problems related to antimicrobial treatment failure, few studies have directly examined bacterial strains before and after treatment failure [6, 7, 8, 9]. Therefore, we aimed to determine the presence of different strains by comparing the bacterial isolates from samples collected before and after treatment.
2. Material and methods

2.1. Strains and clinical data

In total, 677 strains of *S. pyogenes* were isolated from patients who visited Daido Hospital between 2013 and 2017. Of these, we investigated 38 *S. pyogenes* isolates obtained from 19 patients as a bacterial strain isolated from the same patient. As the study criteria, the pairs of two *S. pyogenes* species isolated from the same patient at intervals of 2 weeks or longer were included in this study. Daido Hospital is a 404-bed private general hospital in the central region of Japan. We used the medical records appended to clinical species for the analysis of clinical features of the infection. We considered several isolates from the same anatomical region of the same patient as one isolate per patient for the analysis in this study. All Streptococcus isolates were identified using standard conventional biochemical methods or the VITEK®2 system (BioMérieux, Durham NC, USA) according to instruction manual. The VITEK®2 system is an automatic bacterial test device that can identify and test for drug susceptibility. Bacteria were cultured in Trypticase soy agar with 5 % sheep blood (Nippon Becton Dickinson Co. Ltd, Tokyo, Japan) at 37 °C under 5 % CO₂ for 24 h. The cultured bacteria were measured after adjusting to McFarland No. 0.5 with 0.45 % saline. Our experimental design was approved by the ethics committee of Daido Hospital (ECD2018-028). Informed consent was obtained from all individual participants in this study.

2.2. Antimicrobial susceptibility analysis

*S. pyogenes* isolates were examined for susceptibility to 10 antibiotics—amoxicillin, cefotiam, ceftazidime, ceftriaxone, fosfomycin, meropenem, clarithromycin, clindamycin, minocycline, and ciprofloxacin. Minimal inhibitory concentrations were mainly determined at the clinical laboratory of Daido Hospital using the broth microdilution method with the VITEK®2 system according to instruction manual. This automated susceptible test was based on the Clinical Laboratory Standard Institute (CLSI) breakpoint [10]. *S. pneumoniae* ATCC® 49619 (ATCC, VA, USA) was used as quality control. Inoculum of bacterial colony was adjusted equivalent to a 0.5 McFarland standard after overnight culture on Trypticase soy agar with 5 % sheep blood. Bacteria were incubated at 37 °C under 5% CO₂ for 24 h.

2.3. Random amplified polymorphic DNA analysis

For the genotyping of *S. pyogenes* DNA polymorphisms, the random amplified polymorphic DNA (RAPD) assay was performed according to Seppälä’s modified method [11]. To extract the bacterial chromosomes, the bacteria were cultured in Todd Hewitt broth (Becton, Dickinson and Company, Franklin Lakes NJ, USA) with 0.2% yeast extract (Becton, Dickinson and Company) at 37 °C under 5 % carbon dioxide for 24 h. A Wizard® Genomic DNA Purification Kit (Promega Co, Madison WI, USA) was used to extract the bacterial chromosomes. Ex Taq (Takara Bio Inc., Shiga, Japan) was used as the polymerase chain reaction (PCR) enzyme. The PCR products were further subjected to 1% agarose gel electrophoresis.

3. Results

3.1. Time of bacterial isolation

Nineteen patients met the criteria for this study. At first, the time of bacterial isolation in these patients was also analyzed. In seven and six patients, the bacteria were isolated within 14 days–28 days and within 28–84 days of infection, respectively. In three patients, the bacteria were isolated within 84–168 days or after ≥168 days of infection (Figure 1).

3.2. Patient age and bacterial site distribution

The age distribution of the patients is shown in Figure 2. The age of the patients ranged from 3 months to 9 years; seven patients were 5 years old. In 16 patients, the bacteria were isolated from samples from the same site. The oral cavity, nasal cavity, and skin were the major isolated sites. The bacteria were isolated from samples from different sites in three patients. All of them were isolated from the nasal discharge samples (Figure 3).
Figure 1 Isolation interval.

Figure 2 Age distribution.

Figure 3 Site distribution. (A) - The number of patients with isolated from the same site (B) - The number of patients with isolated from the different site.
3.3. Distinguishing isolated bacteria by drug susceptibility testing and genetic methods

3.3.1. Antimicrobial susceptibility

![Figure 4 Patterns of antimicrobial resistance.](image)

The drug susceptibility of 38 S. pyogenes to four antibiotics was shown.

![Figure 5 Change in antimicrobial susceptibility.](image)

The change of drug susceptibility of S. pyogenes isolated from 19 patients to four antibiotics was shown.

