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Note

Isolation of multidrug-resistant *Haemophilus influenzae* harbouring multiple exogenous genes from a patient diagnosed with acute sinusitis

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ABSTRACT

In paediatric patients, β-lactams and macrolides are widely used to treat acute otitis media and sinusitis, which are often caused by either *Streptococcus pneumoniae* or *Haemophilus influenzae*. However, resistant isolates have emerged and are becoming more prevalent. *H. influenzae* generally acquires antimicrobial resistance by mutation or by expression of β-lactamase. In this study, we isolated *H. influenzae* from a paediatric patient diagnosed with acute sinusitis. This strain harboured multiple exogenous resistance genes: $bla_{\text{TEM-1}}$, *mef*(A) and *tet*(M). DNA sequencing suggested that both *mef*(A) and *tet*(M) had been transferred from *S. pneumoniae* or another Streptococcus. This typical outpatient had not been exposed to excessive levels of antibiotics and had no underlying diseases, strongly suggesting that this type of resistant isolate could become more prevalent.

The prevalence of invasive infections caused by *Haemophilus influenzae* type b has decreased since the introduction of the Hib vaccine. However, non-invasive infections such as respiratory infections and acute otitis media still occur because they are mainly caused by non-typeable *H. influenzae*. This is therefore an important causative pathogen, along with *Streptococcus pneumoniae*. In general, *H. influenzae* infections are treated using β-lactams; however, β-lactam resistant strains are already prevalent. The mechanisms underlying this drug resistance are divided into two major groups: the production of β-lactamase, and genetic mutation of the bacterial *ftsI* gene resulting in amino acid substitutions in penicillin binding protein 3 [1]. Furthermore, isolates showing resistance against non-β-lactams, such as macrolides and quinolones, have also emerged [2,3]. Recently, our group provided the first report of a clinical *H. influenzae* isolate harbouring both the macrolide efflux protein gene *mef*(A) and the tetracycline efflux protein gene *tet*(M) [4]. This type of resistant isolate has never been reported previously in other countries, and the likelihood of it spreading remains unclear. In the present study, we isolated *H. influenzae* harbouring exogenous resistant genes from a patient with acute sinusitis in a hospital that was located in a different region from previous study.

A 5-year-old female with a weight of 15.3 kg and a height of 106.4 cm had no underlying disease and was diagnosed with mumps due to parotid and submaxillary gland swelling. Ten days after this diagnosis, cough, nasal congestion and sputum occurred. A further 10 days later, she was diagnosed with acute sinusitis at Yokohama Rosai Hospital; chest and sinus radiography revealed multiple shadowing in the right lung, around the bronchi, and at maxillary sinuses. This patient underwent treatment with cefditoren pivoxil (150 mg/day) for 7 days, followed by amoxicillin (600 mg/day) for 7 days. Both *H. influenzae* and *S. pneumoniae* were isolated from a nasal swab taken upon first presentation to Yokohama Rosai Hospital.

Routine susceptibility testing using both VITEK® 2 and frozen plates for *Haemophilus* (Eiken Chemical Co. Ltd, Tokyo) indicated that *H. influenzae* showed resistance to penicillin, ampicillin, cefaclor and erythromycin, whereas *S. pneumoniae* showed resistance to erythromycin, clarithromycin and tetracycline (Table 1). This *H. influenzae* isolate was named as 2017-Y3 and sent to the microbiology laboratory at the Tokyo University of Pharmacy and Life Sciences for the following analyses.

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Table 1
Antimicrobial susceptibility of *Haemophilus influenzae* and *Streptococcus pneumoniae* isolated at same timing by routine test at the hospital laboratory.

