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Original Article

Cefotaxime-non-susceptibility of *Haemophilus influenzae* induced by additional amino acid substitutions of G555E and Y557H in altered penicillin-binding protein 3☆

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ABSTRACT

Cefotaxime-non-susceptible *Haemophilus influenzae* has rarely been isolated from clinical specimens. Although several reports have shown that amino acid (AA) alteration in penicillin-binding protein 3 (PBP3), encoded by the *ftsI* gene, reduces activity of cefotaxime, precise mechanisms conferring the non-susceptibility have been unclear. We analyzed the *ftsI* gene of two clinically isolated cefotaxime-non-susceptible *H. influenzae* strains, 16-11 and 20-07 (minimum inhibitory concentrations [MICs]: 16 and 8 μg/mL, respectively), and found that their deduced AA sequences of PBP3 included two AA substitutions of G555E and Y557H in addition to previously described AA alterations. To clarify whether the two additional substitutions are requisite for cefotaxime non-susceptibility, we produced transformants of Rd KW20 (cefotaxime MIC: ≤0.06 μg/mL) with the *ftsI* gene of 16-11. Cefotaxime MICs against transformants M1 and M2, of which deduced PBP3s were altered with that of 16-11 entirely and partially (only the N-terminal side up to the AA position 519), were 8 and 0.25 μg/mL, respectively. We also produced M2-555/7 through site-directed mutagenesis inducing additional substitutions of G555E and Y557H into the PBP3 of M2, against which cefotaxime MIC was 8 μg/mL. These findings show that the additional substitutions of G555E and Y557H in PBP3 with previously described alterations cause cefotaxime non-susceptibility. An additional substitution of either G555E or Y557H alone in altered PBP3 reduced cefotaxime activity but the elevation of MICs were within the category of susceptibility. To our knowledge, this is the first study clarifying a genetic factor in the PBP3 causing cefotaxime non-susceptibility among *H. influenzae* strains.

1. Introduction

Haemophilus influenzae is a small, pleomorphic, facultatively anaerobic Gram-negative bacterium exclusively recovered from humans. The organism, predominantly found in the upper respiratory tract, causes pneumonia, otitis media, sinusitis, conjunctivitis, and bacteremia. More serious infection due to the organism, including epiglottitis, osteomyelitis, arthritis, and meningitis, are noteworthy, especially in regions where vaccination against the serotype b strain is not widely provided [1].

Ampicillin is a classical antibiotic used for treatment of *H. influenzae* infection [2,3]. However, strains resistant to the drug, conferred by production of either TEM-1 or ROB-1 beta-lactamase and/or amino acid alteration in the transpeptidase domain of the penicillin-binding protein 3 (PBP3), have been prevailing worldwide since 1970s [4]. Cefotaxime and ceftriaxone, antibiotics in the third-generation cephalosporin class, are highly active to the ampicillin-resistant strains. To date, *H. influenzae* clinical isolates considered non-susceptible to cefotaxime and ceftriaxone have extremely rarely been reported [5–10]. Thus, administration of these drugs has been recommended in cases of serious *H. influenzae* infection until susceptibility of causative organisms to ampicillin is confirmed [11–13].

Similarly to ampicillin, cefotaxime and ceftriaxone have high affinity to the PBP3 of *H. influenzae* [14], so that the alteration of the

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protein also reduces *anti-H. influenzae* activities of these antibiotics. Strains having amino acid substitutions of M377I, S385T, L389F, and N526 in the PBP3 demonstrated approximately 50- to 100-fold increases in minimum inhibitory concentrations (MICs) of cefotaxime and ceftriaxone compared with those having unaltered PBP3 [15,16]. An analysis with site-directed mutagenesis of the *ftsI* gene, the one encoding the PBP3 of *H. influenzae*, showed that substitutions of S385T and L389F in addition to R517H and/or N526K were associated with the increase in MICs of cephalosporins including cefotaxime [17]. However, the elevated MICs of cefotaxime described in these studies did not exceed 2 μg/mL, the breakpoint for susceptibility according to the description of the Clinical and Laboratory Standards Institute [18]. To our knowledge, what amino acid alteration in the PBP3 reduces activity of cefotaxime to the level of non-susceptibility has not been determined yet.

