Scholars Academic Journal of Biosciences
Abbreviated Key Title: Sch Acad J Biosci
ISSN 2347-9515 (Print) | ISSN 2321-6883 (Online)
Journal homepage: https://saspublishers.com/sajb/

OPEN ACCESS

Integrative Biology

Detection of Mycolic Acid Implying the Presence of Mycobacterium Tuberculosis $H_{37}Rv$ in Host

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DOI: 10.36347/sajb.2021.v09i08.001 | **Received:** 18.12.2020 | **Accepted:** 05.01.2021 | **Published:** 09.08.2021

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Abstract **Review Article**

Tuberculosis is a widespread infectious disease that affects millions of people worldwide every year. The bacterium, responsible for causing tuberculosis is *Mycobacterium tuberculosis* $H_{37}Rv$ (*Mtb*) which belongs to *Mycobacteriaceace* family. It is a gram positive bacteria and highly aerobic in nature which enters the host by the means of inhalation. Several tests are performed for the diagnosis of tuberculosis such as chest X-rays, culture, tuberculin skin test, interferon gamma release assays and histopathology. These techniques are expensive as well as take a lot of time in confirming the presence of bacteria. A cost and time effective way to diagnose the disease could be with the help of lipids present in the cell wall of *Mycobacterium tuberculosis*. The cell wall skeleton is chemically composed of three covalently linked components: peptidoglycan, arabinogalactan and mycolic acids. All these components can be exposed by rupturing the cell wall by suitable means. After their exposure mycolic acids can be extracted. *Mtb* contains three types of mycolic acids i.e. alpha, methoxy and keto mycolic acids. These lipids consist of several functional groups which are expected to give positive result for the organic qualitative analysis. These functional groups are alcohols, carboxylic acids, ketone and ether. Each functional group has different tests which one can perform in order to confirm its presence. This review is describing several chemical tests and these can be done by using mycolic acid as sample. The chemical reactions can impart colour, show effervescence or can show precipitation for identification which in turn will confirm the presence of *Mycobacterium tuberculosis* in the patient.

Keywords: *Mycobacterium tuberculosis* $H_{37}Rv$ (*Mtb);* Mycolic Acid (MAs); Extraction of Mycolic Acid; Functional Groups; Chemical tests.

Abbreviations: *Mycobacterium tuberculosis* $H_{37}Rv$ (*Mtb);* Mycolic Acid (MAs); Dinitrophenylhydrazine (DNPH).

INTRODUCTION

Tuberculosis is one of the deadliest diseases across the world [1]. In 2017, aggregate of 6.4 million new cases of tuberculosis were reported to WHO from all over the world. This figure represents 64% of the estimated 10 million new cases happened in 2017. The top three countries accounted were India (26%), Indonesia (11%) and Nigeria (9%). Worldwide, from 2000-2018 usual decline in TB cases was 1.6% and it was 2.0 % in 2017 & 2018. From 2015 to 2018 the total decrease in TB deaths was only 11% which is less than End TB strategy milestone of 35% by 2020 (WHO report 2018, 2019). The bacteria responsible for causing Tuberculosis, is Mycobaterium tuberculosis (Mtb) which belongs to Mycobacteriaceae family. It is a gram positive bacteria and highly aerobic in nature. It enters the host through inhalation as these pathogens are blown out in the air droplets when exhaled by any Tuberculosis infected person [2, 3]. Presence of Mtb in host expresses symptoms such as cough, chest pain, shortness of breath, fatigue, weight loss, fever and night sweats. Several tests can be performed to detect the presence of Mtb in host. These include chest X-rays, culture, tuberculin skin test, interferon gamma release assays and histopathology [4]. All these detection tests take several weeks to confirm the presence of Mtb in infected person. In this article we are describing a hypothesis on the basis of some chemical method of diagnosis which is both time as well was cost effective. Therefore the idea is to detect Mtb by the means of mycolic acids, present in it. Using suitable methods, we can rupture the capsule of bacteria and mycolic acid can be exposed. After this various chemical tests can be performed in order to confirm the presence of Mtb in the sample.

