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preceding viral illness [2,3]. Leucopenia has also been a finding in cases during the current pandemic of influenza A (H1N1) infection [8–10]. Our patient had leucopenia with mild neutropenia and lymphopenia.

The disease is self-limiting and, when the myositis develops, the patient is already at the early convalescent phase of the viral illness. Therefore, antivirals are not usually indicated. Only supportive treatment and follow-up aiming to monitor full recovery of the patient are required [17]. In our case, the patient was already afebrile at the time of admission and he recovered fully in less than 48 h.

BACM is an acute, self-limiting condition with an excellent prognosis, which occurs during the acute convalescent phase of viral illnesses, mainly influenza A and influenza B infections, and requires no therapeutic intervention. Correct diagnosis by considering the characteristic clinical and laboratory findings as well as the history of the preceding viral illness can prevent unnecessary diagnostic procedures and reassure both the parents and the patient of the excellent prognosis.

Transparency Declaration

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Spontaneous pneumomediastinum complicating pneumonia in children infected with the 2009 pandemic influenza A (H1N1) virus

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Abstract

We report two occurrences of spontaneous pneumomediastinum (SPM) complicating pneumonia in Japanese children infected with the novel influenza A (H1N1) virus (IV). General practitioners especially should suspect possible SPM when examining and treating children with the novel influenza accompanied by status asthmaticus or wheezing. The presented patients illustrate the specific clinical and radiological signs associated with SPM complicating pneumonia in children infected with A(H1N1)v.

Keywords: A (H1N1) virus, children, influenza, pneumonia, spontaneous pneumomediastinum

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A novel influenza A (H1N1) virus (IV), was identified as the cause of outbreaks of febrile respiratory infection ranging from self-limiting to severe illness in Mexico, the USA, Canada, and elsewhere in the spring of 2009 [1]. Triple-reassortant IV containing genes from avian, human and swine influenza viruses emerged and became enzootic among swine herds in North America during the late 1990s [2]. Clinical aspects of the first 11 patients sporadically infected with IV were reported to the CDC in the USA, from December 2005 to February 2009 [2]. Accordingly, surveillance was implemented for human infections with influenza A viruses that could not be subtyped. Specimens were sent to the CDC RT-PCR confirmatory testing for IV. A total of 642 confirmed cases of IV infection were identified from 15 April to 5 May. Sixty percent of patients were aged 18 years or younger, indicating that children may be particularly susceptible to IV [1].

Eighteen cases of pneumonia and confirmed IV infection were identified among 98 patients hospitalized for acute respiratory illness from 24 March to 24 April in Mexico, including five patients aged 15 years or younger [3]. All patients had fever, cough, dyspnoea, increased serum lactate dehydrogenase levels, and bilateral patchy pulmonary shadows on roentgenograms. Other common findings were increased creatine kinase levels and lymphopaenia. Mechanical ventilation was required for 12 patients, and seven patients died.

We report two IV-infected Japanese children with spontaneous pneumomediastinum (SPM) complicating pneumonia.

IV was confirmed in nasopharyngeal-swab specimens using real-time RT-PCR as previously described [4–6]. IV-specific primers for amplification (87 bp) of the nonstructural protein 1 gene (accession number FJ966086) included a sense primer, 5′- GCGAACTTCAGTGTAATC-3′, and a reverse primer, 5′- AATTTCTCCAACTATTGCTC-3′; the specific molecular beacon probe used was 6-carboxyfluorescein-CGCGATGACCTTGATACTACTAAGGGCTTATCGCG-black hole quencher 1. IV-associated pneumonia was based on evidence of both influenza-like illnesses with opacities on chest radiographs and laboratory-confirmed IV infection [3].

Case 1

A previously healthy 6-year-old girl with a 2-day history of cough was referred because of fever and dyspnoea. Respiratory distress was evident from tachypnoea (respiratory rate, 48/min) and supraclavicular and intercostal inspiratory retraction (Table 1). Bilateral expiratory rhonchi were apparent on auscultation, together with insufficient vesicular sounds. The patient required oxygen supplementation (5 L/min) because percutaneous oxygen saturation (SpO_2) was 84% when breathing room air. A chest roentgenogram on admission indicated air leakage into the mediastinum, together with bilateral hyperaeration and a right middle-lobe infiltrate (Fig. 1). She was treated with continuous inhalation of a bronchodilator and intravenous prednisolone (10–30 mg daily for 4 days), as well as antiviral therapy. On day 3 after admission, the radiographic SPM findings had resolved.

Case 2

An 8-year-old boy with a past history of allergic rhinitis had fever and cough for 1 day, and presented with chest pain and dyspnoea. Tachypnoea (36/min) was accompanied by supraclavicular and intercostal inspiratory retraction (Table 1). Vesicular sounds were insufficient over the left hemithorax, with neither rhonchi nor rales. The patient required oxygen supplementation (3 L/min) because SpO_2 was 91% when breathing room air. Chest roentgenography on admission indicated air leakage into the mediastinum and neck, as well as bilateral hyperaeration and a left lower lobe infiltrate (Fig. 1). The patient was treated with continuous inhalation of a bronchodilator and intravenous administration of both aminophylline (0.6 mg/kg/h for 7 days) and prednisolone (10–30 mg daily for 3 days), together with antiviral therapy. On day 4 after admission, there was no evidence of SPM.

Nineteen IV-infected children with pneumonia (n = 16) or bronchitis (n = 3) were admitted to our department from 9 August to 25 September. Clinical and laboratory features, treatment, and outcomes are shown in Table 1. The two

TABLE 1. Clinical and laboratory features, treatments, and outcomes of pneumonia or bronchitis in 19 children infected with 2009 pandemic influenza A (H1N1) virus

Patient no.	Age (years)	Gender	Underlying disease	Time from onset to admission (days)	Main symptom	Body temperature (°C)	Heart rate (beats/min)	Respiratory rate (/min)	SpO_2 (%)	Inspiratory (%) retraction	Auscultation finding	Respiratory disease	WBC (lymphocyte count) (× 10^9 cells/L)	CRP (mg/dL)	LDH/CK (IU/L, IU/L)	Antiviral therapy	Anti-biotic	Duration of oxygen supplementation (days)	Duration of admission (days)	Outcome
1 (SPM)	6	F	Healthy	3	Respiratory distress	39.2	156	48	84	Remarkable	Rhonchi, poor vesicular sounds	Pneumonia	10.9 (0.4)	4.72	193/86	Oseltamivir	MINO	6	13	Recovered
2 (SPM)	8	M	Allergic rhinitis	2	Chest pain	39.9	132	36	91	Remarkable	Poor vesicular sounds	Pneumonia	9.2 (0.6)	3.65	200/75	Oseltamivir	CEZ	5	10	Recovered
3	1	M	Healthy	2	Cough	38.9	126	48	ND	Negative	Normal	Bronchitis	15.6 (6.9)	6.88	424/126	Oseltamivir	PIPC	0	4	Recovered
4	4	F	Healthy	7	Cough	39.7	144	42	96	Negative	Rales	Pneumonia	7.7 (1.9)	1.03	309/45	None	CEZ	0	4	Recovered
5	9	M	Healthy	2	Respiratory distress	40.1	153	60	92	Positive	Rales	Pneumonia	9.7 (0.6)	3.15	236/110	Oseltamivir	CEZ	2	6	Recovered
6	8	F	Healthy	2	Cough	39	150	42	94	Positive	Rales, poor vesicular sounds	Pneumonia	8.7 (0.4)	2.48	252/112	Oseltamivir	ABPC/SBT	2	8	Recovered
7	10	M	Asthma	1	Respiratory distress	38.8	102	54	84	Positive	Rhonchi, poor vesicular sounds	Pneumonia	10.2 (0.8)	3.64	218/108	Oseltamivir	ABPC/SBT	4	8	Recovered
8	8	M	Healthy	2	Cough	39.9	108	36	93	Negative	Poor vesicular sounds	Pneumonia	5.0 (0.3)	2.43	340/71	Oseltamivir	ABPC/SBT	2	7	Recovered
9	6	M	Cough-variant asthma	2	Respiratory distress	39.8	153	66	86	Positive	Poor vesicular sounds	Pneumonia, atelectasis	16.1 (0.5)	2.82	276/57	Oseltamivir	ABPC/SBT	11	18	Recovered
10	11	F	Healthy	2	Cough	39.6	146	48	94	Positive	Poor vesicular sounds	Pneumonia	7.1 (0.6)	0.38	184/55	Oseltamivir	CTX	3	6	Recovered
11	12	F	Healthy	2	Respiratory distress	38.3	134	48	92	Positive	Rhonchi, poor vesicular sounds	Pneumonia	11.3 (0.4)	2.99	215/ND	Oseltamivir	CEZ	4	6	Recovered
12	6	F	Asthma	2	Respiratory distress	39.6	157	28	90	Positive	Poor vesicular sounds	Pneumonia	10.0 (0.8)	1	288/208	Oseltamivir	None	5	8	Recovered
13	12	F	Allergic rhinitis	2	Respiratory distress	39.6	159	36	92	Positive	Poor vesicular sounds	Pneumonia	14.5 (0.3)	3.26	253/137	Oseltamivir	CEZ	3	6	Recovered
14	8	M	Asthma	2	Respiratory distress	38.7	124	42	87	Positive	Rhonchi, poor vesicular sounds	Pneumonia	6.1 (0.3)	0.75	275/87	Oseltamivir	CEZ	2	6	Recovered
15	12	F	Asthma	1	Respiratory distress	39.1	170	48	91	Positive	Rales, poor vesicular sounds	Bronchitis	22.7 (0.3)	1.17	245/86	Oseltamivir	CEZ	5	12	Recovered
16	8	M	Asthma	2	Respiratory distress	39.4	150	52	89	Positive	Rales, poor vesicular sounds	Pneumonia	17.2 (0.5)	1.18	242/249	Oseltamivir	CEZ	7	10	Recovered
17	4	M	Asthma	3	Respiratory distress	37.3	138	48	93	Positive	Rhonchi	Bronchitis	7.9 (2.8)	2.47	221/ND	Oseltamivir	CEZ	3	6	Recovered
18	8	M	Healthy	5	Cough	38.1	102	30	96	Negative	Normal	Pneumonia	3.4 (1.4)	0.1	307/64	Oseltamivir	CAM	0	5	Recovered
19	13	M	Healthy	4	Cough	38	104	24	ND	Negative	Normal	Pneumonia	7.2 (1.0)	6.78	384/1931	Oseltamivir	ABPC/SBT	0	7	Recovered

