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In-vitro Effect of Iron on the Growth Density of *Mycobacterium tuberculosis* and Drug Susceptibility Testing

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

This work was carried out to study the effect of iron on the growth density of *Mycobacterium tuberculosis* as well as drug susceptibility testing. Fifty two smear-positive acid fast bacilli out of 100 sputum specimens were obtained from patients who were referred to National Health Laboratory, Federal Ministry of Health, Sudan. The smear positive specimens were cultured onto control LJ medium and other three sets of LJ medium containing ferrous sulfate (iron) with different concentration; 100 mg/l, 200 mg/l and 400 mg/l. The growth was graded as negative (0 = no growth), +1 (1-19 colonies), +2 (20-100 colonies) +3 (100-200 colonies), +4 (200-500 colonies) and +5 (more than 500 colonies). At the same time, proportion method was applied to test susceptibility of mycobacterial isolates (20 isolates) to anti-tuberculosis drugs; isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and streptomycin (STM) in the presence and absence of ferrous sulfate. Chi squire test was used to analyze categorical variables and the significance level was set at P = .05. Collectively, the results showed that the growth density of *M. tuberculosis* increased significantly when ferrous sulfate was added to LJ medium. Resistance to the four first line anti-tuberculosis drugs (INH, RIF, EMB, STM), INH and EMB were also respectively increased from 10% to 15%, 20% to 40% and 10% to 15% in the presence of ferrous

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sulfate. Contrary to other findings, it was observed that addition of ferrous sulfate to LJ medium enhanced the activity of STM while no effect was seen on RIF and multi-drug resistance (INH and RIF).
The study concluded that the growth density of *M. tuberculosis* can be enhanced by supplementing LJ medium with 100-200 mg/l iron. This would be useful in recovery of the organisms from specimens with a low bacterial load particularly in laboratories in low income setting. Supplementation of LJ medium with iron must be avoided if drug susceptibility testing is required.

Keywords: Mycobacterium tuberculosis; iron; growth; culture; drug susceptibility.

1. INTRODUCTION

Tuberculosis (TB) is the cause of highest number of deaths due mostly to a single infectious agent namely, *Mycobacterium tuberculosis*. It is expected that during 2000-2025, one billion individual will be newly infected, 200 million will get TB and 40 million are likely to die if control programs are not improved [1]. In developing countries, direct microscopy of sputum specimens for detecting acid-fast bacilli (AFB) remains the standard diagnostic procedure [2]. Culture of *M. tuberculosis* is still the gold standard for diagnosis of TB but growth of micro-organism is sometime weak due to scant presence of AFB in clinical specimens. Studies have shown that *M. tuberculosis* requires iron for essential metabolic pathways like most other micro-organisms [3-7]. It is worth mentioning that the importance of iron as a component of ingredients of culture medium for the growth and multiplication of the tubercle bacilli has been started since 1950s [8]. On the other hand, the bactericidal activity of anti-tuberculosis drugs; isoniazid (INH) and ethambutol (EMB) was largely reduced or inhibited in the presence of high level of iron [9-10]. The impact effect of iron on streptomycin (STR) efficacy, other anti-tuberculosis drugs and growth density of *M. tuberculosis* cultured from sputum specimens was not well documented in the literature. In present study, efforts were made to study the effect of iron on the growth density of *M. tuberculosis* in Lowenstein-Jensen (LJ) medium supplemented with different concentrations of ferrous sulfate as well as to study the effect of iron on drug susceptibility testing.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Specimens

One hundred sputum specimens were collected from suspected patients with pulmonary tuberculosis who were referred to National Health Laboratory, Federal Ministry of Health, Sudan, during October 2005 - October 2006.

Specimens that showed acid fastness on microscopy by Ziehl-Neelsen stain were decontaminated by Petroff's method, centrifuged at 3000 g for 15 minutes and supernatant was discarded. The pellet was re-centrifuged as described above and finally three drops were inoculated by sterile Pasteur pipette onto LJ medium for growth of *M. tuberculosis* [1].

The study was approved by the national ethical review committee and informed consent was obtained from patients.

2.2 Identification of Mycobacterial Isolates

The growth of mycobacterial isolates were tested conventionally for nitratase, 68°C labile catalase, susceptibility to para-nitro benzoic acid and thiophene-2-carboxylic acid hydrazide [1]. In addition, the previous described method for DNA extraction and molecular identification of *M. tuberculosis*; duplex polymerase chain reaction (DPCR) and multiplex polymerase chain reaction (MPCR) were performed to confirm the conventional results [11-13].