In 2014 and 2015, we obtained two clinical *H. influenzae* strains non-susceptible to cefotaxime. Characterization of these strains demonstrated that their deduced PBP3s had two amino acid substitutions of G555E and Y557H in addition to amino acid alterations described in previous investigations [4,7,17,19]. Thus, we examined whether the additional amino acid substitutions of G555E and Y557 were associated with the reduced *anti-H. influenzae* activity of cefotaxime to the level of non-susceptibility.

2. Materials and methods

2.1. Bacteria and their characterization

The strains 16-11 and 20-07, identified as cefotaxime-non-susceptible *H. influenzae* with the disk diffusion test in a routine microbiological examination in a clinical laboratory, were isolated from the sputa of patients hospitalized in the same ward of a community hospital in Japan between 2014 and 2015. Capsular types of the strains were determined with *anti-H. influenzae* capsule antisera purchased from Denka Seiken Co., Ltd. (Gosen, Japan). Beta-lactamase production was assayed using Cefinase™ paper discs (Becton Dickinson and Co., Sparks, MD, USA). The TEM-1- and ROB-1-encoding genes were detected with polymerase chain reaction (PCR) [20]. Sequence types of the strains were determined according to a previous publication [21] and using *Haemophilus influenzae* MLST Databases [22]. Strains M1, M2, and M3 were transformants of *H. influenzae* Rd KW20 (American Type Culture Collection: 51907) with full-length *ftsI* gene fragments of 16-11. Strains M2-555 and M2-555/7 were produced from M2 using site-directed mutagenesis as described below. Strains M3-1 and M3-2 were produced through introduction of full-length *ftsI* gene fragments of M3 into Rd KW20 (Fig. 1). All strains were stored in 10% skim milk at −85 °C and cultured twice on chocolate agar broth (Becton Dickinson and Co.) overnight at 37 °C in 5% CO_2 before experiments.

2.2. Determination of MICs

MICs of ampicillin, cefotaxime, and ceftriaxone were measured with the broth microdilution method according to the description of Japanese Society of Chemotherapy [23] using Dry Plate (Eiken Chemical, Tokyo, Japan) and interpreted according to the descriptions of the Clinical and Laboratory Standards Institute [18]. The assays were repeated three times per strain and the median values were adopted.

2.3. Amplification and purification of full-length ftsI gene fragments

A few well-separated colonies of cells on chocolate agar broth were suspended in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH = 8.0), boiled for 10 min, and centrifuged for 10 min. One microliter of the supernatant was used as a template for amplification of the full-length *ftsI* gene with PCR [17]. The amplified DNA fragments were purified with QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction.

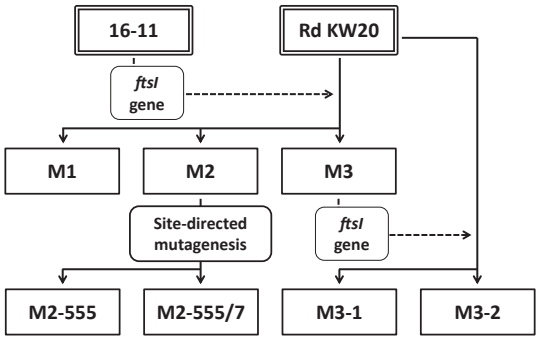

Fig. 1. The scheme of producing transformants (indicated in single-line boxes) in the present study.

2.4. Nucleotide sequencing and analysis of deduced amino acid sequences

The PCR-amplified and purified *ftsI* gene fragments were sequenced using primers listed in Table 1, BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and 3130xl Genetic Analyzer (Applied Biosystems). Amino acid sequences of PBP3 were deduced from the nucleotide sequences and compared with that of *H. influenzae* Rd KW20 (GenBank accession number: NC000907).

