

## Reviewer's Comments

### Determination of Rifampicin Mono-Resistance *Mycobacterium tuberculosis* in the National Tuberculosis Control Programme in Sana'a city-Yemen: A Significant Phenomenon in War Region with High Prevalence TB

#### Abstract

The current study was undertaken in Sana'a city, Yemen, which has one of the highest rates of Tuberculosis (TB) in the Region. We retrospectively determined the prevalence of true RIF's mono-resistance, using the gold standard of molecular assays comparing with culture based phenotypic drug susceptibility testing (DST), and determine the associated risk factors of contracting RIF gene among Yemeni TB patients. Patient's data were collected in a predesigned questionnaire which included demographic information and the relevant risk factors associated with RIF. All sputum specimens received were routinely cultured, and, underwent phenotypic drug susceptibility testing. DNA sequences specific for MTB and RIF were detected by automated molecular test. The total end treatment rate was 5.94% of the total cases (11/185). MTB gene was positive for all cases which indicate more sensitive than sputum examination; 3.2% of total patients show RIF's genes positive. Higher rate of RIF with male (5.4%), and low in female patients (1.8%). There was higher rate of RIF with age group 36-45 years (8.7%) and >15 years (7.1%). There was significant rate of RIF gene with after food drug taken (RIF=13.3%, with OR=6.4), and with DOTs program (RIF=10%, with OR=6.4). In addition, there was significant rate of RIF gene with one dose regimen (11.8%, OR=5.5). In conclusion, this study reveal the presence of RIF-*M.tuberculosis* in Sana'a, Yemen and most of them were observed among patients who default treatment. This is considered as a threat to TB control programme and it is recommended that strategies should be put in place in order to ensure patients' compliance and monitoring of patients' response to TB treatment.

**Keywords:** TB, Rifampicin resistant (RIF), *Mycobacterium tuberculosis* (MTB), Sana'a, Yemen.

#### INTRODUCTION

Globally in 2015, an estimated 480 000 people developed multidrug-resistant TB (MDR-TB). TB incidence has fallen by an average of 1.5% per year since 2000. This needs to accelerate to a 4–5% annual decline to reach the 2020 milestones of the "End TB Strategy". An estimated 49 million lives were saved through TB diagnosis and treatment between 2000 and 2015. Ending the TB epidemic by 2030 is among the health targets of the newly adopted Sustainable Development Goals<sup>1</sup>.

When TB is detected and effectively treated, the disease is largely curable. However, in 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360 000 of whom were Human Immunodeficiency Virus (HIV) positive<sup>2</sup>.

In Yemen, TB is considered one of the major infectious diseases recorded in the national disease list. A rapidly increasing population, poor quality health services, very low annual income of individuals and the whole country's poor economic status plus the Saudi-UAE's aggression is the most important factors responsible for the high incidence of TB in the country<sup>3,4</sup>.

**Comment [D1]:** Although the Author had a lot of work, in my opinion, in some paragraphs of the manuscript, the text is too detailed. It causes that it is incomprehensible. I suggest that the Author should avoid unnecessary information. Moreover, the Author failed to make it clear what is the most important in the whole manuscript. Therefore, the aim of the paper should be more clearly stated.

**Comment [D2]:** - The introduction contains repeats of the same information, it should be corrected.  
- The keywords in methodology and abstract are different. It should be corrected.

**Comment [D3]:** Plz write scientific writing I, we you kind of words not used

**Comment [D4]:** Is this odd ratio????  
Plz mention clearly first time

**Comment [D5]:** Tuberculosis (TB).

**Comment [D6]:** 1-Which sampling technique was used?  
2-What was criteria for age in patient selection?  
3-Which statistical test was used?

Drug-resistant *Mycobacterium tuberculosis*, including multidrug-resistant *Mycobacterium tuberculosis* (MDR- *Mycobacterium tuberculosis*, defined as resistance to at least isoniazid and rifampicin, the two most important first-line anti-TB drugs) and extensively drug resistant TB (XDR- *Mycobacterium tuberculosis*, defined as MDR- *Mycobacterium tuberculosis*, plus resistance to any fluoroquinolones, such as ofloxacin or moxifloxacin, and to at least one of three injectable second-line drugs, amikacin, capreomycin, or kanamycin) has emerged as a serious threat to global health<sup>5</sup>.