ABPC, ampicillin; CAM, clarithromycin; CEZ, cefazolin; CK, creatine phosphokinase; CRP, C-reactive protein; CTX, cefotaxime; F, female; LDH, lactate dehydrogenase; M, male; MINO, minocycline; ND, not determined; PIPC, piperacillin; SBT, sulbactam; SPM, spontaneous pneumomediastinum; SpO_2, percutaneous saturation of oxygen under room air; WBC, white blood cell count. Clinical and laboratory findings were recorded on admission.

patients with SPM represented 10.5%. To the best of our knowledge, this is the first report of SPM complicating pneumonia in IV-infected children.

A rare disorder, SPM typically is triggered by respiratory infection and inflammation. It follows intrathoracic pressure increases leading to rupture of alveoli or pneumatoceles near the mediastinal pleura with air leakage along vessels. Additionally, SPM can complicate pulmonary emphysema, with air drainage through the interstitium to the hilum, mediastinum, neck, and skin [7].

Pneumonias complicated by SPM most notably include *Pneumocystis jirovecii* pneumonia (PCP) [8]. High-resolution chest computed tomography in a PCP patient was reported to show SPM associated with peripheral air-trapping in the right middle lobe [8]. Two patients presenting with tension SPM, which is often fatal, both had AIDS-related PCP [9]. SPM subsequent to seasonal influenza is relatively rare, even though a patient with influenza virus bronchiolitis complicated by SPM has been described [10]. Our observations suggest a possible high prevalence of SPM in IV-infected children that may reflect a characteristic pathology in the respiratory tract.

In children, SPM is observed most commonly in status asthmaticus, bronchiolitis or bronchitis with casts [11,12], but it may also occur in any patient during a Valsalva manoeuver related to coughing, forceful vomiting or wheezing [13]. Interestingly, SPM has occurred in status asthmaticus associated with influenza [14]. Clinicians, therefore, should consider the possibility of SPM when examining and treating children with both novel influenza and wheezing or status asthmaticus.

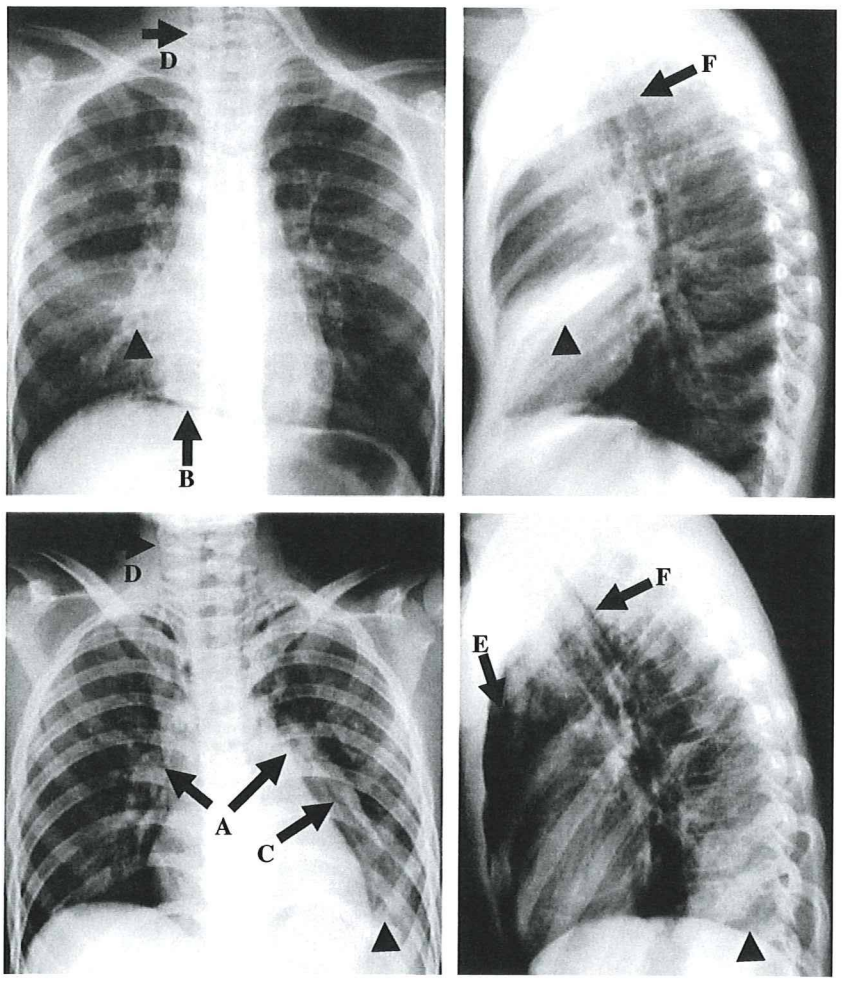

FIG. 1. Chest roentgenograms showing spontaneous pneumomediastinum (arrows) complicating pneumonia in two children (case 1, upper row; case 2, lower row) infected with influenza A (H1N1) virus. The left images are frontal (posterior-anterior) views, and the right images are lateral views. Arrowheads indicate pulmonary infiltrates in each patient. A, spinnaker sail sign (angel wing sign); B, continuous diaphragm sign; C, vertical lucent streak on the left side of the heart; D, subcutaneous emphysema; E, retrosternal emphysema; F, posterior superior mediastinal emphysema.

In summary, SPM in children generally resolves spontaneously with aggressive supportive care. Our patients illustrate the specific clinical and radiologic signs associated with SPM complicating pneumonia in IV-infected children. Surveillance results concerning paediatric deaths (n = 36) associated with the 2009 pandemic IV infection have been reported for April to August in the USA [15]. Clinicians should also be aware that an early diagnosis of influenza can allow for prompt initiation of antiviral therapy for children with an increased risk of severe illness.

