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# Susceptibility Testing of *Mycobacterium frederiksbergense* Strains Isolated from Alfalfa Plants against Antibacterial Compounds

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## Abstract

**Mycobacteria constitute vast population of bacteria that are found in animals, plants and outer environments. Tuberculosis mycobacteria are a group that causes very dangerous diseases for human and animals. *Nontuberculous mycobacteria* are mainly environmental and are pathogenic in certain circumstances. There are reports of finding mycobacteria in plant tissues, which can be effective to survey in case of different infectious disease issues. In a previous study, we found mycobacteria strains in alfalfa vascular tissues and identified them to species level using 16S rRNA gene sequence analysis, causing some symptoms in plant tissues and aerial parts. As these rapidly growing mycobacteria (RGM) have the potential to cause opportunistic infections in human tissues, one of the most important issues for dealing with these agents is to find the best and most effective drug to treat them. Here, we conducted some assays to investigate the MIC of 7 antibiotics and two antibacterial agents against eight *Mycobacterium frederiksbergense* alfalfa isolated strains, in order to find the susceptible and resistant ones. We performed susceptibility testing based on broth microdilution method using Mueller hinton broth as the medium used. Results were different among the strains and different resistant and susceptible reactions were observed among different strains against each drug tested, but ethambutol resulted in similar high MIC for all of them. This variability could be attributed to many factors, such as plant tissue composition and developmental stage from which the strains have been isolated, existence and nature of drug targets and other factors such as test conditions and environmental causes. These results can bring us some insights into design and develop new drugs for treating these kinds of organisms, based on the variations in susceptibility reactions and the plant composition effect on them.**

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## Keywords

***Nontuberculous mycobacteria*, Opportunistic Infections, *Mycobacterium frederiksbergense*, Broth Microdilution**

## 1. Introduction

Mycobacteria are a group of bacteria that some of their species are very pathogenic to human and some animals. This genus of bacteria is divided into different categories, *Tuberculosis mycobacteria* which are very slow growing and nontuberculous which are slow and rapidly growing. *Mycobacterium tuberculosis* and *Mycobacterium leprae* are two very dangerous species that are the agents of lethal diseases, like tuberculosis and leprosy. *Nontubercolous mycobacteria* are mainly environmental [1] and occasionally have been observed for opportunistic infections in human tissues, especially hospital born infections [2]. Also, some of them are environmentally important, for example in bioremediation reactions [3]. Moreover, there are some reports of finding rapidly growing mycobacteria or RGM in plant species. In a recent study, we found *Mycobacterium* strains in alfalfa vascular tissues [4]. Other reports are as interference of *Mycobacterium* species with growth of Scots pine seedlings [5] and finding mycobacteria as hidden endophytes in shoot of rock plant [6]. Having indicated above, fast growing mycobacteria being belonged to "Group IV" of the Runyon classification are exceedingly being dissected as etiological factors of some infectious diseases, mostly pulmonary in patients having cystic fibrosis (*Mycobacterium abscessus*) and either public attained (*Mycobacterium fortuitum*) or cutaneous putrefaction accompanying health care issues (*Mycobacterium chelonae*) [7]. There is a big limitation on the antibiotic range for treatment of infections followed by these organisms and they are not strong. Consequently, new drugs are urgently needed to treat infections due to rapidly growing mycobacteria. Recently, tigecycline and to a less extent linezolid have been shown to have some *in vitro* activity against rapidly growing mycobacteria [8] [9].

Antimicrobial susceptibility testing is important for species that are considered clinically significant. The Clinical and Laboratory Standards Institute (CLSI) recently published guidelines and recommendations for testing of *Nontuberculous mycobacteria*. Broth micro dilution was recommended for isolates of rapidly growing mycobacteria [10]. Reliable analyzing of RGM, esp. strains that have been proven to have pathogenic effects on human tissues, is very important. One of the important issues about these types of mycobacteria is susceptibility testing of these agents against different antibacterial compounds for introducing most effective antibiotic. Plant associated mycobacteria which are either identified in the plant tissues or have some interaction to be infective for plants or have the potential to cause infections in animal cells, can consequently be good candidates for investigation in different analyses. As mentioned above, we identified some isolates of mycobacteria in alfalfa vascular tissues and also, some symptoms were observed in stem vascular inner tissues of alfalfa following *Mycobacterium* inoculation which should be practiced more for confirmation of these observations. As a result, strains that could cause some infections in human tissues and also have some effects on plants structure and growth may have some characteristics that can grow and colonize in both plant and animal tissues.

