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Emergence of Optochin Resistance among *Streptococcus pneumoniae* in Japan

Masaki Nagata[1,2], Osamu Ueda[1,3], Takeo Shobuike[1], Tetsuro Muratani[4,5], Yosuke Aoki[2], Hiroshi Miyamoto[1*]

[1]Division of Microbiology, Department of Pathology and Microbiology, Faculty of Medicine, Saga University, Saga, Japan
[2]Division of Infection Control and Prevention, Saga University Hospital, Saga, Japan
[3]Marketing Department, Siemens Healthcare Diagnostics Kabushiki Kaisha, Tokyo, Japan
[4]Department of Clinical Laboratory, Kyurin Medical Laboratory, Fukuoka, Japan
[5]Hibiki Research Group for Clinical Microbiology, Fukuoka, Japan
Email: [*]miyamoth@cc.saga-u.ac.jp

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ABSTRACT

The optochin susceptibility test is a key method for differentiating *Streptococcus pneumoniae* from other α-hemolytic streptococci; however, optochin-resistant (Opt^r) *S. pneumoniae* have been reported in the last two decades. In this study, we investigated the isolation frequency of Opt^r *S. pneumoniae* in the North Kyushu area of Japan, and biochemically and genetically characterized Opt^r *S. pneumoniae* clinical isolates. Seven (0.68%) out of 1032 *S. pneumoniae* isolates collected by the North Fukuoka Infectious Diseases Working Group were found to be Opt^r *S. pneumoniae*. Resistant strains had MICs of optochin 2- to 64- fold higher than susceptible strains, possessed different antimicrobial resistance profiles, and belonged to different serotypes. All the seven Opt^r isolates had mutations in the nucleotide sequence code for subunit c of F_0F_1 ATPase. Three isolates had mutations in codon 48 (deduced amino acid substitution of valine with phenylalanine) and two isolates had mutations in codon 49 (substitution of alanine with threonine or serine). Of the remaining two isolates, one had mutation in codon 50 (substitution of phenylalanine with leucine) and the other had mutation in codon 44 (substitution of methionine with isoleucine, which was a novel mutation in this position). From these results, we identified the mutation in the H^+-ATPase subunit c gene (*atp*C) of *S. pneumoniae*, which was not recognized earlier, and determined that Opt resistance among Japanese pneumococcal isolates is not related to a specific pneumococcal serotype or antimicrobial resistance profile. Furthermore, the results indicate that when α-hemolytic streptococci resistant to optochin are isolated from patients with invasive infectious diseases, such as meningitis and pneumonia, we should perform additional examinations such as bile solubility tests or PCR assays before confirming isolates as viridans streptococci. This is the first report of the characterization of Opt^r *S. pneumoniae* in Japan.

Keywords: Optochin; *Streptococcus pneumoniae*; H^+-ATPase; MALDI-TOF MS; 16S rRNA

1. Introduction

Streptococcus pneumoniae colonize the nasopharynx in 20% - 40% of children and 5% - 10% of adults at any time, and cause serious infectious diseases, such as pneumonia, septicemia, meningitis, and otitis media [1]. Five phenotypic characteristics are classically used in the clinical laboratory for the presumptive identification of *S. pneumoniae*: Gram stain morphology, colony morphology, type of hemolysis, optochin susceptibility, and agglutination with anti-pneumococcal polysaccharide capsule antibodies. Accurate identification is important for ensuring the correct diagnosis and treatment of patients because of the increasing frequency of resistance to penicillin and other antibiotic agents.

The optochin susceptibility test is one of the most important methods for differentiating *S. pneumoniae* from other α-hemolytic streptococci. Optochin (ethylhydrocupreine hydrochloride) is a quinine analog. It was introduced as a therapeutic agent for treatment of lobar pneumonia in early 20th century. However, its use resulted in severe side effects and a study reported that 4.5% of patients treated with optochin experienced loss of vision [2]. Thus, it was stopped being used as a therapeutic agent. Later, in 1915, optochin was found to be useful for differentiating *S. pneumoniae* from other α-hemolytic streptococci [3]. The optochin susceptibility test was found to be highly satisfactory and less time-consuming for the identification of *S. pneumoniae* compared to bile solubility test; hence, it was adopted in clinical laboratories in 1955 [4]. In 1987, Kontiainen and

[*]Corresponding author.

