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Hemodialysis waters as a source of potentially pathogenic mycobacteria (PPM)

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ABSTRACT

Water plays a notorious role in the epidemiology of nontuberculous mycobacterial infection in humans, as it is one of the natural sources for transmission of this group of organisms. Nosocomial infections with nontuberculous mycobacteria (NTM) are most commonly associated with the contamination of hospital water distribution systems. The aim of this study is to isolate and identify NTM in the water supply of hemodialysis centers in Tabriz, Iran. A total of 65 water samples were collected from hemodialysis centers at four hospitals between May 2017 and October 2017. The samples were filtered through 0.45 μm pore size membranes and decontaminated with 0.01% cetylpyridinium chloride. The sediment was inoculated onto Löwenstein–Jensen medium and incubated for 8 weeks. PCR was used to speciate the bacteria and sequence analysis of the *16S rRNA* and *hsp65* genes were used. Seven NTM species known for causing human disease were isolated including *Mycobacterium fortuitum* (4), *Mycobacterium gordonae* (4), *Mycobacterium mucogenicum* (3), *Mycobacterium abscessus* (2), *Mycobacterium chelonae* (2), *Mycobacterium simiae* (2) and *Mycobacterium kansasii* (1). This indicates that the immunocompromised patients and transplant recipients in Tabriz hemodialysis centers are at risk of infection, which calls for more effective water disinfection procedures.

Keywords: Potentially pathogenic mycobacteria; Hemodialysis; Water

1. Introduction

Nontuberculous mycobacteria (NTM), refers to a species of the genus *Mycobacterium* other than *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. More than 160 different species of NTM have been identified. Although most NTM species are saprophytes, about one-third of these species have been associated with human diseases [1,2]. Water is one of the most important sources of potentially pathogenic mycobacteria [1,3]. There are three important characteristics that contribute to the survival, colonization, and persistence of NTM in water distribution systems. These are tolerant to a wide range of pH, chlorine resistance, and the ability to form biofilms [4,5]. Isolation of NTM from potable water samples was first reported in the early 1900s [6]. Hemodialysis centers necessitate water reservoirs in hospitals [7]. Unlike tuberculosis, transmission of NTM to humans has mostly been associated with environmental sources and nosocomial infections. Furthermore, NTM are most commonly associated with the contamination of hospital water distribution systems [8]. The most common diseases caused by NTM are lymphadenitis in children and pulmonary diseases in adults. Children with cancer, solid organ transplant recipients and immunocompromised people are the most at risk [9]. The most frequent *Mycobacterium* species present in potable water and hospital water distribution systems are *Mycobacterium avium*, *Mycobacterium chelonae*, *Mycobacterium*

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fortuitum, *Mycobacterium gordonae*, *Mycobacterium kansasii* and *Mycobacterium xenopi* [4,10].

NTM were isolated from clinical specimens of hospitalized patients, which were sent to the Mycobacteriology Research Center (MRC) in Tabriz University of Medical Sciences (TUOMS) for speciation. Despite its prevalence, this is the first research of this kind for NTMs in northwestern Iran. The present study was a research project at the TUOMS. The University intends to launch a bone marrow transplantation (BMT) and kidney transplantation center with 300 beds. This study was performed with the aim of surveying the risk of NTM infections in this area and to determine the prevalence of NTM in the water supplies of hemodialysis centers in Tabriz.

Molecular techniques, including sequence analysis of the conserved genes such as *16S rRNA* and the 65 kDa heat shock protein gene (*hsp65*), have been applied for the detection and identification of mycobacteria, including *M. tuberculosis* and NTM [11–13]. Therefore, in this study, sequence analysis of the *16S rRNA* and *hsp65* genes were used to identify NTM species.

2. Materials and methods

2.1. Collection of samples

A total of 65 water samples (each 500 mL) were collected from hemodialysis centers at four hospitals between May 2017 and October 2017 in Tabriz, Iran. Sterile dark flasks were used for sample collection; each sample was collected after running the water about 2 min. Samples were transported to the laboratory and processed on the day of collection.