Mycolic Acids in Mycobaterium tuberculosis

Mtb has a waxy coating of mycolic acids on its surface which makes it hydrophobic and alcohol

insoluble, but is soluble in ether due to its nonpolar characteristics. Mycolic Acids (MAs) are α-alkyl-β-hydroxy (with respect to carboxyl group) long chain fatty acids [4]. They exist as homologous series which differs by 28 atomic mass units. There are three different forms of MAs found in Mtb; they are α-mycolic acid, methoxy-mycolic acid and keto-mycolic acid. The α -mycolic acid is present in highest concentration i.e. more than 70% whereas methoxy- and keto-mycolic acid are present in low concentrations i.e. up to 10-15%. The α-MA is cis, cis-dicyclopropyl fatty acid. There are two structural variations in this MA where the number of methyl groups between the two cyclopropane rings differs. Methoxy-MA and keto-MA exists in the form of cis and trans isomers (as shown in Figure-1) [1].

Extraction of Mycolic Acid

The cell wall of mycolic acid is readily defined. It contains cell wall skeleton and heterogeneous assembly of wall associated molecules, lipids and polypeptides. Mycobacterial walls are broken typically using mechanical stress. After that purification is done by the means of differential centrifugation or density gradients to remove unbroken cells, fragments of plasma membrane and cytoplasmic material. Because of these strong forces, the capsule from the wall is also removed and leaves only traces of capsular polysaccharides, arabinomannan and glucan.

The material that is leftover after the removal of all non-covalently bound wall-associated substances is the Cell Wall Skeleton. The cell wall skeleton is chemically composed of three covalently linked components: peptidoglycan, arabinogalactan and mycolic acids [5]. It can be generated by treating crude wall preparations with nucleases, detergents (such as hot sodium dodecyl sulphate), proteases and organic solvents. The cell wall skeleton is a giant molecule which outlines the shape of the mycobacterial cell. To study every part of the cell wall separately, several methods can be used to dissect the cell skeleton and retrieve the constituents. Treating cell wall with 0.1M dilute HCl acid separates peptidoglycan from mycoloyl arabinogalactan, however treating with hot alkali eliminates MAs and also separates the peptidoglycan from the arabinogalactan. Other way is to use warm, dilute methanolic alkali to extract Mas [6].

Functional Groups in Mycolic Acids

Qualitative Analysis in Organic Chemistry helps us to detect several functional groups present in our sample. It is usually done to identify our unknown sample. Here we are trying to detect the presence of MA in our sample (patient sputum) by performing various functional group tests. After rupturing the cell membrane of Mtb, it is expected that MA will be exposed (as shown in Figure-2). The different functional groups present in MA are: alcohol, carboxylic acid, ketone and ether. Since we already know the functional groups present, we will perform the specific tests which confirm the presence of that particular functional group (as shown in Figure-3). If the sample gives positive result for all the tests performed, presence of Mtb will be confirmed.

Test for Hydroxyl (-OH) Group

Sodium Metal Test

It is a preliminary test to detect the presence of alcoholic group. Sodium metal reacts readily with both alcohol and water and replace some parts of hydrogen. Heat is liberated due to the evolution of hydrogen gas. When small amount of sodium metal is added to the sample containing MA, it will react with the alcoholic group present in it and releases hydrogen gas giving brisk effervescence [7].

$$2ROH + 2Na \longrightarrow 2RO^{-}Na^{+} + H_2\,(g)$$

(white) (brisk effervescence)

The by-product formed in the above equation is sodium alkoxide, a white solid, which decomposes by water according to the equation.

$$RO^{-}Na^{+} + H_2O \longrightarrow ROH + NaOH$$

As stated above as well, sodium reacts with water also therefore it is necessary to dry the sample first which can be done by using anhydrous calcium sulphate. There are certain substances which are not alcohol but contain hydroxyl group and therefore react with sodium metal [7]. Therefore this test cannot be used as confirmatory test.

Acetyl Chloride Test

Acyl chlorides are a group of organic compounds which reacts easily with alcohols. Acetyl chlorides are mostly used for this test. When acetyl chloride is reacted with water, acetic acid and hydrochloric acid gas is formed.

$$H_2O + CH_3COCl \longrightarrow CH_3COOH + HCl(g)$$

When acetyl chloride is reacted with alcohol, ester, a fruity smelling compound, and hydrochloric acid gas is formed.

$$ROH + CH_3COCl \longrightarrow CH_3COOR + HCl(g)$$

(fruity)

The hydrochloric acid gas formed is detected by taking a glass rod dipped in ammonium hydroxide and bringing it in vicinity of gas evolving from the reaction mixture. Due to their interaction white fumes of ammonium chloride can be seen.

$$HCl + NH_4OH \longrightarrow NH_4Cl(g) + H_2O$$

(white)

Again some compounds contain hydroxyl group but are not alcohol, therefore can give positive result for the test [7].