Sivonen [5] first reported the identification of two clinical isolates of optochin-resistant (Opt^r) *S. pneumoniae* in blood samples taken from a 74-year-old man with pneumonia and liver cirrhosis and an 8-month-old child with sepsis from otitis media. Since then, the emergence of Opt^r *S. pneumoniae* has been reported from the United States [6-8], Israel [9], Portugal [10,11], Brazil [12], and Argentina [13]. In addition, mutations of the gene encoding subunit c of the Fo complex of transmembrane H^+-ATPase were reported to be responsible for optochin resistance [14]. There have been no reports of Opt^r *S. pneumoniae* in Japan.

The purpose of this study is to investigate the isolation frequency of Opt^r *S. pneumoniae* in Japan, report the biochemical and genetic characteristics of the isolates, and alert clinical microbiologists of the presence of these strains in the community.

2. Materials and Methods

2.1. Bacterial Strains

A total of 1032 presumptive *S. pneumoniae* isolates were collected by the North Fukuoka Infectious Diseases Working Group (NFIDWG, Fukuoka, Japan). These clinical isolates were recovered mainly from the nasopharynx and sputum samples obtained from 138 medical clinics and hospitals participating in NFIDWG. Each isolate was confirmed to be *S. pneumoniae* based on its Gram stain morphology, colony morphology, type of hemolysis, optochin susceptibility, and agglutination with anti-pneumococcal polysaccharide capsule antibodies. In cases where optochin resistance was suspected, we conducted further examinations such as the bile solubility test, determination of the presence of the major autolysin gene (*lyt*A) by PCR [15], determining bacterial profiles by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and sequencing of the 16*S rRNA* genes (1480 to 1485 bases). *S. pneumoniae* R6 ATCC BAA-255 (uncapsulated derivate of D39) was used as the reference strain.

2.2. Optochin Susceptibility

Optochin disks (5 μg each; Eiken Co., Ltd., Tokyo, Japan) were placed on 5% sheep blood agar plates (Eiken) streaked with the test isolate. The diameter of inhibition zones around the disk was measured after 18 - 24 h incubation at 35˚C - 37˚C in a 5% CO_2 atmosphere. Bacterial isolates with a diameter measuring ≥13 mm were tentatively identified as optochin-sensitive, while isolates with a diameter < 13 mm were identified as optochin-resistant. The MICs of optochin (Sigma Co. LLC, St. Louis, MO, USA) for 1031 isolates were determined using the plate dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) containing 5% defibrinated sheep blood (Kohjin Bio Co., Ltd., Saitama, Japan) and an inoculum size of 10^4 CFU of bacteria. Cell growth was evaluated after incubation for 24 h at 37˚C in a 5% CO_2 atmosphere. Bacterial isolates were considered optochin resistant when MICs were ≥4 μg/ml, based on the result of our present study (**Figure 1**) and a previous report by Pikis *et al.* [8].

2.3. Bile Solubility, Latex Agglutination, and Capsular Serotyping

Bile solubility and latex agglutination tests were performed using the Slidex Pneumo-Kit (Nippon bio- Mérieux Co., Ltd., Tokyo, Japan) based on the methods described by Whatmore *et al.* [16]. Capsular serotyping (the Neufeld Quellung test) was performed using each type or group serum (Statens Serum Institut, Copenhagen, Denmark) at the National Institute of Infectious Diseases, Tokyo, Japan.

2.4. Biotyping

Biotyping was performed using API 20 Strep V7.0 (Nippon bioMérieux) and an automated VITEK 2 Gram Positive Identification (GPI) Card (Nippon bioMérieux) at the Central and Clinical Laboratories in Saga University Hospital by medical technologists who are specialists in clinical microbiology. Cultures were grown anaerobically on 5% sheep blood agar (Eiken) at 35˚C - 37˚C for 22 - 26 h and suspensions were prepared for API 20 Strep V7.0 according to the manufacturer's instructions. The result was determined by matching data with an API 20 Strep profile list. For preparing the GPI Card, colonies were picked and suspended in a 3.0-ml-sterile salt solution (pH 4.5 - 7.0), which was equivalent to a McFarland's 0.50 - 0.63 standard according to the manufac-

