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Original Article

Molecular epidemiology, antimicrobial susceptibility, and characterization of fluoroquinolone non-susceptible *Streptococcus pyogenes* in Japan☆

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ABSTRACT

Streptococcus pyogenes (Group A streptococci: GAS) are known to cause a wide variety of human illnesses, some of which can be life-threatening. Usually, penicillin is the first-choice agent for the treatment of GAS infections. For patients with penicillin or beta-lactam antibiotics allergies, macrolide drugs are recommended as an alternative therapy. However, an increased prevalence of macrolide-resistant GAS (MRGAS) has been reported in many countries. Furthermore, fluoroquinolone non-susceptible GAS has been reported. The present study was focused on determining the features of fluoroquinolone non-susceptible strains collected from children with pharyngotonsillitis in the southwestern areas of Japan. To reveal the characteristics of fluoroquinolone non-susceptible GAS, we investigated the MIC, T-serotype, *emm* typing, and PFGE of 298 GAS strains isolated in the Fukuoka southwest area of Japan between 2011 and 2013. We determined that fluoroquinolone non-susceptibility shows a MIC to tosufloxacin of ≧1 μg/ml.

We identified 33 (11.1%) fluoroquinolone non-susceptible GAS strains. In these strains, 6 T-serotypes and 9 *emm*/MLST patterns were detected. The predominant combinations were *emm*6/ST382 (14 strains, 42.4%) and *emm*89/ST101 (5 strains, 15.2%). PFGE classified 10 pulsotypes, and each was quite different.

These results showed that fluoroquinolone non-susceptible GAS strains have a variety of origins. The usage of fluoroquinolone drugs could have a negative effect on the antimicrobial drug sensitivity of GAS in Japan. Considering such a situation, continuous monitoring of quinolone non-susceptible GAS is necessary.

1. Introduction

Group A streptococci (GAS) is one of the most significant pathogens in pediatric medicine. GAS primarily causes upper respiratory infections, but also causes impetigo, mastoiditis, and life-threatening diseases such as sepsis, necrotizing fasciitis, septic arthritis, and toxic shock syndrome. Also, post-infectious manifestations of pharyngotonsillitis such as rheumatic heart disease and rheumatic fever are also severe immune-mediated complications. GAS infections are a significant cause of morbidity and mortality worldwide [1,2].

Penicillin is normally the first choice for the treatment of GAS infections. When late-onset allergies are considered, however, it is believed that 15% of all patients have a penicillin allergy [3], and some of them have cephalosporin antibiotics allergies, as well. For patients with penicillin or beta-lactam antibiotics allergies, macrolide drugs are recommended as a first-line therapy. However, increases in the prevalence of macrolide-resistant GAS (MRGAS) have been reported in many countries. Actually, the prevalence of macrolide resistance has recently reached 32.8% in Spain, 40% in Belgium, 98.4% in China, and 22.8% in Greece [4–7]. The proportion

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of macrolide-resistant GAS is reported to be 30–40% in Japan [8]. It is apparent that many people have a risk of treatment failure for a GAS infection due to antimicrobial resistance.

There are reports of fluoroquinolone-resistant streptococci, as well as reports showing fluoroquinolone-resistant *Streptococcus pneumonia* [9]. Genes such as *gyrA* and *parC* have been associated with fluoroquinolone resistance in some reports. The *gyrA* gene encodes the target protein for fluoroquinolone and *parC* is related to the elimination mechanism for fluoroquinolone [10]. Fluoroquinolone non-susceptible GAS is believed to operate in the same manner. Only a few countries have recently reported cases of fluoroquinolone non-susceptible GAS [11–13].

Since 2010 in Japan, tosufloxacin (TFLX) has been available for children being treated for bacterial pneumonia and acute otitis media. After the introduction of pneumococcal conjugate vaccine and *H. influenzae* type b conjugate vaccine, non-typeable *H. influenzae* infections have increased, as in other countries. A further problem is the high rate of a beta-lactamase negative ampicillin resistant (BLNAR) strain that has reached a prevalence of approximately 40–50% in Japan. Reflecting such an antimicrobial resistant state, TFLX has become a common antibiotic for children. This situation poses the risk of inducing fluoroquinolone resistance in the microbiome. Appropriate usage of antibiotics is recommended at the points where antimicrobial resistance is generated. On the other hand, surveillance, and monitoring are also recommended for the detection of new antimicrobial-resistant organisms. The present study was focused on determining the features of fluoroquinolone non-susceptible isolates collected from children with pharyngotonsillitis in the southwestern areas of Japan over the past 3 years following the permitted usage of TFLX for children.