Ceric Ammonium Nitrate Test

Ceric ammonium nitrate acts as an oxidising agent for many functional groups such as alcohols, phenols and esters. It is a complex compound having six nitrate ions surrounding cerium atom in bidentate manner. With alcohols, one Ce-O bond of cerium nitrate is replaced by another Ce-O bond of alcohol which leads to the formation of Alkoxy cerium (IV) complex along with ammonium nitrate [8].

$$2ROH + (NH_4)_2Ce(NO_3)_6 \longrightarrow (ROH)_2Ce(NO_3)_4 + 2NH_4NO_3$$

(red)

Ceric ammonium nitrate is an orange coloured complex which after reacting with alcoholic group present in MA becomes deep red in colour due to the formation of the complex which confirms the presence of hydroxyl group.

Use of excess of ceric ammonium nitrate for the test should be avoided as the red colour will disappear. Also the intensity of red colour decreases with time and the end result is colourless solution of cerium (III). This is because an electron is transferred to cerium (IV)-alcohol complex forming cerium (III) and an organic product [8].

$$\text{complex} \longrightarrow \text{cerium(III)} + \text{organic product}$$

(red) (colourless)

Test for Carboxyl (-COOH) Group

Litmus Test

Litmus is used as a pH indicator [21]. It could be either a paper strip or solution. It analysis the pH of a substance by changing colour where red means acidic in nature and blue means basic in nature. Mycolic acid itself has word 'acid' in its name. Presence of carboxyl group (and to some extent alcoholic group as well) makes it acidic in nature. Its presence can be detected by litmus test. When blue litmus is added to the solution of MA in ether, it is expected to change its colour to red which indicates the acidity of the sample. But this is not a confirmatory test as there could be a lot of functional groups which makes a compound acidic in nature. In fact pH of water ranges from 6.5-8.5; hence there is a possibility that water could be responsible for the red colour of litmus. Therefore it is necessary to first dry the sample with the help of anhydrous calcium sulphate and then perform the test.

Sodium Bicarbonate Test

Sodium Bicarbonate, also known as baking soda is an amphoteric compound. It reacts with acids to give carbonate salt, water and carbon dioxide. It is used as leavening agents in baking (leavening is defined as the baking action because which the volume of the dough or batter is increased during baking). It aerates the dough or batter containing acidic ingredients due to the evolution of carbon dioxide gas which is trapped inside the batter creating voids and making in light in weight [10].

$$RCOOH + NaHCO_3 \longrightarrow RCOO^-Na^+ + H_2O + CO_2(g)$$

Since MAs have carboxyl group present in it, making it is acidic in nature, it is expected that it will react with sodium bicarbonate followed by the evolution of carbon dioxide gas with brisk effervescence.

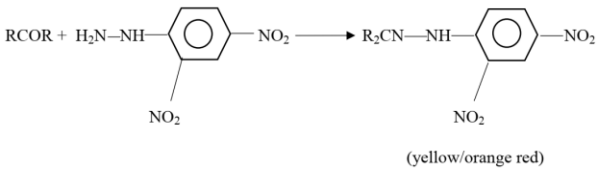
Test for Keto (-CO) Group

2,4-dinitrophenylhydrazine Test

2,4-dinitrophenylhydrazine (DNPH) is a complex reagent that is used for the detection of aldehydes and ketones. Keto-MA has a ketonic group present in its structure which can be detected by the means of this test (since there is no aldehydic group present in any of the three types of MA, the positive result will indicate the presence of keto group only). Ketones react with acidic solution of DNPH to form sparingly soluble hydrazone derivates [11].

The acidic solution of 2,4-DNPH can be formed by given method:

Add 2g of 2,4-DNPH in 100ml of methanol and slowly add 4ml of concentrated sulphuric acid. The mixture will become warm and solid will be dissolved. If necessary filter the solution [12].



(yellow/orange red)

2,4-DNPH solution is yellow in colour. After reacting with ketonic group present in MA, crystalline yellow or orange red precipitate of 2,4-dinitrophenylhydrazone is expected to develop which confirms its presence.