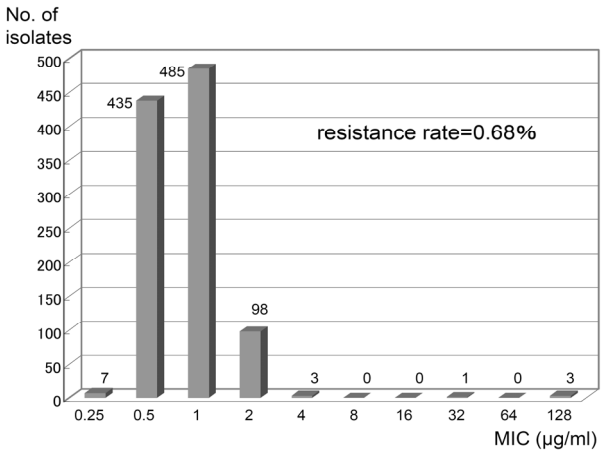

Figure 1. Distribution of MICs of Optochin in *Streptococcus pneumoniae* clinical isolates (1032 strains). The MICs are determined by the plate dilution method.

turer's instructions.

2.5. PCR for the Major Autolysin Gene (*lyt*A)

The *lyt*A gene was detected by PCR using primers described by Ubukata *et al.* [15]. Using a Biometra T Gradient Thermocycler (Biometra GmbH, Goettingen, Germany), thirty cycles of DNA amplification were performed as follows: denaturation at 94˚C for 15 s, annealing at 50˚C for 30 s, and extension at 72˚C for 30 s. The presence of an amplified 273-bp sequence of the autolysin gene indicated the presence of the *lyt*A gene. The DNA size marker was 100 Base-Pair Ladder DNA (Pharmacia Biotech Co., Ltd., Tokyo, Japan).

2.6. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Identification

Identification of isolates by MALDI-TOF MS was performed on a Microflex LT instrument (Bruker Daltonics GmbH, Leipzig, Germany) with FlexControl (version 3.0) software (Bruker Daltonics) for the automatic acquisition of mass spectra in the linear positive mode within a range of 2 - 20 kDa. Colonies were examined by both direct deposition on MSP 96 target plates (Bruker Daltonics) and after a formic acid-acetonitrile extraction step according to the manufacturer's instructions. According to the criteria proposed by the manufacturer, a result was considered valid (accurate identification to the species level) when the score was >2.0.

2.7. Antibiotic Susceptibility

The MICs to 12 antimicrobial agents were determined using the microdilution broth method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotics used in this test were as follows: benzylpenicillin (PCG), ampicillin (ABPC), cefazolin (CEZ), cefotiam (CTM), cefotaxime (CTX), cefpodoxime (CPDX), cefditoren (CDTR), panipenem (PAPM), minocycline (MINO), erythromycin (EM), clindamycin (CLDM), and levofloxacin (LVFX).

2.8. Sequencing of 16*S rRNA* Gene

The 16S rDNA (~1.5 kb) was amplified by PCR using the primers 8UA (5'-AGAGTTTGATCMTGGCTCAG-3') and 1485B (5'-TACGGTTACCTTGTTACGAC-3') [17]. The purified PCR product was sequenced directly on both strands using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). The primers 519A (5'-CAGCMGCCGCGGTAA-3'), 519B (5'-ATTACCGCGGCRGCTG-3'), 774A (5'-GTAGTCCACGCTGTAAACGATG-3'), 774B (5'-CATCGTTTACAGCGTGGACTAC-3'), and 907B (5'-CCGTCAATTCMTTTRAGTTT-3') were used as internal primers for sequencing. Homology search with the 16*S rRNA* gene sequences were performed against sequences registered in GenBank/EMBL/DDBJ using a basic local alignment search tool (BLAST).