Rifampicin is one of the most important anti-tuberculosis (anti-TB) antibiotics; it exerts its bactericidal activity by inhibiting the early steps of gene transcription by binding to the  $\beta$ -subunit of RNA polymerase (rpo  $\beta$ ) encoded by the rpo  $\beta$  gene<sup>6</sup>. The control of TB has been impeded by the emergence of drug-resistant *Mycobacterium tuberculosis* strains<sup>7</sup>. The problem of TB has been compounded by the emergence of multi-drug resistance *Mycobacterium tuberculosis* and Human Immunodeficiency Virus (HIV). The development of resistance to rifampicin is due to mutations in a well-defined 81 base pair (bp) (27 codons) central region of the gene that encodes the  $\beta$ -subunit of RNA polymerase (rpo  $\beta$ ). More than 96% of the rifampicin-resistant strains contain a mutation in this 81 bp region of rpo  $\beta$ , thus facilitating a straight forward approach to detecting rifampicin resistance and/or MDR rapidly<sup>8</sup>. Despite low sensitivity in detection of *Mycobacterium tuberculosis*, acid-fast sputum smear remains the main diagnostic method in most countries, especially in resource-limited settings. In HIV infected patients with pulmonary TB, 24–61% have acid-fast negative sputum smear. In 2011, WHO advice the wide use of Xpert *Mycobacterium tuberculosis* /Rifampicin assay, a fully automated diagnostic molecular test using real-time polymerase chain reaction (PCR) technology to simultaneously detect *Mycobacterium tuberculosis* and rifampicin resistance mutations in the (rpo B) gene<sup>9</sup>. Xpert *Mycobacterium tuberculosis* /Rifampicin is an automated polymerase chain reaction (PCR) test (molecular test) utilizing the GeneXpert<sup>®</sup> platform<sup>9,10</sup>. Xpert<sup>®</sup> MTB/RIF is a single test that can detect both *Mycobacterium tuberculosis* complex and rifampicin resistance within two hours after starting the test, with minimal hands-on technical time. The Xpert MTB/RIF limit of detection, “the lowest number of colony forming units per sample that can be reproducibly distinguished from negative samples with 95% confidence”<sup>11</sup>, is five genome copies of purified DNA per reaction or 131 colony forming units per mL in *Mycobacterium tuberculosis* spiked sputum<sup>10</sup>. In comparison, identification of TB bacilli by microscopic examination requires at least 10,000 bacilli per ml of sputum<sup>12</sup>. Xpert MTB/RIF detects both live and dead bacteria<sup>13</sup>.

The first nationwide prevalence survey on resistance to the four major anti-tuberculosis drugs in Yemen showed a relatively low prevalence of drug-resistant cases, but a high prevalence of multidrug resistance among new cases<sup>14</sup>. The current study aim's was to determine prevalence of true RIF's mono-resistance, using the gold standard of molecular assays comparing with culture based phenotypic drug susceptibility testing (DST), and determine the associated risk factors of contracting RF gene among Yemeni TB patients.

## MATERIALS AND METHODS

This cross-sectional laboratory study conducted among pulmonary patients attending the National Tuberculosis Control Programme (NTCP) in Sana'a city, Yemen. During the period from March, 2017 until December, 2017. Data was taken from each subject and collected in a pre-designed questionnaire which