In this study, we aimed to perform some susceptibility testing on a number of *Mycobacterium* isolates from alfalfa against some antibacterial agents that have been tested in clinical isolates of this species.

## 2. Materials and Methods

### 2.1. Bacterial Strains

Eight rapidly growing mycobacteriastrains were estimated in this experiment of plant origin, which previously isolated from alfalfa plants and identified to the species level as *Mycobacterium frederiksbergense*, using 16s rRNA gene sequence analysis [4]. They were purified and cultured by streaking on *Corynebacterium* agar (BioChemika, Germany) plates and 2 days fresh cultures in 30˚C were used for susceptibility testing. Two strains, BCG (1173P, Pasteur Institute of Iran) and *Mycobacterium smegmatis* strain (PTCC 1307, Iranian Research Organization for Science and Technology) were used as reference strains.

### 2.2. Antibacterial Agents

The mentioned 8 isolates were evaluated for their susceptibility against nine antibacterial compounds as the fol-

lowing: ethambutol, phenylglyoxal, isoniazid (Sigma, Germany), juglone, streptomycin (GibcoInvitrogen, USA) [11]-[14], ciprofloxacin, amikasin,imipenem and doxycycline (ParsDaru, Iran), which are commonly applied to treat NTM[1] infections except juglone, which has been shown to have antibiotic effects on both fungi and bacteria [15].

### 2.3. Susceptibility Testing

Susceptibility testing was performed using broth microdilution method as the standard procedure for RGM [16]. Inocula were prepared from 48 hours broth cultures and wereadjusted to McFarland 0.5 turbidity by sterile distilled water and then diluted moreto reach a density of $1.5 \times 10^5$ cfu/ml, when applied to the microplates, bacterial densitywas to $1.5 \times 10^4$ cfu/ml. Mueller Hinton broth (Sigma, Germany)was used as the medium in this assay. Dosages of the compounds that were used in this study were 0.5 mg/ml for ethambutol andphenylglyoxal, 0.125 mg/ml for streptomycin, 0.062 mg/ml for isoniazid and juglone, 0.015 mg/ml for imipenem and doxycycline, and 0.007 - 0.015 mg/ml for amikasin and ciprofloxacin tothe first well. DMSO[2] (Sigma, Germany) was applied as negative control to investigate the antibacterial activity of the compounds assayed. The microplates were incubated for 72 hours in 30˚C.

## 3. Results

Eight isolates from different fields in Hamadan province were analyzed. Results were examined visually based on the turbidity of bacterial cells in each column and the well without visual growth in each column was regarded as the MIC of each compound against mycobacterial isolates. The results are shown in **Table 1**.

Results presented in **Table 1** indicate different susceptibility patterns among the 8 isolates against each drug tested, except ethambutol, which had the same MIC against all of the strains of *Mycobacterium frederiksbergense*. This variability fell into 3 groups for 5 of the antibiotics, 2 for three of them and MIC of ethambutol was the same for all the isolates.

The most powerful drug on these strains was ciprofloxacin. Among them, isolates 12, 32 and 53 showed highest susceptibility against ciprofloxacin and its MIC was 0.01 µg/ml. The other 5 isolates with more resistant reactions were included in a MIC range of 0.03 to 0.06 µg/ml.

The other antibiotic, to which most susceptibilities were observed was doxycycline, MIC against isolates 16 (1), 38, 24 and 14 was 0.06 µg/ml, whilst for 3, 12 and 53 was 0.12 µg/ml and for 32, 0.24 µg/ml. Amikasin with less effect had a MIC of 0.12 for 5 strains, except 16 (1), 24 and 53 with MIC's 0.97, 0.48 and 0.06, respectively and here 53 is regarded as the most susceptible one. For other 6 compounds, imipenem, streptomycin,