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Transparency Declaration

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cific. The high plasma creatinine level in the newborn sometimes reflects the mother's plasma creatinine level (*9*). However, kidney function of the mother of the newborn was within normal limits at the time of Caesarean section; plasma creatinine level of 0.7 mg/dL. An elevated plasma creatinine level is observed frequently in premature infants due to immaturity of the kidney tissue and will usually decrease within a few weeks. Oseltamivir was administered with dose adjustment based on the infant's estimated glomerular filtration rate. The recommended dose of oseltamivir for glomerular filtration rate <30 mL/min/1.73 m^2 is 2–3 mg/kg/day, based on preliminary data obtained by a National Institutes of Health–funded Collaborative Antiviral Study Group (*10*). The success of our management strategy for this case suggests early treatment with oseltamivir can prevent severe illness in newborns with perinatal influenza A pandemic (H1N1) 2009 infection.

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Bronchial Casts and Pandemic (H1N1) 2009 Virus Infection

To the Editor: In the late 1990s, triple-reassortant influenza A viruses containing genes from avian, human, and swine influenza viruses emerged and became enzootic in swine herds in North America (*1*). The first 11 human cases of novel influenza A virus infection were reported to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) from December 2005 through February 2009 (*1*). In response to those reports, surveillance for human infection with nonsubtypeable influenza A viruses was implemented.

In the spring of 2009, outbreaks of febrile respiratory infections caused by a novel influenza A virus (H1N1) were reported among persons in Mexico, the United States, and Canada (*2*). Patient specimens were sent to CDC for real-time reverse transcription–PCR (RT-PCR) testing, and from April 15 through May 5, 2009, a total of 642 infections with the virus, now called pandemic (H1N1) 2009 virus, were confirmed. Of those 642 patients, 60% were ≤18 years of age, indicating that

children may be particularly susceptible to pandemic (H1N1) 2009 (*2*).

Children and adults with preexisting underlying respiratory conditions, such as asthma, are at increased risk for complications from infection with pandemic (H1N1) 2009 virus. One possible complication is plastic bronchitis, a rare respiratory illness characterized by formation of large gelatinous or rigid branching airway casts (*3*). Plastic bronchitis is a potentially fatal condition induced by bronchial obstruction from mucus accumulation resulting from infection, inflammation, or vascular stasis (*4*). We report a case of bronchial casts that caused atelectasis of the right lung of a child infected with influenza A pandemic (H1N1) 2009 virus.

A 6-year-old boy with asthma and a 1-day history of fever and cough was referred to a hospital pediatrics department because of dyspnea. Clinical examination at hospital admission found respiratory distress, as shown by tachypnea (respiratory rate 66 breaths/min) and inspiratory retraction, deficient vesicular sounds over the right lung field, elevated blood levels of immunoglobulin E (1,770 IU/mL) and a reduced number of lymphocytes (483 cells/μL), and radiographic evidence of atelectasis of the right lung and hyperinflation of the left lung without air leakage (Figure, panel A). Pandemic (H1N1) 2009 virus infection was confirmed by real-time RT-PCR, as described (*5*), of an endotracheal aspirate. Real-time PCR ruled out *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, Legionella pneumophila, Chlamydophila pneumoniae, S. pyogenes*, respiratory syncytial viruses A and B, seasonal influenza viruses A and B, parainfluenza viruses 1–3, rhinovirus, enterovirus, human metapneumovirus, human bocavirus, and adenovirus (*6*). While the patient was breathing room air, his percutaneously monitored oxygen saturation was 86%; respiratory support by mechanical ventilation was then initiated. Mucus casts were extracted by intratracheal suction (Figure, panel B). The patient was treated with an inhaled bronchodilator, intravenous methylprednisolone (20–60 mg/day for 7 days), and antiviral (oseltamivir) and antimicrobial (ampicillin/sulbactam) drugs.

On hospital day 2, chest radiographs showed that atelectasis of the right lower lobe had partially resolved (Figure, panel C). A histologic examination of casts (May-Giemsa stain; Figure, panel D) indicated a mucoid substance containing a predominantly eosinophilic infiltrate (>90% of cells). The patient's respiratory condition during 11 days of oxygen supplementation gradually improved, and he was discharged on hospital day 18.

Plastic bronchitis is related mainly to respiratory, cyanotic cardiac (post-Fontan), and hematologic (sickle cell anemia) diseases. A diagnosis of plastic bronchitis is determined on the basis of clinical findings (pointing to allergic and asthmatic, cardiac, or idiopathic etiologies) and pathologic findings (inflammatory vs. noninflammatory) on examination of casts (*3*). Inflammatory casts contain fibrin, eosinophils, and Charcot-Leyden crystals; noninflammatory casts contain mucin and exhibit vascular hydrostatic changes. The case presented here was the allergic-inflammatory type of plastic bronchitis.

Various treatments for plastic bronchitis have been described and vary from cast removal by expectoration or by bronchoscopy (*7,8*). Other interventions involve cast disruption by tissue plasminogen activator or urokinase and prevention of cast formation by use of mucolytic agents, steroids, or anticoagulants. However, evidence remains anecdotal because too few plastic bronchitis patients are available for clinical trials. Details of steroid dosage will need to be clarified for pandemic (H1N1) 2009 virus–infected children with respiratory distress from bronchitis and pneumonia.

In Iran during 1998–2001, avian influenza (H9N2) infection among broiler chickens resulted in 20%–60% mortality rates on affected farms (*9*). Macroscopic examination of specimens from infected chickens showed extensive hyperemia of the respiratory tract, followed by exudate and casts extending from the tracheal bifurcation to the secondary bronchi. Light microscopy indicated severe necrotizing tracheitis. Pandemic (H1N1) 2009

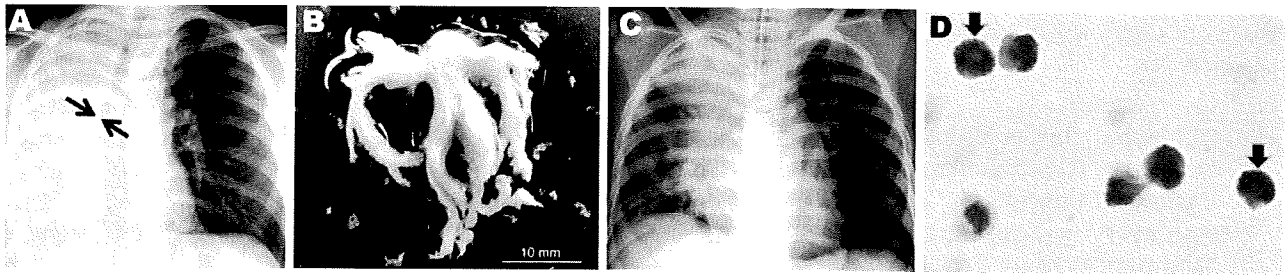

Figure. A) Chest radiograph obtained at hospital admission from a child infected with influenza subtype H1N1 virus. The image shows atelectasis of the right lung and hyperinflation of the left lung; arrows indicate obstruction of the right main bronchus. B) Macroscopic bronchial casts extracted by intratracheal suction. C) Chest radiograph obtained on hospital day 2, indicating partial resolution of atelectasis of the right lower lobe. D) Light micrograph of casts, characterized by predominant eosinophil infiltration (>90% of cells) (May-Giemsa stain, original magnification ×1,000). Arrows indicate typical eosinophil granules. A color version of this figure is available online (www.cdc.gov/EID/content/16/2/344-F.htm).

can produce similar airway cast formation in humans; severe respiratory distress reflects extensive obstruction of the respiratory system.

Healthcare providers should be aware of the possibility of bronchial casts when examining children with influenza (H1N1) infection accompanied by atelectasis. Steroids can be administered early in infection to avoid cast formation, and antiviral drug therapy and respiratory support can be used for influenza (H1N1)–infected children in whom airway casts have developed.