included demographic information, detailed clinical history and risk factors associated with Rifampicin-mono-resistant *Mycobacterium tuberculosis*. The patients were categorized to; (i) patients that had previously received treatment in the same or another clinic but default after some time (ii) patients whose treatment history could not be assessed, (iii) patients that visit the clinic for the first time and (iv) patients who had relapse after completion of normal treatment plan. Three sputum samples were collected from each patient, then one tested for acid fast bacilli, the second mixed with 2 ml of buffer for genetic test (Genexpert), and the third specimen for culture based phenotypic drug susceptibility testing (DST). The **GeneXpert System** equipped with GX2.1 software/computer/printer/barcode wand-reader and operator manual (Cepheid Inc, Sunnyvale, and USA). The GenXpert MTB/RIF is an automated molecular test which detects DNA sequences specific for MTB and RIF's resistance by polymerase chain reaction with fully integrated sample processing in patients suspected of drug sensitive or multidrug resistant pulmonary tuberculosis. Also, the prevalence of true RIF mono-resistance, using the gold standard of molecular assays (The GenXpert MTB/RIF) was comparing with culture based phenotypic drug susceptibility testing (DST). In addition, we determine the associated risk factors of contracting RF gene among Yemeni TB patients as described in result tables 4 and 5.

## RESULTS

Only 7.6% of tested individual were children  $\leq 15$  years old, most of them were adults in age groups 16-25 years (28.1%), 26-35 years (27.6%), 36-45years (12.4%) and  $\leq 46$ years (24.3%) (table 1). Total end treatment rate was 5.94% of the total cases (11/185). 2.7% of total patients show primary infection T.B (low) with low contagious, 24.3% show severe infections T.B with highly contagious, and 30.8% show very severe infections T.B with highly contagious (table 2). MTB gene was positive for all cases which indicate more sensitive than sputum examination; 3.2% of total patients show RIF's genes positive, while 96.8% of total were sensitive to rifampicin (table 3). Higher rate of RIF with male (5.4%) with associated OR equal to 3.1 times, while RIF rate was 1.8% in female patients. There was higher rate of RIF with age group 36-45 years and  $>15$  years in which the rates of RIF gene were 8.7% and 7.1% respectively (table 4). There was significant rate of RIF gene with after food drug taken equal to 13.3%, with OR equal to 6.4 times, and with DOTs program equal to 10%, with OR equal to 6.4 times. In addition, there was significant rate of RIF's gene with one dose regimen (11.8%), with OR equal to 5.5 times (table 4, 5)

## DISCUSSION

There have been some good achievements over the past decade in tuberculosis control in the countries of the WHO Eastern Mediterranean Region including Yemen. These countries have addressed the challenge of tuberculosis through implementation of the directly observed treatment, short-course (DOTS) strategy, with the case detection rate reaching 60% for new smear-positive cases, and an 86% treatment success rate. However, multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) pose a threat to global and regional public health security and to efforts to reduce the global and regional burden of tuberculosis. The Beijing Call for Action on Tuberculosis Control and Patient Care and the World Health Assembly resolution WHA62.15 (2009) on prevention and control of multidrug-resistant tuberculosis. Extensively drug-resistant tuberculosis<sup>15</sup> recognizes the challenges posed by multidrug and

**Comment [D7]:** For instance, it seems to me that in the results presented the infract volumes measurement the p-values are also needed, even there were no significant differences. Moreover, it seems to me that the sentence which statistical test was used in the respective analysis should be added in the all parts of described results

Similarly, more details are needed in the statistical analysis in the Results section. The information concerning the statistical test used in the respective analysis should also be added in Figure Titles. Please complete it. The Abbreviations should be explained.

**Comment [D8]:** Do not start a sentence with a number.

**Comment [D9]:** I would suggest beginning the Discussion with the most important findings of this study and then relate to what other studies have shown and a short paragraph on the data on potential mechanism for the reported findings. The conclusion of the whole manuscript should be more clearly written.

**Comment [D10]:** Reference????

extensively drug-resistant tuberculosis (M/XDR-TB) and call for urgent action to address the situation. The exact burden of multidrug resistance is not known in the Region since drug resistance surveys have been conducted in only eight countries of the Region excluding Yemen.

