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ORIGINAL RESEARCH

Prevalence of nontuberculous mycobacteria and high efficacy of D-cycloserine and its synergistic effect with clarithromycin against *Mycobacterium fortuitum* and *Mycobacterium abscessus*

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Background: The prevalence of pulmonary disease caused by nontuberculous mycobacteria (NTM) is reportedly on the rise in the world. Some of the species are resistant to various antibiotics; hence, limited treatment options are available. The aims of this study were to investigate the prevalence of NTM and to determine the effect of D-cycloserine against *Mycobacterium fortuitum* and *Mycobacterium abscessus* isolated from clinical specimens to find out the synergistic effect of D-cycloserine and clarithromycin.

Methods: A total of 95 nonduplicate pulmonary isolates of NTM were collected from three major Regional Tuberculosis (TB) Centers. NTM isolates were identified by conventional tests and PCR sequence analysis of the *rpoB* gene. PCR sequencing of *erm-41* was performed for detecting the inducible resistance to macrolides. In vitro susceptibilities and activities of D-cycloserine-clarithromycin combinations were accessed using the broth microdilution method.

Results: Among 714-positive acid-fast bacilli from TB-suspected cases, 95 isolates were identified as NTM (13.3%). The prevalence of identified isolates was as follows: *M. fortuitum* 46 (48.4%), *Mycobacterium simiae* 16 (16.8%), *Mycobacterium kansasii* 15 (15.7%), *M. abscessus* 7 (7.3%), *Mycobacterium thermoresistibile* 4 (4.2%), *Mycobacterium elephantis* 3 (3.2%), *Mycobacterium porcinum* 2 (2.1%), and *Mycobacterium chimaera* 2 (2.1%). In addition, *rpoB* sequence analysis could identify all NTM isolates. The effect of D-cycloserine was better than that of clarithromycin. The synergistic effect of D-cycloserine with clarithromycin was observed for six (100%) and five (71.5%) strains of *M. fortuitum* and *M. abscessus*, respectively.

Conclusion: In the present study, we demonstrated a wide range of NTM in processed samples from different provinces of Iran. Our observations indicated that D-cycloserine was very active against *M. abscessus* and *M. fortuitum*; hence, D-cycloserine, either alone or in combination with clarithromycin, may be promising for the treatment of *M. abscessus*- and *M. fortuitum*-associated diseases.

Keywords: nontuberculous mycobacteria, in vitro activity, D-cycloserine, *Mycobacterium fortuitum*, *Mycobacterium abscessus*

Introduction

Nontuberculous mycobacteria (NTM) are generally found in the environmental sources including tap water, soil, and dust but in certain circumstances are able to cause disease in humans especially in immunosuppressed conditions.[1] Many opportunistic NTM pathogens are associated with a wide spectrum of localized and

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systemic disorders such as skin and soft tissue infections[2] and pulmonary and extrapulmonary infections. The prevalence of pulmonary disease caused by NTM is reportedly on the rise in the world.[3]

Based on the growth rate, NTM species are categorized into slowly growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM).[4] *Mycobacterium chelonae*, *Mycobacterium peregrinum*, *Mycobacterium abscessus*, *Mycobacterium fortuitum*, and *Mycobacterium smegmatis* group are among RGM that act as opportunistic pathogens of humans and produce various diseases.[5,6] *M. fortuitum* is most often associated with skin and soft tissue infections and hospital-acquired postoperative infections.[7] *M. abscessus* has emerged as an important human pathogen and is responsible for a wide variety of soft tissue and disseminated infections in immunocompromised patients and can complicate lung transplantation.[3] NTM infections, especially *M. abscessus* infection, is particularly problematic, and often, its treatment is costly and lengthy and comprises side effects.[8,9]

The description of the mechanisms of resistance does not limit the therapeutic options.[10] These organisms have not become resistant, and they are intrinsically resistant to most of the antibiotics that are currently available.[11] Also, infections caused by *M. fortuitum* isolates need long-term antibiotic therapy, because of their high resistance to chemotherapeutic agents and disinfectants.[12] The antibiotic of choice for infections caused by *M. fortuitum* is amikacin, but resistance to aminoglycosides and macrolides has also been reported.[12,13] Therefore, there is a great need to develop new treatment regimens for RGM infections, especially infections due to *M. fortuitum* and *M. abscessus*.[14] Combination therapy of oral macrolides and amikacin for 2–4 months has been recommended by other researchers and also by the American Thoracic Society (ATS).[15] After initial therapy, macrolide administration with at least one other antimicrobial agent to which the organism is susceptible should be used for the treatment. In vitro activity of aminoglycosides, such as clofazimine, D-cycloserine, and dapsone, has also been studied for certain SGM species[16]; however, the combined activities of a macrolide with D-cycloserine against *M. abscessus* and *M. fortuitum* have not been evaluated so far. As *M. abscessus* is the most common cause of NTM infections after *M. fortuitum* and *Mycobacterium simiae* in Iran,[17] the aims of this study were to investigate the prevalence of NTM isolated from clinical samples in Iran and to determine the effect of D-cycloserine against *M. abscessus* and *M. fortuitum* to find out the synergistic effect of D-cycloserine and clarithromycin on these organisms.

Methods

Sample collection

In this cross-sectional study, a total of 2,414 pulmonary samples were collected from three major Regional Tuberculosis (TB) Centers in Iran, including Khuzestan (south west), Kermanshah (west), and Hormozgan (south), from February 2016 to January 2018. Thirteen provinces of Iran are covered by these centers. As a part of the centers' policy, referred patients were requested to sign the informed consent in case their samples are used for research purposes apart from routine clinical investigation.