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Methicillin-Resistant *Staphylococcus aureus* ST398, Italy

To the Editor: It has recently become apparent that livestock can constitute a new methicillin-resistant *Staphylococcus aureus* (MRSA) reservoir and be a source of a novel and rapidly emerging type of MRSA. These livestock-associated MRSA clones are nontypeable by use of pulsed-field gel electrophoresis with *Sma*I and belong to sequence type (ST) 398 (*1*). MRSA ST398 clones account for 20% of all MRSA in the Netherlands (*2*), but the emergence of such clones has been described worldwide (*3*). Although ST398 transmission has been reported primarily between animals, persons with occupational exposure to livestock are at higher risk for MRSA carriage than the general population. Even though MRSA ST398 usually causes colonization, several cases of infections of variable clinical relevance, varying from skin and soft tissue infections (*4*) to endocarditis (*5*) and pneumonia (*6*), have been described over the past few years. Most instances of ST398 human carriers have been identified among persons who work at pig farms (*7*). Data regarding MRSA colonization of dairy farmers are less exhaustive and, to our knowledge, only 1 instance of direct transmission between cattle and humans has been proven. MRSA isolates from cows with subclinical mastitis in 2007 in Hungary were indistinguishable from MRSA isolates from the tonsil swab of a farmer who worked with these animals (*8*). We report a case of MRSA ST398 invasive disease in a cattle farmer, as well as a case of MRSA ST398 necrotizing fasciitis.

In early April 2008, a 52-year-old man was admitted to an intensive care unit in Manerbio, Italy, because of severe sepsis and a large ulcerative and

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Nonhemolytic *Streptococcus pyogenes* Isolates That Lack Large Regions of the *sag* Operon Mediating Streptolysin S Production[▽]

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Among nonhemolytic *Streptococcus pyogenes* (group A streptococcus) strains (n = 9) isolated from patients with pharyngitis or acute otitis media, we identified three deletions in the region from the *epf* gene, encoding the extracellular matrix binding protein, to the *sag* operon, mediating streptolysin S production.

In clinical laboratories, the property of beta-hemolysis on a blood agar plate is a characteristic used to preliminarily detect pyogenic streptococci such as *Streptococcus pyogenes* (group A streptococcus [GAS]), *Streptococcus agalactiae* (group B streptococcus), and *Streptococcus dysgalactiae* subsp. *equisimilis* (12). GAS produces two hemolysins: oxygen-dependent, labile streptolysin O, encoded by the *slo* gene (8), and oxygen-stable streptolysin S (SLS), encoded by the *sag* operon extending from the *sagA* gene to *sagI* (5). SLS, a potent cytolytic toxin produced by nearly all strains of GAS, is responsible for the zone of hemolysis surrounding GAS colonies grown under routine CO_2 culture conditions.

The *sagA* gene, which is positioned upstream in the *sag* operon, encodes a prepropeptide consisting of 53 amino acid (aa) residues, including a Gly-Gly proteolytic cleavage site that has been predicted to release a propeptide of 30 aa from a 23-aa leader sequence. The propeptide is considered to be the structural element of SLS. The remaining genes in the operon have features consistent with export functions, posttranslational modification of the SLS peptide, and a possible immunity protein (3).

Rarely, nonhemolytic variants of GAS have been isolated from patients with pharyngitis (6, 10), pneumonia (13), sepsis (2, 14), and cellulitis (11). These isolates were probably not producers of SLS, but the molecular cause had previously not been explained. Recently, based on mutational analysis, it was reported that all genetic components of the *sag* operon are required for the expression of functional SLS as an important virulence factor in the pathogenesis of invasive infection (3). In this study, we aimed to determine the reason for nonhemolysis by GAS clinical isolates at the molecular level.

A total of 1,690 samples, including throat swabs (n = 1,513) from patients with pharyngitis/tonsillitis and middle ear fluid (n = 177) from patients with acute otitis media (AOM), were sent to our laboratory by clinical physicians. Real-time PCR was immediately carried out routinely, in parallel with culturing, for all clinical samples on the day they were received. The real-time PCR used in this study was an application of the methods using molecular beacon probes and primers that we had constructed to detect six pathogens, including GAS, in samples from patients with respiratory tract infection (9). The set of primers and the probe for 16S rRNA genes used for the identification of GAS are as follows: sense primer, 5′-GAGA GACTAACGCATGTTAGTA-3′; reverse primer, 5′-TAGTT ACCGTCACTTGGTGG-3′; and probe, 6-carboxyfluorescein–CGCGATCGCGACGATACATAGCCGACCTGGAT CGCG–Black Hole Quencher 1. DNA extraction with the Extragen II kit (TOSOH, Tokyo, Japan) and subsequent DNA amplification with the Mx3000P system (Stratagene, La Jolla, CA) were performed by our protocol (9). On the following day, when no colonies with hemolysis were observed on the blood agar plate, Gram staining and reexamination by real-time PCR were carried out for some nonhemolytic colonies having different shapes, regardless of the positive PCR results for GAS on the preceding day. Next, colonies were confirmed to have characteristics of GAS by (i) an agglutination test for Lancefield group A antigen (Streptex; Mitsubishi Chemical Medience, Tokyo, Japan), (ii) use of the API Strep system (bioMérieux, Tokyo, Japan), and (iii) evaluation for the pyrrolidonyl arylamidase reaction (Oxoid, Hampshire, United Kingdom) in accordance with the *Manual of Clinical Microbiology* (12).

We finally identified nine nonhemolytic GAS strains from among 818 clinical isolates (1.1%) obtained from patients with pharyngitis/tonsillitis or AOM between November 2006 and March 2009. Colonies of these GAS isolates remained nonbeta-hemolytic under aerobic, 5% CO_2, and anaerobic conditions. Representative examples are shown in Fig. 1. The clinical and epidemiologic features of these isolates, including the *emm* type, the sequence type determined by multilocus sequence typing (MLST), and the type of deletion in the *sag* region, are listed in Table 1.

The *emm* types of these strains were determined based on DNA sequence homology by comparison of sequences with entries in the CDC database using the Streptococci Group A Subtyping Request Form Blast 2.0 Server (http://www.cdc. gov /ncidod/biotech/strep/strepblast.htm). DNA sequences approx-

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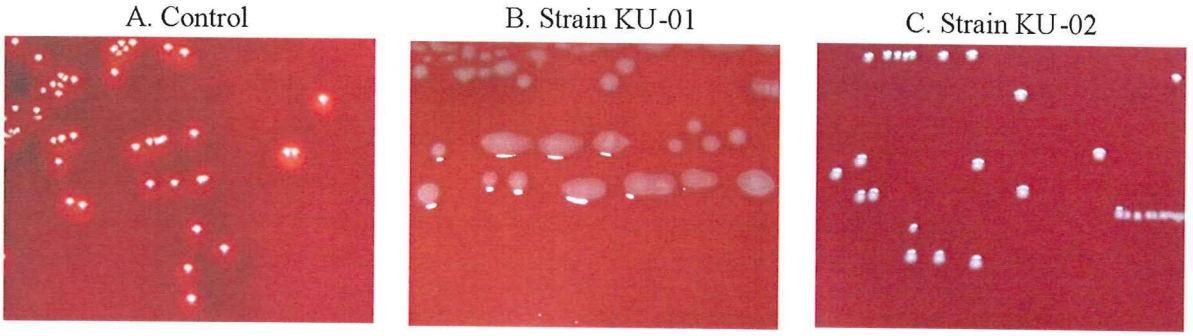

FIG. 1. Nonhemolytic *S. pyogenes* colonies grown on 5% sheep blood agar plates for 18 h at 37°C. (A) Control strain; (B and C) nonhemolytic strains.

imately 14,000 bp in length, extending from the *epf* gene, encoding an extracellular matrix binding protein, to the *sag* operon, encoding SLS, were determined for all nonhemolytic GAS strains. Primers used initially for long-DNA-fragment amplification included a sense primer, 5′-TGTGGATGCCGT TTAGAACA-3′, and a reverse primer, 5′-GAATAGCGACA CGCCTTAGC-3′.

For MLST of the nine GAS strains, DNA sequences from seven housekeeping loci were determined by the methods described by Enright et al. (4), and the sequence results were compared with the data in the *S. pyogenes* database (http://spyogenes.mlst.net/misc/info.asp).

