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Genetic mutations in the quinolone resistance-determining region are related to changes in the epidemiological profile of methicillin-resistant *Staphylococcus aureus* isolates

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ABSTRACT

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important causative agent of nosocomial infections. Mutations in the quinolone resistance-determining regions (QRDRs) of the *gyr* and *par* genes have been described. This study aimed to characterise phenotypic and genotypic fluoroquinolone resistance in 69 MRSA isolates of different clonal lineages from hospitals in Rio de Janeiro, Brazil.

Methods: QRDR mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes were detected by DNA sequencing. Minimum inhibitory concentrations (MICs) for ciprofloxacin and moxifloxacin were determined by broth microdilution. The occurrence of associations between mutations and MICs among the different clonal lineages of MRSA isolates was then verified.

Results: Most isolates from the USA400/ST1/SCC*mec* IV lineage, but mainly USA100/ST5/SCC*mec* II isolates, which have been more recently found in Rio de Janeiro hospitals, showed different patterns of mutations, including double mutation in the QRDR of *parC* (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) and/or *gyrA* (Ser-84→→→Leu and/or Glu-88→→→Lys) associated with higher moxifloxacin and ciprofloxacin MICs (MIC_{90}, ≥8→mg/L and 256→mg/L, respectively). On the other hand, all USA800/ST5/SCC*mec* IV and the BEC/ST239/SCC*mec* III isolates, which have disappeared from hospitals, showed single mutations in *parC* (Ser-80→→→Phe) and *gyrA* (Ser-84→→→Leu or Glu-88→→→Gly) and lower fluoroquinolones MICs (MIC_{90}, ≥2→mg/L and ≥16→mg/L).

Conclusion: This study highlights an increase in the number and types of mutations in the QRDRs of*gyrA* and *parC* associated with high fluoroquinolones MICs that may be related to changes in the epidemiological profile of MRSA isolates from hospitals in Rio de Janeiro.

1. Introduction

Staphylococcus aureus is one of the leading causative agents of nosocomial infections worldwide [1], and methicillin-resistant *S. aureus* (MRSA) account for approximately 48% of isolates in Latin American nations [2]. Molecular analysis of MRSA isolates has shown a variety of circulating lineages in hospitals, such as EMRSA-15 (ST22-IV), which replaced two previous dominant clones in Portugal, namely the Iberian (ST247-I) and Portuguese (ST239-III variant) clones [3]; and in China, where two pandemic MRSA clones are disseminated in hospitals, namely the Brazilian endemic clone (BEC/ST239-III) and the New York/Japan clone (ST5-II) [4]. In Rio de Janeiro, clonal lineages such as USA400 (ST1) and the Pediatric clone (USA800/ST5), both carrying staphylococcal cassette chromosome *mec* (SCC*mec*) type IV, as well as the ST5-II (USA100 or New York/Japan clone) have been replacing the BEC (ST239-III), which was prevalent in Brazilian hospitals until the early 2000s [5]. Recently, we described possible dissemination of

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the USA100 (ST5-II) lineage in a teaching hospital, comprising vancomycin-intermediate *S. aureus* (VISA), heteroresistant VISA (hVISA) and daptomycin-non-susceptible *S. aureus* isolates from bloodstream infections [6].

Fluoroquinolones are frequently prescribed in clinical practice [7]. Their mechanism of action consists of blocking the replication of bacterial DNA by binding to type II topoisomerases and topoisomerase IV [8]. Among staphylococcal isolates, topoisomerase IV is the primary target for these drugs and resistance involves two mechanisms: alterations of the quinolone resistance-determining region (QRDR) sequence in the *gyr* and *par* genes; and the presence of efflux pumps encoded by *nor* genes [9]. High rates of quinolone resistance have already been described for *S. aureus* isolates, mainly among MRSA [10]. Moreover, our group previously showed the relationship between the minimum inhibitory concentration (MIC) for ciprofloxacin and the type of mutation in the *parC* gene in *S. aureus* isolates [11]. In the current study, mutations in the QRDRs of the *gyr* and *par* genes and their relationship with fluoroquinolone MICs were investigated. Moreover, we describe for the first time a relationship between this resistance and clonal lineages of MRSA isolates from hospitals in Rio de Janeiro, Brazil.