**Table 1.** MIC's (µg/ml) of the 9 tested drugs and antibacterial compounds against the eight isolates.

| Compounds | Isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 12 | 16(1) | 38 | 14 | 32 | 24 | 53 | *M. smegmatis* (PTCC1307) | BCG (1173P) |
| Ethambutol | 15.62 | 15.62 | 15.62 | 15.62 | 15.62 | 15.62 | 15.62 | 15.62 | 0.094 | 0.8 |
| Isoniazid | 1.95 | 1.95 | 1.95 | 1.95 | 1.95 | 1.95 | 0.48 | 3.90 | 8 | 0.05 |
| Juglone | 1.95 | 1.95 | 1.95 | 1.95 | 1.95 | 3.90 | 1.95 | 1.95 | 1.50 | 0.78 |
| Phenylglyoxal | 31.25 | 31.25 | 31.25 | 31.25 | 62.5 | 62.5 | 62.5 | 31.25 | >62.5 | >125 |
| Streptomycin | 3.90 | 3.90 | 3.90 | 1.95 | 3.90 | 1.95 | 1.95 | 3.90 | 1.5 | 0.12 |
| Amikasin | 0.12 | 0.12 | 0.97 | 0.12 | 0.12 | 0.12 | 0.48 | 0.06 | 0.12 | 0.015 |
| Imipenem | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 | 0.48 | 0.48 | 0.12 | 25 | 20 |
| Ciprofloxacin | 0.03 | 0.01 | 0.03 | 0.03 | 0.03 | 0.01 | 0.06 | 0.01 | 0.05 | 1.5 |
| Doxycyclin | 0.12 | 0.12 | 0.06 | 0.06 | 0.06 | 0.24 | 0.06 | 0.12 | 0.07 | 0.09 |

[1]*Nontuberculous mycobacteria*.
[2]Dimethyl Sulfoxide.

juglone, isoniazid, ethambutol and phenylglyoxal the susceptibility patterns was consistent with more resistant reactions and for two compounds ethambutol and phenylglyoxal, most strains were very resistant with high MIC's. AS a whole, isolates 53 and 24 showed the most susceptibility against all the compounds, except ethambutol for which the MIC was the same and isolate 3 was regarded as the most resistant bacterium. The results are shown in **Table 1**.

## 4. Discussion

Mycobacteria constitute a group of bacteria that are very important in regard of their impact on human health. Two major diseases influencing human tissues are tuberculosis and leprosy out of which tuberculosis is under strict focus, because of its high lethality in populations. Each year millions of people in the world die of tuberculosis, so there are many programs and projects for controlling these organisms. One of the main disease controlling projects that today are being performed in many clinical studies is searching for the most effective compounds and antibiotics on *Mycobacterium tuberculosis.* It has been under organized research now and before. Many researchers have performed analyses of the susceptibility of this bacterium against different compounds or under various trials to invent novel and fast protocols to best combat this terrible disease. Birinci *et al* (2002) in a study compared three susceptibility testing methods, MGIT, E-test and the proportion method and found no statistically significant difference between them [17]. Their results showed that MGIT and E-test methods are more applicable than the proportion method, because reaching the final result takes more time in proportion method than the two others. Martin *et al.* (2003) in an effort to examine second line drugs for suppression of multidrug resistant (MDR) TB, introduced a new susceptibility test method by using colorimetric method that was very faster, less expensive and more cost-effective than the proportion method and demonstrated that the results are well correlated with the proportion method [18].

Besides *Mycobacterium tuberculosis* which is very significant and falls into the slow growing category of mycobacteria, are the rapidly growing mycobacteria that are environmental. Occurrence of rapidly growing or atypical mycobacteria in different environments, soil, water [19], plants [6] [4] [20], human tissues (causing some infections) [19] indicates expanded ubiquity of these microorganisms. Some RGM species may be potential agents to initiate opportunistic infections in humans, such as *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium abscessus* group [19]. *Mycobacterium abscessus* and *Mycobacterium fortuium* are the agents of nontuberculous disorders in human that cause pulmonary infections. Some species like *Mycobacterium smegmatis* have been also depicted to give rise to rare pulmonary infections in human [21] [22].

As a rapidly growing environmental *Mycobacterium* species, *M. frederiksbergense* was found in alfalfa plants that had been sampled from different fields but from a same region [4]. The bacterium was isolated as an endophyte residing in inner vascular tissues of alfalfa. Also, it was observed that it may have formed some trace on the tissues from which it had been isolated. We aimed to analyze susceptibility reactions of some of the mentioned isolates to a variety of antibiotics and compounds that are currently used for suppressing *M. tuberculosis* and some other drugs. While, there are few reports of identification of *Mycobacterium* sp. in different plants and no previous documents for alfalfa, so considering different biological aspects of these alfalfa tissue isolated strains could be of great importance, especially that there are many reports of isolation of these rapidly growing species from human infected tissues and observation of opportunistic infective behavior for some of these species. In an article they have been shown to be potent of causing some cutaneous infections in humans [23]. Regarding the results of this experiment, these eight isolates presented different susceptibility responses against the antibiotics and compounds. Ranging from resistance to susceptible reactions, and also this variety was observed between isolates against one type of antibiotic. All the strains had the same susceptibilities against ethambutol. The most variable MIC belonged to amikasin, for which 4 different MIC values (ranging from 0.06 to 0.97 µg/ml) for the isolates were obtained. The other two antibiotics, to which the isolates showed most varied susceptibility values, were ciprofloxacin and doxycycline and 3 different MIC values were observed (ranging from 0.01 to 0.06 µg/ml forciprofloxacin and 0.06 to 0.24 for doxycycline). Imipenem and streptomycin was the two latter antibiotics having varied MIC values with three and two different values. The most powerful antibiotics against these isolates were ciprofloxacin, doxycycline and amikasin with MIC's ranging from 0.01 to 0.48 µg/ml. Juglone was also effective against these strains with low MIC's.