The preliminary proposal of the work was approved in joined Institutional Review Board (IRB) and Ethics Committee (Code: IR.AJUMS.REC.1396.103) of the Ahvaz Jundishapur University of Medical Sciences, Iran, and the necessary permission was granted for sample collection.

Phenotypic identification

The isolates were inoculated into Lowenstein–Jensen (LJ) medium and incubated at 37°C. All LJ tubes were examined daily for 30 days and twice weekly for 4 weeks thereafter. The mycobacterial isolates were subjected to phenotypic identification tests including growth characteristics, colony morphology, pigment production, niacin production, Tween 80 hydrolysis, semiquantitative catalase test, salt tolerance, arylsulfatase test, growth on MacConkey agar, heat-stable catalase (68°C), iron uptake, urease production, and tellurite and nitrate reduction.[18]

Molecular identification

DNA extraction and PCR amplification

Chromosomal DNA was extracted from mycobacterial colonies grown on LJ medium using an extraction and purification QIAamp DNA Mini Kit (Qiagen NV, Venlo, the Netherlands) according to the manufacturer's protocol. DNA concentrations and purity were determined using the NanoDrop One instrument (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm and used as template in PCR amplification.

A 751 bp fragment of the *rpo*B gene was amplified by using two specific primers of *Myco*F (5′-GCAAGGTCACCCCGAAGGG-3′) and *Myco*R (5′-AGCGGCTGCTGGGTGATCATC-3′) as previously described.[19] PCR amplification was performed in a final volume of 20 µL comprising 10 µL of Taq DNA Polymerase Master Mix RED (Ampliqon, Copenhagen, Denmark), 0.5 µL of each primer at

10 µM, 3 µL of extracted DNA (50 ng), and 6 µL of ddH_2O. The amplification was performed in a thermocycler (C1000 Touch; Bio-Rad Laboratories Inc., Hercules, CA, USA), with the following program: initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing 64°C for 30 seconds, and extension at 72°C for 90 seconds with a final extension at 72°C for 5 minutes. The PCR products were separated by electrophoresis on a 1.2% agarose gel (EMD Millipore, Billerica, MA, USA), stained with the SYBR Safe DNA Gel Stain (Thermo Fisher Scientific), and the DNA bands were visualized by a UV transilluminator (Uvidoc, gel documentation system; Jencons Scientific Inc., Cambridge, UK). The PCR Clean-Up Kit (Vivantis Technologies Sdn. Bhd., Subang Jaya, Malaysia) was used to purify PCR amplicons according to the manufacturer's instructions.

Analysis of sequence data

The sequence analyses based on the *rpoB* gene were performed in both directions (Bioneer Corporation, Daejeon, South Korea). The sequences were trimmed at both the 5′ and 3′ ends to include the most corresponding gene fragment sequences of mycobacteria collected in NCBI Nucleotide database. All the obtained sequences were analyzed by the Chromas software ver 2.6.

Phylogenetic analysis

The sequences were aligned using the ClustalW format in the MEGA 6.0 program Mega software (version 5.2).[20] The methodology for phylogenetic trees was constructed using the neighbor-joining method[21] and verified by the maximum likelihood method with 1,000 bootstrap replications. The phylogenetic tree was rooted with *Mycobacterium tuberculosis* and used *Nocardia asteroids* as an outgroup.

PCR assay for *erm*-41 gene

PCR amplification for the *erm-41* gene was performed on extracted DNA by using a set of primers of *erm*F (5′-GAC CGG GGC CTT CTT CGT GAT-3′) and *erm*R1 (5′-GAC TTC CCCGCA CCG ATT CC-3′), described by Shallom et al,[22] and the amplification protocol as described earlier for the *rpoB* gene. The target fragments were amplified using a thermal cycler (C1000 Touch), with following conditions: 95°C for 2 minutes, followed by 35 cycles of 95°C for 1 minute, 62°C for 1 minute, and 72°C for 90 seconds and a final extension at 72°C for 10 minutes. The electrophoresis of PCR products was performed on 1.5% agarose gel, and the gels were stained with the SYBR Safe DNA Gel Stain and were then visualized by the UV transilluminator. The PCR products were sent to Bioneer Co. for sequencing. The sequences obtained were analyzed using the BLAST algorithm at the NCBI website (http://blast.ncbi.nlm.nih.gov/).

Antimicrobial agents

D-Cycloserine and clarithromycin were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). For stock solution's preparation, potency of each antibiotic was calculated according to the CLSI guideline.[23] In brief, D-cycloserine and clarithromycin were dissolved in water and dimethyl sulfoxide (DMSO) at a concentration of 1,024 and 256 mg/L, respectively, to serve as stock solutions. Each stock solution was filtered using a sterilized 0.22 µm syringe filters (Sartorius, Melbourne, VIC, Australia). Stock solutions were stored at –80°C prior to each experiment. *Staphylococcus aureus* ATCC 29213 and *M. abscessus* ATCC 19977 were used as the reference strains for the determination of minimum inhibitory concentrations (MICs).

