**Original article**

# A method to survey heat labile anti-tuberculosis drugs

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**Abstract**

**Introduction and objective:** The appearance of resistance to anti-tuberculosis drugs has generated research to find new and more effective drugs. Löwenstein-Jensen medium (LJ) is frequently used for culturing strains of *Mycobacterium tuberculosis*. A group of antimicrobial substances used in treating tuberculosis is sensitive to heat and cannot be used on LJ medium. Research now aims to setup a modified method for evaluation of heat labile drugs in LS medium.

**Materials and methods**: In this study, we investigated culturing *M. tuberculosis* for 48h on Middlebrook 7H9 broth medium with antituberculosis drugs and re culturing on LJ medium (without antibiotic) and incubating for 40 days.

**Results:** Our results after 48h of contact of the strains with antibiotic were comparable with the standard method of culture on Middlebrook 7H10 agar medium containing antibiotic. Therefore, 48h is a suitable time for primary contact between mycobacterium and heat labile antibiotics.

**Conclusion:** This modified method can be applied to LJ medium instead of expensive Middlebrook 7H10 agar medium for evaluation of heat labile anti tuberculosis drugs.

**Keywords:** Löwenstein-Jensen medium, Heat labile anti-tuberculosis drugs, *Mycobacterium tuberculosis*

## Introduction

Tuberculosis (TB) has a long and continuing history of causing worldwide morbidity and mortality. The emergence of multidrug-resistant TB (MDR-TB), defined as resistant to at least isoniazid (INH) and rifampin (RIF), the two principal first line anti-TB drugs, poses an important threat to TB control. MDR-TB reduces responses to standard short-course chemotherapy with first-line anti-TB drugs, leads to higher mortality and treatment failure rates, and increases the period of transmissibility of the disease [1-3]. Multidrug-resistant tuberculosis is an increasing problem worldwide [4]. MDR-TB is associated with significant mortality [5] and has resulted in serious institutional outbreaks [6].

Rapid diagnostic assays for MDR-TB should address these problems by enabling early isolation and treatment of patients with this disease [7-8]. Rifampin resistance

is an excellent marker for multidrug-resistant *M. tuberculosis*, as 90% of rifampin-resistant *M. tuberculosis* strains are also isoniazid resistant and, hence, are classified as multidrug resistant [9]. Over 400,000 MDR-TB cases emerge every year, 50% amongst new TB cases, and 50% in previously treated TB patients [10]. Approximately 5–7% of these cases are expected to have extensively drug-resistant TB (XDR-TB) [11]. MDR-TB and XDR-TB are associated with an extremely high mortality, especially in the human immunodeficiency virus (HIV) co infected [12-13]. Therefore, rapid detection and new anti tuberculosis drug are need to treat multidrug resistant tuberculosis [14].

Resistance against 11 anti-TB drugs has also been reported [15]. One of the health priorities of the WHO is to find new anti-TB agents [16]. The conventional microbiological methods for drug susceptibility testing have been well tested and their strengths as well as limitations are known. Among the conventional methods, the three widely known approaches are proportional, resistance ratio, and absolute concentration methods. Proportion method has been considered the most reliable and is taken as a reference for comparing any method. These conventional microbiological procedures, though quite robust, take several months (can be reduced by direct testing in specimens with sufficient number of bacteria) and as a result the search for alternatives has been accelerated in recent years [17].

The anti-TB effect of tested drugs are carried out mainly using the standard method with Middlebrook 7H10 agar and Löwenstein-Jensen (LJ) medium in the laboratory [18]. The diagnostic medium most often used is LJ. However, because this medium requires heat for preparation (90°C/2h), heat labile drug substances like Allicin (garlic extract) cannot be evaluated by the above method [19-21]. Additionally, the lengthy incubation period required to culture mycobacterium colonies cause uncertainty relative to contact of bacteria with appropriate concentrations of antimicrobial substances. Middlebrook 7H9 broth medium eliminates this problem but needs an automated system to record growth speed of bacterium which is expensive and not available in many less equipped laboratories. By combining the two aforementioned methods, drug sensitivity of *M. tuberculosis* can be assessed without these drawbacks. This method may be used in all of research centers and laboratories to assess bacterial growth in a short period.

## Materials and methods

### *Bacterial strains*

Four strains of *M. tuberculosis* resistant to various drugs (INH, RIF, ETB, SM) isolated from pulmonary patients referring to pulmonary disease centers (National research institute of tuberculosis and lung disease- IRAN) were used in this study. In addition, a standard strain of *M. tuberculosis* (H37RV) was also used. The isolated strains were identified based on standard diagnostic methods [17]. The isolated strains were immediately maintained after isolating and identifying in skim milk medium at a temperature of -80$^{o}$C for the next stages.

### *LJ medium*

The combination of glycerol, asparagin, egg and the starch of potato with of malachite green was used to make LJ medium [22]. Since garlic extract (Allicin) is sensitive to the temperatures greater than 58°C, it was added to the medium at a temperature of 45°C [23]. Middlebrook 7H10 agar medium (Difco Co.) was used and each 180ml was complemented with 20ml of rich oleic acid, cow albumin, dextrose, and citrate (OADC) [24]. To prepare dilutions of antibiotics, Middlebrook 7H10 agar and Middlebrook

7H9 broth containing dilutions of four antibiotics (2µg/ml isoniazid, 4µg/ml streptomycin, 2µg/ml ethambutol and 4µg/ml rifampin) were prepared according to WHO and CDC standards [25].