Sodium Bisulphite Test

Ketones and aldehydes react with saturated solution of sodium bisulphite to give crystalline sodium bisulphite addition complex (since there is no aldehydic group present in any of the three types of MA, the

positive result will indicate the presence of keto group only) [12].

$$RCOR + NaHSO_3 \longrightarrow R_2C(OH)SO_3^-Na^+$$

(white)

In the above reaction an equilibrium is maintained but by using excess of bisulphite reagent reaction will be completed giving white crystalline sodium bisulphite addition product because if reacting with ketonic group present in MA.

Test for Methoxy (-OR) Group

Zeisel Test

Zeisel determination or Zeisel test is a method by which alkoxy (ether) group is detected. Number of methoxy groups can also be counted by this method. In this method the sample is heated up to 140^{o}C with a mixture of acetic acid and hydriodic acid in a test tube. The compound breaks into methoxy iodide (gaseous state) and respective alcohol. The evolved methoxy iodide comes in contact with a paper above the test tube saturated with silver (III) nitrate. After reacting silver iodide is formed which imparts yellow colour and hence confirms the presence of methoxy group [13].

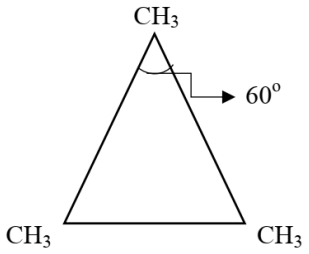
$$ROCH_3 + HI \longrightarrow ROH + CH_3I + AgNO_3 \longrightarrow AgI$$

Test for Cyclopropane Ring

Bromine Water Test

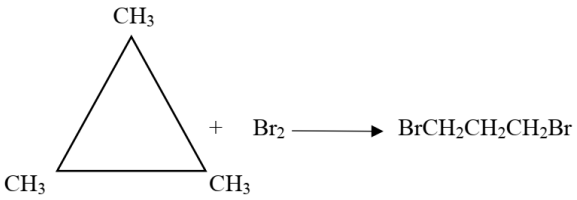
Cyclopropyl ring is not a functional group. It a cyclic hydrocarbon ring present in all the three types of MAs. Bromine water test is used to detect unsaturated hydrocarbons. By electrophilic addition mechanism, bromine (Br_2) breaks the double bond of unsaturated hydrocarbon and get attached to the respective carbons. Bromine loses its original red brown colour and gives colourless solution.

In cyclopropane, the three bonds between each carbon atoms are in bent shape increasing the angle strain from 109.5 ^{o} to 60^o.



Angle strain between the bonds of a cyclopropane ring

Because of this angle strain cyclopropane reacts with bromine water and the bonds are broken to give alkyl halide [14].



Similarly MA is expected to react with Bromine water and decolourise it due to the presence of cyclopropane rings in all the three types.

Figure Legends

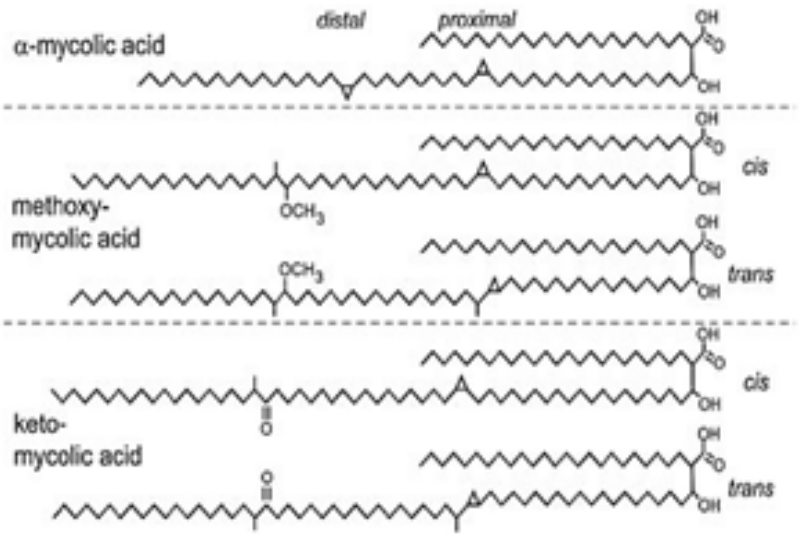


Fig-1: Chemical structures of mycolic acids present in Mtb

This figure is representing the mycolic acids which is depicting α-MA and methoxy- and keto- MA with cis and trans conformations (https://cmr.asm.org/content/18/1/81.short).