The simplified DPCR assay was used to differentiate *M. tuberculosis* complex (MTC) and nontuberculous mycobacteria (NTM) by using a single gene, the RNA polymerase β subunit-encoding gene (*rpoB*). The 235-bp and 136-bp DNA sequences are, respectively, specific for MTC and NTM. The MPCR was used as a rapid and easy tool to differentiate *M. tuberculosis* within MTC. The technique targets *M. tuberculosis*-specific 262-bp DNA sequence.

2.3 Growth of Mycobacterial Isolates on Iron Containing Medium

To establish what amounts of iron might be necessary for growth of *M. tuberculosis*; LJ medium was first prepared with as little iron as possible [8]. Three sets of LJ slope containing ferrous sulfate (iron; Sigma-Aldrich Company) with different concentration; 100 mg/l, 200 mg/l and 400 mg/l were prepared. Mycobacterial suspension equivalent to McFarland tube No.1 (1 mg/ml bacillary suspension) was made and diluted to 0.01 mg/ml by 10 fold dilutions [14]. An amount of 10 µl of diluted bacillary suspension was inoculated onto control and those different concentrations of iron containing medium. The slants were incubated at 37ºC for up to eight weeks. The growth density was graded as explained earlier [15]; negative (0 = no growth), +1 (1-19 colonies), +2 (20-100 colonies) +3 (100-200 colonies), +4 (200-500 colonies) and +5 (more than 500 colonies). To ensure the quality of culture reading more than one researcher was asked to re-read the growth of all mycobacterial isolates and the results were matched for comparison.

2.4 Growth of Mycobacterial Isolates on Drug Containing Medium

Only 20 bacterial isolates were selected randomly to be tested for drug susceptibility. The proportion method [1] was used to test susceptibility of mycobacterial isolates to the first line drugs of tuberculosis; INH, RIF, STR and EMB (Sigma-Aldrich Company). Two slopes of LJ medium containing INH 0.2 mg/l, STR 4 mg/l, RIF 40 mg/l, and EMB 2 mg/l were prepared, one containing 400 mg/ml of ferrous sulfate and the other free of ferrous sulfate. A loopful of bacterial suspension (10 µl) was inoculated onto control, drug containing medium and drug containing medium supplemented with ferrous sulfate. Resistance was expressed as the percentage of colonies on drug containing medium in comparison to the growth on drug free medium at the critical concentrations [1]. The usual criterion for resistance is 1% of growth for all drugs.

2.5 Statistical Analysis

The data were analyzed (SPSS software, version 13) step-by-step, and cross tabulation was done to explain possible relationship between the variables. Chi squire test (χ^2) for categorical variables was calculated and the significance level was set at $P = .05$ to find out

the association between the variables. McNemar's test was used to compare the results of drug susceptibility testing.

3. RESULTS

3.1 Growth and Identification of Mycobacterial Isolates

Only 52 out of 100 sputum specimens were smear-positive AFB and revealed characteristic colonies of *M. tuberculosis* upon LJ culture. All mycobacterial isolates were positive for nitratase, negative for 68ºC labile catalase, susceptible to para-nitro benzoic acid and resistant to thiophene-2-carboxylic acid hydrazide. Differential identification by DPCR showed specific band size 235 bp for MTC. Differentiation within the species of MTC by MPCR proved that all isolates were *M. tuberculosis*.

3.2 Effect of Iron on the Growth Density of *M. tuberculosis*

The effect of ferrous sulfate on the growth density of *M. tuberculosis* was detailed in Table 1. A significant increase was observed in the growth density (+3, +4 and +5) when ferrous sulfate concentration was increased to 100 mg/l (P = .01), 200 mg/l (P = .00) and 400 mg/l (P = .00). It was noticed that the significant increment of +4 and +5 growths was found on LJ medium containing 200 mg/l and 400mg/l ferrous sulfate.

