Characterization of mutations in streptomycin-resistant *Mycobacterium tuberculosis* clinical isolates in the area of Barcelona

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Objectives: To determine the proportion and type of mutations in *Mycobacterium tuberculosis* isolates resistant to streptomycin, and their relationship with the level of resistance and with the epidemiological molecular pattern of the isolates.

Methods: Sixty-nine streptomycin-resistant isolates from a *M. tuberculosis* strain collection (1995–2005) from Barcelona were studied. The MIC of streptomycin for each isolate was determined using the proportions method with Middlebrook 7H11 medium. The entire *rpsL* gene and two specific fragments of the *rrs* gene (the 530 loop and the 912 region) were sequenced. IS*6110*-restriction fragment length polymorphism and spoligotyping were performed in each isolate.

Results: Twenty-six (26/69, 37.7%) streptomycin-resistant isolates presented a mutation in either the *rpsL* gene and/or the *rrs*530 loop, with no mutation in the *rrs*912 region. Seventeen (24.6%) isolates showed *rpsL* mutations (codons 43 and 88) associated with high MIC levels. Nine (13.0%) isolates had alterations in the *rrs* gene (A513T, A513C and C516T). Nineteen isolates (19/64, 29.7%) were classified into seven clusters (containing 2–5 isolates per cluster). Nineteen different spoligotype patterns were found. All the LAM3 spoligotype isolates (10/67, 14.9%) were associated with a C491T change in the *rrs* gene, being also observed in all LAM3 streptomycin-susceptible isolates.

Conclusions: Mutations in the *rpsL* and *rrs* genes were detected in 37.7% of streptomycin-resistant *M. tuberculosis* isolates. High-level resistance was associated with mutations in the *rpsL* gene, whereas wild-type isolates showed low MIC levels. The presence of the C491T substitution in the *rrs* gene in streptomycin-susceptible and -resistant isolates demonstrates that this change is an epidemiological marker associated with LAM3 sublineage.

Keywords: streptomycin resistance, *rpsL*, *rrs*, *M. tuberculosis*

Introduction

Streptomycin, an aminocyclitol glycoside antibiotic, was the first drug to be used in the treatment of tuberculosis (TB), in 1948.[1] Since then, following the use of streptomycin and other antituberculous drugs such as isoniazid, rifampicin, ethambutol and pyrazinamide, TB drug resistance has become an important problem throughout the world. The WHO has reported that the proportion of resistance to at least one antituberculous drug ranges from 0% to 56.3% in new cases and from 0% to 85.9% in previously treated cases. Likewise, 10.9% (range: 8.0%–13.7%) and 20.1%

(range: 12.2%–28.0%) of new and previously treated cases, respectively, are resistant to streptomycin around the world.[2] The latest incidence rate of TB reported in Barcelona was estimated at 25.4 per 100000 inhabitants, with the rate of resistance to any drug being 10.9% (7.4% and 14.2% in Spanish- and foreign-born patients, respectively). Streptomycin resistance was observed in 3.6% of Spanish-born patients and in 8.6% of foreign-born patients.[3] During the study period (1995–2005), the incidence of TB in Barcelona decreased from 55.9 to 30.1 per 100000 inhabitants,[3] whereas the rate of streptomycin resistance increased from 1.31% in 1995–97[4] to 4.8% in 2003–04.[5]

Streptomycin acts on the ribosome, inhibiting the translation of mRNA and thereby disrupting protein synthesis.[6] Mutations associated with streptomycin resistance in *Mycobacterium tuberculosis* have been identified in the *rpsL* and *rrs* genes, which encode the ribosomal protein S12 and 16S rRNA, respectively.[7,8] More than half of the streptomycin-resistant clinical isolates present mutations associated with these genes[9–13] (http://www.tbdreamdb.com/SM.html[14]). The most common mutations have been detected in codons 43 and 88 in the *rpsL* gene, and in two specific regions, the 530 loop and the 912 region, in the *rrs* gene.[7,8,15–17] There is a strong correlation between the level of resistance and the type and the position of the mutations.[11,12,17–19]

The use of genotyping techniques has allowed investigation of the characteristics and transmission dynamics of *M. tuberculosis* isolates. Thus, specific mutations in different genes associated with isoniazid, rifampicin or streptomycin resistance have also been observed in isolates included in the same IS*6110*-restriction fragment length polymorphism (RFLP) clusters and/or in different spoligotyping families [in particular, the Beijing and Latin-American–Mediterranean (LAM) strain families].[12,20–23]

Because of the lack of data regarding streptomycin resistance in our area, the objectives of this study were (i) to determine the different molecular mechanisms of streptomycin resistance and their relationship with the level of resistance, and (ii) to investigate the correlation between streptomycin resistance mutations and the IS*6110*-RFLP and spoligotype patterns in a collection of resistant *M. tuberculosis* isolates from Barcelona, Spain.