Drug susceptibility testing

Broth microdilution method was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines,[23] using 96-well round bottom microtiter plates. Briefly, the colonies of *M. fortuitum* and *M. abscessus* clinical isolates were harvested from growth on Luria-Bertani (LB) agar and suspended in 2 mL of sterile saline in tubes containing glass beads and mixed by vortexing. The mycobacterial suspensions were adjusted to McFarland 0.5 and inoculated into the cation-adjusted Mueller-Hinton broth (CAMHB) medium (Sigma-Aldrich Co.), containing twofold dilutions of antibiotics. The final drug concentration was from 2 to 256 mg/L for each antibiotic. The prepared MIC plates were incubated aerobically at 30°C. MICs were measured at day 3 or 4 in comparison to control sample well without antibiotic showing sufficient bacterial growth. The MIC was defined as the lowest drug concentration at which bacterial growth was not visualized. To detect the inducible macrolide-resistant *M. abscessus* isolates, MIC measurements were carried out at days 3 and 14 for clarithromycin.

Since to date, there are no reference MIC data for D-cycloserine, *M. fortuitum*, and *M. abscessus* isolates to determine the MIC breakpoints (susceptible, intermediate, and resistant), we referred to the MIC breakpoints of D-cycloserine (≤16, 32, and ≥64 µg/mL) for *Mycobacterium avium* complex proposed by Huang et al.[16] The MIC breakpoints (susceptible, intermediate, and resistant) of ≤2, 4, and ≥8 µg/mL, respectively, for clarithromycin were interpreted according to the CLSI guideline.[23]

The synergistic effects testing between clarithromycin and D-cycloserine

The synergistic effects of clarithromycin and D-cycloserine against six randomly selected *M. fortuitum* strains, and all of the seven *M. abscessus* strains were determined by the checkerboard approach using the broth microdilution method described elsewhere.[24,25]

Briefly, colonies were harvested from the LB agar and transferred to a CAMHB with glass beads for vigorous mixing on a vortex for 1 minute. After mixing, the mycobacterial suspensions were adjusted to the McFarland standard 5.0 (further diluted 1:200 in CAMHB). Clarithromycin and D-cycloserine were prepared as working solutions in 2.5 mL of CAMHB. For each isolate, 11 concentrations of clarithromycin (0.062–64 µg/mL) in the horizontal wells were tested for synergy against eight concentrations of D-cycloserine (0.25–32 µg/mL) in the vertical wells, using 96-well round bottom microtiter plates. After adding drug concentrations in wells, 100 µL of mycobacterial suspensions were added to each well of the microplate containing twofold diluted antimicrobials and, then, the plates were sealed by parafilm and incubated at 37°C for 3 days. Also, each drug was tested alone for two species within the range of concentrations from 0.062 to 64 µg/mL, at twofold serial dilutions. MIC measurements were carried out at day 3 of incubation.

The fractional inhibitory concentration index (FICI) method was calculated as previously described,[26]

$$\text{FICI} = \text{FICA} + \text{FICB} = (\text{MIC}_{\text{A}}\ \text{combination/MIC alone}) + (\text{MIC}_{\text{B}}\ \text{combination/MIC alone})$$

where A represents D-cycloserine and B represents clarithromycin. An FICI of ≤0.5 was interpreted as synergism, an FICI of 0.5 < FICI ≤ 4 was interpreted as indifference, and an FICI of >4 was interpreted as antagonism between the two drugs.

Results

A total of 2,414 pulmonary samples were examined during the period of study. Of these, 714 samples were positive for acid-fast bacilli by the Ziehl–Neelsen (ZN) staining and culture method. Among 714 isolates, 619 (86.69%) isolates were identified as *M. tuberculosis* complex (MTC) based on biochemical and molecular tests and 95 isolates were identified as NTM (13.3%). The samples were obtained from TB-suspected patients who were referred to the three major Regional TB Centers covering 13 provinces of Iran.

Of the 95 NTM-positive isolates, 55 (57.9%) isolates were belonged to male patients and 40 (42.1%) isolates were from females, with the mean age of 47.4±19.9 years. Coughing, fever, phlegm, and weight loss were the most common signs and symptoms of NTM-positive patients (Table 1).

Phenotypic identification

According to the growth rate, the isolates were classified into two groups (RGM and SGM). On the basis of growth characteristics, morphological, and biochemical properties, which are presented in Table 2, *M. fortuitum* (38 isolates) was the most frequent isolate, followed by *M. simiae* (10 isolates), *Mycobacterium kansasii* like (nine isolates), and *M. chelonae* (nine isolates). Among the 95 NTM isolates studied, only 76 (80%) strains were detected by phenotypic tests and the remaining isolates were unidentifiable.

Molecular identification

According to the sequencing of the *rpoB* gene, all 95 isolates belonged to eight different species and were clearly delineated (Figure 1A), including five RGM and three SGM. The most prevalent NTM species isolated from different provinces were *M. fortuitum* 46 isolates (48.4%), *M. simiae* 16 isolates (16.8%), and *M. kansasii* 15 isolates (15.7%). These were followed by *M. abscessus* seven isolates (7.3%), *Mycobacterium thermoresistibile* four isolates (4.2%), *Mycobacterium elephantis* three isolates (3.2%), *Mycobacterium porcinum* two isolates (2.1%), and *Mycobacterium chimaera* two isolates (2.1%) (Figures 2 and 3). As indicated in Table 3 and Figure 2, the majority of NTM were isolated from

Table 1 Demographic characteristics of nontuberculous mycobacteria-positive patients

Variable	Sample proportion, n (%)
Age (years), mean ± SD	47.4±19.9
Age groups (years)	
1–20	4 (4.2)
21–30	6 (6.3)
31–40	15 (15.8)
41–50	20 (21.05)
51–60	17 (17.9)
>61	33 (34.73)
Gender (male/female)	
Male	55 (57.9)
Female	40 (42.1)
Symptoms	
Cough	95 (100)
Phlegm	90 (94.7)
Fever	53 (55.7)
Weight loss	46 (48.42)
Night sweats	23 (24.2)

Kermanshah, Hormozgan and Khuzestan provinces. There was no significant correlation in the distribution of NTM in different regions.