Agent	*Haemophilus influenzae*		*Streptococcus pneumoniae*	
	MIC (μg/mL)	Decision	MIC (μg/mL)	Decision
Penicillin G	>2	nd	≤0.06	Susceptible
Ampicillin	>4	Resistance	≤0.06	Susceptible
Amoxicillin	nt	nd	≤0.06	Susceptible
Cefaclor	>16	Resistance	nt	nd
Cefmetazole	>2	nd	nt	nd
Latamoxef	0.5	Susceptible	nt	nd
Flomoxef	4	Resistance	nt	nd
Cefotaxime	0.5	Susceptible	0.25	Susceptible
Ceftriaxone	nt	nd	0.25	Susceptible
Cefixime	0.25	Susceptible	nt	nd
Cefpodoxime	1	Susceptible	nt	nd
Cefterame	0.5	Susceptible	nt	nd
Imipenem	nt	nd	≤0.03	Susceptible
Erythromycin	>8	nd	≥1	Resistance
Clarithromycin	nt	nd	≥1	Resistance
Tetracycline	nt	nd	≥16	Resistance
Minocycline	1	Susceptible	nt	nd
Chloramphenicol	1	Susceptible	≤2	Susceptible
Vancomycin	nt	nd	≤1	Susceptible
Fosfomycin	4	Susceptible	nt	nd
ST	nt	nd	≤10	Susceptible
Norfloxacin	<0.06	Susceptible	nt	nd
Levofloxacin	<0.06	Susceptible	1	Susceptible
Meropenem	nt	nd	≤0.06	Susceptible
Refampicin	nt	nd	≤0.25	Susceptible
Quinupristin/Dalfopristin	nt	nd	0.5	Susceptible

nt, not tested; nd, not decided.

H. influenzae 2017-Y3 was classified as a non-typeable *H. influenzae* by PCR method [5]. To clarify the mechanism underlying its antimicrobial susceptibility and resistance, we repeated our measurement of MIC using the broth microdilution method, according to the guidelines of the Clinical & Laboratory Standards Institute (Table 2) [6]. This isolate showed resistance to both penicillins and macrolides. In addition, its susceptibilities to amoxicillin-clavulanate and cephalosporins were reduced by more than 2-fold, as compared to the susceptible control isolate ATCC49247. Screening for known resistance mechanisms using PCR revealed *bla*$_{\text{TEM-1}}$, *mef*(A) and *tet*(M). Furthermore, sequencing analysis of the gene encoding penicillin binding protein 3, a β-lactam target protein, identified two resistance-related amino acid substitutions, Ser385Thr and Asn526Lys. This indicated that this isolate contained β-lactamase-positive and amoxicillin-clavulanate-resistant *H. influenzae*. To investigate the origins of *mef*(A) and *tet*(M), we performed sequencing from *mef*(A) to *tet*(M) and conducted a BLAST search. *mef*(A) was located at a distance of 3314 bp from *tet*(M). The sequence of *mef*(A) indicated 100% homology to those of *S. pneumoniae* ST556 (GenBank accession no. CP003357) and *Streptococcus oralis* FDAARGOS_367 (GenBank accession no. CP023507), with 99% homology to *H. influenzae* 2014-102 (GenBank accession no. LC168847). The sequence of *tet*(M) also showed 99% homology to these strains; however, it included a 1-bp frameshift deletion, strongly suggesting that Tet(M) protein would not be transcribed successfully. The MIC of tetracycline was consistent with a loss of *tet*(M) function (Tables 1 and 2). In addition, we analysed several macrolide resistance-related molecules (L4, L22, AcrR and 23S rRNA), but identified no relevant mutations or amino acid substitutions. Multilocus sequence typing was performed to analyse the relationship between *H. influenzae* 2017-Y3 and *H. influenzae* 2014-102, a strain that was previously reported to harbour *mef*(A) and *tet*(M) [4,7]. *H. influenzae* 2017-Y3 was classified as ST1658, which differed at all loci from the previous isolate (ST478), indicating that 2017-Y3 could be genetically distinct from the previous isolate.

Unfortunately, the *S. pneumoniae* strain that was isolated at same time was not stored and could not therefore be analysed molecularly.

In the present study, we encountered a multidrug-resistant *H. influenzae* harbouring multiple exogenous resistant genes; this represented the second such isolate to be identified worldwide. This strain was isolated from a Japanese hospital that was located in a different geographical region from the previous study site. Furthermore, the strain was β-lactamase-positive and amoxicillin-

Table 2
Antimicrobial susceptibility and molecular characteristics of *Haemophilus influenzae* 2017-Y3.

Strain	ST	MIC (μg/mL)								Resistant gene			Amino acid substitution[b]			
		AMP	AMX/CVA	CDN	CTX	CLR	AZM	LVX	MIN	β-lactam	Macrolide	Tetracycline	PBP3	AcrR	L4	L22
2017-Y3	1658	32	2/1	0.125	0.5	64	16	0.016	0.063	*bla*$_{\text{TEM-1}}$	*mef*(A)	*tet*(M)[c]	S385T N526K	None	V189I	None
2014–102[a]	478	64	0.5/0.25	≤0.063	≤0.063	128	64	0.016	8	*bla*$_{\text{TEM-1}}$	*mef*(A)	*tet*(M)	None	None	None	None

AMP, Ampicillin; AMX/CVA, Amoxicillin/clavulanic acid; CDN, cefditoren; CTX, cefotaxime; CLR, Clarithromycin; AZM, Azithromycin; LVX, Levofloxacin; MIN, Minocycline.
[a] These data were referred from previous study.
[b] Compared to *Haemophilus influenzae* Rd.
[c] This factor would not be transcribed successfully because of a frameshift mutation.

clavulanate-resistant and classified into a different ST, suggesting that it emerged independently of the previously reported strain.