2. Material and methods

2.1. Bacterial isolates

From 2011 to 2013, a total of 298 GAS isolates were submitted to our study from four clinics (Shindo children's clinic, Nagai children's clinic, Ikezawa children's clinic, and Tsumura clinic) and two general hospitals (Kurume University Hospital and St. Mary's Hospital) in the southwestern area of Japan. These strains were revived from frozen stock and cultured on sheep blood agar plates at 37 °C. We identified them as *Streptococcus pyogenes* according to their colony morphologies and by the results from β-hemolysis on blood agar, the bacitracin test, the BinaxNOW® Strep A test (Alere Medical Co. Ltd. Chiba, Japan), and 16S rRNA polymerase chain reaction (PCR) analysis [14].

2.2. Antimicrobial susceptibility

We investigated the antimicrobial susceptibilities of these strains by measuring their Minimum inhibitory concentrations (MICs). MICs were determined using the broth dilution method, which was performed in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). As a control, the reference strain *Streptococcus pneumoniae* ATCC 49619 was included. GAS strains were tested against 7 antibiotics: penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan); amoxicillin (SigmaAldrich Co., LLC, Tokyo, Japan); erythromycin (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan); clarithromycin (Taisho Pharmaceutical Co., Ltd., Osaka, Japan); azithromycin (Pfizer Japan Inc., Tokyo, Japan); clindamycin (Pfizer Japan Inc.); and, tosufloxacin (FUJIFILM Toyama Chemical Co., Ltd., Tokyo, Japan). Susceptibility results were categorized according to criteria established by the CLSI [15] and by the Japanese society of chemotherapy. Then, the fluoroquinolone non-susceptible strains were selected as the study population. For the present study, the presence of fluoroquinolone non-susceptibility was assigned a MIC to TFLX of ≧1 μg/ml. Based on this standard, a total of 33 fluoroquinolone non-susceptible strains were selected for analysis.

2.3. T-serotype and emm type

The T-serotypes were identified using a slide agglutination test with type-specific antiserum (DENKA SEIKEN Co., Ltd, Tokyo, Japan). Then, *emm* sequencing was performed in accordance with the protocol of the CDC International Streptococcal Reference Laboratory (http://www.cdc.gov/streplab/M-ProteinGene-typing.html).

2.4. MLST analysis

All fluoroquinolone non-susceptible strains were assessed by MLST in accordance with the protocol on the MLST website. The primers of 7 housekeeping genes (i.e., *gki*, *gtr*, *muri*, *mutS*, *recP*, *xpt*, and *yiqL*) were based on information from the website [16]. The sequence types were assigned using the MLST website.

2.5. PFGE analysis

We performed PFGE analysis by referencing the *Clostridium botulinum* (https://www.cdc.gov/pulsenet/pdf/c-botulinum-protocol-508c.pdf) method with slight modifications [17]. In brief, chromosomal DNA was digested overnight at 30 °C with the restriction enzyme *Cfr*9I (Thermo Fisher Scientific K.K., Tokyo, Japan). The electrophoresis conditions were 20 h at a 120° angle and 6 V/cm using a CHEF Mapper system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We performed PFGE using CHEF DNA Size Standard ladder (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The DNA bands were stained with ethidium bromide and photographed. The interpretation of the PFGE patterns was based on criteria described by Tenover et al. [18]. *Cfr*9I profiles were coded alphabetically, and for closely related pulsotypes (differences in 2 or 3 bands), a number was added. PFGE profiles were analyzed using Quantity One® software version 4.6.3 (Bio-Rad Laboratories, Inc. Hercules, CA, USA) and employing the unweighted pair group method with an arithmetic mean, a Dice coefficient, and a position tolerance of 1% [19].

2.6. Fluoroquinolone resistance gene

For All fluoroquinolone non-susceptible strains, we investigated the presence of fluoroquinolone resistance gene. We performed PCR for *gyrA* and *parC* gene by referencing the method described by S. Yan et al. [20], and analyzed amino acid arrangement.

2.7. Ethical statement

We made this study available to the public on our home page, which required approval through the ethics committee of the Kurume University School of Medicine (No. 18241).