In addition, all the *M. abscessus* isolates confirmed as *M. abscessus* subsp. *abscessus* by the replication and sequencing of the *erm-41* gene. Additionally, sequence analysis revealed that all *M. abscessus* strains showed mutation at position 28 in the *erm-41* gene (Figure 1B). Sequencing of *erm-41* and *rpoB* genes could identify all *M. abscessus* subsp. *abscessus* and showed valuable sensitivity and specificity, but *erm-41* was limited to the identification of *M. abscessus* species. *M. fortuitum* and *M. abscessus* isolates were then selected for further characterization.

In vitro susceptibilities to D-cycloserine and clarithromycin

For *M. abscessus* and *M. fortuitum*, MICs were determined for two antibiotics and their susceptibility patterns are shown in Table 4. From total *M. fortuitum* strains, 10 (21.7%), 10 (21.7%), and 26 (56.5%) isolates were susceptible, intermediate, and resistant, respectively, to clarithromycin. All *M. abscessus* isolates in this study were resistant to clarithromycin.

For D-cycloserine, 41 (89.1) of the *M. fortuitum* isolates were susceptible to D-cycloserine and all *M. abscessus* isolates were susceptible to this antibiotic. Thus, D-cycloserine showed good activity against both *M. fortuitum* and *M. abscessus*, while clarithromycin showed lower activity, inhibiting only 21.7% (n=10) of the *M. fortuitum* isolates and none of *M. abscessus* isolates. Moreover, two (4.4%) of *M. fortuitum* strains were co-resistant to both antibiotics.

In vitro activities of D-cycloserine–clarithromycin combinations

The combination of clarithromycin + D-cycloserine showed good activity against *M. fortuitum* isolates. So, the well in which more than 90% of isolates were killed after colony-forming unit (CFU) enumeration, was determined as MIC value (Table 5). The synergistic effect of D-cycloserine with clarithromycin was observed for six (100%) *M. fortuitum*, and combination results showed at least a 2.4-fold decrease in the concentration of each drug compared to either drug alone. These results suggested that this novel combination is active against clarithromycin-resistant *M. fortuitum* with FICI values ranging from 0.375 to 0.5.

Among inducible macrolide-resistant *M. abscessus* isolates, the synergistic effect of D-cycloserine with clarithromycin was observed for five (71.5%) isolates, by FICI values ranging from 0.375 to 0.5. The other two (28.5%) *M. abscessus* strains showed indifferent interaction with the drugs' combination by FICI value of 0.75 (Table 5). Nevertheless, antagonism was not observed for both species. Our combination results showed at least a 2.6-fold decrease in the concentration of each drug when needed to achieve >90% inhibition as compared to the concentration needed to achieve the same level of inhibition when used alone.

Discussion

Infections due to NTM are a serious problem for immunocompromised patients, and its prevalence among clinical isolates is greatly on the increase in recent years worldwide.[27] The antibiotic choices for the treatment of NTM infections are different from those for TB, as NTM is naturally resistant to the classical antituberculous drugs. Additionally, among NTM, RGM shows resistance to most of the currently available antibiotics in different geographical regions of the world. By the improvement in microbiological and laboratory techniques, more RGM has been identified[28]; therefore, identification of NTM species and determination of drug susceptibility pattern are crucial.[29–31] In the current study, only 76 (80%) strains were assigned to a species or complex by phenotypic tests. By the *rpoB* sequence analysis, all NTM isolates (100%) were identified and clearly delineated (Figure 3). According to the sequence analysis, the *rpoB* gene showed high discriminatory power for species' identification and all isolates were clustered with their reference strains. In concordance with our study, other previous reports demonstrated the efficacy of molecular tests such as *rpoB* sequencing. They introduced the technique as one of the most common techniques currently used for NTM identification, which is significantly more accurate than the phenotypic tests.[32,33]

Our findings indicated that the phenotypic tests alone cannot be applied as an efficient method for the identification of NTM species. The phenotypic method is time consuming and costly, and its results are very variable compared to those of molecular tests.[33]

Based on the sequences' analyses of *erm-41* and *rpoB* genes, all *M. abscessus* isolates were identified more correctly and this indicated that the *erm-41* and *rpoB* genes had valuable sensitivity and specificity for *M. abscessus* group identification. Further studies with more diverse selection of *Mycobacterium abscessus* group should be conducted to verify the discriminatory power of these genes for identification.[34]

In the present study, we demonstrated that *M. fortuitum*, *M. abscessus*, *M. simiae*, and *M. kansasii* are the most common RGM and SGM species in samples of patients from