2.2. Filtration, decontamination, and culture of samples

About 400 mL of samples were filtered through membranes with 0.45 μm pore size and 30 mm diameter (Millipore, PES Syringe Filter, Orange Scientific, Belgium). Each filter was aseptically transferred into a separate sterile 15 mL tube containing 2 mL sterile distilled water. Each sample was decontaminated with cetylpyridinium chloride 0.01% (CPC 0.01%) for 30 min. For each tube, 2 mL 0.01% cetylpyridinium chloride (CPC, cetylpyridinium chloride monohydrate for synthesis; Merck 84008, Merck KGaA, Darmstadt, Germany) was added. The tubes were vortexed for 15 min at 3,000 rpm, membranes were aseptically removed, and the tubes were centrifuged at 6,000 rpm for 15 min at room temperature. The supernatant was discarded and 400 μL SDW was added to the sediment to dilute the residual CPC. The sediment was inoculated onto Löwenstein–Jensen medium and incubated at 30°C for 8 weeks. Mycobacterial growth was controlled weekly. The Ziehl–Neelsen staining technique was used to confirm the suspected colonies to be acid–alcohol-resistant bacilli.

2.3. DNA extraction from cultures

A loop-full of mycobacterial cells were suspended in 500 μL of 1X TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0), homogenized and vortexed. Afterwards, the suspension was boiled at 100°C for 20 min and centrifuged at 15,000 rpm for 10 min. After centrifugation, the supernatants were used for PCR amplification.

2.4. PCR amplification of the 16S rRNA and hsp65 genes

In this study, the sequence analysis of the *hsp65* and *16S rRNA* genes was used to identify NTM. A 921-bp fragment of the *16S rRNA* gene and 441-bp fragment of the *hsp65* gene were amplified with primer sets according to Harmsen et al. [14] and Telenti et al. [12], respectively. Primer sets are shown in Table 1. PCR reactions were performed using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 U μL^{-1} Taq polymerase, 0.5 μM of each of the primers, DNA template and nuclease-free water. PCR cycle conditions for amplification of the two genes (*hsp65* and *16S rRNA*) were as follows: 95°C for 4 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, and then final extension at 72°C for 10 min. To control the accuracy of the PCR, DNA from *M. tuberculosis* H37Rv and nuclease-free water (SinaClon, BioScience) were used as positive and negative control, respectively. Three μL of the PCR products (amplicon) were analyzed by electrophoresis on 1.5% gel agarose. After electrophoresis and gel staining with GelRed™ DNA stains, the fragments were visualized under UV light in the gel documentation system (Gel Doc, ATP Co; Fig. 1).

PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Germany) and analyzed the sequencing results for *16S rRNA* and *hsp65* genes by Sanger sequencing (Macrogen Corp., Korea). DNASTAR Lasergene software (version 7.1) and GenBeans (version 5.1). A representation of the direct sequencing of PCR-amplified *hsp65* gene of the isolate *Mycobacterium abscessus*, obtained by Sanger sequencing is shown in Fig. 2. The sequences were compared with similar sequences of the organisms in the Gene Bank using the BLAST online software from the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1
Primer sets for amplification of the *hsp65* and *16S rRNA* genes

Target gene	Name	Primer type	Primer sequence	Amplified size	Reference
16S rRNA	16S27f	Forward	AGAGTTTGATCMTGGCTCAG	921-bp	[14]
	16S907r	Reverse	CCGTCAATTCMTTTRAGTTT		
hsp65	Tb11	Forward	ACCAACGATGGTGTGTCCAT	441-bp	[12]
	Tb12	Reverse	CTTGTCGAACGCATACCCT		