2. Materials and methods

A total of 69 MRSA isolates were selected, including 67 ciprofloxacin-resistant isolates from adult patients admitted to six hospitals in Rio de Janeiro between 2004 and 2012. The isolates, which are part of our laboratory collection, were obtained from different clinical sources, including 39 (56.5%) from blood, 13 (18.8%) from nasal swabs and 17 (24.6%) from other sources. Isolates were previously characterised for antimicrobial susceptibility by the disk diffusion test according to Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines, for SCC*mec* type by multiplex PCR [12], and the genetic background by pulsed-field gel electrophoresis (PFGE) [13] and multilocus sequence typing (MLST) [14]. Overall, 32 isolates (46.4%) belonged to the USA100 (ST5-II) lineage, 12 (17.4%) to the BEC (ST239-III) lineage, 13 (18.8%) to the USA400 (ST1-IV) lineage and 9 (13.0%) to the USA800 (ST5-IV) lineage as well as 3 isolates (4.3%) carrying the SCC*mec* IV related to ST97 (Table 1).

In this study, MICs to ciprofloxacin (CIP) and moxifloxacin (MFX) (Sigma-Aldrich, St Louis, MO, USA), often used in local hospitals in Brazil, were determined by the broth microdilution method according to CLSI guidelines [15] in duplicate for all 69 MRSA isolates. *Staphylococcus aureus* ATCC 29213 was used as a susceptible control for broth microdilution as recommended by the CLSI [15]. MICs for CIP and MFX required to inhibit the growth of 90% (MIC_{90}) and 50% (MIC_{50}) of the isolates were also determined. Bacterial DNA was obtained using guanidine isothiocyanate [16] and the presence of *gyrA*, *parC*, *gyrB* and *parE* genes was assessed by PCR [8,17]. PCR was performed in a 25→μL reaction containing 1→μL of bacterial DNA, 1ÿ PCR buffer (10→mM Tris→HCl, 25→mM KCl), 5→mM $MgCl_2$, 0.2→μM of each primer, 200→μM dNTP and 1 U of *Taq* DNA Polymerase (Biotools, Madrid, Spain).

PCR products of each gene were purified using a GTX PCR and Band Purification Kit (GE Healthcare, Amersham, UK) according to the manufacturer→s instructions and were sequenced on an automated DNA MegaBACE 1000 Sequencing System (AP Biotech, Cambridge, MA, USA) using a DYEnamic ET Dye Terminator System (GE Healthcare). The sequences obtained were analysed using BioEdit v.7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and ExPASy (Bioinformatics Resource Portal) to identify possible mutations in comparison with sequences already deposited in GenBank (*S. aureus* NCTC 8325, GenBank accession no. **NC_007795**).

Fisher→s exact test was used to determine the significance of the association between mutation genotypes, MICs and each *S. aureus* clonal lineage. This test was performed because two variables were used (the type of genetic mutation and MICs) within each clonal lineage with a small sample size. Significance was established at 5% (P→<→0.05).

Table 1
Characteristics of 69 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from hospitals in Rio de Janeiro, Brazil.

Clonal lineage/ST (*n*)	SCC*mec* type	Hospital	Clinical source (*n*)	Ciprofloxacin[a]		Moxifloxacin[b]	
				MIC_{90}/MIC_{50}	MIC range (±S.D.[c])	MIC_{90}/MIC_{50}	MIC range (±S.D.[c])
USA100/5 (32)[d]	II	H1, H2	Blood (28) Tracheal aspirate (2) Nasal swab (1) Wound (1)	256/128	4 to ≥256 (±117.61)	32/4	≤0.5→64 (±18.05)
BEC/239 (12)[d]	III	H1, H2, H3, H4	Blood (4) Tracheal aspirate (6) Nasal swab (1) Bronchoalveolar lavage (1)	128/16	16→128 (±48.40)	4/2	≤0.5→4 (±1.36)
USA400/1 (13)[d]	IV	H1, H2, H5	Blood (5) Tracheal aspirate (1) Nasal swab (3) Bronchoalveolar lavage (1) Pleural fluid (1) Wound (1) Urine (1)	>256/256	32 to ≥256 (±98.77)	8/4	1→8 (±2.49)
USA800/5 (9)[d]	IV	H1, H2, H6	Blood (2) Tracheal aspirate (1) Nasal swab (6)	16/16	4→32 (±7.05)	2/1	1→2 (±0.44)
ST97 (3)[e]	IV	H1	Nasal swab (2) Pleural fluid (1)	1/1	1 to ≥256 (±147.22)	<0.5/<0.5	≤0.5→8 (±4.33)

ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*; MIC, minimum inhibitory concentration (in mg/L); $MIC_{50/90}$, MIC required to inhibit the growth of 50% and 90% of the isolates, respectively; S.D., standard deviation; CLSI, Clinical and Laboratory Standards Institute.
[a] Resistance breakpoint for ciprofloxacin: MIC→≥→4→mg/L (CLSI, 2013).
[b] Resistance breakpoint for moxifloxacin: MIC→≥→2→mg/L (CLSI, 2013).
[c] Based on MICs from all *S. aureus* isolates within the specific clonal lineage.
[d] All isolates were resistant to ciprofloxacin.
[e] Two isolates were susceptible to quinolones.

3. Results

Among the 69 MRSA isolates, the MIC_{50} and MIC_{90} values for CIP were 32→mg/L and 256→mg/L, respectively, whilst for MFX they were 2→mg/L and 32→mg/L. Two ST97 isolates were susceptible to the quinolones with MICs of <2→mg/L. CIP and MFX MIC data based on clonal lineage of the 69 MRSA isolates are detailed in Table 1.

Investigation of mutations in *gyrA*, *gyrB*, *parC* and *parE* genes was carried out for all 69 MRSA isolates, including USA100 (*n*→=→32 isolates), BEC (*n*→=→12), USA400 (*n*→=→13), USA800 (*n*→=→9) and ST97 (*n*→=→3) (Table 2). Among the four QRDR genes sequenced, no mutations were found in *gyrB* or *parE*. For the *gyrA* gene, mutations at positions 84 (Ser→→→Leu) and 88 (Glu→→→Gly/Lys) of the amino acid sequences were found; and for the *parC* gene, mutations at positions 80 (Ser→→→Phe/Tyr) and 84 (Glu→Gly/Lys) were identified. They were assigned the GenBank accession nos. **MF460985**→**MF460990**. Specific mutation patterns were associated with certain clonal lineages as well as with fluoroquinolones MICs (*P*→<→0.05). The types and number of mutations were used to cluster the isolates into five groups. Two isolates (66.6%) from ST97 did not present mutations and were clustered in Group 1. All BEC isolates were clustered in Group 2, presented MICs ranging from ≤0.5→4→mg/L for MFX and from 16→128→mg/L for CIP, and were associated with single mutation patterns in *gyrA at* codon 84 (Ser→Leu) and in *parC* at codon 80 (Ser→→→Phe) (*P*→<→0.0001). Among the USA800 isolates, low MICs were found for MFX (1→2→mg/L) and CIP (4→32→mg/L). In this group, which was named Group 3 and clustered only USA800 isolates (*P*→<→0.0001), a single mutation was found in the *gyrA* gene (Glu-88→→→Gly) and this mutation was different from the one found among BEC isolates, whilst the mutation in the *parC* gene was the same (Ser-80→→→Phe). The USA400 isolates presented MFX MICs ranging from 1→8→mg/L and all CIP MICs were ≥32→mg/L. For 11 (84.6%) of the 13 USA400 isolates, one type of mutation in the *gyrA* gene (Ser-84→→→Leu) and a double mutation in the *parC* gene (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) were found and were associated with high MFX and CIP MIC_{90} values (≥4→mg/L and ≥128→mg/L, respectively) and were clustered in Group 4 (*P*→<→0.0001). Finally, USA100 isolates showed MICs ranging from ≤0.5→mg/L to 64→mg/L for MFX and from 2→mg/L to >256→mg/L for CIP. Most of USA100 (ST5-II) isolates (17/32; 53.1%) had double mutations in the QRDR of *parC* and/or *gyrA* genes. For 8 (25.0%) of the USA100 isolates, double mutations were detected both in *gyrA* (Ser-84→→→Leu and Glu-88→Lys) and *parC* (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) and these isolates were clustered in Group 5 (*P*→=→0.0013). Group 5 isolates showed the highest fluoroquinolones MIC (≥32→mg/L for MFX and ≥128→mg/L for CIP). Furthermore, isolates of the USA100 lineage were the most distributed among the groups and were also clustered in Group 2 (15 isolates) and Group 4 (9 isolates) according to their mutation patterns.