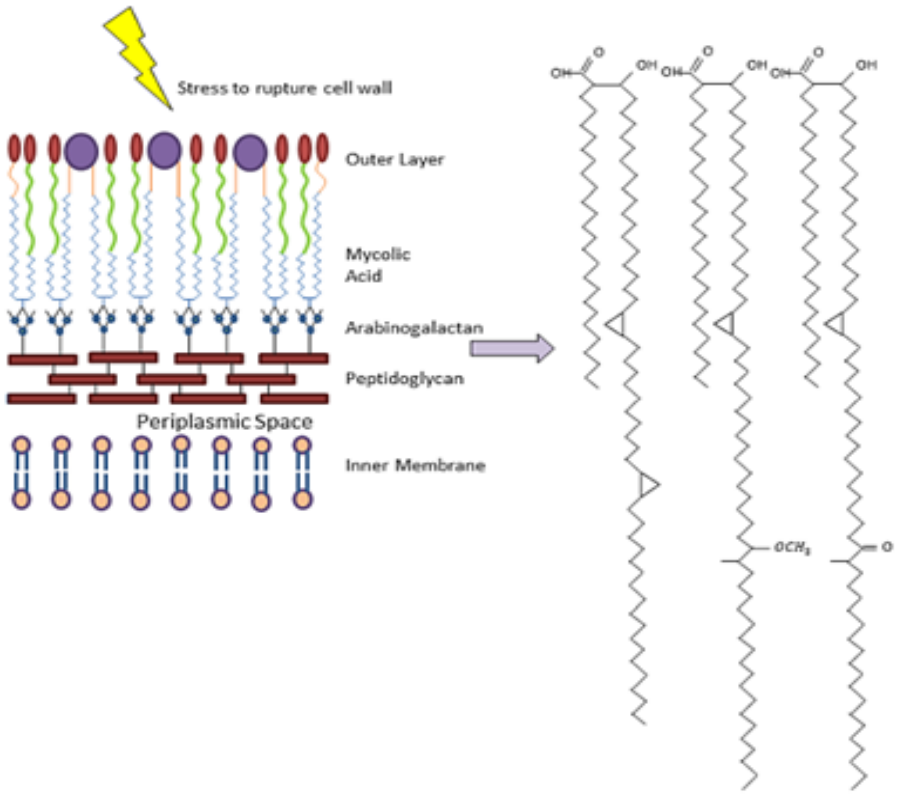


Fig-2: Exposure of Mycolic Acids

In this figure, Mycobacterial walls are broken using mechanical stress. The material leftover after the removal of non- covalently bound substances is the cell wall skeleton which contains three covalently linked components: peptidoglycan, arabinogalactan and mycolic acids.