Figure 2 depicts three types of DNA deletions in the region from the *epf* gene to the *sag* operon that were identified among the strains. Deletion type 1 was exhibited by one strain (strain KU-01; *emm1.0*) that lacked a 1,358-bp segment extending from the 3′-terminal region of the *epf* gene to the 5′-terminal region of the *sagB* gene. Six other strains (strains KU-02 to strain KU-07; *emm12.0*) with deletion type 2 lacked a 7,503-bp segment extending from the middle region of the *epf* gene to the *sagD* gene. The remaining two strains (KU-08 and KU-09; *emm1.0*) represented deletion type 3 and had discontinuous deletions in two regions: a 1,709-bp segment in the *epf* gene and another segment of 6,615 bp extending from the middle region of the *epf* gene to the *sagD* gene. All these deletions encompassed the region of the promoter and the *sagA* gene encoding the precursor of SLS.

Two types of *sag* regions have been identified using a DNA database for GAS genomes. In one, the ordinary type, the genes are aligned beginning with the *eno* gene, encoding enolase, and extending through *sagA* to *sagI*. The other type possesses both a *tnp* gene, encoding transposase, and an *epf* gene between the *eno* and the *sagA* genes. GAS strains identified as having *emm2*, *emm3*, and *emm5* represented the former type, while the *emm1*, *emm4*, *emm12*, and *emm28* strains carried the latter type. All nine strains analyzed in this study contained the latter type. Although these unique deletions suggest some associations with a transposon or insertion sequence, such details remain to be clarified.

Transcripts of the *nga* and the *slo* genes are known to be produced by read-through from the *nga* promoter (7). Although the data are not shown here, nucleotide sequences of 4,754 bp in length from the *nga* gene, including the promoter region, to the end of open reading frame of the *slo* gene from the nine strains were identified. No mutations or nucleotide deletions were detected in this region; therefore, the *slo* gene was intact in all strains.

DNA profiles of the nine nonhemolytic strains after pulsed-field gel electrophoresis (PFGE) are shown in Fig. 3. PFGE was performed with the ApaI restriction enzyme (Takara Bio, Kyoto, Japan). The DNA fragments were separated on a 1% agarose gel by using a contour-clamped homogeneous electric field mapper system (Bio-Rad, Tokyo, Japan) for 18 h at 14°C in 0.5× TBE buffer (0.05 M Tris, 0.05 M boric acid, and 1 mM EDTA [pH 8.0]) at 5.7 V/cm with pulse times of 3 to 20 s at an angle of 120° (1). Six strains with *emm12.0* isolated from patients in different regions, i.e., the Chiba and Niigata prefectures, Japan, showed similar DNA restriction patterns. Furthermore, three strains from Gunma prefecture and the Sendai

TABLE 1. Clinical and epidemiologic features of nonhemolytic *S. pyogenes* isolates

Strain no.	Date of isolation	District	Patient			*emm* type	Sequence type	Deletion type involving *sag* operon
			Age (yr)	Gender	Disease			
KU-01	Nov. 2006	Gunma	30	M	AOM	*emm1.0*	28	1
KU-02	Feb. 2008	Chiba	2	M	Pharyngitis	*emm12.0*	36	2
KU-03	Apr. 2008	Chiba	4	F	Pharyngitis	*emm12.0*	36	2
KU-04	May 2008	Chiba	5	F	Pharyngitis	*emm12.0*	36	2
KU-05	May 2008	Chiba	6	M	Pharyngitis	*emm12.0*	36	2
KU-06	May 2008	Chiba	2	F	Pharyngitis	*emm12.0*	36	2
KU-07	Nov. 2008	Niigata	4	M	Pharyngitis	*emm12.0*	36	2
KU-08	Jan. 2009	Sendai	5	F	Pharyngitis	*emm1.0*	28	3
KU-09	Jan. 2009	Sendai	5	M	Pharyngitis	*emm1.0*	28	3

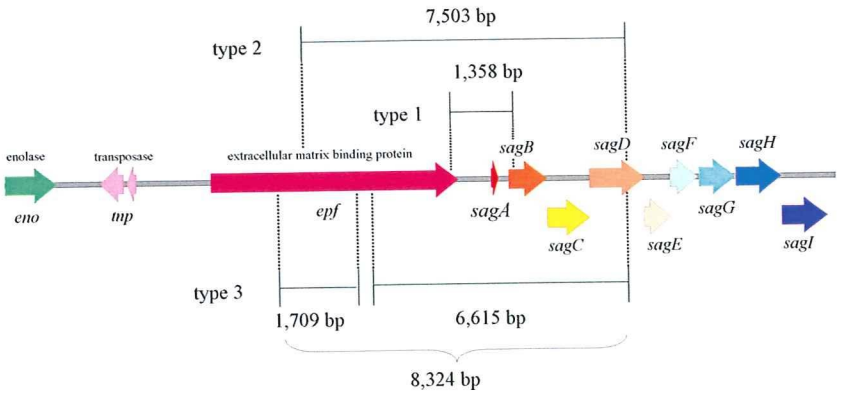

FIG. 2. Three deletion types identified near and/or in the *sag* operon encoding SLS. Large deletions encompassed the regions of the promoter and *sagA*, encoding the precursor of SLS. Deletion type 1, accession number AB518308; deletion type 2, accession number AB518309; deletion type 3, accession number AB518310.

City area identified as *emm1.0* strains showed very similar DNA restriction patterns.

Previously described nonhemolytic GAS strains have included various T antigen types and *emm* types (2, 6, 10, 11, 13, 14). In this study, we analyzed nonhemolytic phenotypes of the *emm1* and *emm12* strains. Evidence suggests that nonhemolytic *emm12* variants spread horizontally among children, considering that several cases occurred in the same area (Chiba prefecture). We also isolated three *emm1* GAS strains, one mucoid type and two nonmucoid types, from samples obtained from different areas. These strains displayed different deletion types in the *sag* operon but showed highly similar PFGE profiles, suggestive of a common origin.

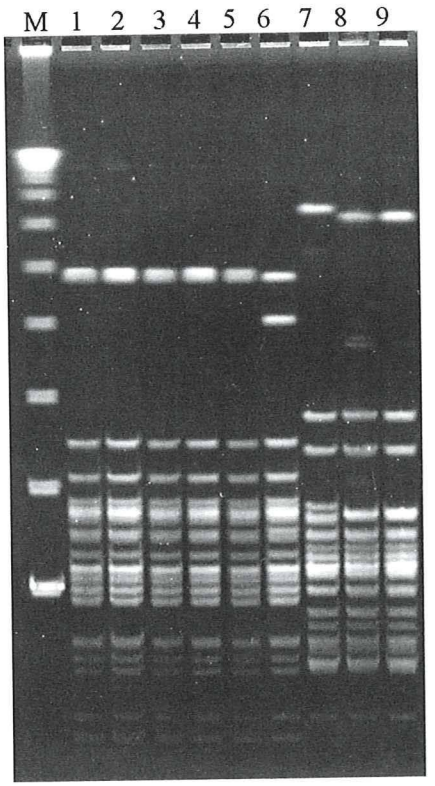

FIG. 3. PFGE patterns for nine nonhemolytic strains. Lanes: M, lambda ladder; 1, KU-02 (*emm12.0*); 2, KU-03 (*emm12.0*); 3, KU-04 (*emm12.0*); 4, KU-05 (*emm12.0*); 5, KU-06 (*emm12.0*); 6, KU-07 (*emm12.0*); 7, KU-01 (*emm1.0*); 8, KU-08 (*emm1.0*); and 9, KU-09 (*emm1.0*).

Emergence of the GAS strains described herein suggests that the routine bioassay poses a risk of missing nonhemolytic GAS colonies on blood agar plates, although nonhemolytic GAS variants are considered to be rare.

Nucleotide sequence accession numbers. The deletion type sequences determined in this study have been deposited in GenBank under the following accession numbers: deletion type 1, AB518308; deletion type 2, AB518309; and deletion type 3, AB518310.