3. Results

3.1. Antimicrobial susceptibility

We collected 298 GAS strains between 2011 and 2013 including 84, 148, and 66 strains collected in 2011, 2012, and 2013, respectively. All 298 GAS strains showed susceptibility to penicillin G and amoxicillin (Table 1). Of the 298 GAS strains, 123 (41.3%) were erythromycin-resistant, and this total included 44 (52.4%), 53 (35.8%), and 26 (39.4%) collected in 2011, 2012, and 2013, respectively. The proportions of strains resistant to clarithromycin,

Table 1
Distribution of minimum inhibitory concentration in isolates.

MIC	≦0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	≧128	Total
PCG	298	0	0	0	0	0	0	0	0	0	0	0	298
AMPC	298	0	0	0	0	0	0	0	0	0	0	0	298
CLDM	74	102	64	8	2	0	0	0	0	0	1	47	298
CAM	64	59	44	8	0	0	9	43	23	2	7	39	298
EM	31	66	67	11	0	0	0	22	46	7	1	47	298
AZM	5	20	66	60	20	4	1	29	35	7	3	48	298
TFLX	32	67	101	65	20	13	0	0	0	0	0	0	298

PCG:penicillin G, AMPC: amoxicillin, CLDM: clindamycin, CAM: clarithromycin, EM: erythromycin, AZM: azithromycin, TFLX: tosufloxacin.

azithromycin, and clindamycin were 41.3, 42.6, and 16.8%, respectively. None of the erythromycin-resistant strains was susceptible to clarithromycin or azithromycin, whereas 72 erythromycin-resistant isolates were susceptible to clindamycin.

Of all strains, 33 (11.1%) were tosufloxacin non-susceptible, including 9 (3.0%), 10 (3.4%), and 14 (4.7%) collected in 2011, 2012 and 2013, respectively. Only 7 (2.3%) GAS strains were non-susceptible to both TFLX and erythromycin (Table 2).

3.2. T-serotype and emm type (emm/T types)

We detected 6 T-serotypes among all 33 fluoroquinolone non-susceptible strains. T6 (10 strains, 30.3%) was predominant, followed, in order, by TB3264 (7 strains, 21.2%), T1 (3 strains, 9.1%), T3 (2 strains, 6.1%), T25 (2 strains, 6.1%), T12 (1 strain, 3.0%), and non-typable (NT) (8 strain, 24.2%). We also detected 6 *emm* types. Among 33 strains, *emm*6 (14 strains, 42.4%) was predominant, followed by *emm*89 (10 strains, 30.3%), *emm*75 (4 strains, 12.1%), *emm*1 (3 strains, 9.1%), *emm*12 (1 strain, 3.0%), and *emm*44 (1 strain, 3.0%).

The predominant *emm*/T types were *emm*6/T6 (10 strains, 30.3%), which was followed, in order, by *emm*89/TB3264 (7 strains, 21.2%), *emm*6/NT (4 strains, 12.1%), *emm*1/T1 (3 strain, 9.1%), *emm*75/T25 (2 strains, 6.1%), *emm*75/NT (2 strains, 6.1%), *emm*89/T3 (2 strains, 6.1%), *emm*12/T12 (1 strain, 3.0%), *emm*44/NT (1 strain, 3.0%), and *emm*89/NT (1 strain, 3.0%).

3.3. MLST, emm typing, and PFGE

MLST analysis was performed for all 33 fluoroquinolone non-susceptible strains. We identified 9 sequence types. The predominant sequence type was ST382 (14 strains, 42.4%), followed by ST101 (5 strains, 15.2%), ST646 (4 strains, 12.1%), ST49 (3 strains, 9.1%), ST28 (3 strains, 9.1%), ST466, ST230, ST194, and ST36 (1 strain, 3.1%, respectively). By combining MLST with *emm* typing, we detected 9 patterns. The major combinations, in order, were as follows: *emm*6/ST382 (14 strains, 42.4%); *emm*89/ST101 (5 strains, 15.2%); *emm*89/ST466 (4 strains, 12.1%); *emm*75/ST49 (3 strains, 9.1%); *emm*1/ST28 (3 strains, 9.1%); and, *emm*89/ST466, *emm*75/ST230, *emm*44/ST194, and *emm*12/ST36 (1 strain each, 3.1% each).

