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Mycobacterium tuberculosis Spoligotypes in Monterrey, Mexico[∇]

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Although tuberculosis is still a public health problem in Mexico, there is little information about the genetic characteristics of the isolates. In the present study, we analyzed by spoligotyping 180 *Mycobacterium tuberculosis* clinical isolates from the urban area of Monterrey, Mexico, including drug-susceptible and drug-resistant isolates. The spoligotype patterns were compared with those in the international SITVIT2 spoligotyping database. Four isolates presented spoligotype patterns not found in the database (orphan types); the rest were distributed among 44 spoligo international types (SITs). SIT53 (clade T1) and SIT119 (clade X1) were predominant and included 43 (23.8%) and 28 (15.5%) of the isolates, respectively. In order to determine if there was a dominant spoligotype in the group of multidrug-resistant isolates, 37 of them were analyzed by IS6110-based restriction fragment length polymorphism assays, and scarce clustering of strains with more than five bands was observed. Fourteen isolates of this multidrug-resistant group presented four bands or less and were distributed in four SITs: SIT53 ($n = 8$), SIT92 ($n = 3$), SIT70 ($n = 2$), and SIT3038 ($n = 1$). When the molecular detection of mutations in the *katG* and *rpoB* genes were analyzed in these isolates with low copy numbers of IS6110, only two isolates shared the same IS6110, spoligotyping, and mutations patterns. When the distribution of the spoligotypes was analyzed by age cohort, SIT119 was predominantly found in patients 0 to 20 years old, especially in males, accounting for up to 40% of the isolates. In contrast, SIT53 was more prevalent in older females. This analysis demonstrates the variability of *M. tuberculosis* isolates in Monterrey and the partial dominance of SIT53 and SIT119 in that area of Mexico.

Despite the efforts to control tuberculosis (TB), it is still one of the most important causes of morbidity and mortality in the world. About 9 million new TB cases and approximately 2 million TB deaths were reported in 2004 (26). Although Mexico is not considered a country with a high tuberculosis burden, the number of cases has remained unchanged in recent years. The city of Monterrey is located in northeast Mexico. In 2005, the population of the city was estimated to be 1,133,814 and its metropolitan area had a population of 3.8 million, making it the third largest city in the country. Every year about 1,000 new cases of tuberculosis are reported in the metropolitan area (Boletín Epidemiológico, Secretaría de Salud, Mexico, www.dgepi.salud.gob.mx), and about 250 cases per year are referred to the Jose E. Gonzalez Hospital. Despite the high number of new cases, the genetic diversity of the *Mycobacterium tuberculosis* isolates in that region is little known.

Diverse techniques have been developed to study the epidemiological distribution of the disease, from case-contact studies to the application of molecular techniques, such as for the determination of variations in specific loci (3, 9), the determination of conserved deletions of long stretches of DNA (14), and analysis of the distribution of single nucle-

otide polymorphisms (6). These methods have allowed the identification of specific outbreaks, the classification of isolates into families, and the spread to or within human populations.

One of the most simple methods for the subtyping of *M. tuberculosis* isolates is spoligotyping (3, 9). This method detects variations in the direct-repeat (DR) locus, which consists of a repeated 36-bp sequence interspersed with nonrepetitive 31- to 41-bp DNA segments called spacer sequences. The DR region is amplified by PCR and the amplicon is hybridized to probes that detect the specific sequences of the spacers. A specific pattern of recognition of the spacers is called a spoligotype. The identification of these allows us to study the phylogeographical distribution of isolates.

In the present work we studied the genetic diversity of *M. tuberculosis* clinical isolates from the Monterrey metropolitan area by analyzing their spoligotypes. A selected group of multidrug-resistant (MDR) isolates was studied by IS6110-based restriction fragment length polymorphism (IS6110-RFLP) assays, and mutations conferring rifampin and isoniazid resistance were characterized.

(This paper fulfills part of the requirements for a master of science in public health for E.M.-T.)