Materials and methods

Study setting, patients and isolates

We recovered all *M. tuberculosis* clinical isolates ($n=69$) resistant to at least streptomycin that had been collected from TB patients (one per patient) attending five university hospitals in Barcelona over an 11 year period (1995–2005). In addition, 30 streptomycin-susceptible isolates, which were collected during the same period, were included for the molecular analysis.

Susceptibility testing

Drug susceptibility testing was carried out for first-line antituberculous drugs using the radiometric BACTEC 460TB system (Becton–Dickinson, MD, USA) or the MGIT 960 system (Becton–Dickinson), according to the specific procedures in each hospital. For the radiometric system, the streptomycin critical concentrations tested were 6 mg/L for isolates collected during 1995–99, and both 2 and 6 mg/L for those collected during 2000–05. For the MGIT system these concentrations were 4 mg/L for isolates collected during 1995–99, and both 1 and 4 mg/L for those collected during 2000–05.[24,25] Twenty-three isolates (23/69) corresponded to the 1995–99 period and 46/69 to the 2000–05 period. MICs of streptomycin for each isolate were determined using the proportional method in Middlebrook 7H11 medium incubated for 4 weeks at concentrations ranging from 2 to 512 mg/L. MICs of ≤16 mg/L, 32–64 mg/L and >64 mg/L were considered to indicate low, intermediate and high levels of resistance, respectively.

DNA extraction and DNA sequencing analysis

According to previous studies,[11,16] the entire *rpsL* gene (GenBank accession number L08011), and the 530 loop (238 bp) and 912 region (240 bp) of the *rrs* gene (GenBank accession number X52917) were sequenced. *M. tuberculosis* strains were suspended in 300 μL of distilled water and inactivated at 95°C for 30 min. Then, 10 μL was used as a template in the first PCR. After purification of the PCR products with the QIAquick PCR purification kit (Qiagen), both DNA chains were sequenced using the ABI PRISM BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Inc., CA, USA) and the ABI PRISM 3700 DNA sequencer (Applied Biosystems).

Nucleotide sequences were analysed using the ClustalW2 programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Genotyping

IS*6110*-RFLP and spoligotyping of *M. tuberculosis* isolates were carried out as described previously.[26,27] Fingerprinting patterns were analysed using BioImage Whole Band Analyzer software, version 3.2.2 (Bio Image, Inc., Ann Arbor, MI, USA). The international SpolDB4 database[20] was used to determine the spoligotype families.

Statistical analysis

Differences between categorical variables were compared with the χ^2 test and Fisher's exact test when necessary, with statistical significance set at $P \leq 0.05$. Potential predictors of having an MIC >16 mg/L were examined at the bivariate level by calculating the odds ratio (OR) with a 95% confidence interval (CI) and the P value. Factors significant at the bivariate level were included in a multivariate logistic regression model using adjusted ORs, with corresponding CIs and P values. Statistical analysis was done using the SPSS v.13.00 (SPSS Inc., USA) statistical package.

Results

Fifty-seven of the 69 isolates (82.6%) belonged to untreated patients. Thirty-three (47.8%) patients were foreign-born and from 19 different countries [most frequently Peru ($n=4$) and Pakistan ($n=4$)]. Latin America was the most represented geographical area (16/33, 48.5%). No significant association was found between having received treatment and having a resistant mutation, and between previous treatment and MIC level of streptomycin. Nevertheless, we observed a statistical trend between foreign-born and multidrug-resistant (MDR) patients ($P=0.076$), as well as between being foreign-born and having a mutation in *rpsL* and/or *rrs* genes ($P=0.056$). Furthermore, isolates with MIC values >16 mg/L were more frequently associated with foreign-born compared with Spanish-born patients ($P<0.001$). No differences were observed in the proportion of mutated isolates before and after the year 2000.