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NOTE

Clinical aspects of invasive infection with *Streptococcus dysgalactiae* subsp. *equisimilis* in elderly patients

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Abstract The number of patients with severe invasive infections (mainly exhibiting bacteremia) with *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) has been increasing worldwide. We herein report the clinical aspects of invasive infections (cellulitis, pneumonia, and urosepsis) occurring with SDSE in 13 elderly patients (mean age 84 years, range 69–99 years) diagnosed at a hospital for elderly individuals during the period January 2005–June 2009. Ten subjects had underlying diseases, including neurologic disorders, diabetes mellitus, and others. Eleven patients presented to the hospital emergency department, and the most common symptom was high fever or respiratory distress. Primary care and emergency department doctors treating elderly patients with high fever should keep in mind invasive SDSE infection as a differential diagnosis, especially when an elderly person has underlying illnesses. To detect SDSE in elderly subjects, blood cultures should be obtained before the administration of antimicrobials because, as we found, the patients' symptoms were limited.

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Streptococcus dysgalactiae subsp. *equisimilis* (SDSE) was proposed in 1996 as a new taxon involved in human streptococcal infections [1]. This microorganism has Lancefield group C or G antigen, exhibits strong β-hemolysis, and shows streptokinase activity in human plasminogen and proteolytic activity in human fibrin. We have just completed whole-genome analyses of the original two isolates [GGS_124 (GenBank accession no AP010935) and RE378] of SDSE, demonstrating a rate of overlap between this subspecies and group A streptococcal genomes of 61–63%; overlap between the subspecies and group B streptococcal genomes was 15%. Similarly to group A streptococci, SDSE possesses virulence factors such as M protein, streptolysin O, streptolysin S, streptokinase, hyaluronidase, and C5a peptidase. SDSE has been established as possible normal flora of the skin, oropharynx, and gastrointestinal and genitourinary tracts. This pathogen was identified in respiratory tract specimens from patients with noninvasive SDSE diseases [2]. Invasive SDSE infections, mainly exhibiting bacteremia, are now being observed increasingly worldwide [3, 4]. Invasive infections represent the isolation of SDSE from a normally sterile site (i.e., blood, cerebrospinal fluid, joint fluid, ascites, or pleural effusion) [3, 4]. Here we report clinical aspects of invasive infections caused by SDSE in 13 elderly patients diagnosed at a hospital for elderly individuals between January 2005 and June 2009.

Streptococcal isolates with Lancefield group C or G antigen and β-hemolysis were isolated from blood specimens, and detailed information was collected from the hospital clinical laboratory database. SDSE was speciated

based on results of biologic tests, including bacitracin resistance, pyrrolidonyl-arylamidase test (negative), Voges–Proskauer test (negative), and β-D-glucuronidase test (positive). In addition, the *emm* gene, which encodes M protein—a major bacterial virulence factor—was typed, as previously described [4, 5]. To assess the similarity of isolates, DNA profiles after digestion with the restriction enzyme *Sma*I were compared by pulsed-field gel electrophoresis (PFGE) [6]. Patients' clinical features were reviewed using medical records. Clinical data (age, underlying disease, onset situation, main symptom, and vital signs) and laboratory data [white blood cell count (WBC), neutrophil count, C-reactive protein (CRP), platelet count (Plt), hemoglobin (Hb), and others] were obtained at the time blood cultures were performed for each patient. Also, the diagnosis of invasive infection was reviewed by a physician expert in infectious diseases.

Thirteen strains of SDSE and the corresponding elderly patients were studied. The clinical features and outcomes for invasive SDSE infections are shown with microbiologic data in Table 1. Twelve isolates had Lancefield group G antigen. The *emm* genotypes (*stG10.0* and others) and *Sma*I-digested PFGE DNA profiles for 7 isolates (numbers 7–13) varied between strains, suggesting the possible invasion of a different colonized strain in each patient rather than the clonal expansion of a specific subpopulation of isolates. The mean age of the infected patients was 84 years (range 69–99 years); 7 were female; and 10 had underlying diseases including neurologic disorders, diabetes mellitus, and others. Eleven patients with SDSE infections presented to the hospital emergency department, indicating that the infection was mainly community-acquired. The most common symptom was high fever or respiratory distress, without other complaints. Mean body temperature and heart rate were 38.9°C (range 36.4–40.1°C) and 93 beats/min (range 72–120 beats/min). Cellulitis, pneumonia, and urosepsis ($n = 4$ for each) constituted the main invasive infections. In subjects with pneumonia, chest roentgenograms disclosed patchy infiltrates. Mean WBC, neutrophil count, CRP, Plt, and Hb were 12.2×10^9 cells/l (range $5.7–19.2 \times 10^9$ cells/l), 10.9×10^9 cells/l (range $4.6–15.6 \times 10^9$ cells/l), 6.27 mg/dl (range 0.35–28.34 mg/dl), 210×10^9 cells/l (range $59–510 \times 10^9$ cells/l), and 11.2 g/dl (range 7.4–13.4 g/dl), respectively. As treatment, β-lactam antibiotics including penicillins or cephalosporins were administered parenterally in all subjects. No patients died of an invasive infection; any deaths involved other causes. No recurrent SDSE infections were found. The clinical aspects of the invasive SDSE infections in the elderly subjects were considered to be characterized as follows; onset in older-age patients, presence of underlying illnesses, community-acquired onset, and limited patients' symptoms.

In a recent report from Finland considering non-necrotizing bacterial cellulitis, SDSE was observed most often, being isolated in 22% of cultures from either skin lesions or blood [7]. Also, pneumonia and thoracic empyema caused by SDSE were reported from Japan [8, 9]. Similar to the findings of previous reports [8], chest radiographs in our patients with pneumonia indicated patchy infiltrates. The pulmonary lesions may have been induced by the aspiration of SDSE as possible normal flora of the oropharynx, because the functions of both swallowing and coughing are reduced in the elderly. The mortality of invasive group G streptococcal infections was reported previously as 8–18% [1, 10, 11]. Active laboratory-based surveillance for invasive SDSE, *Streptococcus pyogenes* (GAS), and *S. agalactiae* (GBS) infections was conducted for 1 year (August 2006–July 2007) in 142 medical institutions participating in the Invasive Streptococcal Disease Working Group established at the 19th annual meeting of the Japanese Society for Clinical Microbiology, in order to compare the clinical aspects of invasive infections caused by the three species [12]. While 231 invasive SDSE infections were identified, 97 other patients had GAS and 151 had GBS. All patients with invasive SDSE infection were adults (median age 75 years), while GBS infected some patients 4 months old or younger in addition to adults. Underlying diseases were present in 78.8% of the patients with invasive SDSE illnesses; underlying medical conditions were less frequent in subjects with GAS infections than in patients with SDSE or GBS. In addition, all cases of SDSE, GAS, or GBS diseases were community-acquired and 42 patients (18%) with SDSE infections presented to the hospital emergency department, a fraction similar to the proportions of subjects presenting to the emergency department with infections involving the other two bacterial groups.

There are some limitations regarding the clinical features of the SDSE invasive infections reported in the elderly subjects in our study. There may have been a selection bias for the enrolled patients, because our institute is already established as one of the expert hospitals for elderly individuals. In order to clarify risk factors in the subjects susceptible to SDSE, we did not design a setting for control patients, who were frequency matched to the case subjects by age and gender.