MDR-TB is defined as resistance to both isoniazid (INH) and rifampicin (RIF), with or without resistance to other anti-tuberculosis drugs<sup>16</sup>. Unlike most other bacteria, resistance in *Mycobacterium tuberculosis* (MTB) develops primarily through mutations in chromosomal genes. These mutations develop spontaneously and are sustained in the bacterial population mainly through selective pressure with inappropriate treatment<sup>17,18</sup>. The exact burden of multidrug resistance in Yemen is not known, due to the limited number of national drug resistance surveys and surveillance, so that our study was one of uncommon study in Yemen and the RFR rate in our study was 3.2% (table 2). This rate (3.2%) was slightly higher than 2% of the mean world rates that reported by WHO in the fourth WHO global report on drug resistance, *Anti-tuberculosis drug resistance in the world*<sup>15</sup>. Furthermore, our MDR-TB rate (3.2%) is higher than that reported from Lebanon, Morocco and Oman in which the rates were from 0.5% in Lebanon and Morocco to 1.3% in Oman<sup>19</sup>. Additionally, our result was similar to that reported from Yemen previously in which the rate was 2.9%, but lower than that from Jordan (5.4%) among new cases of TB reported<sup>19</sup>.

Comment [D11]: current

Comment [D12]: current study

The resistance to RIF in our study is largely attributed to nucleotide substitutions in an 81-bp core region of the *rpoB* gene as confirmed by Telents<sup>17,18</sup> and Pietzka *et al.*<sup>20</sup>. In contrast, resistance to INH occurs by mutations in several genes, in particular *inhA* and *katG*, and to a lesser extent in *ahpC*, *oxyR*, *kasA*, *furA* and *ndh*<sup>18,21</sup>. RIF's resistance in our study (3.2%) is regarded as a proxy for MDR-TB as a large proportion of RIF resistant strains have INH resistance as described by Caws *et al.*<sup>22</sup>, Ramaswam and Mussen<sup>23</sup>, Rieder *et al.*<sup>24</sup>, Aziz<sup>25</sup>, Sau *et al.*<sup>26</sup>. What's more, theoretically, if RIF's resistance and MDR-TB were perfectly correlated, then detection of MDR-TB in Yemen would be sufficient with a single rapid test that detects RIF's resistance. As well, similarly, other parts of the world have also reported that RFR is not uncommon. Sanders *et al.*<sup>27</sup> reported that levels of drug resistance in Bujumbura are higher than average for Africa and most worrying was the appearance of MDR-TB and RFR in new cases. Traore *et al.*<sup>28</sup> studied a large number of MTB complex clinical isolates from diverse countries to detect RFR. He reported a median of 4.6% RFR with the highest prevalence in Western Europe. Pablos-Mendez *et al.*<sup>29</sup> performed anti-TB drug resistance surveillance (WHO) in 19 different countries and reported RFR in all. A median of 1.5% was noted, with the highest rate of 6.9% being reported from both Thailand and Dominican Republic. In comparison to the above studies, we report a similar range of RFR.

In the current study, males were more likely to have RFR than females. This result is similar to that reported from Africa in which males have shown higher prevalence rates of RIF than females<sup>19</sup>. However, to the best of our knowledge, gender as a specific risk factor for RIF has not previously been documented in Yemen by others<sup>30</sup>.

Although using age as risk factor is RIF, in the current study RIF was more likely to occur in the 36–45 year age group. It is well recognized that TB is more common in this age group and that RIF is more common in 3<sup>rd</sup> and 4<sup>th</sup> decades of life of patients as previously highlighted<sup>31–33</sup>. Our findings however are in contrast to Mukinda *et al* who reported RIF higher in >45 year age group<sup>34</sup>. In spite of that the countries in

the Region including Yemen have been able to make a remarkable improvement in addressing the challenge of tuberculosis control through implementation of DOTS during the past decade in public health facilities; including primary health care centers with the treatment success rate for 2008 was 87%. In our study there was significant association between RF resistant positive cases and positive DOTS treatment in which the associated OR was 6.4 times, with CI=1.1-38 and PV=0.02 (table 5). This negative effect for DOTS it can be explained by that resistance is a reflection of the mismanagement of tuberculosis cases. This mismanagement includes wrong diagnosis and delay of diagnosis, wrong or interrupted treatment, and the misuse of tuberculosis medicines, both first-line and second-line, such as through poor adherence to standardized treatment by private care providers, unregulated sale of anti-tuberculosis medicines and utilization of tuberculosis medicines of unknown quality. In addition, the difficulty of patients to reach centers for treatment due to the war situation in Yemen.