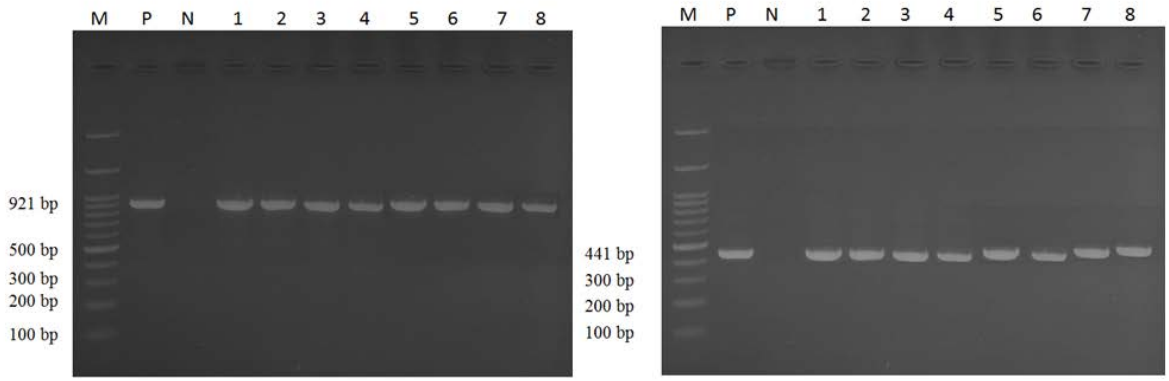

Fig. 1. Agarose gel electrophoresis of the PCR products obtained after amplification. Figure to the left is 921-bp of the *16S rRNA* gene and figure to the right is 441-bp of the *hsp65* gene (M: Marker, P: positive, N: Negative).

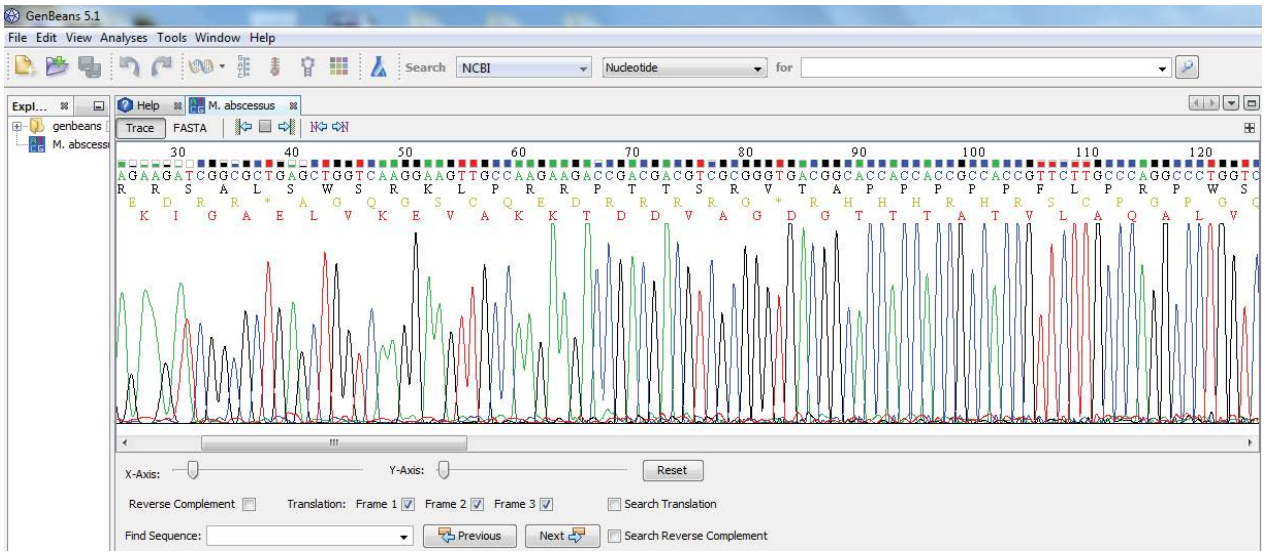

Fig. 2. Graph of the direct sequencing of PCR-amplified *hsp65* gene of the isolate *M. abscessus* obtained by Sanger sequencing.

2.5. Statistical analysis

Descriptive tests such as frequency and mean were applied to analyze the data using SPSS v.22 software (SPSS Inc., Chicago, IL, USA).