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MATERIALS AND METHODS

Mycobacterial strains and DNA isolation. The *M. tuberculosis* isolates ($n = 180$) were obtained from the urban area of Monterrey, mostly from patients attending either the Laboratorio Estatal de Salud Pública or the tuberculosis

TABLE 3. Descriptions of predominant shared types found in this study and their worldwide distribution in the SITVIT2 database

SIT (clade) ^a	Octal no.	Total no. (%) in this study	% in this study compared to no. in SITVIT2 database	Distribution (%) in regions with ≥5% of a given SIT ^b	Distribution (%) in countries with ≥5% of a given SIT ^c
20 (LAM1)	67777607760771	5 (2.78)	0.74	AMER-N, 25.48; AMER-S, 24.29; AFRI-S, 13.26; EURO-S, 11.77; EURO-W, 8.49; CARIB, 6.41	USA, 23.70; BRA, 14.75; NAM, 9.24; PRT, 7.30; VEN, 6.26
47 (H1)	77777774020771	4 (2.22)	0.34	AMER-N, 21.76; EURO-W, 20.91; EURO-S, 14.39; AMER-S, 10.67; EURO-E, 8.72	USA, 20.15; AUT, 10.67; ITA, 7.62; BRA, 6.10; CZE, 5.00
52 (T2)	77777777760731	5 (2.78)	0.82	EURO-W, 22.50; AMER-N, 19.70; EURO-S, 7.72; ASIA-W, 7.72; EURO-E, 6.57; EURO-N, 6.24; AFRI-M, 5.42	USA, 17.57; BEL, 7.22; FRA, 6.57; ITA, 5.09
53 (T1)	77777777760771	43 (23.89)	0.91	AMER-N, 19.58; AMER-S, 13.83; EURO-W, 12.75; EURO-S, 10.06; ASIA-W, 8.65; AFRI-S, 5.93	USA, 17.23; ZAF, 5.79; ITA, 5.18
73 (T)	77773777760731	4 (2.22)	2.16	AMER-N, 22.16; EURO-S, 20.54; AFRI-S, 14.05; EURO-W, 12.97; AMER-S, 10.81	USA, 19.46; ITA, 18.38; ZAF, 14.05
92 (X3)	70007677760771	8 (4.44)	2.13	AFRI-S, 50.27; AMER-N, 25.00; AMER-S, 9.84; EURO-N, 5.32	ZAF, 50.27; USA, 22.34; BRA, 5.85
119 (X1)	77776777760771	28 (15.56)	2.77	AMER-N, 70.26; AFRI-S, 14.13	USA, 62.25; ZAF, 14.13; MEX, 7.31
211 (LAM3)	776137607760771	6 (3.33)	8.00	AMER-N, 66.67; AMER-S, 14.67; EURO-S, 10.67	USA, 42.67; MEX, 24.00; BRA, 10.67; ESP, 6.67
478 (X2)	61776777760601	4 (2.22)	12.90	AMER-N, 80.65; CARIB, 9.68; EURO-N, 6.45	USA, 61.29; MEX, 19.35; TTO, 6.45
1211 (S)	57637777760771	9 (5.0)	69.23	AMER-N, 84.62; EURO-N, 7.69; EURO-S, 7.69	MEX, 76.92; ESP, 7.69; USA, 7.69; SWE, 7.69

^a The predominant shared types (SITs) were defined as SITs representing 2% or more strains in a given data set (i.e., four or more strains in this study).

^b The worldwide distribution is reported only for regions with ≥5% of a given SIT compared to their total number in the SITVIT2 database. The definition of macrogeographical regions and subregions is according to the United Nations (<http://unstats.un.org/unsd/methods/m49/m49regin.htm>); Regions: AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania) are subdivided into E (eastern), M (middle), C (central), N (northern), S (southern), SE (southeastern), and W (western). Furthermore, CARIB (Caribbean) belongs to the Americas, while Oceania is subdivided into four subregions: AUST (Australasia), MEL (Melanesia), MIC (Micronesia), and POLY (Polynesia). Note that in our classification scheme, Russia has been attributed a new subregion by itself (northern Asia) instead of being included among the rest of Eastern Europe. This reflects its geographical localization and is also due to the similarity of specific *M. tuberculosis* genotypes circulating in Russia (a majority of which are Beijing genotypes) to those prevalent in Central, Eastern, and Southeastern Asia.