In conclusion, based on the detailed information of invasive SDSE infection in our observations, primary care and emergency department doctors treating elderly patients with high fever should keep in mind invasive infections caused by SDSE as a differential diagnosis, especially when the elderly person has underlying illnesses, although we note that no characteristic features giving clues to the diagnosis were found in the present study. To detect this

Table 1 Clinical features and outcomes of invasive infections with *Streptococcus dysgalactiae* subsp. *equisimilis* in 13 elderly patients

Patient no.	Blood culture date	Lancefield group	*emm* Type	Age	Gender	Underlying disease	I or O	Main symptom	Body temperature (°C)
1	2005/5/1	G	*stG652.0*	76	F	Breast cancer	O	Respiratory distress	36.4
2	2005/7/12	G	*stG2078.0*	74	M	Diabetes mellitus	I	Fever	39
3	2006/3/31	C	*stC6979.0*	83	F	Angina pectoris	O	Fever	38.7
4	2006/5/11	G	*stG10.0*	83	M	Stroke	O	Fever	39.2
5	2006/9/18	G	*stG652.1*	69	M	Frontotemporal dementia	O	Fever	39.7
6	2007/7/4	G	ND	87	F	Diabetes mellitus	O	Fever	38.8
7	2007/8/16	G	*stG10.0*	91	M	Healthy	O	Fever	38.5
8	2007/8/21	G	*stG10.0*	99	F	Healthy	O	Fever	38.2
9	2007/10/2	G	*stG6792.4*	90	M	Hypothyroidism	O	Fever	40.1
10	2007/10/9	G	*stG245.0*	81	F	Angina pectoris	O	Fever	40.1
11	2007/10/13	G	*stG652.0*	91	F	Healthy	I	Respiratory distress	39.9
12	2008/11/30	G	*stC46.0*	83	M	Pulmonary emphysema	O	Respiratory distress	37.2
13	2009/3/4	G	*stG2078.0*	85	F	Parkinson disease	O	Fever	39.8

Patient no.	Heart rate (beats/min)	Invasive infection	WBC (neutrophil count $\times 10^9$ cells/l)	CRP (mg/dl)	Plt ($\times 10^9$ cells/l)	Hb (g/dl)	BUN/Cr/CK (mg/dl, mg/dl, IU/l)	Antibiotic	Outcome
1	104	Urosepsis	12.9 (ND)	28.34	510	12.7	71.8/2.6/777	IPM/CS	Died of cancer
2	98	Urosepsis	5.7 (4.6)	8.84	183	10.7	10/0.5/ND	PIPC	Died of aspiration
3	98	Cellulitis	8.1 (8)	0.35[a]	148	12.3	25.3/1/111	CMZ	Recovered
4	105	Septic arthritis	14.3 (13.7)	10.48	209	11.4	15.3/0.9/198	PIPC/TAZ	Died of heart failure
5	77	Urosepsis	6.8 (6.3)	2.05	231	12.6	12/0.5/96	ABPC	Recovered
6	90	Urosepsis	14.9 (13.7)	7.31	289	12.3	17.4/0.6/13	CTM	Recovered
7	72	Cellulitis	13.7 (12.2)	1.59	168	11.4	21.7/0.8/25	CEZ	Recovered
8	96	Pneumonia	13.9 (12.6)	0.49[a]	104	9.6	35.4/0.8/79	PIPC	Died of stroke
9	91	Pneumonia	11.3 (9.8)	1.82	163	7.4	35.1/1.2/133	ABPC/SBT	Recovered
10	88	Cellulitis	17.4 (15.2)	2.33	216	13.4	17.4/0.7/104	PIPC	Recovered
11	120	Pneumonia	10.4 (9.5)	4.42	149	8.8	19.9/0.5/98	CZOP	Recovered
12	88	Pneumonia	19.2 (15.6)	0.37[a]	59	13.4	15.5/0.9/77	PIPC	Recovered
13	77	Cellulitis	10.3 (9.9)	13.09	299	10	32.5/1/667	ABPC/SBT	Recovered

Clinical and laboratory findings were recorded when blood cultures were performed

I or O Inpatient or outpatient, *WBC* white blood cell count, *CRP* C-reactive protein, *Plt* platelet count, *Hb* hemoglobin, *BUN* blood urea nitrogen, *Cr* creatinine, *CK* creatine phosphokinase, *ND* not determined, *IPM/CS* imipenem/cilastatin, *PIPC* piperacillin, *CMZ* cefmetazole, *TAZ* tazobactam, *ABPC* ampicillin, *CTM* cefotiam, *CEZ* cefazolin, *SBT* sulbactam, *CZOP* cefozopran

[a] CRP was increased later, 1 or 2 days after blood cultures were obtained

pathogen in elderly subjects, blood cultures should be obtained before the administration of antimicrobials because, as we found, the patients' symptoms were limited to high fever or respiratory distress.

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ORIGINAL ARTICLE

Diverse mutations in the *ftsI* gene in ampicillin-resistant *Haemophilus influenzae* isolates from pediatric patients with acute otitis media

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Abstract To clarify molecular changes in β-lactamase-nonproducing, ampicillin-resistant (BLNAR) *Haemophilus influenzae*, which is increasing in pediatric patients with acute otitis media (AOM) in Japan, we identified amino acid (aa) substitutions in penicillin-binding protein 3 for the BLNAR strains. Of 191 *H. influenzae* strains isolated from middle ear fluid of pediatric AOM patients between October 2005 and March 2008, BLNAR strains determined by PCR accounted for 49.2%. Of the BLNAR strains, 91.5% possessed 4 aa substitutions: Met377Ile, Ser385Thr, Leu389Phe, and either Asn526Lys or Arg517His. Additionally, the emergence of BLNAR strains possessing a new aa substitution of Val329Ala in the conserved aa motif of Ser327-Thr-Val-Lys, or Val511Ala adjacent to the conserved aa motif of Lys512-Thr-Gly, was noted. Transformation of the *ftsI* gene into the Rd reference strain (ATCC 51907) demonstrated that these two aa substitutions reduced susceptibility to amoxicillin more than to cephalosporins. Pulsed-field gel electrophoretic profiles of BLNAR strains were highly diverse. These results suggested that inadequate antibiotic use may increase BLNAR strains by selecting mutations in the *ftsI* gene and that such use may have favored the new aa substitutions.

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Introduction

Haemophilus influenzae is an important pathogen causing respiratory tract infection, pneumonia, acute otitis media (AOM), and meningitis. Two well-known mechanisms are implicated in the resistance of *H. influenzae* to ampicillin (AMP). One is the enzymatic hydrolysis of β-lactam agents resulting from the production of TEM-1 and ROB β-lactamases [1–4]; the other is decreased affinity of penicillin-binding protein (PBP) 3 for β-lactam antibiotics reflecting amino acid substitutions in the enzyme [3, 5]. Strains with alterations in PBP3 are termed β-lactamase-nonproducing, AMP-resistant (BLNAR) *H. influenzae*. In addition, strains demonstrating both mechanisms are termed β-lactamase-producing, amoxicillin-clavulanic acid-resistant (BLPACR) *H. influenzae*.

Amino acid substitutions in PBP3 surrounding the conserved Lys512-Thr-Gly (KTG) and Ser379-Ser-Asn (SSN) motifs are responsible for β-lactam resistance [5–9]. Single substitution of Asn526Lys or Arg517His was commonly found in BLNAR isolates with intermediate resistance to AMP (low-BLNAR). Additional amino acid substitutions, Met377Ile, Ser385Thr, and/or Leu389Phe, were characterized by higher than intermediate resistance to AMP (BLNAR).

In the United States, the prevalence of BLNAR strains was reported to be less than 5% in a recent study [10]. Another recent study reported a relatively constant prevalence of BLNAR strains, about 9%, in most of Europe [11]. In Japan and Spain, however, a marked increase in the number of BLNAR isolates has been observed [12–14].

Currently, an increase in intractable or recurrent AOM caused by BLNAR has become a great concern in pediatrics and otolaryngology in Japan [15, 16]. Despite the high reported prevalence of BLNAR strains from AOM, their evolutionary molecular changes in the *ftsI* gene have not been clarified. In this study, we characterized amino acid substitutions in PBP3 and their correlation with antibiotic susceptibilities in *H. influenzae* isolated from pediatric patients with AOM.

Materials and methods

Strains

In Japanese medical institutions, 191 clinical *H. influenzae* strains were isolated from middle ear fluid collected from pediatric patients with AOM by puncture or incision of the tympanic membrane between October 2005 and March 2008. The middle ear fluid samples were sent to our laboratory, where we immediately carried out bacterial culture and species identification based on the requirement of β-NAD (V factor) and hemin (X factor). Additionally, PCR was performed on all isolates, as described below, to identify species, β-lactamase genes, and *ftsI* mutations.

Polymerase chain reaction (PCR)

We performed PCR for *H. influenzae* using six sets of primers described previously [7]. The six targets were the 16S rRNA gene identifying species [17], the TEM-1 β-lactamase gene [18], the ROB-1 β-lactamase gene [19], an amino acid substitution of Asn526Lys in the *ftsI* gene [20], an amino acid substitution of Ser385Thr in the *ftsI* gene [5], and the Hib-specific *capB* locus [21]. PCR cycling conditions using lysates extracted from colonies of isolates included 35 cycles at 94°C for 15 s; at 53°C for 15 s; and at 72°C for 15 s. On the basis of the PCR results, the resistance class was described by attaching "g" to indicate genetic identification as opposed to other biologic assays, yielding designations such as gBLNAR, gLow-BLNAR, gBLPAR, and gBLNAS.