Of 69 streptomycin-resistant isolates, 26 (37.7%) presented a mutation in either the *rpsL* gene and/or the 530 loop in the *rrs* gene. No mutation was found in the *rrs*912 region. Seventeen (24.6%) isolates showed *rpsL* mutations and 9 (9/69, 13.0%) had alterations in the *rrs* gene (Table 1). In addition, 10 streptomycin-resistant isolates presented a C→T change at position 491 in the *rrs* gene. All the *rpsL* mutated isolates had MIC values >16 mg/L ($P<0.0001$). Drug susceptibility testing determined that 8 isolates (8/26, 30.7%) with a mutation were monoresistant to streptomycin, 7 isolates (7/26, 26.9%) were polyresistant and 11 isolates (11/26, 42.3%) were MDR. Forty-three (62.3%) streptomycin-resistant isolates had wild-type alleles of both genes. Twenty-eight of these (65.1%) were

Table 1. Characteristics of the *M. tuberculosis* streptomycin-resistant isolates

Type and location of mutations		Mutation frequency (%)	MIC (mg/L)	Type of resistance	No. of isolates
rpsL gene	*rrs*530 loop				
43 (AAG→AGG; Lys→Arg)	—	11.6	>512	M	3
43 (AAG→AGG)	—		>512	P	4
43 (AAG→AGG)	—		>512	MDR	1
88 (AAG→AGG; Lys→Arg)	—	13	>512	M	1
88 (AAG→AGG)	—		512	MDR	5
88 (AAG→AGG)	—		32	MDR	1
88 (AAG→ACG; Lys→Thr)	—		512	MDR	1
88 (AAG→CAG; Lys→Gln)			128	M	1
—	A513T	10.1	512	M	1
—	A513T		512	P	2
—	A513T		32	M	1
—	A513T		16	M	1
—	A513T		16	MDR	1
—	A513C		16	MDR	1
—	C516T	2.9	512	MDR	1
—	C516T		64	P	1
—	—	62.3	4	M	8
—	—		4	P	3
—	—		4	MDR	1
—	—		8	M	4
—	—		8	P	3
—	—		8	MDR	2
—	—		16	M	14
—	—		16	P	2
—	—		16	MDR	4
—	—		32	M	2

M, monoresistant; P, polyresistant; MDR, multidrug resistant.

monoresistant to streptomycin and 16.3% of the isolates (7/43) were MDR (Table 1).

IS*6110*-RFLP typing was performed in 92.7% (64/69) of the isolates. Forty-five (45/64, 70.3%) unique patterns were identified and 19 isolates (19/64, 29.7%) were classified into seven clusters (Figure 1): Clusters I, III, IV, VI and VII contained two isolates each; Cluster II contained five isolates; and Cluster V contained four isolates. The isolates included in Clusters I, II, V and VII were monoresistant to streptomycin, with a low MIC level and wild-type alleles in both studied genes. Cluster III isolates were MDR with a high MIC level. Cluster IV isolates were monoresistant (with wild-type alleles) and MDR (with a mutation in the *rpsL* gene). Cluster VI isolates had a low MIC level and presented a mutation at codon 88 in the *rpsL* gene. No statistical difference was found between cluster and non-cluster isolates.

Spoligotyping analysis was performed in 97.1% (67/69) of the isolates. According to the spoligotype database, SpolDB4,[20] 19 different families were identified. Sublineages T1 and LAM3 were the largest, with 11 and 10 isolates, respectively. Beijing family isolates presented mutations in the *rpsL* gene (two isolates at codon 88 and one isolate at codon 43) (Figure 1).

Thirty streptomycin-susceptible isolates were analysed in order to clarify the role of the C491T change. Twenty-one isolates belonged to the LAM family: 14 isolates were LAM3 sublineage; 6 were LAM9; and 1 was LAM2. The remaining nine isolates were identified as T1 sublineage (five isolates), Beijing family (two isolates), and H3 and X3 (one isolate each one) sublineages. Only the LAM3 sublineage isolates presented the C491T change.

Discussion

To our knowledge, the present study is the longest prospective study on streptomycin resistance. The present findings reveal that 37.7% of the streptomycin-resistant isolates had a mutation in the *rpsL* or *rrs* genes. Several studies in other European countries and the USA have reported higher overall rates of streptomycin-resistant isolates associated with a mutation (44%–67%),[9,11,12,17,22] whereas, in Northern India, no mutations were found in any of the streptomycin-resistant isolates.[28] In contrast, Japan, China and Latvia have reported the highest frequencies, with 77.8%, 85.2% and 85%, respectively.[10,13,29]