^c The three-letter country codes are according to http://en.wikipedia.org/wiki/ISO_3166-1_alpha-3; the countrywide distribution is shown only for SITs with ≥5% of a given SIT compared to their total number in the SITVIT2 database.

family of spoligotypes to be second in frequency, producing 28.8% of the cases: X1, 20%; X2, 2.77%; and X3, 6.1%. Within the LAM lineage we found 26 cases (14.4%). Among the families with more than 10 isolates was the Haarlem family, which comprised 7.7% of the isolates ($n = 14$). Unexpectedly, three isolates belonging to the Manila family (two SIT19 isolates and 1 orphan SIT isolate) were observed. In order to determine if the two SIT19 isolates were clonally related, we performed RFLP-IS6110 analysis and observed identical spoligotypes and IS6110 patterns; however, the patients from whom these isolates were recovered had no epidemiological links. We also detected the TBD1 gene fragment associated with this ancestral lineage by PCR. None of the patients with the Manila spoligotype were of Asian origin or had traveled to places where this clade is common. Isolates belonging to SIT1 or the Beijing family were not found among the 180 clinical isolates analyzed.

A description of the predominant spoligotypes in our study (patterns shared by 2% or more of the isolates) and their worldwide distribution in the SITVIT2 database (Table 3) showed that a total of 10 SITs predominated (representing 116/180 isolates, or 64.4% of all isolates); and corresponded to the following (in decreasing order): SIT53-T1 ($n = 43$, 23.8%), SIT119-X1 ($n = 28$, 15.56%), SIT1211-S ($n = 9$, 5.0%), SIT92-X3 ($n = 8$, 4.44%), SIT211-LAM3 ($n = 6$, 3.33%),

SIT20-LAM1 ($n = 5$, 2.78%), SIT52-T2 ($n = 5$, 2.78%), SIT47-H1 ($n = 4$, 2.22%), SIT73-T ($n = 4$, 2.22%), and SIT478-X2 ($n = 4$, 2.22%). Interestingly, the bulk of these spoligotypes predominated in North America, including SIT119 and SIT53, the most predominant spoligotypes, which represented 70.3% and 19.6% of all reported cases in SITVIT2 database from North America, respectively (62.25% and 17.23% from the United States, respectively). Interestingly, the strains belonging to the LAM lineage (SIT20 and SIT211) were most commonly found in the Americas (North and South America), while the 3rd-highest proportion of predominant spoligotypes was from Europe. Lastly, four spoligotypes represented more than 5% of the worldwide recruitment of a given spoligotype from Mexico (Table 3) and corresponded to SIT119-X1 (7.3%), SIT211-LAM3 (24.0%), SIT478-X2 (19.35%), and SIT1211-S (76.92%).

IS6110-RFLP analysis of multidrug-resistant isolates and molecular characterization of mutations associated with resistance. In order to determine if any specific RFLP types predominated within our population of MDR strains, we performed IS6110-RFLP analysis with 37 multidrug-resistant isolates (Fig. 1) collected from 1998 to 2003 for an MDR characterization study in Monterrey. Only four of the isolates with four or more copies of IS6110 were identical by both typing methods. When we checked their epidemiological data,