Table 1. Growth density of *M. tuberculosis* onto different concentration of ferrous sulfate containing medium

Growth density	Number of *M. tuberculosis* cultures onto different LJ medium (%)			
	Control	100 mg/L	200 mg/L	400 mg/L
0	1 (1.9 %)	0 (0%)	0 (0 %)	1 (2 %)
+1	13 (25 %)	6 (11.5)	7 (13.5 %)	5 (9.6 %)
+2	21 (40.4%)	18 (34.6 %)	5 (9.6 %)	6 (11.5 %)
+3	16 (30.8 %)	20 (38.5 %)*	18 (34.6 %)*	9 (17.3 %)
+4	1 (1.9 %)	8 (15.4 %)*	20 (38.5)*	22 (42.3 %)*
+5	0 (0%)	0 (0 %)	2 (3.8 %)*	9 (17.3 %)*
Total	(100%)	(100%)	(100%)	(100%)

** Significant increase*

3.3 Resistance to Anti-tuberculosis Drugs

A total of 10% of isolates (2/20) was resistant to the four first line anti-tuberculosis drugs in the absence of ferrous sulfate. This number was increased to 15% (3/20) when the drug containing medium was supplemented with ferrous sulfate. The percentage of multi-drug resistance (resistance to INH and RIF) was found 20% whether ferrous sulfate was added to the drug containing medium or not.

Resistance of *M. tuberculosis* to each anti-tuberculosis drug was given in Table 2. Resistance to RIF was not affected by presence or absence of ferrous sulfate, whereas resistance to the remaining drugs was changed, although it was not significant.

Table 2. Comparison of resistance of *M. tuberculosis* to anti-tuberculosis drugs before and after addition of ferrous sulfate 400 mg/l to the LJ medium

	Resistance	
Drug	**LJ medium free of ferrous sulfate**	**LJ medium supplemented with ferrous sulfate**
Isoniazid	4 (20 %)	8 (40 %)
Rifampicin	4 (20 %)	4 (20 %)
Streptomycin	7 (35 %)	6 (30 %)
Ethambutol	2 (10 %)	3 (15 %)

4. DISCUSSION

Data presented here proved that the growth density of *M. tuberculosis* was enhanced and increased significantly by the addition of iron to LJ medium (Table 1). The importance of iron as major factor on the growth of *M. tuberculosis* and hence the increase occurrence of tuberculosis infection was demonstrated by several workers. Raghu et al. [9] reported that the addition of iron enhances the in vitro growth of *M. tuberculosis*. Gangaidzo et al. [4] reported a significant association between exposure to high levels of dietary iron and the presence of pulmonary tuberculosis in patients. Similarly, Lounis et al. [16] and Gomes et al. [17] clearly showed that the outcome of experimental infections of *M. tuberculosis* and *M. avium* in mice was considerably worsened by iron supplementation.

As well, a total of 100% of growth of mycobacterial isolates was obtained in this study by supplementing LJ medium with 100 mg/l and 200 mg/l ferrous sulfate compared to 98.1% of growth in control medium. It may be useful to supplement LJ medium with iron to improve the diagnosis as well as the growth of *M. tuberculosis* particularly in low income settings laboratories. Method involving the use of a centrifuge is more efficient than simple decontamination and culture of sputum directly onto medium that used in these laboratories. Hence, supplementation of LJ medium with iron may overcome disadvantage of direct method and promote the growth of *M. tuberculosis*. Even though, a proportion of 2% of reduction in growth was observed here in LJ medium supplemented with 400 mg/l ferrous sulfate. This reduction of growth was probably as a result of uncontrolled iron acquisition because excess iron can be extremely toxic to *M. tuberculosis* [18]. Building on these results, optimal growth of *M. tuberculosis* can be achieved by supplementing LJ medium with 100-200 mg/l ferrous sulfate.

The finding of drug susceptibility testing in this study does not necessarily reflect the prevalence of the drugs resistance in the country but might be resulted by the fact that the specimens were collected from different clinical cases accidentally. Prevalence of drugs resistance should be studied in country-wide surveillance to evaluate the true situation.

This study assessed the effectiveness of all first line anti-tuberculosis drugs in the presence and absence of iron, which were not well documented in the literature. The concentration of 400 mg/l ferrous sulfate containing medium was selected for drug susceptibility testing because it revealed highest density of mycobacterial growth.

As expected, the growth of *M. tuberculosis* isolates in the presence of INH increased after addition of ferrous sulfate to LJ medium. This result supported Lounis and his co-workers [10] who showed that the bactericidal activity of INH was reduced in iron loaded mice compared to the normal-iron mice. Correspondingly, Sritharan et al. [19] reported that the

peroxidase activity of the catalase-peroxidase katG was abolished in-vitro upon iron limitation, resulting in the failure of the activation of the prodrug INH to an active form.

