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Original

Monitoring Quinolone Resistance Due to Mutations in GyrA and ParC in *Haemophilus Influenzae* (2012-17)

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Abstract: Knowing recent drug-resistant bacteria trends is important for proper antibacterial drug use to improve the prognosis of patients with infectious diseases and for public health. Because multiple quinolone antibacterial agents are simultaneously adopted in hospitals in Japan, we examined whether minimum inhibitory concentrations (MICs) against *Haemophilus influenzae* differ among quinolones. We determined MICs of six different quinolone antibacterial agents and performed molecular genetic analysis. We investigated β-lactamase-producing and β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae* using the nitrocefin method in parallel. Overall, 144 clinical *H. influenzae* strains isolated at the Showa University Hospital between 2012 and 2017 were subjected to MIC determination for penicillin/quinolone antibacterial agents using the Clinical and Laboratory Standards Institute broth microdilution method. Amino acid mutations in the quinolone resistance-determining regions were analyzed in the isolates showing an MIC value ≥ 0.25 μg/ml of quinolone antibacterial agents. BLNAR isolates increased from 2016 onward. Among quinolone antibacterial agents, all isolates remained susceptible to sitafloxacin. However, for moxifloxacin (MFLX), strains with an MIC value = 0.5 μg/ml were detected every year since 2013 except in 2015. Amino acid mutations were investigated in 17 isolates (11.8%) with MFLX MIC value ≥ 0.25 μg/ml and confirmed in 11 isolates (7.6%), of which 9 contained GyrA mutations. The results demonstrated that MFLX was useful for predicting the presence of amino acid mutations and 0.25 was an appropriate MIC threshold for this purpose. This screening procedure may be effective for reducing the inappropriate use of quinolones and controlling the emergence of drug-resistant *H. influenzae*.

Key words: quinolone resistance, minimum inhibitory concentrations, moxifloxacin, *Haemophilus influenzae*

Introduction

Haemophilus influenzae is the second most common causative bacterium of community-acquired pneumonia, following *Streptococcus pneumoniae*, and has taken the leading position after

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pneumococcal vaccines became popular[1]. β-lactam antibiotics have previously been used for the treatment of community-acquired pneumonia. In the 2000s, however, β-lactamase-producing ampicillin-resistant bacteria have increasingly been detected in Canada[2], Japan, and some other countries. Moreover, β-lactamase-negative ampicillin-resistant (BLNAR) bacteria have increasingly been detected in Japan recently[3].

Meanwhile, fluoroquinolones are clinically effective against these bacteria[4], and in particular, have frequently been prescribed to outpatients with respiratory and otolaryngologic infections. Accordingly, reports on quinolone-resistant *H. influenzae* have started emerging in the beginning of 2000. In Spain, low-sensitive strains to ciprofloxacin (CPFX) accounted for 43% of *H. influenzae* strains in 2007[5]. In Taiwan, the levofloxacin (LVFX) resistance rate has reportedly increased from 2.0% in 2004 to 24.3% in 2010[6]. These data illustrate that the prevalence of quinolone resistance among *H. influenzae* isolates is highly variable depending on when the test is conducted even within the same country or within the same institution. Furthermore, high-level BLNAR strains have been reported to exhibit reduced sensitivity toward fluoroquinolones[7].

Fluoroquinolone resistance is primarily induced through mutations in quinolone resistance-determining regions (QRDRs) of GyrA gene encoding DNA gyrase or ParC gene encoding topoisomerase IV, which cause three-dimensional structural alterations in the respective enzymes and decreased the affinity between fluoroquinolones and these replication enzymes[8]. In the case of *H. influenzae*, drug resistance has been shown to be strongly related to serine and aspartic acid mutations at positions 84 and 88 of GyrA and of serine at position 84 of ParC[9]. However, only a few previous studies have investigated mutations in QRDRs in the same institution over time and have compared MICs among different quinolones. In this study, we tested sensitivity levels to quinolone and penicillin antibacterial agents over 6 years in *H. influenzae* strains that were isolated at the same institution without effects from clinical departments, diseases, or specimens, and analyzed genetic mutations in QRDRs in strains with MIC value of ≥ 0.25 μg/ml of quinolones. Next, we assessed whether antibacterial efficacies varied among quinolone antibacterial agents or whether the bacteria tended to be resistant.