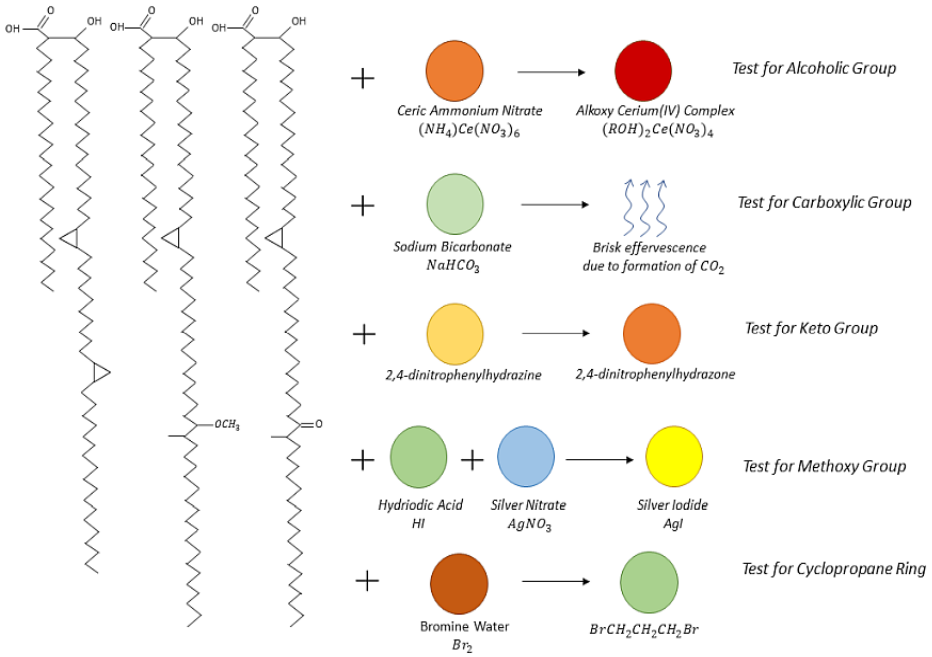
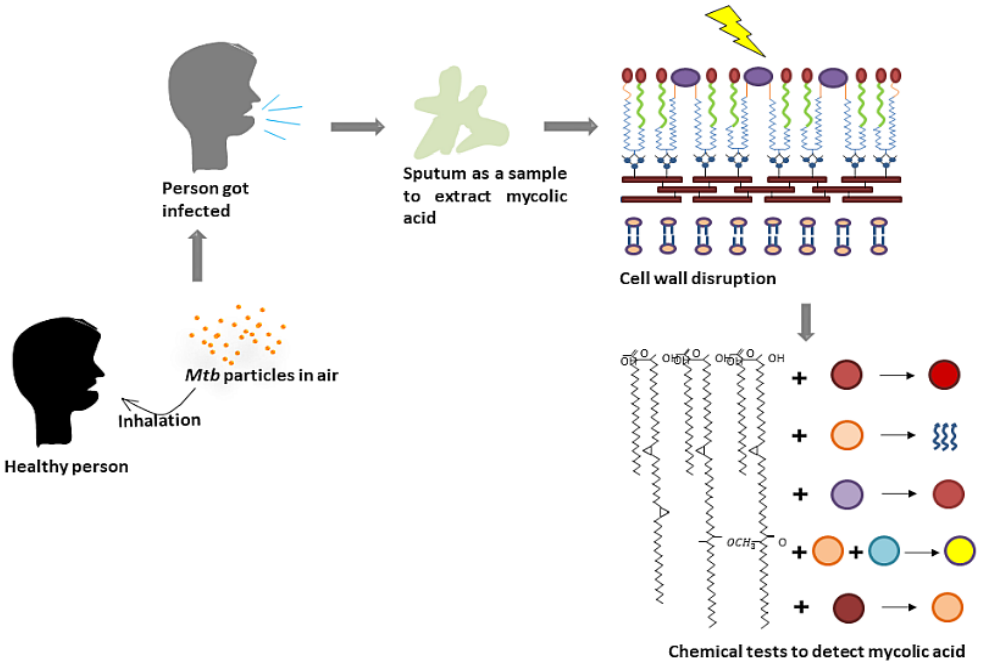


Fig-3: Detection of functional groups in Mycolic Acids

In this figure, qualitative analysis has been shown with the help of various specific functional group tests to confirm the presence of mycolic acids in the sample.

Highlights

1) Tuberculosis is one of the deadliest diseases across the world.
2) Several tests are performed for the diagnosis of tuberculosis.
3) Detection of Mycobacterium tuberculosis $H_{37}Rv$ by the means of mycolic acids.
4) Qualitative analysis by various specific functional group tests to confirm the presence of mycolic acid.



Graphical Abstract

Graphical representation of mycobacterial transmission in healthy person and detection tests for mycolic acid: an indicator of mycobacterium existence.

CONCLUSION

Tuberculosis is a widespread infectious disease that affects millions of people worldwide every year. Due to the alarming rate of the spread of tuberculosis, implementation of new strategies for the diagnosis has become a primary and necessary step. One such way of diagnosis could be with the help of mycolic acids, present in the cell walls of the tuberculosis causing bacteria i.e. Mtb. For the diagnosis, first step would be to expose the contents of cell wall, including mycolic acids, by some suitable means. After the exposure of mycolic acids, specific functional group tests can be performed on the sample which on giving positive result will confirm the presence of Mycobacterium tuberculosis. This could give an easy and cost effective way to detect Tuberculosis.

ACKNOWLEDGMENT

The author acknowledges financial support from the Department of Science and Technology-SERB, Council of Scientific and Industrial Research-Institute of Genomics and Integrative Biology under the research project GAP0145 (SERB-DST Grant no: EEQ/2016/000514).

Conflicts of interest: Authors declare no conflict of interest.

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