Unaffected resistance of RIF after addition of ferrous sulfate to the LJ medium probably caused by lack of RIF to any metal ion properties [16]. On the other hand, the increased growth of mycobacterial isolates after addition of ferrous sulfate to EMB containing medium might indicate impairment of the drug by iron [9]. Contrary to other findings, it was found that addition of ferrous sulfate enhanced the activity of STM.

5. CONCLUSION

The most significant value in this study is that iron in LJ medium promotes growth of *M. tuberculosis* which would be useful in recovery of the organisms from specimens with a low bacterial load. This is of especial value in laboratories in low income setting. The finding of increased resistance when isolates were tested on iron containing medium was interesting but not of much significance. However, supplementation of LJ medium with iron must be avoided if drug susceptibility testing is required.

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COMPETING INTERESTS

Authors have declared that no competing interests exist

REFERENCES

1. WHO. Global Tuberculosis control; surveillance, planning. WHO report; 2005. Geneva, World Health Organization (WHO/HTM/TB/2005. 349) 2005.
2. Enarson DA, Rieder HL, Arnadottir T, Trébucq A. Management of Tuberculosis. A Guide for Low Income Countries. 5th ed. Paris, International Union Against Tuberculosis and Lung Disease; 2000.
3. Woodridge KG and Williams PH. Iron uptake mechanisms in pathogenic bacteria. FEMS Microbiol. Rev. 1993;12:325-48.
4. Gangaidzo IT, Moyo VM, Mvundura E, Aggrey G, Murphree NL, Khumalo H, et al. Association of pulmonary tuberculosis with increased dietary iron. J. Infect. Dis. 2001;184:936-39.
5. Hantke K. Iron and metal regulation in bacteria. Curr Opin Microbiol. 2001;4:172–7.
6. Hobson RJ, McBride AJA, Kempsell KE and Dale JW. Use of an assayed promotor–probe library for the identification of macrophage-regulated genes in *Mycobacterium tuberculosis*. Microbio. 2002;148:1571-79.
7. Ratledge C. Iron, mycobacteria and tuberculosis, Tuberc. 2004;84:110-130.
8. Ratledge C. Iron acquisition by the genus *Mycobacterium*: History, mechanisms, role of siderocalin, anti-tuberculosis drug development. Editor B. Rowe Byers. 2013;4-5.
9. Raghu B, Sarma GR, Venkatesan P. Effect of anti-tuberculous drugs on the iron-sequestration mechanisms of mycobacteria. Indian J Pathol. Microbiol. 1995;38:287-92.

10. Lounis N, Maslo C, Truffot-Pernot C, Grosset J, Boelaert JR. Impact of iron loading on the activity of isoniazid or ethambutol in the treatment of murine tuberculosis. Int. J. Tuberc. Lung Dis. 2003;7:575-79.
11. Kim B, Hong S, Lee K, Yun Y, Kim E, Park Y, et al. Differential Identification of *Mycobacterium tuberculosis* Complex and Nontuberculous Mycobacteria by Duplex PCR Assay Using the RNA polymerase Gene (rpoB). J. Clini Microbio. 2004;42:1308-12.
12. Bakshi CS, Shah DH, Verma R, Singh RK, Malik M. Rapid differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* based on a 12.7-kb fragment by a single tube multiplex-PCR. Vet. Microbio. 2005;109:211–16
13. Bakshi CS, Shah DH, Verma R, Singh RK, Malik, M. Corrigendum to ‘‘Rapid differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* based on a 12.7-kb fragment by a single tube multiplex-PCR’’. Vet. Microbio. 2007;123:282.
14. Fujiki A. TB bacteriology examination to stop TB. The Research Institute of Tuberculosis. Japan. 2001;31-33.
15. WHO. Laboratory services in tuberculosis control. Part III, culture. WHO/TB/98.258. 1998;77.
16. Lounis N, Truffot-Pernot C, Grosset J, Gordeuk VR, Boelaert JR. Iron and *Mycobacterium tuberculosis* infection. J. Clin. Virol. 2001;20:123-26.
17. Gomes MS, Boelaert JR, Appelberg R. Role of iron in experimental *Mycobacterium avium* infection. J. Clin. Virol. 2001;20:117-22.
18. Rodriguez GM Control of iron metabolism in *Mycobacterium tuberculosis*. Trends in Microbio. 2006;14:320-27.
19. Sritharan M, Yeruva VC, Sivasailappan SC, Duggirala S. Iron enhances the susceptibility of pathogenic mycobacteria to isoniazid, an anti-tubercular drug. World J Microbiol. Biotechnol. 2006;22:1357-64.

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