Materials and Methods

Bacterial strains

We used a total of 144 *H. influenzae* strains that were clinically isolated at the Showa University Hospital between 2012 and 2017 and stored in our laboratory. These *H. influenzae* isolates were obtained from 90 men and 54 women (age, 0–99 years; median, 43 years), including 52 patients from the department of pediatrics, 35 from the department of respiratory medicine, 14 from the department of emergency medicine, and 4 from the department of otolaryngology. *H. influenzae* strains were isolated from respiratory (n = 130), otolaryngologic (n = 6), blood (n = 3), pus (n = 3), and other (n = 2) samples.

β-Lactamase-producing ability test

Each bacterial strain was cultured on chocolate agar medium (Nippon Becton-Dickinson,

Tokyo, Japan) at 35℃ in 5% CO_2 for 22 hours[8]. Next, drops of nitrocefin (Kantokagaku, Tokyo, Japan) were added to *H. influenzae*. The strain was considered capable of producing β-lactamase when a red color developed[10].

Drug susceptibility assay

Susceptibility to antibacterial agents was measured the Clinical and Laboratory Standards Institute broth microdilution method using 96-well plates[11]. As a basal medium, liquid Haemophilus Test Medium (HTM) medium (Eiken, Tokyo, Japan) was added to each well and was supplemented with Hematin (15 μg/ml) and NAD (15 μg/ml) (Eiken Chemical, Class Ⅰ bacteria test series, frozen plates). Each strain was cultured on a chocolate agar medium at 35℃ in 5% CO_2 for 22 hours, and then a bacterial suspension was prepared at a concentration adjusted to be similar to that of McFarland standard (bioMèrieux), 0.5 using physiological saline. Finally, each well was inoculated with a bacterial suspension (5×10^5 CFU/ml). The plate was incubated at 35℃ in 5% CO_2 for 22 hours, and MIC values of test drugs for these bacteria were measured.

The following test drugs were used: aminobenzylpenicillin (ABPC), sulbactam/ampicillin (SBT/ABPC), clavulanic acid/amoxicillin (CVA/AMPC), levofloxacin (LVFX), ciprofloxacin (CPFX), garenoxacin (GRNX), moxifloxacin (MFLX), tosufloxacin (TFLX), and sitafloxacin (STFX). We determined the MICs of these drugs and calculated a mean value for each of six quinolone antibacterial agents. Finally, we intercompared between the six agents.

Analysis of QRDR amino acid sequences

H. influenzae strains showing a MFLX MIC value of ≥ 0.25 μg/ml in the drug susceptibility assay were selected as test strains and allowed to grow, as previously described by Shoji *et al*[12]. Genomic DNA was extracted using Sepa-Gene (EIDIA Co., Ltd., Tokyo, Japan) from the bacterial cells of the test strains and were allowed to aggregate by adding 99.5% alcohol, centrifuged at 8,000 × g to precipitate, and washed with 70% alcohol. Using the resulting DNA sample as a template, genes encoding QRDRs were amplified by PCR. For amplification, a mixture solution of *Taq* polymerase (Roche, Mannheim, Germany) and primer[13] was used in a T100 thermocycler (BIORAD, CA, USA) under PCR conditions as previously described by Shoji *et al*[12].

For purification/separation and extraction, the entire PCR product was subjected to electrophoresis in 2% agarose gel (TAKARA, Shiga, Japan) under 120 V/h for 1 hour in Tris-borate-EDTA (NIPPON GENE, Tokyo, Japan) buffer. The gel was then stained with ethidium bromide to detect the target PCR product bands (GyrA : 375bp, ParC : 370bp), and the detected bands were excised. Thereafter, DNA was extracted and purified from the excised gel segments using a GenElute Minus EtBr Spin Column (SIGMA-ALDRICH, St, Louis, USA) and concentrated with ethanol. For direct sequencing using the resulting DNA samples as templates, PCR amplification was performed using Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems Japan, Tokyo, Japan). The PCR primers described above were used here as sequence primers.