A temporal distribution of 51 (73.9%) quinolone-resistant MRSA isolates selected from the two largest hospitals evaluated in this study is shown according to the QRDR mutation group in Table 3. They were isolated between 2004 and 2012 and belong to BEC (*n*→=→5 isolates), USA800 (*n*→=→8), USA400 (*n*→=→10) and USA100 (*n*→=→28) clones. In 2004, BEC isolates were found in both hospitals, with single mutations (Group 2), as well as USA100 and USA400 isolates, both presenting Group 4 mutations at Hospital 2. The following year (2005), USA800 isolates presenting single mutations in *gyrA* and *parC* genes (Group 3) were found in both hospitals, together with USA400 isolates presenting mutations of Groups 2 and 4. These USA400 isolates were observed in four

Table 2
Quinolone resistance-determining region (QRDR) mutation groups in *parC* and *gyrA* genes and fluoroquinolone minimum inhibitory concentrations (MICs) in 69 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from different lineages.

Mutation group (no. of isolates)	Mutation types in *parC* and *gyrA*	Drug	Clonal lineage (no. of isolates) at MIC (in mg/L) of:									
			≤0.5	1	2	4	8	16	32	64	128	≥256
Group 1 (*n*→=→2)	None	CIP	→	ST97 (2)	→	→	→	→	→	→	→	→
		MFX	ST97 (2)	→	→	→	→	→	→	→	→	→
Group 2 (*n*→=→29)	*parC* (Ser-80→→→Phe) *gyrA* (Ser-84→→→Leu)	CIP	→	→	USA100 (1)	USA100 (1)	USA100 (5)	BEC (6) USA100 (8)	BEC (1) USA400 (2)	BEC (2)	BEC (3)	→
		MFX	BEC (1) USA100 (1)	BEC (4) USA100 (10) USA400 (2)	BEC (3) USA100 (4)	BEC (4)	→	→	→	→	→	→
Group 3 (*n*→=→9)	*parC* (Ser-80→→→Phe) *gyrA* (Glu-88→→→Gly)	CIP	→	→	→	USA800 (1)		USA800 (7)	USA800 (1)	→	→	→
		MFX	→	USA800 (7)	USA800 (2)	→	→	→	→	→	→	→
Group 4 (*n*→=→21)	*parC* (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) *gyrA* (Ser-84→→→Leu)	CIP	→	→	→	→	→	→	→	→	USA100 (1) USA400 (2)	USA100 (8) USA400 (9) ST97 (1)
		MFX	→	USA100 (1)	→	USA100 (6) USA400 (7)	USA100 (2) USA400 (4) ST97 (1)	→	→	→	→	→
Group 5 (*n*→=→8)	*parC* (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) *gyrA* (Ser-84→Leu and Glu-88→→→Lys)	CIP	→	→	→	→	→	→	→	→	USA100 (2)	USA100 (6)
		MFX	→	→	→	→	→	→	USA100 (6)	USA100 (2)	→	→

MIC, minimum inhibitory concentration; CIP, ciprofloxacin; MFX, moxifloxacin.
The Clinical and Laboratory Standards Institute (CLSI, 2013) uses the following MIC breakpoints for susceptibility (S) and resistance (R): for CIP, S, ≤1→mg/L, and R, ≥4→mg/L; and for MFX, S, ≤0.5→mg/L, and R, ≥2→mg/L.