3. Results and analysis

The results of this study indicated that the water supplies of hemodialysis centers in the examined hospitals were colonized by NTM. Eighteen NTM colonies comprising 11 (61.1%) RGM (Rapidly growing mycobacteria) and 7 (38.9%) SGM (slowly growing mycobacteria) colonies were isolated from 65 collected samples. Of those, five NTM were isolated from hospital A, four NTM were isolated from hospital B, two NTM were isolated from hospital C and seven NTM were isolated from hospital D. A number of samples (A9, B4, D6 and D13), have more than one NTM species (Fig. 3). The prevalence of NTM in hospital D compared with other hospitals was high (7/19, 26.3%), and hospital C low (2/16, 12.5%). In hospital D, three *M. gordonae* species were isolated which were genetically different (Fig. 3). In our study, seven NTM species including four RGM species and three SGM species were recovered. *Mycobacterium avium* complex, which are most commonly associated with human diseases, were not found in any of the samples. Instead, other potentially pathogenic mycobacteria including *M. abscessus*, *M. chelonae*, *M. fortuitum* and *Mycobacterium mucogenicum* from rapidly growing mycobacteria, and *M. gordonae*, *Mycobacterium simiae* and *M. kansasii* from slowly growing mycobacteria which are related with human disease [8] were isolated. *M. fortuitum* and *M. gordonae* were the most prevalent, each with 22.2%. The number and percentage of other NTM species that were isolated from hemodialysis water samples are shown in Table 2.

In the present study, 18 NTM were recovered from water supplies of hemodialysis centers in Tabriz, northwest of Iran. For identification to the species level, partial sequence analysis of the *hsp65* and *16S rRNA* genes were used. The results of this study show that the *hsp65* gene sequencing has a high genetic heterogeneity compared with *16S rRNA* gene and can be used to identify the species which cannot be

Table 2
Number and percentage of NTM species that isolated from hemodialysis water samples at four hospitals in Tabriz city

Species or group	Species isolated from hospital A	Species isolated from hospital B	Species isolated from hospital C	Species isolated from hospital D	Total number (%) of isolates
Slowly growing mycobacteria (SGM)					7/18 (38.9%)
M. gordonae	0	1	0	3	4/18 (22.2%)
M. kansasii	1	0	0	0	1/18 (5.6%)
M. simiae	0	0	1	1	2/18 (11.1%)
Rapidly growing mycobacteria (RGM)					11/18(61.1%)
M. abscessus	0	2	0	0	2/18 (11.1%)
M. chelonae	1	0	0	1	2/18 (11.1%)
M. fortuitum	2	1	1	0	4/18 (22.2%)
M. mucogenicum	1	0	0	2	3/18 (16.7%)
Total	5	4	2	7	18/18(100%)
Number of collected samples	17	13	16	19	65
Number (%) of positive samples	4 (23.5%)	3 (23%)	2 (12.5%)	5 (26.3%)	**14/65 (21.5%)**

obviously differentiated by analysis of the *16S rRNA* gene. For example; *M. mucogenicum* was not distinguishable from *Mycobacterium phocaicum* using *16S rRNA* gene sequencing. However, by using the *hsp65* gene, these NTMs were distinguished from each other. Unlike *hsp65* gene, partial sequence analysis of the *16S rRNA* gene did not seem to be sufficient for recognizing NTM species.

The molecular phylogenetic and molecular evolutionary analysis of these species based on the *hsp65* gene sequences were conducted using MEGA 7.0 software [35] and the representative result is shown in Fig. 3. *Mycobacterium tuberculosis* strain H37Rv sequence was used as an out-group species. The NTM isolates represented in this study are indicated by a star (*).

4. Discussion

NTM are emerging pathogens in hemodialysis patients [7]. NTM infections in these patients are associated with water sources used in reprocessing hemodialyzers [8]. Basically, the source of water consists of municipal drinking water, which is purified by various techniques. Therefore, the quality of water used in hemodialysis centers is very important. Also, it is significant for hospitals to consider municipal drinking water as a source for these infections. The first hemodialysis NTM prevalence happened in 1982 in a dialysis center in Louisiana. *M. abscessus* and *M. mucogenicum* were causative organisms [15].

In the present study, 14 out of 65 samples (21.5%) were contaminated with NTM. Totally, 18 NTM colonies were isolated. Of this, 11 (61.1%) NTM were RGM and 7 (38.9%) NTM were SGM. We isolated pathogenic NTM species such as *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. mucogenicum*, *M. kansasii* and *M. simiae* from hemodialysis water supplies. These *Mycobacterium* species are responsible for various infections in humans [8,16–18].