2.9. Cloning and DNA Sequence Analysis of H^+-ATPase Subunit c Gene

The H^+-ATPase subunit c gene (*atp*C) of *S. pneumoniae* was amplified by PCR using primers (sense primer: 136 5'-TAGCGGTTAAAAGTTGACAA-3'; antisense primer: 437 5'-CCCTTTTCTTCTCGTTCC-3') described by Cogné *et al.* [18]. After initial denaturation at 95˚C for 2 min, 25 cycles of DNA amplification were performed as follows: denaturation at 95˚C for 1 min, annealing at 54˚C for 2 min, extension at 72˚C for 2 min 30 s, and a final extension at 72˚C for 7 min 30 s. The expected 302-bp fragment was purified using PCR purification columns (GenElute Minus EtBr Spin Columns; Sigma Chemical Co., St Louis, MO, USA) and cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transformation was performed using competent *E. coli* TOP10 cells provided by the manufacturer. A total of three to five white colonies were randomly selected from each clone library for sequence analysis. To prepare a template for sequence analysis, a partial fragment of the cloning vector (PCR II) containing an inserted PCR product was amplified using M13Forward (5'-GTAAAACGACGGCCAG-3'), M13Reverse (5'-CAGGAAACAGCTATGAC-3'), and AmpliTaq Gold DNA polymerase. Primers and dNTP were eliminated from the PCR mixture using an ExoSAP-IT Kit (USB, Cleveland, OH, USA) according to the manufacturer's instructions and a 1 μl aliquot was used as a template for the sequencing reaction. Sequencing reactions were performed using the M13 primers and a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). The nucleic acid sequences were determined using a 3130xl Genetic Analyzer (Applied Biosystems).

2.10. Genetic Transformation

Transformation in *S. pneumoniae* R6 was performed as described by Muñoz *et al.* [19]. *S. pneumoniae* R6 was grown in C medium plus yeast extract (C + Y) [20] to the late exponential phase and frozen at −80˚C after the addition of glycerol to 15%. Frozen stock (200 μl) was added to 4 ml of C + Y medium and incubated for about 2 h to reach competence for transformation. The competent *S. pneumoniae* R6 cells were diluted 10-fold in C + Y medium and a cloned PCR fragment (*atp*C gene) of *S. pneumoniae* SPJ661 was added at final concentrations

of 0.1 mg/ml to 500 ml of the diluted cultures. These were then incubated at 30˚C for 1 h and at 37˚C for at least 2 h to allow expression of optochin resistance. Samples were then plated in 100 μl volumes on Mueller-Hinton agar (Difco Laboratories) containing 5% defibrinated sheep blood (Kohjin Bio) and 2 μg/ml optochin (Sigma).

2.11. Nucleotide Sequence Accession Numbers

Nucleotide sequence data of partial sequences of *atp*C genes were deposited in the DDBJ database under the accession numbers AB569578 to AB569584.

3. Results

3.1. Isolation Frequency and Biochemical Characteristics of Optochin-Resistant *S. pneumoniae*

A total of 1032 strains conclusive or presumptive as *S. pneumoniae* were collected by NFIDWG from patients with invasive diseases, such as pneumonia, septicemia, meningitis, and otitis media. Of these, 1025 isolates (99.32%) exhibited an optochin MIC of ≤2 μg/ml and seven isolates (0.68%) exhibited an MIC of ≥4 μg/ml (three isolates, 4 μg/ml; 1 isolate, 32 μg/ml; three isolates, ≥128 μg/ml) using the plate dilution method (**Figure 1**, **Table 1**). **Table 1** shows that three of the seven optochin-resistant strains were isolated from the nasal discharges of children, while four strains were isolated from the sputum of elderly individual persons. As shown in **Table 2**, all isolates presented typical Gram staining characteristics, α-hemolysis on blood agar plates, bile solubility, and agglutination reactions with sera targeting pneumococcal capsular polysaccharides. In addition, all isolates contained the *lyt*A gene and exhibited autolysis. However, they were not confirmed as *S. pneumoniae* using API Strep 20 V.7.0 because they had biochemical alterations with regards to optochin resistance, a loss of inulin and starch utilization, and a loss of arginine hydrolysis and pyrrolidone arylamidase (**Table 3**). **Table 2** shows that they were identified as *S. pneumoniae* using the extraction method based on MALDI-TOF MS analysis. Mass spectra were not obtained for strain SPJ1298 using the direct method because it produced mucoid colonies. Mass spectra were also not obtained for another three isolates probably because of autolysis, when the colonies were analyzed on day 1 after being cultured on blood agar plates (**Table 2**). Isolates belonged to different serotypes (**Table 2**) and exhibited different antimicrobial resistance profiles (**Table 1**). Although all Opt^r isolates were susceptible to PCG and LVFX, six of the seven isolates were resistant to EM (**Table 1**). In addition, five isolates were CPDX intermediates or CPDX resistant, and four isolates were resistant to CLDM (**Table 1**). Finally, all isolates were confirmed to be *S. pneumoniae* by the 16*S rRNA* gene analysis (**Table 2**).