Next, we examined the drug susceptibility of the isolated 38 bacteria. All strains were susceptible to β-lactams. The 23 (61%) bacteria were clarithromycin resistant; 15 (40%) bacteria were ciprofloxacin resistant; and 11 (29%) and 10 (26%) bacteria were clindamycin and minocycline resistant, respectively (Figure 4). In addition, we investigated the changes in drug susceptibility between the strains from the first and second quarantine periods (Figure 5). In 14, 13, and 15 patients, there was no change in the susceptibility to clarithromycin, clindamycin, and minocycline, respectively. Four, five, and three patients showed reduced susceptibility to these antibiotics, respectively. Only one patient had S. pyogenes which was resistant to these antibiotics.
3.3.2. DNA fingerprinting

According to the results of the susceptible testing, seven patients showed consistent results on drug susceptibility tests and 12 showed inconsistent results. Among these 12 patients, nine patients showed a greater decrease in drug susceptibility than the other three patients. Genetic mismatch was confirmed by RAPD analysis in all 7 patients with consistent drug susceptible results (Figure 6). Thus, the *S. pyogenes* isolates from the same patients were of different strains.

![Figure 6 RAPD patterns of consistent drug susceptibility in isolates from seven patients.](image)

4. Discussion

In this study, we performed antimicrobial susceptible tests and genetic studies to compare the bacterial strains before and after antimicrobial treatment. The results confirmed that the strains were different before and after treatment in all patients. Hence, we speculated that the re-isolation of the *S. pyogenes* strains was due to bacterial reinfection with a different strain rather than due to treatment failure and that the first antimicrobial treatment might have been effective. *S. pyogenes* infection spreads rapidly from person-to-person. Thus, after *S. pyogenes* is successfully eradicated from a patient, it may be transmitted again from the surroundings.

In our study, the bacterial re-isolation rate from patients who met the criteria was almost 70% within 3 months. As the average drug resistance rate of the isolated bacteria was also about 40%, this result is not remarkably high compared to previous report [1]. In addition, changes in the not all drug resistance rate of the re-isolated bacteria showed an upward trend. Since no penicillin-resistant strain has been confirmed, there seems to be no problem in the actual treatment of bacterial infections. Therefore, inappropriate drug selection is not always considered a matter of grave concern.

As a screening method for distinguishing the bacterial strains, we first examined the drug susceptibility patterns because drug resistance in *S. pyogenes* is mainly due to external gene acquisition, such as via a bacteriophage, and bacterial genetics rarely change in the short period before and after treatment [12, 13].

Several studies have been conducted on *S. pyogenes* reinfection. In a 2001 Swedish study on *S. pyogenes*, analysis of four patients with recurrent pharyngeal tonsillitis revealed the same bacterial T-type and arbitrarily primed PCR patterns [9]. In a 2011 Japanese study on patients with recurrent *S. pyogenes* infection, isolates from 11 patients showed different pulse field gel electrophoresis (PFGE) patterns, and isolates from 38 showed the same PFGE patterns. In addition, after recurrence, ≥50% bacteria acquired macrolide resistance [7]. In a Japanese study conducted from 2012 to 2014, the PFGE patterns of bacterial strains in 11 patients with recurrent pharyngeal inflammation with a 40-day isolation interval were the same. In addition, the PFGE patterns of the bacterial strains in six patients with recurrent pharyngeal inflammation collected at an interval of ≥40 days were the same [6]. However, in a Chilean study, macrolide resistance, prevalence of the *prtF1* gene, and biofilm formation were not as-sociated with *S. pyogenes* reinfection. However, the frequency of reinfection with FCT-4-type *S. pyogenes* was high. Since FCT-4-type *S. pyogenes* possesses two fibronectin-binding proteins, there may be a causal link between bacterial reinfection and adhesion molecules [14]. Moreover, the methods of bacterial genetic analysis were different between these studies and our study. We suggest that the cause of recurrent symptoms of pharyngeal tonsillitis was not recurrent infection in carrier patients but reinfection with
different strains. In addition, our results showed that the macrolide resistance rate exceeded 50%, similar to the finding of a previous report [1]. The change in drug susceptibility due to macrolide resistance acquired by the bacteria after reisolation was only 5%. Hence, further observational studies are needed in this regard. As a limitation of our research, we did not examine the emm and prtF1 genes of the isolated strains; hence, we could not compare this point with the results of previous studies.

We used the RAPD genetic testing method to distinguish between bacterial strains. Usually, PFGE and whole genome sequencing (next-generation sequencing) are used for genetic sequencing of bacterial strains [15, 16]. These methods have high susceptibility and specificity, but the cost of the equipment is extremely high, and the process takes a long time. Hence, its versatility in clinical settings is low. Although the RAPD method is less accurate than other methods, it yielded acceptable results in this study [8]. In addition, previous studies have also identified reinfection in cases of recurrent infection with S. pyogenes using the RAPD method [17].

5. Conclusion
According to our results, S. pyogenes isolates from the same patients contained different strains, indicating that in many cases, reisolation of S. pyogenes is not due to treatment failure, but due to infection with other clinical strains. Thus, even if S. pyogenes is detected after antimicrobial treatment, we recommend that antimicrobial treatment should be restart.

Compliance with ethical standards

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Disclosure of conflict of interest
All authors declare no conflict of interest of regarding the publication of this paper.

Statement of informed consent
Informed consent was obtained from all individual participants included in this study.

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