2.5. Production of ftsI gene-transformed H. influenzae strains

All colonies of recipient cells, having grown on chocolate agar broth at 37 °C in 5% CO_2 for 12 h, were wiped off with a cotton swab, suspended in 10 mL of sterilized and pre-chilled water, and centrifuged at 2800×*g* for 15 min at 4 °C. The precipitate cells were washed with sterilized water and SG buffer (10% glycerol, 10% sucrose) once each at 4 °C and suspended in 100 μL of SG buffer. Forty microliters of the recipient cell suspension was mixed with 1 μL of solution containing purified full-length *ftsI* gene fragments in a cuvette with a 0.1-cm electrode gap and given an electric pules of 1.8 kV using MicroPulser™ (Bio-Rad Laboratories, Hercules, CA, USA). The cells were immediately suspended in 1 mL of sBHI (brain heart infusion broth supplemented with 15 mg/L of nicotinamide adenine dinucleotide and 15 mg/L of haematin) [15], and incubated at 37 °C for 1.5–24 h with vigorous shaking. A portion of the sBHI suspension was spread on Haemophilus Test Medium Agar [24] containing 0.1–0.5 μg/mL of cefotaxime (Sigma-Aldrich, St. Luis, MO, USA) and incubated at 37 °C in 5% CO_2 for 24 h. Organisms growing on the medium agar were re-cultured on chocolate agar broth and confirmed whether their *ftsI* genes were transformed appropriately through sequence analyses as described above.

2.6. Site-directed mutagenesis to produce M2-555 and M2-555/7

A plasmid pBS-M2-*ftsI* was constructed through inserting a full-length *ftsI* gene fragment of M2 into the blunted *Pst*I site of pBlueScript II SK (+) (Stratagene, La Jolla, CA, USA). Plasmids pBS-M2-*ftsI*-555 and pBS-M2-*ftsI*-555/7, of which one and two nucleotides in the *ftsI* gene portion of the pBS-M2-*ftsI* were mutated to yield amino acid substitution(s) of G555E alone and G555E and Y557H in the PBP3 of M2, respectively, were constructed using

Table 1
Primers used for amplification, sequencing and site-directed mutagenesis of the *ftsI* gene.

Use	Primer	Position[a]	Reference
PCR	5′-CTCGTTATCCGTTACAGCAG-3′	−297	[17]
	5′-GCCAAACCGTGTGATGAAAC-3′	2383	[17]
Sequencing	5′-GTTGCACATATCTCCGATGAG-3′	732	[15]
Forward	5′-GTTAATGCGTAACCGTGCAATTAC-3′	959	[22]
Reverse	5′-GGAGTCACACCACGGTTAC-3′	1139	This study
	5′-ACCACTAATGCATAACGAGGATC-3′	1618	[22]
	5′-CAGCTGCTTCAGCATCTTGC-3′	1743	[15]
Site-directed mutagenesis[b]	5′-AAAGCAGAAGAATATTATGGTGGTGCG-3′	1682	This study
G555H substitution	5′-ATATTCTTCTGCTTTTGGATCATTGAT-3′	1645	This study
G555H and Y557H substitutions	5′-AAAGCAGAAGAACATTATGGTGGTGCGGTTTCT-3′	1690	This study
	5′-ACCATAATGTTCTTCTGCTTTTGGATC-3′	1651	This study

[a] Numbers refer to the position of the 3′-end nucleotide in the *ftsI* gene of *H. influenzae* Rd KW20 (accession no.: L42023).
[b] Nucleotides mismatched with those in the *ftsI* gene of M2 to produce mutagenesis were indicated with underlines.

PrimeSTAR Mutagenesis Basal Kit (Takara Bio Inc., Shiga, Japan), primers listed in Table 1, and *Escherichia coli* DH5α as recipient cells. Full-length fragments of the *ftsI* gene with the nucleotide mutation(s) in the pBS-M2-*ftsI*-555 and pBS-M2-*ftsI*-555/7 were amplified with PCR and introduced into M2 with electroporation as described above. The strains having the *ftsI* genes encoding PBP3 of M2 and the additional amino acid substitution(s) of G555E alone and G555E and Y557H were designated as M2-555 and M2-555/7, respectively.

3. Results

3.1. Characteristics of cefotaxime-non-susceptible clinical strains

The strains 16-11 and 20-07 were both non-typeable with *anti-H. influenzae* capsular antisera. Sequence types of the strains were ST1779 and ST1905, respectively, indicating that these strains were not *Haemophilus haemolyticus*. 16-11 produced beta-lactamase and possessed the TEM-1-encoding gene; while 20-07 was negative for beta-lactamase production. Both strains, having the *ftsI* gene with an identical nucleotide sequence, were resistant to ampicillin (MICs: >4 μg/mL), non-susceptible to cefotaxime (16 and 8 μg/mL against 16-11 and 20-07, respectively), and susceptible to ceftriaxone (2 μg/mL). The deduced amino acid sequence of their PBP3 contained 20 amino acid substitutions including S385T, L389F, R517H, G555E, and Y557H but not N526K (Table 2).