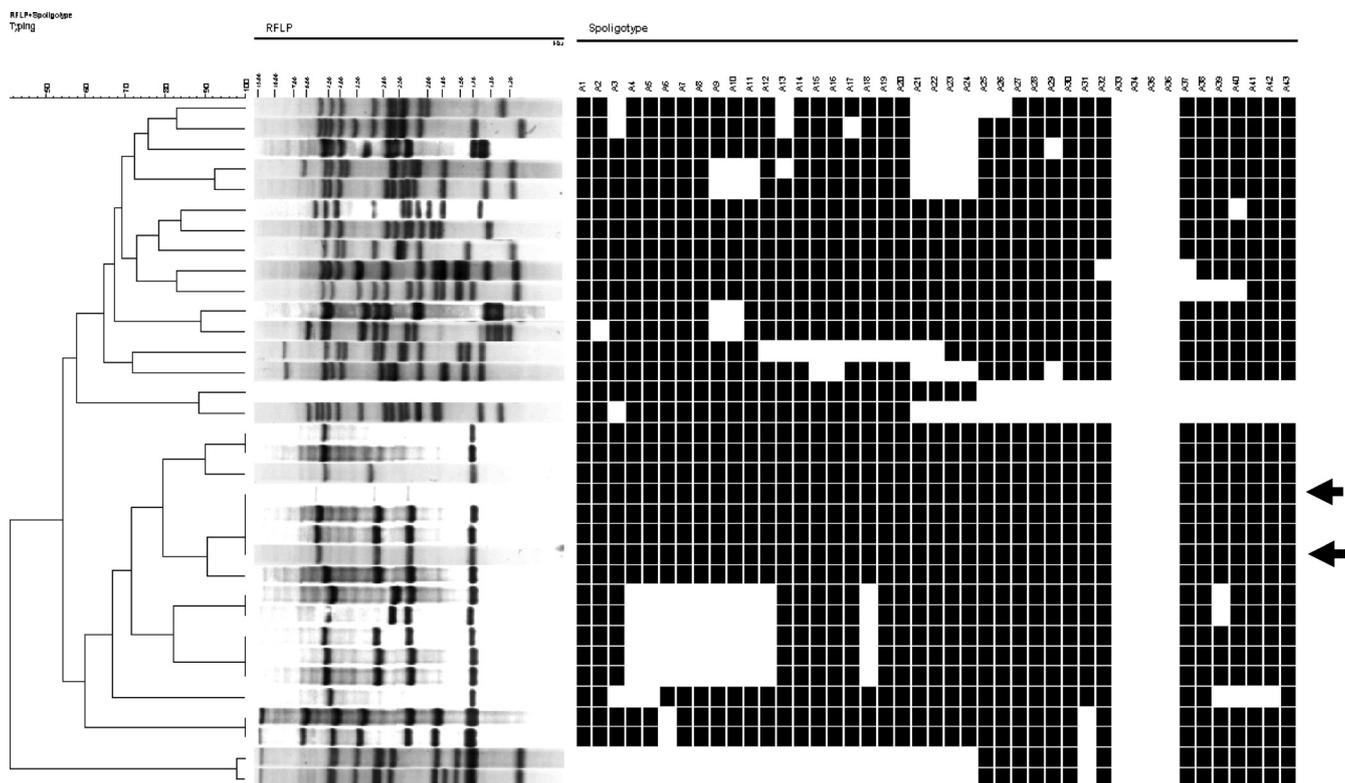


FIG. 1. IS6110-RFLP and spoligotyping analyses of 37 drug-resistant isolates from Monterrey, Mexico. Isolates are aligned according to their RFLP-IS6110 patterns. Arrows on the right indicate MDR isolates 528-98 and 67-99, which share an IS6110-RFLP pattern, spoligotype, and mutations in the *katG* and *rpoB* genes.

we observed that they could not be considered close contacts. We observed the abundance of isolates with four or more copies in this group (14 of 37, 38%) (Fig. 1); they were distributed in four SITs: SIT53 (8/14, 57%, T1 subclade), SIT70 (2/14, 14.2%, X3 subclade), SIT92 (3/14, 21.4%, X3 subclade), and SIT3038 (1/14, 7.1%, unknown subclade). When these low-copy-number were analyzed for the presence of mutations in the *katG* and *rpoB* genes (Table 4), we found point mutations in the *rpoB* gene in 71% (10 of 14) of the isolates and point mutations in the *katG* gene in 50% (7 of 14) of the

isolates. We found the Ser315Thr mutation in the *katG* gene in 5 (35%) of the 14 isolates. The S531L and H526Y mutations predominated in the *rpoB* gene (Table 4). Only two isolates shared the same IS6110-RFLP patterns, spoligotypes, and mutation patterns, suggesting a link between these cases.

Distribution of predominant spoligotypes by gender, age, and drug susceptibility. Table 5 shows the distributions of SIT53 and SIT119 by several ranges of ages and by gender. SIT53 was more prevalent than all other spoligotypes in women, being isolated from more than 30% of the patients

TABLE 4. Descriptions of the mutations associated with isoniazid and rifampin resistance in the IS6110 low-copy-number isolates