## CONCLUSION

Significant number of our patients ended first line treatment due no response, most of them in the first 2 months of starting treatment. MTB gene was positive for all TB cases which indicate high sensitive rate than sputum examination. Significant rate of RIF's genes positive was recorded in our study. There was a significant high rate of RIF's gene with previous TB infection. Great risk of develop RIF gene with DOTs program of treatment. The emergence of RF-resistant TB strains in Yemen would make the treatment of TB infections more difficult and increase the complications of tuberculosis. As accurate susceptibility data are crucial for appropriate therapy decisions, we recommend that microbiology laboratories screen for RF in clinical isolates before starting treatment. Currently, no single Yemen surveillance study can provide estimates of the burden of all factors lead to wide spread of TB drug resistant across acute care TB patient populations.

## ACKNOWLEDGMENTS

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## CONFLICT OF INTEREST

"No conflict of interest associated with this work".

## AUTHOR'S CONTRIBUTION

This research work is part of M.Sc. thesis. The candidate is the first author (RMH) who conducted the works and the experiments and wrote up the thesis. The corresponding author (HAA) supervised the experimental work, revised and edited the thesis draft and the manuscript. (BMJ) was co-advisor of the work and helped in revised and edited the thesis draft and the manuscript and in the laboratory works.

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8. <http://www.who.int/tb/publications/latent-tuberculosis-infection/en/>.

### Comment [D13]:

Do not use words 'we' 'our' etc

### Comment [D14]:

Do not use words 'we' 'our' etc

**Comment [D15]:** •References should be described in accordance with Editorial's requirements.

- There are a lot of errors editorial.
- Moreover, the weakness of this manuscript is that it does not offer any newer available information.
- It seems to me that such study should provide the evidences from the most recent literature data available, e.g. 2016, 2017, 2018, 2019.
- Therefore, the References list should be supplemented by the newest literature data.
- In the presented manuscript (in References Section) I do not know what the publication year is????!

**Comment [D16]:** 1-Mention access date for web referenes

- 2-Mention web link for reference 4, 15, 16, 19, 25
- 3-Correct Reference 24
- 4- EMHJ, write full

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**Comment [D17]:** 15 and 16 Both are WHO references  
plz follow uniformity

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Comment [D18]: Plz put latest references

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**Table 1:** The age and sex distribution of T.B patient included in Rifampicin mono-resistance study, at the National Tuberculosis Control Programme in Sana'a city, Yemen.(n=185).

**Comment [D19]:** Table-1 title must be 'Demographics of study participants/patients'

**Comment [D20]:** Make it as scientific writing N% don't put number separately and %age severalty

Age group	Male (n=74)		Female (n=111)		Total(185)	
	N (%)	N (%)	N (%)	%	N (%)	%
≤ 15 year	2 (2.7)	2.7	12 (10.8)	10.8	14 (7.6)	7.6
16-25 year	19 (25.7)	25.7	33 (29.7)	29.7	52 (28.1)	28.1
26-35 year	18 (24.3)	24.3	33 (29.7)	29.7	51 (27.6)	27.6
36-45 year	10 (13.5)	13.5	13 (11.7)	11.7	23 (12.4)	12.4
≤ 46 year	25 (33.8)	33.8	20 (18)	18	45 (24.3)	24.3
<b>Total</b>	<b>74 (100)</b>	<b>100</b>	<b>111 (100)</b>	<b>100</b>	<b>185 (100)</b>	<b>100</b>
Mean age	38.6yrs		31.1		34.15 yrs	
S.D	17.9 yrs		14.8		16.5 yrs	
Medial	35 yrs		30yrs		30 yrs	
Mode	20 yrs		30 yrs		30 yrs	
Min	1 yrs		1 yrs		1 yrs	
Max	75 yrs		70 yrs		75 yrs	

**Table 2:** The laboratory result of sputum specimens for AFB, MTB gene and RIF's gene for T.B. patients under treatment.