*Bacterial contact with antibiotics*

A dilution equivalent to number one turbidity of Macfarland tube was prepared from colonies of bacteria after growing each of the strains on LJ medium. Dilutions of $10^{-1}$, $10^{-3}$, and $10^{-5}$ bacteria per milliliter was prepared from this tube and added to media containing antibiotics. In samples of Middlebrook 7H9 broth media, they were sampled after inoculating bacteria in 12, 24 and 48 hours, and bacteria were washed 3 times with PBS in order to omit antibiotic from the surface of bacterium. Then, it was cultured again on LJ medium until the capability of the growth and characteristics of the colonies were investigated on the medium during the maintenance time period. The results were read after 28 and 41 days.

## Results

The characteristics of the selected strains and antibiogram results with standard method in Middlebrook 7H10 agar medium and incubating for 41 days show in table 1. In modified method, after contacting of mycobacteria with selective antibiotics (RIF, INH, ETB and SM) in Middlebrook 7H9 broth media for 12h and subculture that in LJ medium. It was seen that strains A, B and C, had no changes in comparison with the standard antibiogram method. Whereas, strains of D and E completely showed resistance to antibiotics (Table 2). However, after 24 hours of primary exposure with antibiotics, the resistance of strain E revealed similar results with standard antibiogram method (table 3). Strain D remained uniformly resistant. Table 4 shows the obtained results after 48h of contact of the strains with antibiotic, the obtained results correspond with standard results. Therefore, 48h is a suitable time for contact between mycobacteria and heat labile antibiotics

**Table 1:** The characteristics of the selected strains and antibiogram results with standard method in Middlebrook 7H10 agar medium and incubating for 41 days

| *Strains* | RIF | INH | ETB | SM |
|---|---|---|---|---|
| *M. tuberculosis* (A) (H37RV) | S | S | S | S |
| *M. tuberculosis* (B) | R | R | R | R |
| *M. tuberculosis* (C) | S | S | S | S |
| *M. tuberculosis* (D) | R | S | S | R |
| *M. tuberculosis* (E) | S | S | S | S |

**Table 2:** The results of contacting bacterium with antibiotics in Middlebrook 7H9 broth medium for 12 hours and passage on LJ medium and incubating for 41 days

| *Strains* | RIF | INH | ETB | SM |
|---|---|---|---|---|
| *M. tuberculosis* (A) (H37RV) | S | S | S | S |
| *M. tuberculosis* (B) | R | R | R | R |
| *M. tuberculosis* (C) | S | S | S | S |
| *M. tuberculosis* (D) | R | R | R | R |
| *M. tuberculosis* (E) | R | R | R | R |

**Table 3:** The results of contacting bacterium. with antibiotics in Middlebrook 7H9 broth medium for 24 hours and passage on LJ medium and incubating for 41 days

| *Strains* | SM | ETB | INH | RIF |
|---|---|---|---|---|
| *M. tuberculosis* (A) (H37RV) | S | S | S | S |
| *M. tuberculosis* (B) | R | R | R | R |
| *M. tuberculosis* (C) | S | S | S | S |
| *M. tuberculosis* (D) | R | R | R | R |
| *M. tuberculosis* (E) | S | S | S | S |

## Discussion

Tuberculosis is one of the oldest diseases the human being has been in struggle against it. Tuberculosis discovered in 1882 by Robert Koch, is still a major health problem today, not only the third world

countries, but also in the industrialized world. This disease has resurfaced again after a period of reduction [26].The disease after a period of fall again escalates and today, the human society despite improvements in many areas of life has many troubles with this disease. Still the civilized world is suffering from this disease that was forced to experience a new challenge called aids. AIDS has caused diversified disease. It is right in the case of atypical *M. tuberculosis* which has more complicated the problem. Today, drug resistance has become a major medical crisis in some countries, and is increasing the number of individuals afflicted [27].

**Table 4:** the results of contacting bacterium with antibiotics in Middlebrook 7H9 broth medium for 48 hours and passage on LJ medium and incubating for 41 days

| *Strains* | SM | ETB | INH | RIF |
|---|---|---|---|---|
| *M. tuberculosis* (A) (H37RV) | S | S | S | S |
| *M. tuberculosis* (B) | R | R | R | R |
| *M. tuberculosis* (C) | S | S | S | S |
| *M. tuberculosis* (D) | R | S | S | R |
| *M. tuberculosis* (E) | S | S | S | S |

In order to get rid of the problem of tuberculosis the discovery of new anti-tuberculosis drugs is significant. For example, the use of herbal and traditional medicine is under consideration today. The use of herbal medicine for treatment of diving diseases has been under consideration since the very old age [20-23]. Some of anti-TB drugs are heat labile. In order to test drug susceptibility testing in LJ medium, we need to modify method. Research now aims to setup a modified method for evaluation of heat labile drugs in LJ media. *M. tuberculosis* is an acid-fast bacterium with *in vitro* cell division period of about 17 to 20 hours [22].

Adding antibiotics to LJ medium always propounds this question of whether or not the antibiotic resistance is stable during the 41-day incubation period required until results can be read. The heat labile antimicrobial substances cannot be tested in LJ medium because this medium needs temperatures of 90°C for coagulation [22,23]. The contact period of bacteria with antimicrobial substances has been estimated to be 48h in this reformed method. On the other hand, with regard to the generation time of the bacteria, the bacterium divides at least twice, may contact antimicrobial substances be effective in different stages of bacterium reproduction. Additionally, the half-life of antibiotics during 48 hours is greater in comparison with time duration of 41 days. Incubation time of bacteria in this method does not require antibiotic in the medium, and naturally, no questions can be raised regarding the half-life of antibiotic. The use of this method provides the possibility to test heat labile antimicrobial substances with minimal facilities in diagnostic laboratories.

## Conclusion

For the evaluation of heat labile anti tuberculosis drugs we need Middlebrook 7H10 agar that is very expensive and inapplicable, approximately. But our modified method suggest that we can apply LJ media because it is cheap and routine for laboratories

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