Isolate identification no.	SIT	Octal no.	Catalase gene		RNA polymerase beta subunit	
			Codon	Amino acid change	Codon	Amino acid change
291-98	53	77777777760771	315	S → T	526	H → Y
528-98	53	77777777760771	315	S → T	526	H → Y
67-99	53	77777777760771	315	S → T	526	H → Y
238-99	53	77777777760771	315	S → T	531	S → L
242-99	53	77777777760771	315	S → T	531	S → L
35-03	53	77777777760771	None	None	None	None
192-03	53	77777777760771	None	None	None	None
227-03	53	77777777760771	None	None	None	None
511-98	3038	61777777760601	None	None	516	D → V
340-98	92	70007677760771	271	T → P	531	S → L
02-99	92	70007677760771	271	T → S	531	S → L
208-99	92	70007677760771	None	None	522	S → Q
434-98	70	70007677760671	None	None	516	D → X
55-99	70	70007677760671	None	None	None	None

TABLE 5. Distributions of SIT53 and SIT119 according to gender and age^a

Age range (yr)	Female patients			Male patients			Both genders		
	No. of patients	% of isolates		No. of patients	% of isolates		No. of patients	% of isolates	
		SIT53	SIT119		SIT53	SIT119		SIT53	SIT119
0-20	6	66.60	16.60	15	0	40	21	20	35
21-40	24	8.30	8.30	43	18.60	16.20	67	15.30	12.30
41-60	12	33.30	16.60	41	24.30	12.19	53	26.40	13.20
>60	7	42.00	0	14	21.40	14.20	21	28.55	9.50

^a We had data for 162 isolates from the 180 patients.

over 40 years old. When the SIT prevalence was classified by the mean age of the patients with that SIT, we observed that SIT53 was more prevalent (mean age, 43.82 years) than the rest of the spoligotypes (mean age, 36.69 years) ($P = 0.012$). SIT119 was more commonly seen in younger patients (mean age, 35.44 years) than the rest of the spoligotypes (41.4 years old) ($P = 0.041$).

In the analysis of the spoligotype distribution by drug susceptibility, SIT53 and SIT119 were also predominant. The prevalence rates of SIT53 isolates susceptible, MDR, and resistant to one drug were 27.9, 15, and 29.4%, respectively. The values for SIT119 were 19.7, 11.6, and 11.7%, respectively. The male/female sex ratio for the total study population ($n = 180$) was 2.4, but for the pansusceptible group it decreased to 2. For the MDR group, the sex ratio increased to 2.75, and for the group resistant to one drug, it was 2.77. Spoligotype SIT1211 was highly prevalent in the multidrug-resistant group, comprising 13.3% of all MDR cases. Of a total of nine SIT1211 isolates, eight were MDR.

DISCUSSION

The most predominant spoligotypes found in our study, SIT53 and SIT119, are not the same previously reported for Mexico in the SITVIT2 database (Table 3), although when we analyzed reports from regions neighboring Monterrey, we observed that SIT53 and SIT119 are quite commonly found. In a study conducted in Houston, TX, SIT1 (S1, Beijing family) was the most common spoligotype found (25% of isolates) (16). However, among a total of 1,429 isolates, isolates of SIT119 (X1 sublineage) were recovered from 110 (7.69%) cases and isolates of SIT53 were recovered from 46 (3.2%) cases. Houston has a very large Mexican population, and that would explain in part the abundance of these spoligotypes.

Quitugua et al. (12), studying isolates from the Mexican border with the United States, analyzed the IS6110-RFLP patterns and spoligotypes of isolates from 313 patients from border cities in Mexico in the states of Tamaulipas (located beside Nuevo Leon and Texas) and Chihuahua, as well as 606 cases from Texas. They predominantly found SIT119 in the border region of Mexico and the United States, as well as in the interior of Texas, among both susceptible and drug-resistant isolates. SIT53 was scarcely found. In that study, 51% of the patients from Texas were Hispanic, and of those individuals, 57% were born in Mexico.