3.2. Genetic Characteristics of Optochin-Resistant *S. pneumoniae*

Table 4 shows that all the seven Opt^r isolates had mutations in the nucleotide sequence coding for subunit c of F_0F_1 ATPase. Three isolates (SPJ48, 492, and 1298) had mutations in codon 48 (GTT to TTT, deduced amino acid substitution of valine with phenylalanine) and two isolates (SPJ743 and 1331) had mutations in codon 49 (GCC to ACC or TCC, substitution of alanine with threonine or serine). Of the remaining two isolates, one (SPJ246) had mutation in codon 50 (TTT to CTT, substitution of phenylalanine with leucine) and the other

Table 1. Characteristics of optochin-resistant *Streptococcus pneumoniae* clinical isolates.

Strain	Source	Patient (y.o)	MIC (μg/ml)											
			optochin	PCG	ABPC*	CEZ*	CTM*	CTX	CPDX	CTDR*	PAPM*	MINO*	EM	CLDM	LVFX
SPJ48	nasal discharge	outpatient (2)	128	2 (S**)	2	4	4	0.5 (S)	1 (I***)	0.5	0.06	8	4 (R****)	0.06 (S)	1(S)
SPJ246	nasal discharge	outpatient (1.5)	4	0.25 (S)	0.5	0.5	2	1 (S)	2 (R)	0.5	0.06	>8	>4 (R)	>4 (R)	1(S)
SPJ492	sputum	inpatient (84)	128	1 (S)	0.5	2	4	1 (S)	2 (R)	0.5	0.06	0.25	0.06 (S)	0.06 (S)	1(S)
SPJ661	sputum	inpatient (89)	4	0.03 (S)	0.06	0.25	0.25	0.03 (S)	0.03 (S)	0.03	0.06	>8	>4 (R)	1 (R)	1(S)
SPJ743	nasal discharge	outpatient (3.5)	32	0.5 (S)	1	1	0.25	0.5 (S)	1 (I)	0.25	0.06	>8	2 (R)	0.06 (S)	1(S)
SPJ1298	sputum	inpatient (76)	128	0.06 (S)	0.12	0.12	0.25	0.12 (S)	0.25 (S)	0.12	0.06	>8	>4 (R)	>4 (R)	2(S)
SPJ1331	sputum	outpatient (66.5)	4	0.12 (S)	0.12	0.5	2	1(S)	2 (R)	0.5	0.06	>8	>4 (R)	>4 (R)	2 (S)

PCG, benzylpenicillin; ABPC, ampicillin; CEZ, cefazolin; CTM, cefotiam; CTX, cefotaxime; CPDX, cefpodoxime; CDTR, cefditoren; PAPM, panipenem; MINO, minocycline; EM, erythromycin; CLDM, clindamycin; LVFX, levofloxacin. *The breakpoints of MICs for ABPC, CEZ, CTM, CTDR, PAPM, and MINO are not determined by CLSI. **S: susceptible, ***I: intermediates, ****R: resistant.

Table 2. Characteristics and identification of optochin-resistant *Streptococcus pneumoniae* clinical isolates.

Strain	Morphology	BS·	LA†	PCR (*lyt*A)	Serotype	VITEK2 GPI	API 20 Strep	MALDI Biotyper		Homology (%) of 16*S rRNA* gene###
								Direct (fresh culture/1 day-culture)##	Extraction	
SPJ48	typical	+	+	+	19	*S. pneumoniae*	NI#	*S. pneumoniae*/NI	*S. pneumoniae*	99.8
SPJ246	typical	+	+	+	14	*S. pneumoniae*	NI	*S. pneumoniae*/NI	*S. pneumoniae*	99.8
SPJ492	typical	+	+	+	23	*S. pneumoniae*	NI	*S. pneumoniae*/*S. pneumoniae*	*S. pneumoniae*	99.9
SPJ661	typical	+	+	+	15	*S. pneumoniae*	NI	*S. pneumoniae*/NI	*S. pneumoniae*	99.9
SPJ743	typical	+	+	+	23	*S. pneumoniae*	NI	*S. pneumoniae*/*S. pneumoniae*	*S. pneumoniae*	99.9
SPJ1298	mucoid	+	+	+	3	*S. pneumoniae*	NI	NI/NI	*S. pneumoniae*	99.9
SPJ1331	typical	+	+	+	14	*S. pneumoniae*	NI	*S. pneumoniae*/*S. pneumoniae*	*S. pneumoniae*	99.9

·bile solubility test; †latex agglutination test; #nonidentifiable; ##bacterial colony from fresh culture or from day 1 after being cultured is used for identification; ###homology to the 16*S rRNA* gene of *S. pneumoniae* ATCC33400 (T).