By combining *emm*/MLST with a T-serotype, we obtained 14 patterns (Table 3) and performed PFGE using the restriction enzyme *Cfr*9I. We detected 10 pulsotypes (Fig. 1). From these results we constructed a dendrogram of the PFGE patterns. PFGE, *emm* typing, and MLST results showed that most GAS isolates with the same PFGE pulsotype were of the same *emm*/MLST types. The exception was *emm*89/ST646.

Table 2
The number of strains which were fluoroquinolone non-susceptible and macrolide-resistant was 7 (2.3%).

	TFLX MIC≦0.5	TFLX MIC≧1	Total
EM MIC≦0.5	149	26	175
EM MIC≧1	116	7	123
Total	265	33	298

3.4. gyrA, parC

We identified the mutations of *par*C in 30 isolates. But in other 3 isolates we could not detect mutations of both genes and all these 3 isolates were classified in pulsotype D. Nine pulsotypes had the mutation in *parC*. Serine-79 was changed to phenylalanine in pulsotype A, B, and F, to tyrosine in pulsotype C, and to alanine in pulsotype E. Aspartic acid-91 was changed to asparagine in pulsotype A, G1, G2, G3, and G4. In all isolates, mutations of *gyrA* were not found. Pulsotype A was revealed to have a double substitution in *parC*.

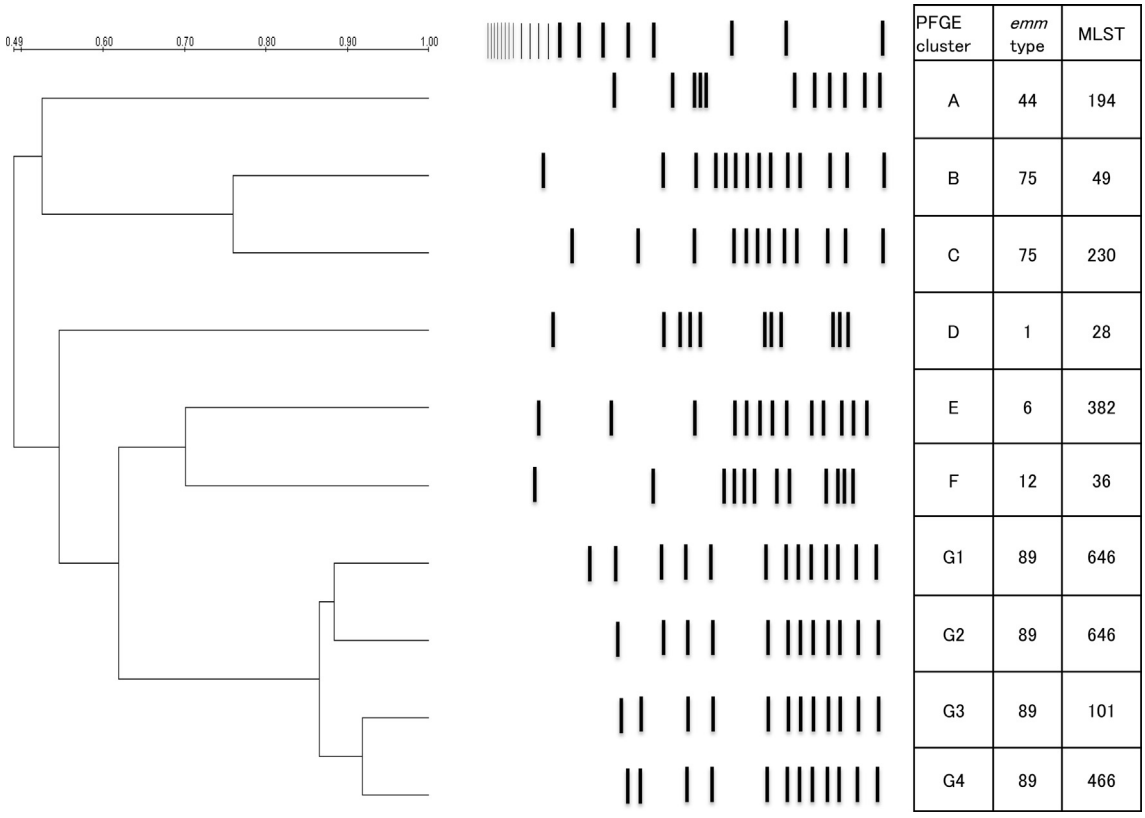
4. Discussion

In our study, 11.1% of the strains were identified as fluoroquinolone non-susceptible GAS. The rate of fluoroquinolone non-susceptible GAS gradually increased each year for the three years measured. This is the first epidemiological report concerning fluoroquinolone non-susceptible GAS in Japan.