Tests	Number/N%	Percentage	Interpretations
<b>AFB</b>			
a) 1-9 BPF	5(2.7)	2.7	primary infection T.B(low) and low contagious
b) +1 BPF	95(24.3)	24.3	Moderate infection T.B and low contagious
c) +2 BPF	57(30.8)	30.8	Severe infections T.B and Highly contagious
d) +3 BPF	49	26.5	Very severe infections T.B and Highly contagious
Negative	24	15.7	The difficulty of coughing and removing sputum is a recent infection with few AFB.
<b>MTB gene</b>			
Positive	185	100	More sensitive than sputum examination
Negative	0	0	Good diagnosis of suspicion
<b>RIF genes:</b>			
RR (Resistant)	6	3.2	Random use of treatment or treatment or use of rifampicin therapy for non-TB disease
SR (Sensitive)	179	96.8	Resistant therapy may be a first-line treatment other than rifampicin, e.g. Isonazoid

AFB= Acid fast bacilli, BPF=Bacilli per field

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Reviewer's Comment

**Table 3:** The frequency of different period of stop first line treatment due no response (failure) of T.B patients, attending NTCB in Sana'a city.

Period of stop previous T.B treatment (failure)	RIF gene positive (n=6)		OR	CI	X <sup>2</sup>	P
	Number	%				
2 Months	5	2.7				
4 Months	0	0				
6 Months	3	1.62				
9 Months	3	1.62				
<b>Total stop (failure)</b>	<b>11</b>	<b>5.94</b>				

**Comment [D21]:** Merge it together put as N%

- All that patient had previous T.B and stopped taking treatment.  
- The shorter the treatment period, the greater the resistance.

**Table 4:** The association of resistant RIF's gene with age and sex of T.B patient under studied.

Characters	RIF gene positive (n=6)		OR	CI	X <sup>2</sup>	P
	No.	%				
<b>Sex:</b>						
1) Male (n=74)	4	5.4	3.1	0.6-17.5	1.8	0.17
2) Female (n=111)	2	1.8	0.32	0.05-1.7	1.8	0.17
<b>Age groups:</b>						
Less than 15yrs (n=14)	1	7.1	2.6	0.3-23	1.7	0.39
16-25yrs (n=52)	2	3.8	1.3	0.2-7.2	0.08	0.77
26-35yrs (n=51)	1	2	0.51	0.058-4.5	0.36	0.54
36-45yrs (n=23)	2	8.7	3.8	0.64-21.8	1.5	0.11
≤46years (n=45)	0	0	undefined		1.99	0.15
Total n=185	6	3.2				

**Comment [D22]:** Chi square values are not separately required can put the p-value and mark with star in bottom can mention the name of test used

OR= Odds ratio, CI=95% confidence interval, X<sup>2</sup>=Chi square, P=probability value

**Comment [D23]:** In discussion the justification is required why author used chi square or fisher exact test

**Table 5:** The associations of Rifampicin Resistance with positive case in the house hold (contacts), time of drugs taken and DOTs.

	Rifampicin Resistance +ve		OR	CI	X <sup>2</sup>	P
	No.	%				
<b>House-hold case (contacts):</b>						
1) Yes (n=29)	0	0	0.0	0.0-4.6	0.25	0.61
2) No (n= 155)	6	3.9	0.0	0.22-1	1.2	0.27
<b>Time and drugs:</b>						
3) Before eat (n=2)	0	0	0	0-170	0.062	0.79
4) After food (n=15)	2	13.3	6.4	1.1-38	5.2	0.02
<b>DOTs:</b>						
5) Yes (n=15)	2	100	6.4	1.1-38	5.2	0.02
6) No (n=2)	0	0	0.0	0.2-10	5.0	0.02
<b>Type of dose:</b>						
7) One dose (n=17)	2	11.8	5.5	1-32.3	4.3	0.03
Two doses (n=0)	0	0	Undefined			
Three doses(n= 1)	0	0	Undefined			

OR= Odds ratio, CI=95% confidence interval, X<sup>2</sup>=Chi square, P=probability value

**Comment [D24]:** In discussion the justification is required why author used chi square or fisher exact test

Reviewer's Comment