Table 3. Biochemical characteristics of optochin-resistant *Streptococcus pneumoniae.*

Characteristics	Positive rates (%)	
	Optochin-resistant isolates	Pooled data·
Acid from		
Arabinose	0	1
Inulin	0	64
Lactose	100	99
Mannitol	0	0
Raffinose	71	87
Ribose	0	3
Sorbitol	0	0
Trehalose	100	98
Starch	0	84
Glycogen	0	10
Hydrolysis of		
Arginine	0	57
Esculin	0	39
Hippurate	0	0
Voges-Proskauer	0	0
Production of		
Alkaline phosphatase	0	3
Alpha-Galactosidase	100	70
Beta-Glucuronidase	0	3
Beta-Galactosidase	100	79
Leucine arylamidase	100	100
Pyrrolidone arylamidase	0	60
Beta-hemolysis	0	1

·Based on the API 20 Strep manual's data (version 7).

(SPJ661) had mutation in codon 44 (ATG to ATT, substitution of methionine with isoleucine, which was a novel type of mutation in this position). To identify the novel mutation (M44I) responsible for optochin resistance, strain R6 was transformed with cloned PCR products containing the M44I mutation. Two transformants (strains R6/R-7 and R6/4-16) were selected and tested for susceptibility to optochin. The transformants did not produce adequate inhibition zones around the optochin disks. Furthermore, when the optochin MICs for the transformants were tested, they were the same as those for the original strain SPJ661 (**Table 4**). Sequence analysis of the transformants confirmed the expected amino acid changes (**Table 4)**.

4. Discussion

S. pneumoniae is a major pathogen that causes fatal infectious diseases such as pneumonia, septicemia, and meningitis, particularly in children and elderly individuals. The increasing frequency of resistance to antibiotic agents necessitates accurate identification for ensuring the correct diagnosis and treatment of patients, especially in Japan where approximately 35% of *S. pneumoniae* strains are penicillin intermediates (PISP) or penicillin resistant (PRSP), and 60% - 70% of the strains are macrolide resistant [21]. The optochin susceptibility test is a key method for differentiating *S. pneumoniae* from other α-hemolytic streptococci, and most clinical microbiology laboratories depend on this test for differentiating streptococci. However, Opt^r *S. pneumoniae* have been reported in the last two decades [6-13]. Both information of the isolation frequency of Opt^r *S. pneumoniae* in Japan and knowledge about the biochemical and genetic characteristics of the Opt^r isolates are required for accurate identification of *S. pneumoniae*, leading to correct diagnosis and treatment of patients.

Table 4. Comparisons of ATPase c-subunit amino acid sequences between optochin-resistant *Streptococcus pneumoniae* and optochin-sensitive R6 strain (ATCCBAA-255).

Strain	MIC of Optochin (mg/ml)	1 10 20 30 33
R6	2	M N L T F L G L C I A C M G V S V G E G L L M N G L F F S V A R Q
R6/R-7 (transformant)	4	- -
R6/4-16 (transformant)	4	- -
SPJ48	128	- -
SPJ246	4	- -
SPJ492	128	- -
SPJ661	4	- -
SPJ743	32	- -
SPJ1298	128	- A* - -
SPJ1331	4	- -
Mutation previously reported		S
[Reference]		[14] This study
*GCA to GCT		