In relation to *S. pneumoniae*, *mef*(A) and its homologue, *mef*(E), are known to be present in the *tet*(M)-containing Tn916 family, and could transfer to other strains via transposition [8]. In addition, this type of transposon is prevalent among *S. pneumoniae* and other Streptococcal bacteria [9]. Our BLAST analysis showed that the *mef*(A) and *tet*(M) sequences of this isolate had high homology to those of Streptococci. These findings suggested that these resistant genes might transferred to *H. influenzae* from *S. pneumoniae* and/or oral Streptococci. In fact, routine susceptibility testing of the *S. pneumoniae* isolated from this patient at the same time showed resistance to both macrolides and tetracycline, but susceptibility to streptogramin. This susceptibility profile and MIC values were identical to the phenotypes of *mef*(A) and *tet*(M) in *S. pneumoniae* [8], suggesting that *S. pneumoniae* has *mef*(A) and *tet*(M) that could transfer to *H. influenzae*. However, this *H. influenzae* strain showed susceptibility to tetracycline, even though it included *tet*(M). The observed 1-bp deletion in *tet*(M) may have occurred during the transfer process, resulting in a loss of function. Unfortunately, we could not conduct molecular analysis of this *S. pneumoniae* strain in order to determine that this transmission had occurred and the evidence of transmission of these genes was limited. However, *mef*(A) and *tet*(M) were previously reported to show transfer among strains via transformation [10]. These findings suggest that *H. influenzae* could have acquired these exogenous resistant genes from *S. pneumoniae*. In addition, *H. influenzae* might become a reservoir of antimicrobial resistant genes.

The patient from which this resistant strain was isolated presented with common acute sinusitis and had not received excessive antimicrobial agent exposure, indicating that this type of resistant strain could emerge in the nasopharynx of any host. Therefore, we should consider the potential for antimicrobial resistance to arise via gene mutation and also via the transfer of exogenous resistance genes. Moreover, emergence of the isolate showing resistance to both macrolides and tetracyclines, which have not been used for first-line therapy, indicates the importance of appropriate use of antimicrobial agents.

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Authorship statement

All authors meet the ICMJE authorship criteria.

Conflicts of interest

None declared.

References

[1] Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother 2001;45:1693–9.

[2] Seyama S, Wajima T, Nakaminami H, Noguchi N. Clarithromycin resistance mechanisms of epidemic beta-lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae* strains in Japan. Antimicrob Agents Chemother 2016;60:3207–10.

[3] Seyama S, Wajima T, Yanagisawa Y, Nakaminami H, Ushio M, Fujii T, et al. Rise in *Haemophilus influenzae* with reduced quinolone susceptibility and development of a simple screening method. Pediatr Infect Dis J 2017;36:263–6.

[4] Seyama S, Wajima T, Suzuki M, Ushio M, Fujii T, Noguchi N. Emergence and molecular characterization of *Haemophilus influenzae* harbouring *mef*(A). J Antimicrob Chemother 2017;72:948–9.

[5] Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. J Clin Microbiol 1994;32:2382–6.

[6] Clinical & Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. Document M100-S25. Wayne, PA: CLSI; 2015.

[7] Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol 2003;41:1623–36.

[8] Montanari MP, Cochetti I, Mingoia M, Varaldo PE. Phenotypic and molecular characterization of tetracycline- and erythromycin-resistant strains of *Streptococcus pneumoniae*. Antimicrob Agents Chemother 2003;47:2236–41.

[9] Palma TH, Harth-Chu EN, Scott J, Stipp RN, Boisvert H, Salomao MF, et al. Oral cavities of healthy infants harbour high proportions of *Streptococcus salivarius* strains with phenotypic and genotypic resistance to multiple classes of antibiotics. J Med Microbiol 2016;65:1456–64.

[10] Santagati M, Iannelli F, Oggioni MR, Stefani S, Pozzi G. Characterization of a genetic element carrying the macrolide efflux gene *mef*(A) in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 2000;44:2585–7.