The RGM species have recently gained increasing attention, because they are important agents of nosocomial infections [19]. Acquired infections due to RGM have been reported in patients on dialysis and receiving kidney transplants [20]. In the present study, the prevalence of the RGM species compared with SGM species was more than 1.5 times (61.1% vs. 38.9%). This alarming fact shows that the hemodialysis water supply can be considered as a reservoir for transmission of NTM among patients with chronic kidney disease. In this study, *M. fortuitum* and *M. gordonae* were the most encountered *Mycobacterium*. These species have been isolated from renal transplant recipients [21–23]. This indicates that the renal transplant recipients in Tabriz hemodialysis centers are the most at risk.

Other studies from Iran have displayed *M. fortuitum* as the dominant species in water and clinical samples [24–27]. Khosravi et al. [28] isolated 8 NTM species from 17 hemodialysis water samples including *M. fortuitum* (n = 3), *Mycobacterium novocastrense* (n = 2), *Mycobacterium senegalense* (n = 2) and *Mycobacterium lentiflavum* (n = 1).

From 13 studies in Iran (during 1992–2014), 480 NTM species were isolated from clinical samples. Of these isolates, 56% (269/480) were SGM and 44% (211/480) were RGM. Among SGM species, *M. simiae* (103/480, 21.4%) and among RGM species, *M. fortuitum* (136/480, 28.3%) was the most prevalent [29].

The results of this study indicate that in water disinfection strategies, NTM should be considered as part of the normal microbiological flora in water supplies of hemodialysis centers, and hemodialysis patients in Tabriz hospitals are at risk of contamination.

All our results were presented to infection control committees and to the head of development committees to raise awareness of the risk of NTM for BMT patients. This survey showed high risk of NTM infection in the study area and awareness is needed for risk of these infections for BMT patients. Therefore doctors have to consider these infections and hospital managers have to improve water quality and screening systems before establishing new transplantation wards in their hospitals.

Contamination of hemodialysis water with NTM has been reported in various countries. Gomila et al. [30] isolated 20 NTM from the distribution system of hemodialysis centers in Spain. In their research, *M. abscessus*, *Mycobacterium*