Regarding the genes associated with streptomycin resistance, the present study showed that 24.6% (17/69) of the isolates had
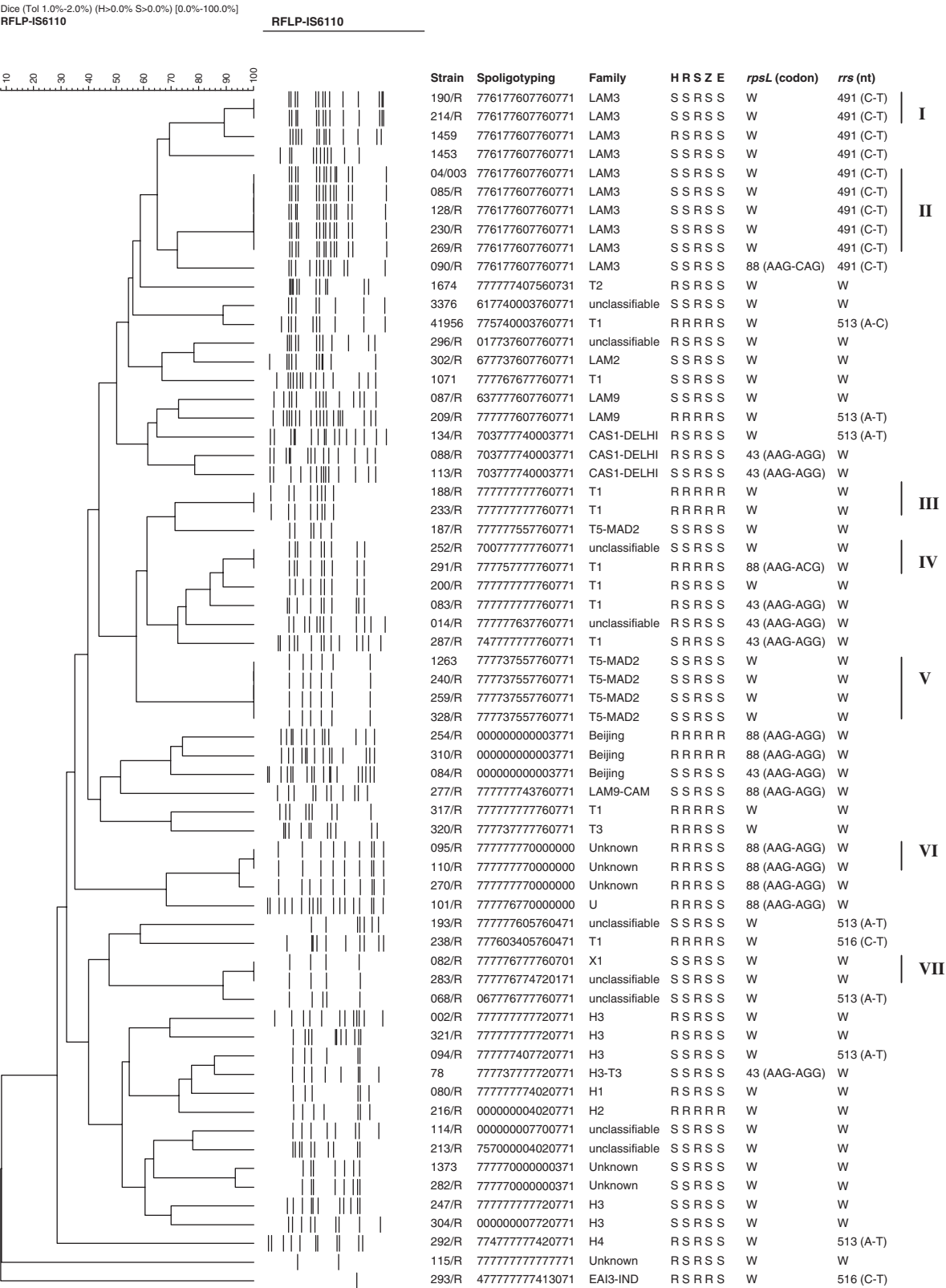
Figure 1. RFLP-IS*6110* profile dendrogram of 64/69 streptomycin-resistant isolates showing, in addition, spoligotyping families and sublineages, *rpsL* and *rrs* gene genotypes, and resistance profiles. H, isoniazid; R, rifampicin; S, streptomycin; Z, pyrazinamide; E, ethambutol.

mutations in the *rpsL* gene and 13.0% (9/69) in the *rrs* gene. We found that mutations in the *rpsL* gene occurred at a similar rate at codons 88 (9/69, 13.0%) and 43 (8/69, 11.6%); this is in contrast to other studies,[6,8,9,19] in which mutations at codon 43 predominated. This difference is especially remarkable in studies from Japan and China, where this last mutation was found in 78% and 91% of isolates, respectively.[13,19] It is interesting to note that all the isolates with a mutation at codon 88 were found in foreign-born patients, particularly those from Latin America, suggesting differences in geographical distribution. Statistical analysis could not establish a significant relationship between foreign-born patients and this mutation, although it did show a strong trend towards this association ($P = 0.058$). Nevertheless, there are few data related to the frequency and type of mutations in Latin American countries to support this relationship.