The resulting sequencing solutions were analyzed using an ABI PRISM 310 genetic analyzer

(Applied Biosystems, Foster City, CA), and nucleotide sequences of the target gene regions were determined. Based on the nucleotide sequence data obtained, amino acid sequences were analyzed using GENETYX Ver.8 (GENETYX, Tokyo, Japan) to locate amino acid mutations in QRDRs.

Statistical analysis

Statistical analysis was performed with SPSS statistics version 23.0 (IBM Japan, Tokyo, Japan). Tukey's honestly significant difference post-hoc test was used to compare the susceptibilities of isolated strain toward quinolones. All statistical tests were two-tailed, and a P-value of < 0.05 was considered significant.

Ethics

This study was approved by the research ethics committee of Showa University School of Health Sciences (Approval No.371).

Results

BLNAR and β-lactamase-producing bacteria

Percentages of BLNAR and β-lactamase-producing bacteria over time are shown in Figure 1. Among all tested strains, BLNAR strains accounted for 38.9% in 2012, 22.2% in 2013, 37.0% in 2014, 15.0% in 2015, 43.5% in 2016, and 63.6% in 2017, and the percentage of BLNAR strains consecutively increased in 2016 and 2017. In 2017, aminobenzylpenicillin (ABPC) -resistant strains, including β-lactamase-producing ones, accounted for 78.8% of all isolates. β-Lactamase-producing amoxicillin/clavulanic acid-resistant *H. influenzae* was not detected.

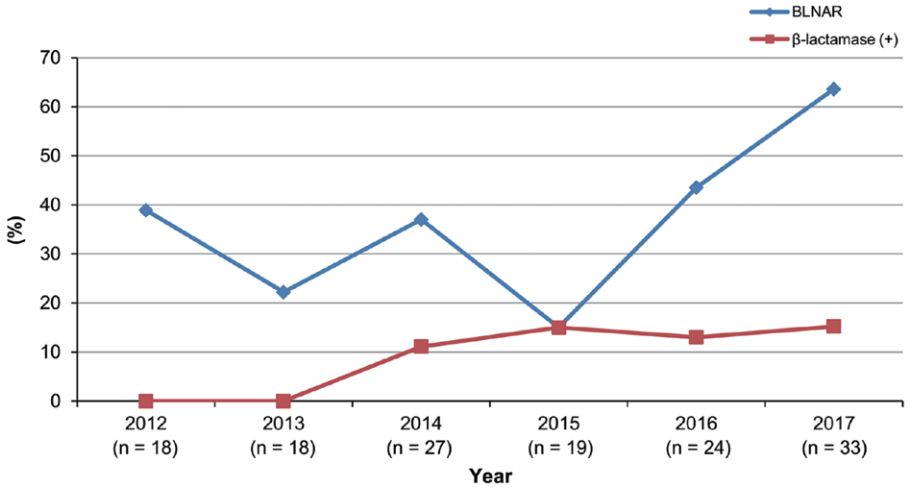

Fig. 1. Percentage of BLNAR and β-lactamase-positive *Haemophilus influenzae* isolates in different years: Percentages of BLNAR isolates increased to be 15.0% in 2015, 43.5% in 2016, and 63.6% in 2017. β-lactamase-producing isolates accounted for 15.2% in 2017.

Susceptibility to quinolones

MIC values of various quinolone antibacterial agents measured with 144 *H. influenzae* isolates are shown (Figure 2). For all quinolones, non-susceptible strains as defined with MIC value of ≥ 2 μg/ml were found. For STFX in particular, no strains showing MIC values of ≥ 0.12 μg/ml were detected during the study period. For MFLX, only 80% of strains had an MIC value of ≤ 0.06.

Comparison of susceptibility to MFLX and other quinolones

A cross-comparison of MIC mean values of six quinolone antibacterial agents against 144 experimental strains indicated that MFLX had significantly higher MIC mean value than STFX and TFLX (p = 0 and 0.02, respectively) (Table 1). For MFLX, one or more isolates with MIC values of ≥ 0.5 μg/ml were detected every year since 2013 except in 2015 (Table 2). This series of results demonstrated that the antibacterial potency of MFLX was lower than that of any other antibacterial agent tested.