Strain	MIC of Optochin (mg/ml)	34 40 50 60 66
R6	2	P D M L S E F R S L M F L G V A F I E G T F F V T L V F S F I I K
R6/R-7 (transformant)	4	- - - - - - - - - - I -
R6/4-16 (transformant)	4	- - - - - - - - - - I -
SPJ48	128	- - - - - - - - - - - - - - F - - - - - - - - - - - - - - - - -
SPJ246	4	- - - - - - - - - - - - - - - - L - - - - - - - - - - - - - - -
SPJ492	128	- - - - - - - - - - - - - - F - - - - - - - - - - - - - - - - -
SPJ661	4	- - - - - - - - - - I -
SPJ743	32	- - - - - - - - - - - - - - - T - - - - - - - - - - - - - - - -
SPJ1298	128	- - - - - - - - - - - - - - F - - - - - - - - - - - - - - - - -
SPJ1331	4	- - - - - - - - - - - - - - - S - - - - - - - - - - - - - - - -
Mutation previously reported		F T, S L
[Reference]		This study [14] [8,12,13] [14]

In this study, seven (0.68%) out of 1032 *S. pneumoniae* isolates collected by NFIDWG were Opt^r *S. pneumoniae* (**Figure 1**). The isolation frequency was similar to those found in the United States [6-8], Israel [9], Portugal [10,11], Brazil [12], and Argentina [13] (0.58% to 2.1%). Optochin resistance among Japanese pneumococcal isolates was not related to a specific pneumococcal serotype or antimicrobial resistance profile (**Tables 1** and **2**). In addition, **Table 3** shows that the biochemical characteristics (loss of inulin and starch utilization and loss of arginine hydrolysis) of these Japanese Opt^r isolates were similar to those of the first clinical Opt^r isolates reported from Finland in 1987 [5]. These results suggest that the emergence of Opt^r isolates may not be by clonal expansion, but may be attributable to spontaneous mutations that are not caused by exposure to any antimicrobial agent. It was also shown that API 20 Strep is not useful for species identification of Opt^r *S. pneumoniae*

because they had the biochemical alterations.

It was reported that point mutations in the *atp*C genes, which encode subunits of F_0F_1-ATPase, conferred optochin resistance on *S. pneumoniae* [8,12,14,18]. On the other hand, there are no reports about mutations in the *atp*C genes among optochin-sensitive *S. pneumoniae* [8, 13,14]. H^+-ATPase is a membrane-bound multimeric enzyme complex responsible for proton translocation across plasma membranes. This enzyme is an F_0F_1 class ion transport ATPase. The F_0 sector of the complex comprises membrane-embedded protein subunits that translocate hydrogen ions, while the F_1 sector of the complex contains the catalytic subunit for ATP hydrolysis and synthesis. Certain amino acid residues in the transmembrane domains are essential for H^+ translocation across the plasma membrane [8,22]. The molecular mechanism determining how optochin interacts with the F_0 portion of ATPase remains unresolved, but the proton-translocating subunits of ATPase may be disrupted in the presence of optochin, resulting in proton pump failure and *S. pneumoniae* cell death. Amino acid changes in the subunit c in Opt^r isolates may prevent optochin from disrupting the proton transport pathway. Only 21 Opt^r clinical strains are known to have been characterized at the molecular level and 10 mutations in the *atp*C gene have been reported [13]. The most frequent modifications in our Opt^r clinical strains (**Table 4**) were found at position 48 in the subunit c where valine was substituted with phenylalanine (V48F), as also reported by Fenoll *et al.* [14]. This mutation may be involved in the high levels of optochin-resistance (strains SPJ48, 492, and 1298 in **Table 4**). The second most common modification was found at position 49 where alanine was substituted with serine (A49S) or threonine (A49T), which was also reported previously [8,12-14]. In both cases, the nonpolar hydrophobic alanine was replaced by a polar uncharged amino acid (serine or threonine) in the α-helix 2 domain. This polarity change may interfere with the binding of optochin to subunit c. We identified a novel mutation, M44I, in the same domain.

This is the first report of the characterization of Opt^r *S. pneumoniae* in Japan. Cortes *et al.* [13] reported that Opt^r strains maintain their virulence as well as optochin-susceptible *S. pneumoniae*. Nunes *et al.* [11] reported a prevalence of approximately 2.1% from a total of 1973 pneumococcal strains isolated during a period of 6 years in Portugal. Thus, when α-hemolytic streptococci resistant to optochin are isolated from patients with invasive infectious diseases, such as meningitis and pneumonia, we should perform additional examinations such as bile solubility tests or PCR assays before confirming isolates as viridans streptococci. Accurate identification and antimicrobial susceptibility testing are crucial for correct diagnosis and treatment of patients.

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