Soini et al. found in Houston that 20.3% of the total cases studied had from zero to four IS6110 copies (17). When they analyzed 377 isolates with equal to or less than four copies, a

total of 72 spoligotypes were found. Of those spoligotypes detected, SIT119 (S3 subclade) was found in 39 cases and SIT53 (S29 subclade) and SIT92 (S27 subclade) were found in 8 cases each. Ramaswamy et al. (13) found that 10 of 50 isolates from Monterrey had less than six copies (13), although they did not perform spoligotyping with those isolates. In our work, we found that 14 (50%) of the 37 drug-resistant isolates had four copies of IS6110 or less; 8 belonged to SIT53, 3 belonged to SIT92, 2 belonged to SIT70, and 1 belonged to SIT3038. Although we did not perform IS6110-RFLP analysis with all 180 isolates, it seems that low-copy-number isolates are abundant in this region. Warren et al. (24) found that many low-copy-number isolates share identical IS6110 insertion points and spoligotypes; in contrast, they failed to demonstrate clustering when they analyzed the isolates by MIRU-VNTR on the basis of 12 loci, suggesting that spoligotype determination and IS6110 positioning are earlier events (24). It is possible that other changes, such as the acquisition of point mutations related to drug resistance, also appear in a later period of time. This was observed in our low-copy-number isolates. When we analyzed the isolates for point mutations in the *katG* and *rpoB* genes, we observed that only two SIT53 isolates (Table 3, isolates 528-98 and 67-99) had identical, IS6110 fingerprinting, spoligotype, and mutation patterns. The point mutations detected were quite similar to those reported previously in drug-resistant isolates in Monterrey (23). Five of the SIT53 low-copy-number isolates shared the same spoligotype and IS6110 pattern; it is possible that these SITs were predominant before the introduction of isoniazid and rifampin and that the isolates acquired point mutations after exposure to these drugs in the 1960s and 1970s.

Beijing strains (SIT1) are very common in many parts of the world, but they are rarely reported in Mexico or in the rest of the Latin American region (10, 15). Although our state (Nuevo Leon) is beside Texas, where a high incidence of Beijing isolates have been reported in cities like Houston (25% of total isolates studied), we did not find any. That may be explained by the small Asian population in Monterrey.

In this study we found three isolates that belonged to the EAI2-Manila family. These ancestral isolates are more commonly found in Asian countries, such as Indonesia or the Philippines, where they account for high percentages of the *M. tuberculosis* isolates (4). In Mexico, a few Manila isolates were previously reported (10). The Philippines was a Spanish colony, governed as a territory of the Viceroyalty of New Spain (Mexico) from 1565 to 1821, that was part of the Spanish East Indies. A galleon transporting spices and materials from the

Far East navigated between the Philippines and Acapulco, Mexico, two times a year. It is thus possible that during this period some cases of tuberculosis were imported from the Philippine islands to Mexico.

In conclusion, SIT53 and SIT119 seem to be very predominant in Texas and northern Mexico. The predominance of SIT53 in Monterrey is consistent with that observed throughout the world, where this SIT is the most abundant, representing 17.85% of all *M. tuberculosis* isolates, and it is predominant in places distant from Mexico, such as Madrid and South Africa (2, 7, 20). SIT119, the second most predominant pattern in Monterrey, belongs to the X family (which includes sublineages X1 to X3), a well-characterized IS6110 family with low band copy numbers prevalent in the United Kingdom, Australia, the United States, South Africa, and former British colonies (2, 20). Historically, until the 1800s, when Texas became part of the United States, Texas and Monterrey belonged to the same region. It is possible that SIT53 and SIT119 were prevalent in that region in past centuries and that the spoligotypes in distant central Mexico may differ, although the spoligotypes from the highly populated region of Mexico City are not available at this time for comparison.

Modern molecular tools have demonstrated the evolution of microorganisms and their association with human migrations. Studies of single nucleotide polymorphisms of *Mycobacterium leprae* have suggested that leprosy originated in Africa and that the Hansen disease cases in the Americas are from European and African descendants as a result of emigration and the slave trade (11). Even though molecular evidence of the presence of *M. tuberculosis* in the pre-Columbian age has been reported (19, 25), controversy over this issue remains. If tuberculosis existed in native populations, the prevalence of specific spoligotypes would shrink significantly, since the population of about 22 million people living in Mesoamerica in 1520 was reduced by 95% by 1600, mainly because of infectious diseases (1). Therefore, the *M. tuberculosis* genetic pool was also reduced by the same proportion. It is possible that isolates with low levels of representation (e.g., those belonging to orphan SITs not found in other places) were predominant at some time, although this deserves further study, perhaps by using spoligotyping of pre-Columbian human remains.

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