Serotyping

Serotypes of *H. influenzae* strains, except for type b, were determined by the agglutination test using antiserum purchased from Becton–Dickinson (Franklin Lakes, NJ, USA).

Antibiotic susceptibility

Susceptibility testing was performed by an agar dilution method [22]. Antibiotics used in this study were AMP, amoxicillin (AMX), cefditoren (CDN), and tebipenem (TBM, a new oral carbapenem; Meiji Seika Kaisha, Tokyo, Japan); cefdinir (CDR; Astellas Pharma, Tokyo, Japan); cefotaxime (CTX; Aventis Pharma, Tokyo, Japan); and meropenem (MEM; Dainippon Sumitomo Pharma, Osaka, Japan). *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control strains.

Sequencing

The 1.0-kb DNA region of the *ftsI* gene corresponding to the transpeptidase domain of PBP3 was amplified from the chromosomal DNA of *H. influenzae* by PCR using a sense primer, 5′-GTTGCACATATCTCCGATGAG-3′, and a reverse primer, 5′-CAGCTGCTTCAGCATCTTGC-3′, as described previously [5]. Amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as templates. Sequencing reactions were carried out using a BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed with an ABI Prism 3130/3130xl genetic analyzer (Applied Biosystems).

Transformation

The Rd strain (ATCC 51907) was transformed with an open reading frame corresponding to the *ftsI* gene, which was PCR-amplified. Transformation was carried out using a cuvette with a 0.1-cm electrode gap and a MicroPulser electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA), as described previously [5]. Conditions for electroporation were 1.8 kV/cm with time constants of 5.8 to 5.9 ms. Colonies grown on selective agar plates containing CTX at 0.016, 0.063, and 0.25 mg/l were selected at random, and antibiotic susceptibilities for the colonies were determined by the agar dilution method as described above. The *ftsI* gene of the colonies was sequenced to confirm gene transfer.

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out according to the method described previously, with some modifications [23]. Chromosomal DNAs extracted from each *H. influenzae* strain were digested with *Sma*I. Electrophoresis was performed using CHEF Mapper (Bio-Rad Laboratories). Separation of DNA fragments was achieved at 6 V/cm at 14°C for 20 h and 18 min. Pulse time, which changed in a lineal manner, was 0.47 to 63.08 s.

Results

Resistance classes and susceptibility

The resistance classes of 191 *H. influenzae* isolates were identified by PCR. AMP-resistant strains were extremely

common, representing 60.2% of all isolates; the proportion of each resistance class was 49.2% for gBLNAR, 6.8% for gLow-BLNAR, 3.7% for gBLPACR II, and 0.5% for gBLPAR. Strains without any resistance genes, i.e., gBLNAS, AMP-susceptible strains, represented 39.8%.

Of all strains tested, only 6 (3.1%) were serotyped as type b ($n = 5$) or type f ($n = 1$); the remaining strains were nontypable (NT).

Table 1 shows the MIC ranges, MIC_{50}s, and MIC_{90}s of seven β-lactam antibiotics for *H. influenzae* strains classified into four resistance groups, excluding gBLPAR, which consisted of 1 strain. The MIC_{90} of the standard antibiotic AMP for gBLNAR, 8 mg/l, was 16 times higher than the value for gBLNAS, 0.5 mg/l. In contrast, the MIC_{90}s of most cephalosporin antibiotics (CDR, CDN, and CTX) for gBLNAR were markedly increased; the MIC_{90} values of CDR, CDN, and CTX were 64, 8, and 32 times higher than the value for gBLNAS, respectively. The *ftsI* gene mutations affected the MICs of cephalosporin antibiotics more than those of AMP. The MICs of TBM and MEM for AMP-resistant strains were affected slightly by *ftsI* gene mutations. The MIC_{90}s of TBM and MEM for gBLNAR were increased 4 to 8 times relative to those for gBLNAS.

Table 1 MIC distributions and resistance classes of *Haemophilus influenzae* strains

Antimicrobial agent and Resistance class	MIC (mg/l) Range	MIC_{50}	MIC_{90}
Ampicillin			
gBLNAS[a] ($n = 76$)	0.063–1	0.25	0.5
gLow-BLNAR ($n = 13$)	0.5–2	1	1
gBLNAR ($n = 94$)	0.5–32	2	8
gBLPACR-II ($n = 7$)	2–>64	16	>64
Amoxicillin			
gBLNAS	0.125–1	0.5	0.5
gLow-BLNAR	0.5–4	2	4
gBLNAR	0.25–64	8	32
gBLPACR-II	2–>64	64	>64
Cefdinir			
gBLNAS	0.031–1	0.25	0.5
gLow-BLNAR	0.5–4	0.5	2
gBLNAR	2–32	8	32
gBLPACR-II	8–16	16	16
Cefditoren			
gBLNAS	0.002–0.063	0.016	0.031
gLow-BLNAR	0.016–0.063	0.031	0.063
gBLNAR	0.031–1	0.25	0.25
gBLPACR-II	0.125	0.125	0.125
Cefotaxime			
gBLNAS	0.004–0.063	0.016	0.031
gLow-BLNAR	0.016–0.125	0.031	0.125
gBLNAR	0.063–4	0.5	1
gBLPACR-II	0.25–0.5	0.5	0.5
Meropenem			
gBLNAS	0.008–0.125	0.063	0.125
gLow-BLNAR	0.063–0.5	0.125	0.25
gBLNAR	0.031–0.5	0.25	0.5
gBLPACR-II	0.063–0.25	0.063	0.25
Tebipenem			
gBLNAS	0.008–0.25	0.063	0.125
gLow-BLNAR	0.031–0.5	0.25	0.5
gBLNAR	0.031–1	0.25	1
gBLPACR-II	0.25–0.5	0.5	0.5

gBLNAS, gLowBLNAR, low-β-lactamase-nonproducing, ampicillin (AMP)-resistant; gBLPACR-II, β-lactamase-producing, amoxicillin-clavulanic acid-resistant-II

[a] g in the strain name denotes genetic identification

Amino acid substitutions in PBP3

Table 2 shows the deduced amino acid substitutions in PBP3 in gLow-BLNAR ($n = 13$), gBLNAR ($n = 94$), and gBLPACR II ($n = 7$) strains. These strains were classified into groups based on the eight amino acid substitutions reported by Hasegawa et al. [24]. Of these eight amino acid substitutions, three substitutions (Arg517His, Asn526Lys, and Ser385Thr) were considered to importantly affect resistance.

All gLow-BLNAR strains commonly possessed Asn526Lys without Ser385Thr, which had been identified frequently among AMP-resistant *H. influenzae* strains isolated in the late 1990 s. On the other hand, all gBLNAR and gBLPACR II strains possessed Ser385Thr and either Asn526Lys or Arg517His. Furthermore, 91.1% (92/101) of the gBLNAR and gBLPACR II strains possessed Met377Ile and Leu389Phe as well.

The emergence of gBLNAR with a Val329Ala substitution in the conserved amino acid motif of Ser327-Thr-Val-Lys (STVK) and gBLNAR, with a Val511Ala substitution adjacent to the KTG motif, which had not been identified in the early 2000 s in Japan, was noted.

Correlation between Val329Ala and Val511Ala and antibiotic susceptibilities

To investigate the effects of Val329Ala in the STVK motif and Val511Ala adjacent to the KTG motif in the *ftsI* gene upon antibiotic susceptibilities, the AMP-susceptible strain Rd was transformed with PCR-amplified *ftsI* gene fragments from gBLNAR with Val329Ala (KU007) and gBLNAR with Val511Ala (KU026). As controls, PCR-amplified *ftsI* gene fragments from gBLNAR without Val329Ala (KU001), gBLNAR without Val511Ala (KU002), and gLow-BLNAR (KU046) were introduced to Rd as well.