Table 2 Phenotypic and molecular characteristics of clinical isolates

Number of isolates	Conventional tests							
	Growth rate (days)	Colony morphology	Pigment production	Growth on MacConkey agar	Urease	Iron uptake	NaCl tolerance	Arylsulfatase (3 days)
21	R	Smooth	N	+	+	+	+	+
1	R	Smooth	N	+	+	+	+	+
2	S	Rough	Y/Sc	+	+	+	–	–
9	R	Smooth	N	+	+	+	–	+
3	R	Rough	N	+	+	+	–	–
1	S	Rough	N	+	–	–	–	–
10	S	Smooth	Y/P	–	–	–	–	–
2	S	Smooth	N	–	+	–	+	+
4	S	Smooth	Y/Sc	–	–	–	–	+
9	S	Smooth	Y/P	–	+	–	–	–
16	R	Smooth	N	+	+	+	+	+
1	R	Smooth	N	+	+	+	+	+
1	S	Smooth	Y/P	–	+	–	+	+
3	R	Smooth	N	+	+	+	+	–
2	S	Smooth	N	–	+	–	–	–
1	R	Rough	N	+	+	+	–	–
6	S	Rough	Y/Sc	–	+	–	–	–
1	R	Smooth	N	+	+	+	+	+
1	S	Smooth	N	+	–	–	–	–
1	R	Rough	Y/P	+	+	+	–	–

Abbreviations: Sc, scotochromogen; SQ, semiquantitative; Y, yellow.

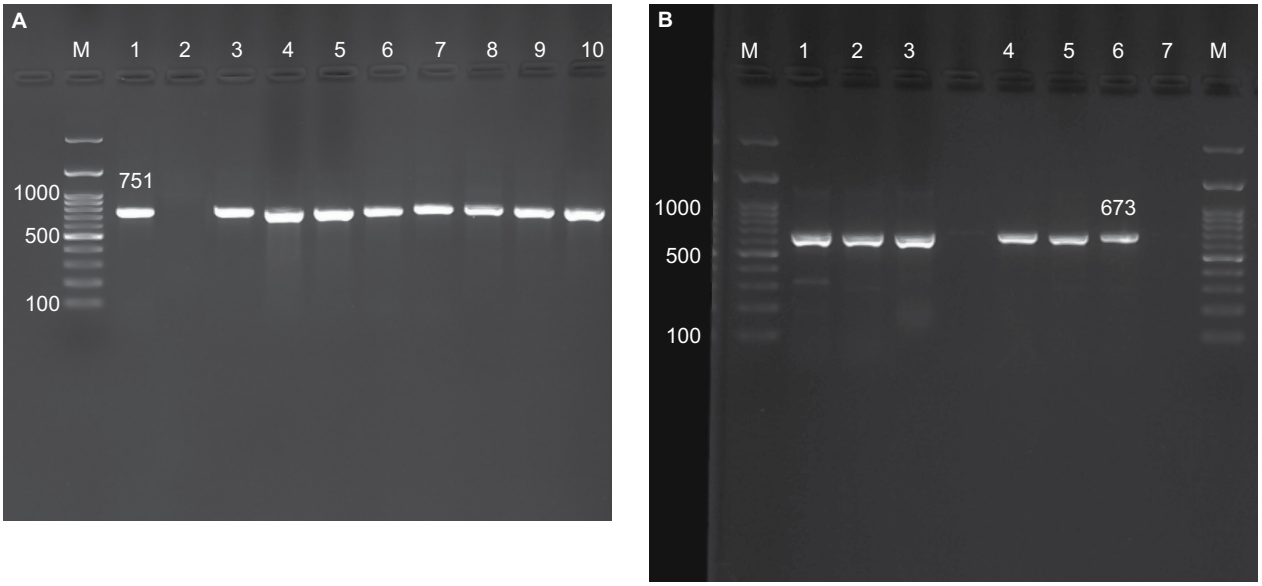

Figure 1 PCR amplification of applied genes for NTM isolates.
Notes: (**A**) amplification of *rpoB* gene; M, DNA size marker; 1, positive control; 2, negative control; 3–10, positive samples. (**B**) amplification of the *erm-41* gene for *M. abscessus* isolates. M, DNA size marker; 1–3 and 4–5, positive samples; 6, positive control; 7, negative control.
Abbreviations: *M. abscessus*, *Mycobacterium abscessus*; NTM, nontuberculous mycobacteria.

different provinces investigated in this study; this finding is in line with other reports from Iran.[17,35,36] Moreover, *M. fortuitum* was reported as the most common NTM, isolated from environmental and clinical sources in the distinctive part of Iran.[37,38]

Several studies indicate a high incidence of *M. simiae* in Iran,[39,40] while according to our results, *M. fortuitum* (48.4%) was the most prevalent NTM in pulmonary samples. We indicated that, in 12 provinces of Iran, some patients with TB misdiagnosis and relevant treatment were actually infected

Conventional tests						Phenotypic tests	*rpoB* sequencing
SQ catalase (>45 mm)	68°C catalase	Tween hydrolysis, 5 days	Nitrate reduction	Tellurite reduction	Niacin production		
+	+	+	+	+	–	*M. fortuitum* like	*M. fortuitum*
+	+	+	–	+	–	*M. fortuitum* like	*M. porcinum*
+	–	+	+	+	–	*M. gordonae*	*M. thermoresistibile*
+	+	+	–	+	–	*M. chelonae*	*M. fortuitum*
+	+	–	–	+	–	*M. chelonae*	*M. abscessus*
–	–	–	–	+	–	*M. avium* complex	*M. chimaera*
+	+	–	–	+	+	*M. simiae*	*M. simiae*
+	+	+	+	+	+	*Mycobacterium* sp.	*M. elephantis*
+	+	–	–	+	–	*M. scrofulaceum*	*M. simiae*
+	+	+	+	+	–	*M. kansasii* like	*M. kansasii*
+	+	–	+	+	–	*M. fortuitum* like	*M. fortuitum*
+	–	–	–	+	–	*Mycobacterium* sp.	*M. abscessus*
–	+	+	+	+	–	*Mycobacterium* sp.	*M. elephantis*
+	–	–	–	+	–	*Mycobacterium* sp.	*M. abscessus*
+	+	–	+	+	–	*Mycobacterium* sp.	*M. simiae*
–	–	+	+	+	–	*Mycobacterium* sp.	*M. thermoresistibile*
+	+	+	+	–	–	*Mycobacterium* sp.	*M. kansasii*
–	+	+	+	–	–	*Mycobacterium* sp.	*M. porcinum*
–	+	–	–	+	–	*M. avium* complex	*M. chimaera*
–	–	+	+	+	–	*Mycobacterium* sp.	*M. thermoresistibile*