Molecular epidemiology studies have reported the rates of fluoroquinolone non-susceptible GAS in Europe [11,12]. These studies revealed that the predominant *emm* types were *emm*6, particularly *emm*6/ST382, and *emm*75 [18]. In the present study, the predominant *emm*/ST type was *emm*6/ST382, which agreed with the results found in European countries. With regard to the PFGE band patterns, *emm*6/ST382 had only 1 pulsotype (pulsotype E), which revealed this to be the most prevalent fluoroquinolone non-susceptible GAS type. These results suggested that a single clonal strain of *emm*6/ST382 might be prevalent worldwide. On the other hand, one report concerning fluoroquinolone non-susceptible GAS

Table 3
We obtained 14 patterns by combining emm/MLST with T-serotype and performed PFGE for each pattern.

emm/MLST	T-serotype	Number
emm6/ST382	T6	10
emm89/ST101	TB3264	4
emm6/ST382	NT	4
emm1/ST28	T1	3
emm75/ST49	T25	2
emm89/ST646	TB3264	2
emm12/ST36	T12	1
emm44/ST194	NT	1
emm75/ST49	NT	1
emm75/ST230	NT	1
emm89/ST101	T3	1
emm89/ST646	T3	1
emm89/ST646	NT	1
emm89/ST466	TB3264	1
		33



PFGE cluster	*emm* type	MLST
A	44	194
B	75	49
C	75	230
D	1	28
E	6	382
F	12	36
G1	89	646
G2	89	646
G3	89	101
G4	89	466

Fig. 1. Dendrogram and PFGE patterns of *Cfr*9I-digested chromosomal DNA, and association with phenotype, *emm* type, and sequence type in fluoroquinolone non-susceptible GAS (n = 33). We used CHEF DNA Size Standard ladder (50–1000 kb). All isolates generated 7 pulsotypes (A–G) and closely related pulsotypes (differences in 2 or 3 bands) were assigned to pulsotype G.

showed *emm*12 to be the predominant type in China [13]. In our study *emm*12 had only one isolate. The Asian results show that the epidemic status of fluoroquinolone non-susceptible GAS should be afforded epidemic status and be closely monitored.

In addition to *emm*6/ST382, eight emm/ST types were identified: *emm*89/ST101, *emm*89/ST466, *emm*75/ST49, *emm*1/ST28, *emm*89/ST466, *emm*75/ST230, *emm*44/ST194, and *emm*12/ST36. That result indicates that a greater variety of fluoroquinolone non-susceptible GAS strains are predominant in Japan compared with that in Europe. The cause is believed to be the level of fluoroquinolone drug usage. In Asian countries, including Japan, a greater amount of fluoroquinolone has been prescribed than in either European countries or the USA [21,22]. We suggest that the usage of fluoroquinolone has had a negative effect on the drug sensitivity of GAS in Asian countries. In Japan, the AMR action plan was devised by the Health, Labor and Welfare Ministry in 2016 and many clinicians have begun to prescribe antibiotics in a more appropriate manner after adopting the AMR action plan. Epidemiological information of antimicrobial-resistant GAS strains is needed following implementation of the AMR action plan. This information is important for the prevention of antimicrobial-resistant GAS infectious diseases.

At the point of non-susceptible mechanisms, almost all pulsotypes were revealed to have some mutations of *parC* gene in this study. These mutations agreed with other reports [23,24], so we thought these mutations caused fluoroquinolone non-susceptibility. In isolates of pulsotype D there were no mutations at *gyr*A and *par*C. We only investigated these 2 genes in this study. These isolates might have other non-susceptible mechanisms such as mutations of *gyr*B and *parE*.

Of all 298 GAS strains, the fluoroquinolone non-susceptible and macrolide-resistant varieties numbered only 7 (2.3%). This result shows that fluoroquinolone drugs could be an alternative to MRGAS for patients with beta-lactam allergies.

In conclusion, the existence of quinolone-resistant streptococcus was confirmed in Japan, and not only is it possible that the epidemic strains of the world are represented, but also that Japan-specific resistant strains are occurring. Revealing the true scope of the problem will require continuous surveillance.

Declaration of Competing Interest

None.

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