3.2. Drug susceptibilities and amino acid substitutions in deduced PBP3 of transformants

MICs of cefotaxime against M1 and M2, transformants of Rd KW20 with the *ftsI* gene of 16-11, were 8 and 0.25 μg/mL, respectively. M1 had deduced PBP3 entirely identical to that of 16-11; whereas deduced PBP3 of M2 included amino acid alteration with that of 16-11 only in the N-terminal side up to the amino acid position 519. M2-555 and M2-555/7, produced through site-directed mutagenesis in the *ftsI* gene of M2 to have additional substitution(s) of G555E alone and G555E and Y557H in their deduced PBP3, were inhibited to grow by 1 and 8 μg/mL of cefotaxime, 4- and 32-fold higher than the MIC against M2, respectively.

M3, another transformants of Rd KW20 with the *ftsI* gene of 16-11, had amino acid alteration in its deduced PBP3 between the positions 141 and 569. The ranges of amino acid alteration in the deduced PBP3 of M3-1 and M3-2, transformants of Rd KW20 with the *ftsI* gene of M3, were narrower than that of M3, between the positions of 239 and 569. A deduced amino acid residue at the position 555 in M3-1 returned spontaneously to glycine, identical to that of Rd KW20. MICs of cefotaxime against M3-1 and M3-2 were 1 and 8 μg/mL, equal to those against M2-555 and M3, respectively.

MICs of ceftriaxone were 1 μg/mL against M1 and ≤0.5 μg/mL against the other transformants (Table 2).

4. Discussion

In the present study, we first determined deduced amino acid sequences of the PBP3 of two clinically isolated cefotaxime-non-susceptible *H. influenzae* strains, 16-11 and 20-07 (MICs: 16 and 8 μg/mL, respectively). The sequences of both strains were identical and contained 20 amino acid substitutions including G555E and Y557H. Next, to clarify which of these amino acid substitutions were requisite to elevate MIC of cefotaxime to the level of non-susceptibility, we produced transformants through introducing the *ftsI* gene of 16-11 into Rd KW20 (susceptible to cefotaxime; MIC: ≤0.06 μg/mL) and compared their deduced amino acid sequences of PBP3 and susceptibility to cefotaxime. A transformant M1, non-susceptible to cefotaxime (MIC: 8 μg/mL), had deduced PBP3 of which the amino acid sequence was completely identical to that of 16-11; in contrast, M2, another transformant of which deduced PBP3 was partially altered with that of 16-11 (only the N-terminal side up to the amino acid position 519), was susceptible to the drug (MIC: 0.25 μg/mL). M2-555/7, produced from M2 through site-directed mutagenesis to have two additional amino acid substitutions of G555E and Y557H, was non-susceptible to cefotaxime (MIC: 8 μg/mL, equal to that against M1). These findings show that presence of amino acid alterations identical to that of 16-11 in the N-terminal side up to the position 519 elevates MIC of cefotaxime within the category of susceptibility and that additional amino acid substitutions of G555E and Y557H in the altered PBP3 cause further reduction in the activity of the drug to the level of non-susceptibility.

The amino acid alterations in the PBP3 are genetically classified into four groups according to the presence of certain amino acid substitution(s) surrounding the SSN and KTG motifs [19]. 16-11 and 20-07, the cefotaxime-non-susceptible clinical strains in the present study, had amino acid substitutions of S385T and R517H in their deduced PBP3, designated as the group III-like [19]. Several reports have described that amino acid alterations in the group III-like were associated with elevated MIC of cefotaxime in the range between 0.03 and 4 μg/mL [7,9,10,15–17,19,25–27], indicating that not only the substitutions of S385T and R517H but also additional factor(s) are indispensable to develop cefotaxime non-susceptibility. However, in these reports, what factors induce cefotaxime non-susceptibility has not been elucidated. To our knowledge, this is the first study clarifying a genetic factor in the PBP3 inducing cefotaxime non-susceptibility among *H. influenzae* strains.

Table 2
MIC and deduced amino acid substitutions in the PBP3.