by *M. fortuitum*. In contrast, *M. simiae* isolates (16.8%) were the second dominant NTM species. These differences may be the result of different geographical distributions of NTM throughout Iran. Umrao et al[41] reported that the predominant species were *M. abscessus* and *M. fortuitum* in Northern Indian population.

Infection with NTM is often misdiagnosed for TB and may be treated as drug-resistant TB. NTM species are resistant to the first and some second lines, resulting in treatment failure that may lead to an increase in mortality and morbidity; therefore, accurate and timely diagnosis is essential. Our study showed that most of the isolates of NTM were obtained from pulmonary samples and thus, there is a need to develop prevention strategies against them.

This study evaluated the activity of D-cycloserine and clarithromycin and the combinations of both antibiotics against *M. abscessus* and *M. fortuitum* clinical isolates. The findings demonstrated that the effect of D-cycloserine alone against *M. abscessus* and *M. fortuitum* was better than that of clarithromycin. Furthermore, we observed the synergistic effect of D-cycloserine–clarithromycin combination against *M. abscessus* and *M. fortuitum*. These results indicate that both D-cycloserine and clarithromycin may serve as an alternative treatment for *M. abscessus*- and *M. fortuitum*-associated infections. However, the D-cycloserine–clarithromycin combination was not effective against the minority of tested *M. abscessus* and 28.5% of the isolates showed an indifferent interaction.

Cycloserine acts by inhibiting cell wall biosynthesis in bacteria. In fact, cycloserine acts against two crucial enzymes important in the peptidoglycan synthesis, including D-alanine:D-alanine ligase (Ddl) and alanine racemase (Alr).[42] Cycloserine also has good capability of penetrating into liver and tissues. Cycloserine was found to have low resistance rate in MTC and also high susceptibility rates against *M. intracellulare* and *M. avium*.[16]

In combination, cycloserine may interrupt cell wall synthesis, thus increasing the penetration of clarithromycin into the bacteria.

Previous studies have demonstrated an in vitro synergistic effect of combination of clofazimine with amikacin or clarithromycin against *M. abscessus*.[43] The only oral drugs used in the treatment of *M. abscessus* are the macrolides and clofazimine,[44] but there is less supportive clinical evidence of its efficacy.[45] We found that two isolates of *M. fortuitum* were resistant to D-cycloserine, while all *M. abscessus* strains were susceptible. The mechanism of resistance to D-cycloserine is unknown in *M. fortuitum*, but overexpression of *ddlA*