The variability and differences in the susceptibilities of these isolates could be attributed to different reasons. Researches about endophytic organisms of plants have shown that different factors involving environmental and

biological, like plant cultivar and age, type of tissues, etc have impacts on them [24]-[28]. Residing in the tissues of different varieties of alfalfa sown in some different fields could have brought some variations in the characteristics of this bacterium. They are isolated from the same plant species, but from different regions, so it may have been a relation between the plant composition variations and their effect on the susceptibility reactions of the strains isolated. Because of their isolation in different regions, there may have been a cause of climatic difference for these susceptibility variations; and the plant developmental stage or genotype from which these strains were isolated. Andreot *et al*. (2010) showed that type of bacterial media, different genotypes of plants and developmental condition, impact bacteria residing in roots of potato and some other endophytes [29]. Also, the interaction or antagonistic effects of other endophytic bacterial species may have affected these results. These varieties are noticed in the reactions of the isolates to the antibiotics to which they are susceptible and very susceptible, so impacts that the environment may have on them cannot be justified to direct them to more resistant types and it may have lead them to more susceptible reactions. These traits could have been the outcome of responses of the isolates against the plant environment and materials from which these strains have been isolated and alterations in genes controlling susceptibility or resistance reactions of the bacteria to the compounds tested. Because of these variations in the susceptibility responses, it would be a good idea to test more compounds and monitor the responses and choose the most susceptible organism and taking them into more investigations. Also, there would exist this possibility to extract some materials from alfalfa inner tissues and use them as some antibacterial compounds to test the susceptibility responses, especially alfalfa varieties from which the most susceptible ones are isolated and those varieties may have led these strains to be more susceptible genetically.

Taking into consideration the menacing role of tuberculosis mycobacteria for the health of world population and the significance and seriousness of finding most fruitful and fastest methods and solutions to challenge this bacterium and discovering novel and less worrisome remedies for infected patients and on the other part, the laboriousness and hazards of working with *Mycobacterium tuberculosis* has forced microbiologists to seek alternative agents and procedures to experiment combating tuberculosis.RGM also could be good candidates for investigation, both for themselves and for being an alternative for the experiments including *Mycobacterium tuberculosis.* Different works have been also done in order to test different antibiotics for susceptibility testing of *Nontuberculous mycobacteria* that may be infectious to human tissues [19] [23] [30]-[31]. As an instance, *Mycobacterium smegmatis* could be a good option and as a model organism in surveying *Mycobacterium tuberculosis*, because it shows similar susceptibility pattern as *M. tuberculosis* to some drugs [32] [33]. Another advantage of working with RGM is their rapid growing and giving more rapid results than tuberculosis.

## 5. Conclusions

According to these observations and previous works, it can be suggested that these environmental bacteria may have potentials in designing new strategies and remedies for medical microbiology especially that they have been found in both plant tissues and observed to have some trace on alfalfa vascular and also they can produce some opportunistic infections in human. Therefore, providing more information for their reactions to various compounds could be used as a model in designing new and more effective drugs against dangerous agents like Tuberculosis, as these phyto RGM grow faster and are less risky than *M. tuberculosis*. Hence, more researches on them could be safer than tuberculosis strains. In this case, investigation of their susceptibility responses and comparison with tuberculosis strains could be very effective and applicable.

By consideration this issue that *Mycobacterium tuberculosis* is a dangerous bacterium and slow growing and working with them in the laboratory is labor intensive, these *M. frederiksbergense* isolates might be investigated for application as model organisms as a replace for *Mycobacterium tuberculosis.* As these rapidly growing mycobacteria show various susceptibilities against the compounds tested, it can be proposed to recognize and isolate the genes responsible for these behaviors and use them as some models to target *Mycobacterium tuberculosis* strains.

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