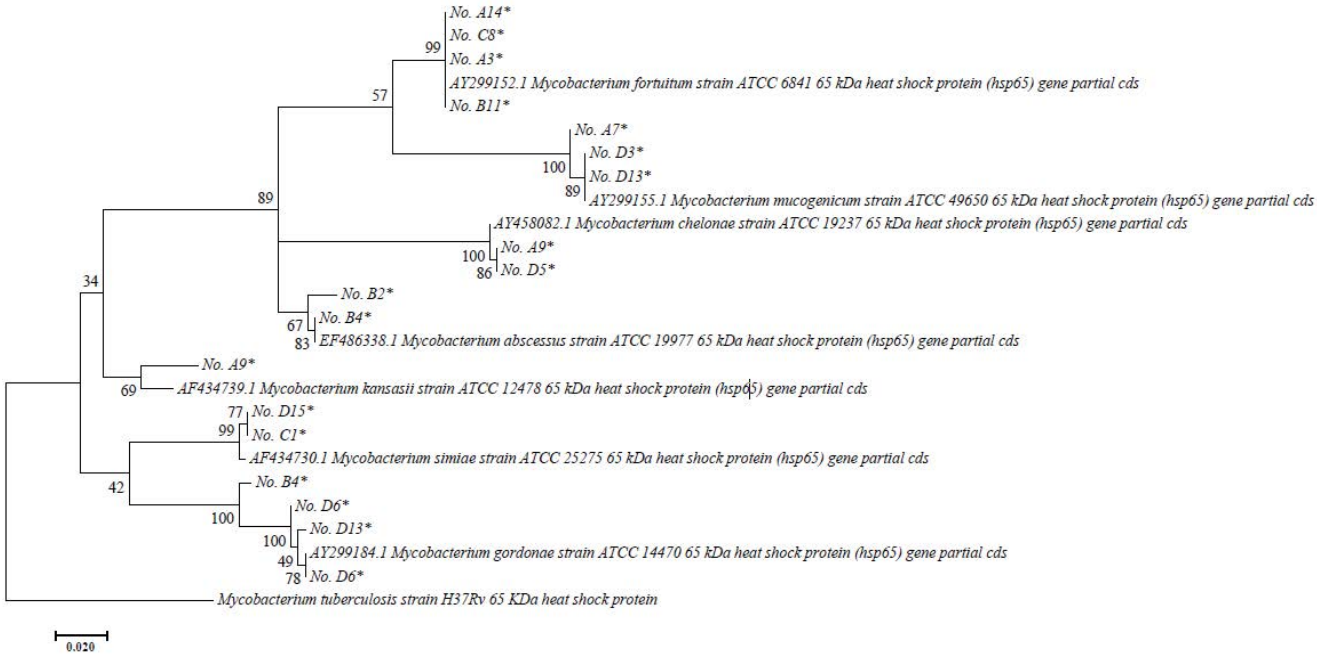

Fig. 3. Phylogenetic tree based on the *hsp65* gene sequences of the isolates from the water supplies of hemodialysis centers in Tabriz, northwest of Iran. A neighbor-joining tree was created, bootstrapped 1,000 times and visualized with MEGA 7.0 software [35]. Bootstrap values are indicated at the nodes. *M. tuberculosis* strain H37Rv was used as the out-group. The scale bar represents a 0.020 difference in nucleotide sequences.

bolletii, *M. chelonae*, *M. fortuitum*, *Mycobacterium immunogenum*, *M. mucogenicum* and *Mycobacterium massiliense* were among the identified NTMs. In another study, (11/110, 10%) NTM including *M. gordonae* ($n = 4$), *M. kansasii* ($n = 3$), *Mycobacterium gastri* ($n = 3$) and *M. lentiflavum* ($n = 1$) were isolated from hemodialysis center water system [31].

Carson et al. [7] also investigated the presence of NTM in a water distribution system at a hemodialysis center in Georgia. These authors isolated 550 NTM, including *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *Mycobacterium scrofulaceum* and *Mycobacterium terrae*. Sartori et al. [32] analyzed 210 samples of water collected from five different hemodialysis centers. In their study, 51 NTM (24.3%) including *M. lentiflavum*, *M. gordonae*, *Mycobacterium szulgai*, *M. kansasii* and *M. gastri* were isolated. The results of several studies show that distribution and frequency of NTM species in various geographical areas is different.

Compared with other molecular techniques, such as PCR restriction enzyme analysis, sequence analysis of the *16S rRNA* gene particularly *hsp65* is the best strategy to identify NTM. Our study showed that sequence analysis of the *hsp65* gene can be called the accurate, reliable and useful means of identification of NTM. Moreover, partial sequence analysis of the *16S rRNA* gene alone is not sufficient for the identification of NTM species. The analysis of more than one gene can be an effective way for differentiation between closely related species.

Escobar-Escamilla et al. [33] concluded that the *hsp65* gene sequencing is a better identification tool to differentiation of *Mycobacterium* species and is useful to complement diagnosis of NTM. These authors used the *hsp65* sequence analysis to recognize 10% of isolates which were not identified by PRA. The sequencing of the *hsp65* gene has been used successfully for differentiation of *Mycobacterium* species [34].

5. Conclusions

From this study and other studies, it is concluded that hemodialysis water supplies may be contaminated with NTM and thus may serve as a source of human disease. We were able to isolate and identify NTM species such as *M. kansasii*, *M. fortuitum*, *M. chelonae* and *M. simiae* in water samples collected from hemodialysis centers in Tabriz, northwest of Iran. This indicates that the immunocompromised patients and transplant recipients in Tabriz hemodialysis centers are at risk for infection, which calls for more effective water disinfection procedures. We recommend continuous monitoring of water supplies in hemodialysis centers, and adoption of effective preventive measures that minimize the exposure of renal transplantation and immunocompromised individuals to contaminated sources.

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