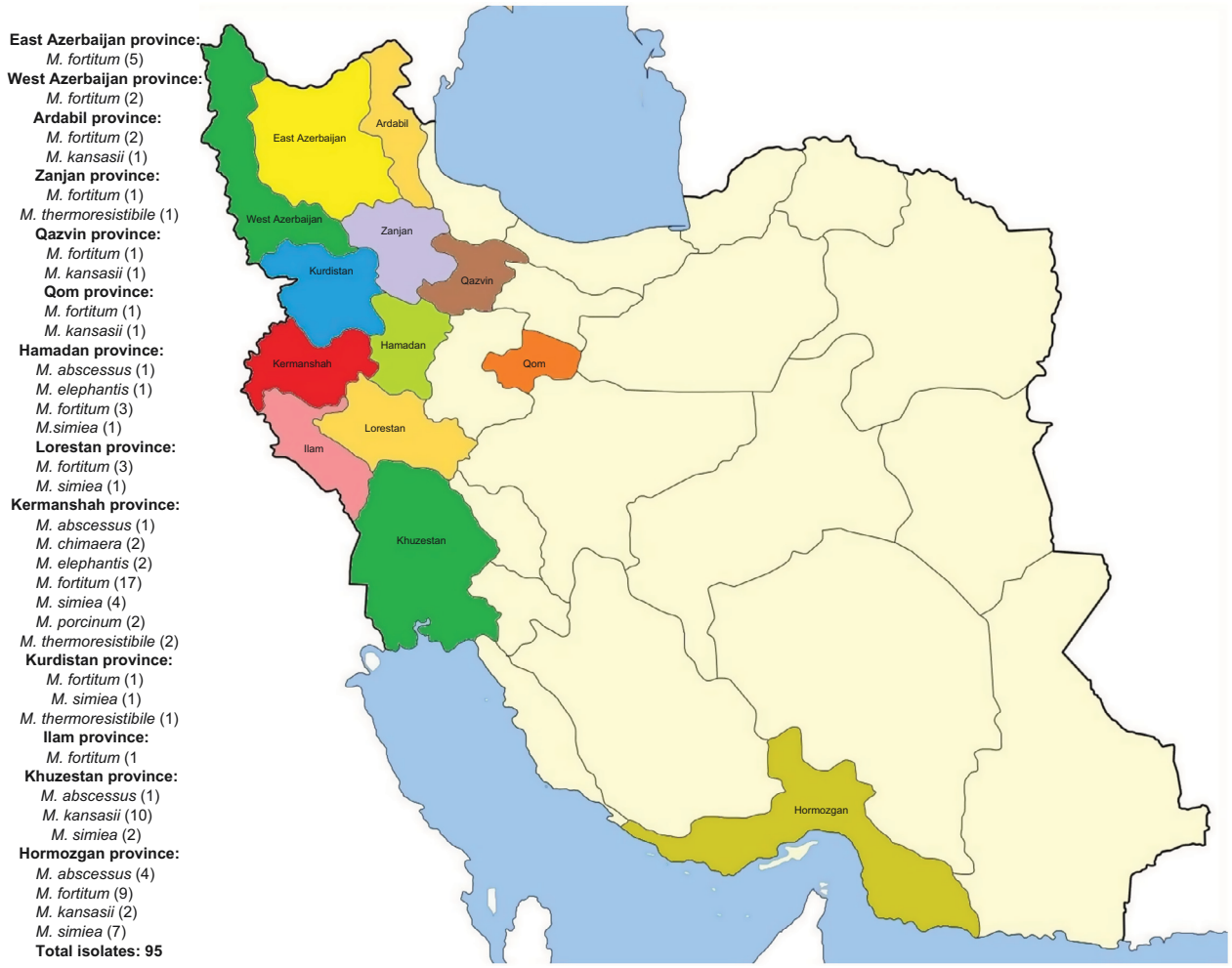

Figure 2 Geographic prevalence of NTM species among the 13 provinces of Iran.
Notes: The regions are marked in different colors. The numbers of isolates and the constituent ratios are added to the side of the map.
Abbreviations: *M. abscessus*, *Mycobacterium abscessus*; *M. chimaera*, *Mycobacterium chimaera*; *M. elephantis*, *Mycobacterium elephantis*; *M. fortuitum*, *Mycobacterium fortuitum*; *M. kansasii*, *Mycobacterium kansasii*; *M. simiae*, *Mycobacterium simiae*; *M. thermoresistibile*, *Mycobacterium thermoresistibile*; NTM, nontuberculous mycobacteria.

and *alr* genes has been reported in D-cycloserine-resistant *Mycobacterium smegmatis*.[46] Another report demonstrated that those mutations in *alr* and *ald* genes and the loss of function in the *ald* gene conferred resistance to D-cycloserine in *M. tuberculosis*.[47] In concordance with our findings, Huang et al recently reported the suitability of D-cycloserine for the treatment of both *M. avium*- and *M. intracellulare*-associated diseases,[16] in addition, Cowman et al reported that cycloserine, tigecycline, and clofazimine might be useful in the treatment of the most resistant SGM.[48] These reports emphasize on the D-cycloserine efficacy against other NTM and *M. tuberculosis* clinical isolates as well. A limitation of two drugs' combination, which was studied for synergy in this work, was small sample size; hence, we suggest to use the method with a larger sample size in future studies to

Table 3 Distribution of clinical NTM strains throughout 13 Iranian provinces

Province	*Mycobacterium* strain number	NTM, n (%)
East Azerbaijan	31	5 (16.1)
West Azerbaijan	14	2 (14.3)
Ardabil	19	3 (15.8)
Zanjan	13	2 (15.4)
Qazvin	21	2 (9.5)
Qom	11	2 (18.2)
Hamadan	59	6 (10.16)
Lorestan	26	4 (15.38)
Kermanshah	223	30 (13.45)
Kurdistan	24	3 (12.5)
Ilam	8	1 (12.5)
Khuzestan	126	13 (10.31)
Hormozgan	139	22 (15.8)

Abbreviation: NTM, nontuberculous mycobacteria.