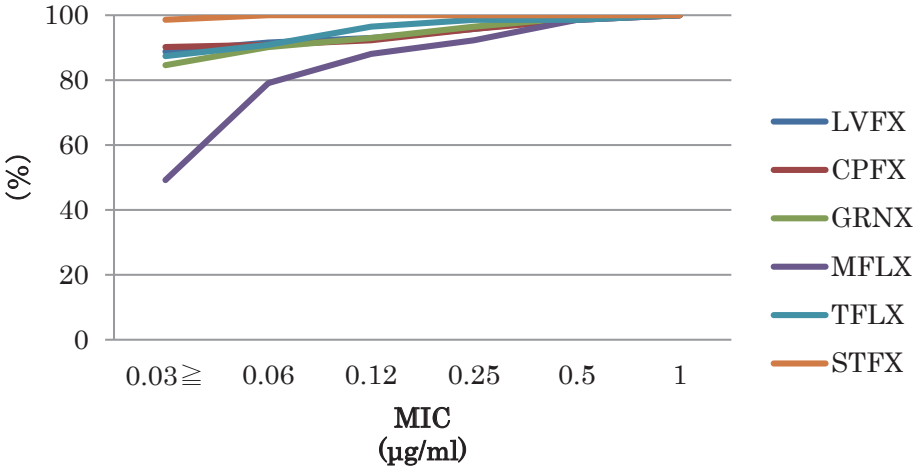

Fig. 2. Cumulative MIC values of fluoroquinolones for *Haemophilus influenzae* isolates

Table 1. MIC values of quinolones against *Haemophilus influenzae* and cross-comparison

			P value					
	MIC50	MIC90	vs. CPFX	vs. GRNX	vs. LVFX	vs. MFLX	vs. STFX	vs. TFLX
CPFX	0.03	0.03	—	1.000	1.000	0.176	0.134	0.966
GRNX	0.03	0.06		—	1.000	0.091	0.245	0.955
LVFX	0.03	0.06			—	0.086	0.256	0.996
MFLX	0.06	0.25				—	0.000*	0.020*
STFX	0.03	0.03					—	0.558
TFLX	0.03	0.06						—

*MFLX had significantly higher MIC average value than STFX and TFLX.

Table 2. Association between low quinolone susceptibility and QRDR mutations in strains isolated at the Showa University Hospital

Strain							Mutation	
	MFLX	CPFX	GRNX	LVFX	TFLX	STFX	GyrA	ParC
2012-1	1	0.25	1	0.25	0.25	0.06	Ser84Leu	-
2012-2	0.25	0.03	0.12	0.06	0.03	0.03	-	Gly82Arg
2013-1	0.5	0.25	0.5	0.25	0.25	0.03	Ser84Leu	-
2014-1	0.5	0.25	0.5	0.25	0.25	0.03	Ser84Leu	-
2014-2	0.5	0.25	0.5	0.12	0.12	0.03	Ser84Leu	-
2014-3	0.5	0.25	0.25	0.25	0.12	0.03	Ser84Leu	-
2014-4	0.25	0.12	0.25	0.12	0.06	0.03	Ser84Leu	-
2014-5	0.5	0.5	0.12	0.5	0.12	0.03	-	-
2014-6	0.25	0.03	0.06	0.06	0.06	0.03	-	-
2014-7	0.25	0.03	0.06	0.03	0.06	0.03	-	-
2015-1	0.25	0.5	0.06	0.5	0.12	0.03	-	-
2016-1	0.5	0.5	0.25	1	0.12	0.03	Ser84Leu	-
2016-2	0.5	0.5	0.12	0.5	0.12	0.03	Ser84Leu	-
2016-3	0.25	0.03	0.06	0.03	0.03	0.03	-	-
2017-1	1	1	0.5	0.5	1	0.06	-	Ser84Ile
2017-2	0.5	1	0.25	0.5	1	0.03	Ser84Leu	Ser84Ile
2017-3	0.5	0.03	0.12	0.03	0.03	0.03	-	-