Table 3
Temporal distribution of 51 fluoroquinolone-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from four lineages isolated from two public hospitals in Rio de Janeiro, Brazil, according to the isolation year and quinolone resistance-determining region (QRDR) mutation group.

Hospital (no of isolates)	Clonal lineage/mutation group[a] (no. of isolates)						
	2004 (*n*→=→7)	2005 (*n*→=→5)	2006 (*n*→=→7)	2008 (*n*→=→6)	2009 (*n*→=→6)	2011 (*n*→=→11)	2012 (*n*→=→9)
H1 (*n*→=→32)	BEC/G2 (1)	USA400/G4 (1) USA800/G3 (1)	USA400/G4 (2) USA800/G3 (5)	BEC/G2 (1) USA400/G4 (1) USA100/G4 (1)	N/A	USA400/G4 (4) USA100/G2 (7)	USA100/G2 (4) USA100/G4 (4)
H2 (*n*→=→19)	BEC/G2 (3) USA400/G4 (1) USA100/G4 (2)	USA400/G2 (1) USA800/G3 (2)	N/A	USA100/G5 (3)	USA100/G2 (2) USA100/G5 (4)	N/A	USA100/G2 (1)

N/A, not available.
[a] G2, one mutation in *parC* (Ser-80→→→Phe) and *gyrA* (Ser-84→→→Leu); G3, one mutation in *parC* (Ser-80→→→Phe) and *gyrA* (Glu-88→→→Gly); G4, two mutations in *parC* (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) and one in *gyrA* (Ser-84→→→Leu); G5, two mutations in *parC* (Ser-80→→→Tyr and Glu-84→→→Lys/Gly) and *gyrA* (Ser-84→→→Leu and Glu-88→→→Lys).

evaluated periods (2005, 2006, 2008 and 2011) at Hospital 1. However, in 2008 the USA100 lineage presenting Group 4 mutations emerged at this institution as well as at Hospital 2, however in this case the mutations belonged to Group 5. USA100 lineage isolates were observed at both hospitals from 2008 to 2012 and, altogether, presented three different groups of mutations (Groups 2, 4 and 5).

4. Discussion

Staphylococcus aureus is an important nosocomial pathogen frequently associated with acquisition of resistance to multiple antimicrobial agents. Therefore, knowledge of its molecular epidemiology and antimicrobial resistance profile is essential for guidance of empirical therapy. Fluoroquinolones have been widely used in clinical practice for the treatment of infections in general since the 1980s [9]. As a result, *S. aureus* isolates present resistance rates to fluoroquinolones ranging from 36% to 48% in Latin America [2]; moreover, among MRSA isolates the rates can reach up to 90% [10]. In Brazil, a study by our group [18] reported that 93% of MRSA isolated from two hospitals in Rio de Janeiro were resistant to fluoroquinolones. Also, De Matos et al. compared multiresistant *S. aureus* SCC*mec* IV from different lineages isolated from Brazilian hospitals and found mutations in the *parC* gene [11]. In the current study, mutations in the QRDR region involving the *gyrA* and *parC* genes were detected and an association with MICs for fluoroquinolones and clonal lineages of MRSA isolates that have been prevalent in Rio de Janeiro hospitals was found. The *gyrB* and *parE* genes were also sequenced since the isolates were multiresistant and showed high fluoroquinolones MICs, however no mutations were found.