Additionally, two, albeit infrequent, mutations involving codon 88 were observed. The AAG→CAG (Lys→Gln) change has been described in other studies[9,11,16,19,23,29] and, recently, the substitution AAG→ACG (Lys→Thr) has also been found in Singapore.[23]

The frequency of mutations in the *rrs* gene observed in the present study (9/69, 13.0%) presented an intermediate rate compared with other studies (ranging from 2.3% to 24%).[7,13,16,29] However, on analysing the two *rrs* gene fragments (the 530 loop and the 912 region) separately, the frequencies of mutations differ greatly (13% versus 0%). These data are similar to those of other studies strongly confirming that mutations in the *rrs*912 region are not common.[11,13,17,29]

The alterations found in the *rrs* gene were located at positions 513 (A→C; A→T) (10.2%) and 516 (C→T) (2.9%). All these changes have previously been reported in several studies[9,10,13,16,29,30] and have also been included in the TB Drug Resistance Mutation Database published in 2009.[14] The C→T change at nucleotide position 491 in the *rrs* gene was observed in all isolates of the LAM3 sublineage. This association has not previously been reported, although Lipin *et al.*[21] found a similar association between the LAM spoligotype family and a mutation in the *rrs* gene located at position 513 (A→C) that was not observed in the present study. Two studies have reported that the change at position 491 in the *rrs* gene could be a sequence polymorphism instead of a mutation associated with streptomycin resistance.[12,31] Both studies reported a relationship between the C491T change and IS*6110*-RFLP clustering. In the present study, we also observed that 7 out of 10 isolates were classified into two different IS*6110*-RFLP clusters (Figure 1). We analysed the 530 loop in the *rrs* gene in 21 streptomycin-susceptible isolates, including several sublineages of the LAM family, and another 9 isolates belonging to several spoligotype families, in order to clarify whether or not there is an association between the C491T change and the LAM family. Interestingly, sequence analysis determined the presence of this change in all the LAM3 sublineage isolates, but not in the remaining isolates.

The Beijing lineage has been associated with drug resistance.[21,32] Recently, Sun *et al.*[23] have reported the association of Beijing isolates with mutation in the *rpsL* gene at codon 43. We only identified three Beijing strains among the isolates studied and one had a mutation at codon 43.

We observed that all the isolates with mutations in the *rpsL* gene were highly resistant to streptomycin (MIC ≥ 512 mg/L). This is similar to the findings of several other studies,[17,18] but in contrast to another study in which no relationship was found between the mutation type and resistance level.[13] Likewise, most of the wild-type isolates had a low MIC (MIC ≤ 16 mg/L).

On the other hand, a correlation between a mutation in the *rrs* gene and the level of resistance could be established according to the point mutation, with mutations at position 516 being related to high-level resistance. This correlation, however, was not clear when the mutation was located at position 513, showing different MICs.

No mutation was found in 62.3% of the streptomycin-resistant isolates. Other studies have reported a lower proportion of genetic alteration in streptomycin-resistant isolates, with 51% in Poland[11] and 53% in Japan[19] being much higher than the 14.8% reported in China[13] and the 33.3% in Lisbon, Portugal.[22] It has been suggested that unknown mechanisms, such as efflux pumps, the *gibB* gene[30] or alterations in the cell envelope, lead to decreased permeability, reduced drug uptake or enhanced efflux.[18] A combination of different phenomena may also explain what occurs in isolates without mutations.

Our data as well as those of others show that there are differences in the frequencies of streptomycin mutations and their gene distribution observed among different geographical areas of the world.[9–13,17,29] Several reasons may explain this: the use of streptomycin in treatment schemes in some countries, as has been suggested by Shi *et al.*;[13] the design of the study; and the study duration. The present study includes all the isolates resistant to streptomycin that were collected during a prospective period of 11 years. This, in our opinion, clearly reflects the real distribution of the streptomycin resistance in the geographical area analysed throughout the study period. Since the year 2000, streptomycin resistance in the hospitals participating in the study has been determined using the low and high concentrations recommended for both equivalent and validated methods. This methodological modification led to the detection of 12 isolates with MIC values of 4 mg/L. In previous studies using higher concentrations, these lower MIC isolates may not have been detected.

In conclusion, streptomycin resistance in the Barcelona area was due to mutations in the *rpsL* and/or *rrs* genes in 37.7% of the streptomycin-resistant isolates. High MIC levels were associated with mutations in the *rpsL* gene, whereas low MIC values were associated with wild-type isolates. Finally, we can conclude that the C491T substitution in the *rrs* gene is an epidemiological marker associated with the LAM3 sublineage.

Transparency declarations

None to declare.

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