Amino acid mutations in QRDRs of GyrA and ParC

We investigated amino acid mutations in QRDRs of GyrA and ParC in 17 strains with low susceptibility to MFLX (MIC ≥ 0.25 μg/ml). Mutations were found in 11 strains and detected every year except in 2015 (Table 2). Of all *H. influenzae* strains isolated in each year, strains with any amino acid mutation accounted for 11.1% in 2012, 5.6% in 2013, 14.8% in 2014, 8.3% in 2016, and 5.3% in 2017 (total 7.6% in the past six years). Only one mutation was found in GyrA (Ser84Leu) in eight strains or in ParC (Ser84Ile or Gly82Arg) in two strains, and one mutation each in GyrA (Ser84Leu) and ParC (Ser84Ile). Both strains with the Ser84Ile mutation (2017-1 and 2017-2) had MFLX MIC ≥ 0.5 μg/ml, CPFX MIC = 1 μg/ml, GRNX MIC ≥ 0.25 μg/ml, LVFX MIC = 0.5 μg/ml, and TFLX MIC = 1 μg/ml, which indicated reduced susceptibility.

BLNAR and amino acid mutations in QRDRs

Of the 11 isolates with any amino acid mutation, 5 (2012-2, 2014-1, 2014-3, 2017-1, and 2017-2) were BLNAR, and all but 2012-2 exhibited reduced susceptibility to fluoroquinolones except for STFX (Table 2).

Discussion

In Japan, no Hib strains have been detected in children with invasive infections after Hib vaccines were introduced in 2008[14]. However, the frequency of isolation of BLNAR strains is

high in some areas : 35.8% in the otorhinolaryngology area in 2012[3], and 36.4% in the pediatric area in 2015[15]; a rate of 36.9% has also been reported for 2016 surveillance isolates in Japan[16]. Additionally, the present study showed that the frequency of isolation of BLNAR strains increased to 63.6% in 2017 (Figure 1). Possible factors contributing to the observed increase in BLNAR include the inappropriate oral cephem use[17] and transmission from children to their parents.

Fluoroquinolone resistance rates of *H. influenzae* previously studied in Japan were extremely low, and the 2016 respiratory specimen surveillance isolates report also stated that the resistance to CPFX and LVFX was found in 0.6% and STFX resistance was found in 0% of isolates[16]. STFX is characterized by the strong inhibitory potencies against both DNA gyrase and topoisomerase IV[18]. The well-preserved STFX sensitivity owing to this characteristic was consistent with that reported in other reports[7, 12] and the present findings.

Meanwhile, the MFLX resistance rate in *H. influenzae* isolates is reportedly 1.1% in 2010[19]; however, it is noteworthy that low susceptibility to MFLX was observed for 11.8% (17 of 144) of isolates collected from 2012 to 2017 in the present study, possibly attributable to the fact that MFLX can be orally administered in Japan, whereas injectable formulations are available in Europe and the United States. Interestingly, the mutant prevention concentration (MPC) of fluoroquinolone for *H. influenzae* is 0.125–0.25 μg/ml for MFLX[20]; this MPC differs substantially from those of LVFX (0.024–0.391 μg/ml), GRNX (0.024–0.391 μg/ml), and STFX (0.003–0.049 μg/ml)[21]. Thus, it is plausible that if certain drugs such as antacids and laxatives are taken, the MFLX level in blood does not adequately increase, and MFLX resistance is acquired if the blood MFLX level remains below MPC for a long period of time. The drug resistance epidemic status in the working area should be understood in this regard as well.

Additionally, MIC values of LVFX and GRNX, which are frequently used in Japan, and MIC value of CPFX, which is often used against *Pseudomonas aeruginosa*, also tended to be elevated. In 2017, two isolates with TFLX MIC = 1 μg/ml (one from a child) were identified. After the recent approval for treatment of respiratory infections in children as an additional indication in Japan, opportunities of prescribing TFLX are increasing, and there is a concern that this led to an increase in the prevalence of TFLX resistance[22].

Considering that MIC value of MFLX is relatively higher than the MIC values of other quinolones (Table 1) and two of six isolates with MFLX MIC value of 0.25 μg/ml had amino acid mutations in QRDRs (Table 2), MIC values of MFLX appear to be more useful than those of other quinolones as a reference to find bacterial isolates with reduced susceptibility to quinolone antibacterial agents. Therefore, we believe that MFLX is suited for MIC assays to screen bacterial isolates with fluoroquinolone resistance, particularly in Japan. Furthermore, considering the reports by Shoji[12] and Perez[5], we propose that an MFLX MIC value of 0.25 μg/ml is an appropriate threshold to screen bacterial isolates for amino acid mutations in QRDRs.