These findings show that most isolates from the same lineage presented the same mutations, even though they were isolated from different hospitals. Also, isolates from USA400 (ST1-IV) and USA100 (ST5-II) lineages were more resistant to fluoroquinolones than isolates from the other lineages evaluated. Interestingly, a greater number of mutations, including double mutations in the QRDR of the *gyrA* and *parC* genes, were also associated with these two lineages. Indeed, Sanfilippo et al. also found single and double mutations in *gyrA* and *parC* as well as quinolone MICs increasing with the number of mutations in the QRDR in *S. aureus* clinical isolates in the USA [8]. Other authors have previously described mutation patterns in *gyr* and *par* genes that are related to a decrease in affinity to fluoroquinolones and higher quinolone MICs in *S. aureus* [17,19,20]. Horii et al. reported increased levofloxacin resistance that resulted from some combinations of mutations in the QRDRs among MRSA isolates [19]. A study in China involving 154 *S. aureus* isolates from bovine clinical mastitis showed that CIP-resistant isolates had the classic mutations at codon 80 in the ParC subunit of topoisomerase IV and codon 84/88 in the GyrA subunit of DNA gyrase [20]. In the current study, we were not only able to confirm the association between the number and types of mutations in the QRDRs of the *gyrA* and *parC* genes and fluoroquinolone MICs in MRSA isolates from Rio de Janeiro, but also to establish an association with the genetic background of these isolates. Specifically, for the USA100 (ST5-II) lineage, some isolates showed double mutations in both genes and this was not found in any of the other MRSA lineages analysed. To the best of our knowledge, this is the first study to report these findings.

The association between the types and number of mutations, fluoroquinolones MICs and clonal lineage showed statistically significant correlations (P→<→0.05) and was found for all groups of MRSA isolates evaluated in this study. All prevalent lineages of MRSA evaluated that presented fluoroquinolones resistance were associated with mutations in the QRDRs of *gyrA* and *parC*. In addition, certain lineages (USA400/ST1-IV and USA100/ST5-II) that presented higher MICs for fluoroquinolones presented double mutations, which could provide a selective advantage allowing their establishment and dissemination in the hospital setting. It is interesting to note that these genetic mutations could be maintained over the period analysed in the study. Moreover, and probably due to the selective pressure of antimicrobials used, an alternation of clonal lineages in the environment was observed. This can be observed in the replacement of the BEC lineage by other clones, such as USA100, as had just been observed in other Brazilian hospitals [21,22]. These results highlight a selective advantage of certain *S. aureus* lineages that allowed their dissemination in the environment as well as the importance of knowledge of local epidemiology for empirical use of fluoroquinolones in patients with MRSA infections. According to Fuzi, an ability to develop favourable mutations in the gyrase and topoisomerase IV genes appears to be a prerequisite for pathogens to retain fitness while showing high-level resistance to fluoroquinolones [23]. The author suggests the determination of fluoroquinolone resistance in emergent MRSA isolates as a way to predict their disseminating capacity in regions where fluoroquinolones are used extensively [23].

In the present study, USA100 (ST5-II) isolates showed specific mutation patterns, including double mutations in the QRDR of *parC* and/or *gyrA* genes associated with higher MFX and CIP MICs. This MRSA lineage has been most recently detected in Brazilian hospitals [6,22] and is associated with multidrug resistance, including resistance to mupirocin [5], vancomycin and daptomycin [6]. USA100 lineage appears to have survival and growth advantages since it has remained for years as a major hospital-associated lineage in diverse geographic areas [24,25].

Fluoroquinolone resistance in *S. aureus* is mostly due to point mutations in the QRDRs, however active efflux of the antibiotics by chromosomally-encoded multidrug resistance transporter proteins, including NorA, NorB and NorC [9,17], can also affect

susceptibility. The *norA* gene, for example, may be responsible for decreased susceptibility to fluroquinolones [26]. Therefore, when coupled with other mechanisms such as target alterations, efflux pumps can produce high levels of fluoroquinolone resistance in *S. aureus* [27,28]. The presence of *nor* genes was not evaluated in the current study and this will be a focus of future studies.

In summary, we showed that isolates of prevalent MRSA lineages from Rio de Janeiro hospitals presented fluoroquinolone resistance associated with mutations in the QRDRs of the *gyrA* and *parC* genes. In addition, certain lineages presented double mutations, which could provide a selective advantage allowing their establishment and dissemination in the hospital setting. Thus, the number and types of mutations in the QRDRs as well as quinolone MICs appear to be associated with changes in genetic backgrounds of MRSA isolates in Brazil.

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Competing interests

None declared.

Ethical approval

Not required.

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