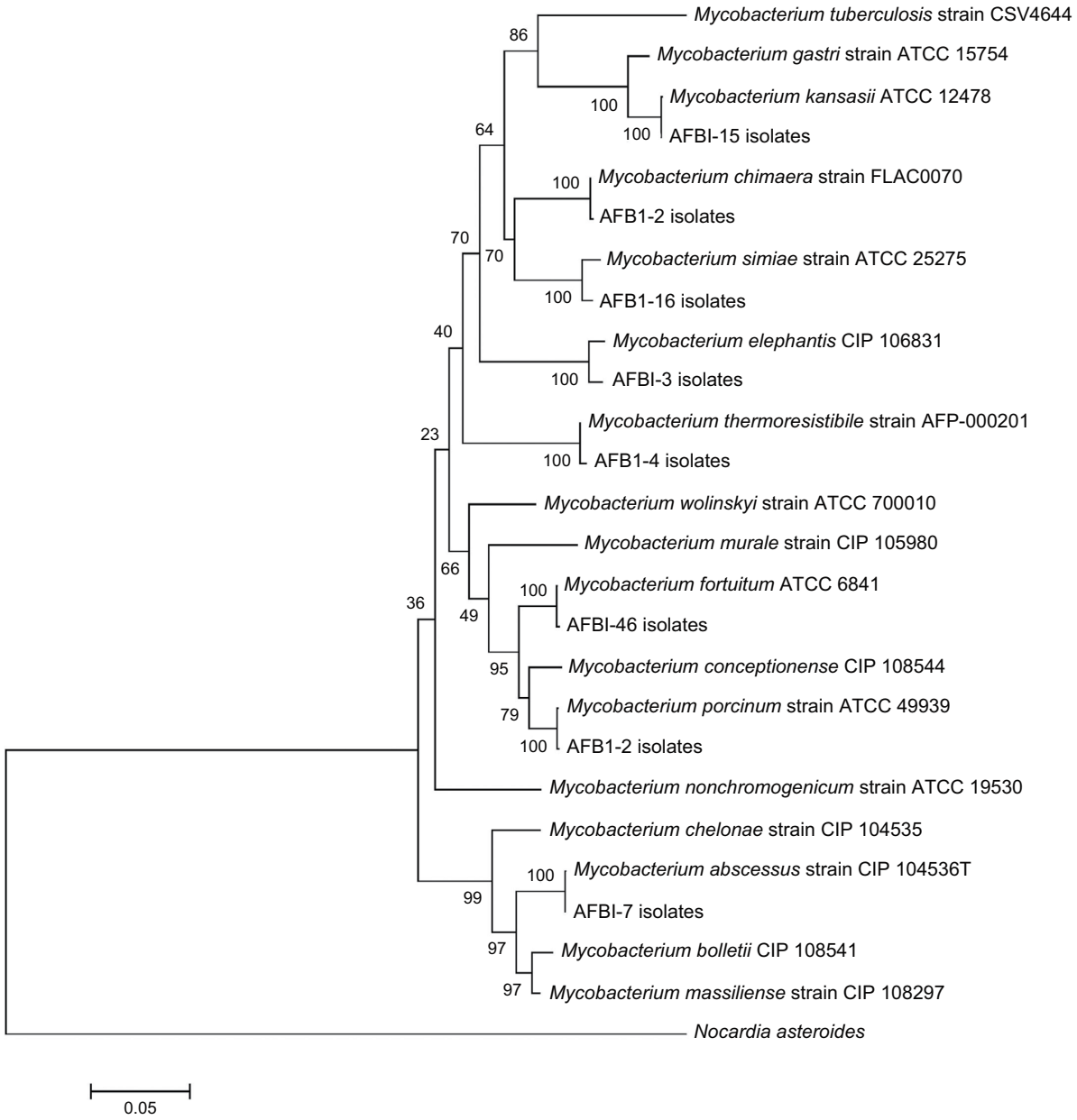

Figure 3 Phylogenetic relationships of 95 clinical and type strains.
Notes: The methodology for phylogenetic trees was inferred using the neighbor-joining method and verified by the maximum likelihood method with 1,000 bootstrap replications. The evolutionary distances were computed using the Kimura 2-parameter method.

Table 4 In vitro activity of tested antimicrobials against *M. fortuitum* and *M. abscessus* clinical isolates

Antimicrobial agent	MIC (μg/L)			Number of isolates (%)			Total
	Range	50%	90%	S	I	R	
M. fortuitum							
Clarithromycin	2–256	8	16	10 (21.7)	10 (21.7)	26 (56.5)	46
D-Cycloserine	2–256	8	32	41 (89.1)	3 (6.5)	2 (4.4)	46
M. abscessus							
Clarithromycin	2–256	8	16	0	0	7 (100)	7
D-Cycloserine	2–256	8	32	7 (100)	0	0	7

Abbreviations: I, intermediate; *M. abscessus*, *Mycobacterium abscessus*; *M. fortuitum*, *Mycobacterium fortuitum*; MIC, minimum inhibitory concentrations; R, resistant; S, susceptible.

Table 5 The synergistic effect test of clarithromycin and D-cycloserine, alone and in combination, against selected clarithromycin-resistant *M. abscessus* and *M. fortuitum* isolates

Strains	MIC (μg/mL)				MIC fold change	FICI in combination	Relationship
	Clarithromycin		D-Cycloserine				
	Alone	Combined	Alone	Combined			
M. fortuitum (n=6)							
12b	8	2	32	8	4	0.5	Synergism
9k	16	4	64	16	4	0.5	Synergism
11k	8	1	16	4	4.8	0.375	Synergism
45k	16	4	64	16	4	0.5	Synergism
43k	8	1	8	2	5.3	0.375	Synergism
50k	16	4	32	16	2.4	0.5	Synergism
M. abscessus (n=7)							
4b	8	2	8	4	2.6	0.75	Indifference
6b	16	4	16	8	2.6	0.75	Indifference
9b	8	2	16	4	4	0.5	Synergism
13k	8	1	8	2	5.3	0.375	Synergism
15k	8	2	16	4	4	0.5	Synergism
884	8	1	8	2	5.3	0.375	Synergism
92	16	4	32	8	4	0.5	Synergism

Abbreviations: FICI, fractional inhibitory concentration index; *M. abscessus*, *Mycobacterium abscessus*; *M. fortuitum*, *Mycobacterium fortuitum*; MIC, minimum inhibitory concentrations.

achieve a better understanding of the synergistic effect of present antibiotics.

Conclusion

The present study showed that the pulmonary samples comprise a wide range of NTM in different provinces of Iran. Based on our observations, we came to the conclusion that D-cycloserine is very active against both *M. abscessus* and *M. fortuitum*. D-Cycloserine, either alone or in combination with clarithromycin, may be promising for the treatment of *M. abscessus*- and *M. fortuitum*-associated diseases. Future clinical trials are needed to confirm our findings.

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Disclosure

The authors report no conflicts of interest in this work.

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