While QRDR mutations in *H. influenzae* occur in GyrA, GyrB, ParC, ParE, and some other proteins, QRDR mutations are found more frequently at Ser84 and Asp88 of GyrA gene and at Ser84, Glu88, and Gly82 of ParC gene than at other positions[7, 12]. In addition, the first and

second step mutations have been reported to occur in GyrA and ParC, respectively[23]. Accordingly, we also focused on GyrA and ParC in the present study. In the case of *H. influenzae*, previous studies have demonstrated that MICs increase when strains have mutations at Ser84 in GyrA QRDRs or at Gly82 or Ser84 in ParC QRDRs[6, 12]. In this study, we analyzed 17 isolates showing MFLX MIC value of ≥ 0.25 μg/ml and found that nine isolates had a leucine mutation at Ser84 of GyrA gene (Table 2), a finding that is consistent with previous reports. Considering the reports on *Escherichia coli*, *Klebsiella pneumoniae*[24] and other bacteria, this finding suggests that GyrA is a likely primary target of quinolones for their antimicrobial activity similarly, against *H. influenzae*.

In this study, 2012-2 and 2017-1 isolates were found to have mutations only in ParC (Table 2). Related to this result, Shoji *et al* obtained a mutant with the mutation only at Ser84 of ParC gene among hot spots in a resistance induction experiment[12]. The MFLX MIC for this mutant was 0.5 μg/ml, indicating low susceptibility. This induction experiment showed that Ser84 is an important mutation site for *H. influenzae* to acquire quinolone resistance, supporting the finding in the clinical isolates in the present study.

While MIC values have been reported to increase as the number of QRDR mutations increases[12, 25], only one isolate was found to have two mutations in the present study. The isolate 2017-2 showed reduced susceptibility to all fluoroquinolones except for STFX, a finding supporting the above MIC changes (Table 2).

Isolate 2014-5 showed a high MIC value (0.5 μg/ml) for not only MFLX but also CPFX and LVFX, although this isolate had no mutations in QRDRs of both GyrA and ParC enzymes (Table 2). A factor presumably involved in this is a drug efflux pump (acrAB)[25] or *H. influenzae*-specific porin[26], the protein structure of which is similar to that of porin (OmpF). Solid evidence was not obtained from the present study, and this point remains to be addressed in future studies.

It is difficult to predict whether the frequency of quinolone-resistant *H. influenzae* isolates will increase in the future. Nevertheless, we consider that an outbreak of quinolone-resistant *H. influenzae* is likely to occur in Japan because the population aged > 65 years is a risk factor for this resistance[6], quinolone-resistant *E. coli* is increasing rapidly over recent years, and quinolone-resistant *E. coli* isolates have mutations in DNA gyrase and topoisomerase IV[24]. In 2015, a global action plan on antimicrobial resistance (AMR) was adopted by the World Health Assembly, and member countries developed national action plans. In Japan, a milestone is to halve the amount of use of broad-spectrum oral antibacterial agents, including oral fluoroquinolones by 2020. Therefore, monitoring the trend of occurrence of fluoroquinolone-resistant *H. influenzae* isolates is likely to have a substantial meaning to AMR countermeasures.

In clinical practice, the presence of a drug resistance gene does not necessarily indicate that the isolate has a reduced susceptibility to that antibacterial agent (i.e., silence mutations)[27]. However, acquisition of quinolone resistance is facilitated by quinolone use. Therefore, the emergence of quinolone-resistant bacteria can be prevented and future medical care against infectious diseases wound benefit if we can eliminate the unnecessary antibacterial drug use by promoting

proper antibacterial drug use. To achieve this and thereby improve the prospects of future medical treatments against infectious diseases, we should preset MFLX in the commercial drug sensitivity panel.

Limitations of this study include that we could not follow clinical courses of cases in which isolates with low quinolones susceptibility were found and that we could not determine the resistance status in other institutions. Close monitoring of changes in drug resistance and clinical characteristics should be jointly performed at multiple sites.

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Conflicts of interest disclosure

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