Strain	Beta- lactamase	MIC (μg/mL)			Amino acid residue[a]																			
		Ampicillin	Cefotaxime	Ceftriaxone	31	141	173	239	350	357	371	377	385	389	517	519	547	555	557	569	586	594	595	603
Rd KW20	−	0.25	≤0.06	≤0.5	P	E	R	A	D	S	T	M	S	L	R	I	V	G	Y	N	A	S	A	E
16–11	+	>4	16	2	S	K	K	E	N	N	I	I	T	F	H	V	I	E	H	S	S	T	T	D
20–07	−	>4	8	2	S	K	K	E	N	N	I	I	T	F	H	V	I	E	H	S	S	T	T	D
M1	−	4	8	1	S	K	K	E	N	N	I	I	T	F	H	V	I	E	H	S	S	T	T	D
M2	−	0.5	0.25	≤0.5	S	K	K	E	N	N	I	I	T	F	H	V	–	–	–	–	–	–	–	–
M2-555	−	1	1	≤0.5	S	K	K	E	N	N	I	I	T	F	H	V	–	E	–	–	–	–	–	–
M2-555/7	−	4	8	≤0.5	S	K	K	E	N	N	I	I	T	F	H	V	–	E	H	–	–	–	–	–
M3	−	4	8	≤0.5	–	K	K	E	N	N	I	I	T	F	H	V	I	E	H	S	–	–	–	–
M3-1	−	1	1	≤0.5	–	–	–	E	N	N	I	I	T	F	H	V	I	–	H	S	–	–	–	–
M3-2	−	2	8	≤0.5	–	–	–	E	N	N	I	I	T	F	H	V	I	E	H	S	–	–	–	–

[a] Amino acid residues not described in this table or indicated with horizontal bars were identical to those of Rd KW20. No strain in this table had deduced amino acid substitution in the conserved motifs (S327-T-Ve-K, S379e-Se-N, and K512-T-G).

We also examined effects of additional amino acid substitution of either G555E or Y557H. Comparison of cefotaxime susceptibility among M2, M2-555, and M2-555/7 (MICs: 0.25, 1, and 8 μg/mL, respectively) showed that the additional substitution of G555E alone increased MIC of cefotaxime but the level was still within the category of susceptibility. Unfortunately, we could not produce a transformant of M2 with the additional substitution of Y557H alone in the present study. Instead, M3-1 and M3-2, transformants of Rd KW20 with the *ftsI* gene of M3, were analyzed. Deduced PBP3 of M3-1 and M3-2 had the identical amino acid alteration with that of 16-11 between the positions 239 and 569, except for that the amino acid residue at the position 555 of M3-1 was unintentionally unaltered to that of Rd KW20. MICs of cefotaxime against M3-1 and M3-2 were 1 and 8 μg/mL, indicating that absence of the substitution G555E decreased MIC of cefotaxime to the level of susceptibility. We finally concluded that an additional amino acid substitution of either G555E or Y557H alone reduced activity of cefotaxime but the additional substitutions of both G555E and Y557H were necessary to develop cefotaxime non-susceptibility.

The clinical strains 16-11 and 20-07 were isolated from patients hospitalized in the same ward at the same period. However, sequence types of the strains were different and only 16-11 produced beta-lactamase, suggesting that these cefotaxime-non-susceptible strains emerged independently. Another possibility is that only the *ftsI* gene of the strains transferred horizontally, as described in another report [28]. We could not confirm how close the patients had contact during their hospitalization. However, horizontal transfer of the gene is plausible because of the complete identity in the nucleotide sequences of their *ftsI* gene. Further genetic examinations on the strains and their *ftsI* genes are necessary to clarify the issue.

In conclusion, we demonstrated that additional amino acid substitutions of G555E and Y557H in the altered PBP3 of the group III-like induce cefotaxime-non-susceptibility to *H. influenzae*. These amino acid positions exist outside the recommended range of the *ftsI* gene for identification of PBP3 mutations [22]. Further analyses including these amino acid substitutions may elucidate the relevance between mutation of the PBP3 and beta-lactam resistance in *H. influenzae* more clearly.

Conflicts of interest

None.

Appendix

The nucleotide sequences of the *ftsI* genes of strains 16-11, 20-07, M1, M2, M2-555, M2-555/7, M3, M3-1, and M3-2 were deposited in DNA Data Bank of Japan (accession numbers: LC455782 to LC455790).

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