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BASIC RESEARCH

Correlation between rpoB gene mutation in *Mycobacterium avium* subspecies *paratuberculosis* and clinical rifabutin and rifampicin resistance for treatment of Crohn's disease

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Abstract

AIM: To investigate overlapping regions of the rpoB gene previously involved with rifamycin resistance in *M. tuberculosis* and seek correlation between rpoB mutations in clinical MAP strains with susceptibility to RIF and RFB.

METHODS: We designed a molecular-based PCR method for the evaluation of rifabutin (RFB) and rifampicin (RIF) resistance based on probable determinant regions within the rpoB gene of MAP, including the 81 bp variable site located between nucleotides 1363 and 1443. The minimum inhibitory concentration (MIC) for RIF was also determined against 11 MAP isolates in attempt to seek correlation with rpoB sequences.

RESULTS: We determined that MAP strain 18 had an MIC of > 30 mg/L and ⩽ 5 mg/L for RIF and RFB respectively, and a significant and novel rpoB mutation C1367T, compared to an MIC of ⩽ 1.0 mg/L for both drugs in the wild type MAP. The 30-fold increase in the MIC was a direct result of the rpoB mutation C1367T, which caused an amino acid change Thr456 to Ile456 in the drug's binding site. In addition, MAP strain 185 contained five silent rpoB mutations and exhibited an MIC comparable to the wild-type. Moreover, our *in vitro* selected mutation in MAP strain UCF5 resulted in the generation of a new resistant strain (UCF5-RIF16r) that possessed T1442C rpoB mutation and an MIC > 30 mg/L and > 10 mg/L for RIF and RFB respectively. Sequencing of the entire rpoB gene in MAP strains UCF4, 18, and UCF5-RIF16r revealed an rpoB mutation A2284C further downstream of the 81 bp variable region in UCF4, accounting for observed slight increase in MIC. In addition, no other significant mutations were found in strains 18 and UCF-RIF16r.

CONCLUSION: The data clearly illustrates that clinical and *in vitro*-selected MAP mutants with rpoB mutations result in resistance to RIF and RFB, and that a single amino acid change in the beta subunit may have a significant impact on RIF resistance. Unconventional drug susceptibility testing such as our molecular approach will be beneficial for evaluation of antibiotic effectiveness. This molecular approach may also serve as a model for other drugs used for treatment of MAP infections.

Key words: Mycobacterium paratuberculosis; Crohn's disease; Rifabutin; Rifampicin; rpoB; Minimum inhibitory concentration

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INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease that detrimentally affects the epithelial lining of the digestive tract and presents with symptoms such as diarrhea, weight loss, abdominal pain, and constipation[1]. Despite the gross similarity of histological and pathological characteristics between CD and the inflammatory intestinal disease found

in cattle with Johne's disease (JD), both diseases remain distinct[1,2]. There is a strong debate currently between CD's potential autoimmune cause and its relation to bacteria, and either concept has yet to be proven. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is known to be the causative agent of JD and has previously been implicated in etiological studies of CD[2-8]. Available information regarding MAP's role in CD pathogenesis has promoted the ongoing studies to continue investigating the relationship between the MAP bacterium and the human bowel disease[3]. A most recent report has shown that antibiotics including rifabutin (RFB) is beneficial to CD patients despite the fact that the clinical trial study met with a few shortcomings regarding drug dosage, duration, and lack of MAP testing for the participating subjects[9]. The latter is best detected using PCR-based assays that amplify regions of the insertion sequence IS900 as shown by many investigators[10-12].

RFB and rifampicin (RIF) are antibiotics that belong to the rifamycin drug family and are very similar in chemical structure. The function of these antibiotics are to inhibit the growth of bacteria, specifically by binding to the beta subunit of RNA polymerase through direct and indirect amino acid interactions, and preventing the production of nascent RNA transcripts[13]. The rpoB gene in prokaryotes encodes the beta subunit, and mutations within this gene result in a higher level of resistance to the rifamycin antibiotics in several bacteria[14-19]. Moreover, mutations within an 81 base pair region in rpoB spanning nucleotides 1276-1356 in *M. tuberculosis* have been shown to contain the majority of alterations relating to rifamycin resistance[20]. In addition, mutations at the beginning of rpoB are associated with rifamycin resistance in some strains of *M. tuberculosis*. However, these occurrences are not as prevalent as the former[15,21].

Previous reports have shown that RFB may potentially serve as a therapeutic agent for the treatment of CD[22-27], and as an effective drug against MAP[28]. In addition, other treatments such as anti-inflammatory agents have been proved to relieve symptoms of CD[29]; however these treatments are not as beneficial as antibiotic therapy[22]. As a result, more attention has been focused on the possible use of antibiotics as an alternative remedy. However, a screening method for RIF and RFB resistant strains of MAP has yet to be established. Therefore, as CD therapy becomes more focused on antibiotics, it is essential to develop a method for susceptibility testing in order to detect and monitor MAP strains for RIF and RFB resistance.

Unlike other prokaryotes, MAP is very fastidious and requires unusual *in vitro* growth conditions, including the addition of *Mycobactin J*. Additionally, MAP cultured from CD samples has shown to lack a cell wall, and this characteristic is a major contributing factor to the complexity of the bacteria's primary isolation process[11,30,31]. Consequently, conventional drug susceptibility tests involving MAP are not reliable and may result in inaccurate results. Therefore, the challenges faced when working with this bacterium must be counteracted *via* alternate approaches that could potentially result in the exposition of significant data in order to effectively treat MAP infections. In this study, we adopted a molecular approach based on PCR amplification, followed by nucleotide sequencing of regions within the rpoB gene of MAP. We attempted to investigate the overlapping regions of the rpoB gene previously involved with rifamycin resistance in *M. tuberculosis*. The ultimate goal is to seek the correlation between rpoB mutations in clinical MAP strains with susceptibility to RIF and RFB.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All clinical strains including UCF3, UCF4, UCF5, UCF7, UCF8, MAP18, MAP185, and 61a were isolated in our laboratory from clinical samples obtained from CD patients[32]. Each MAP isolate was taken from different patients; however the anatomical sources for each overlap. For instance, each MAP isolate was isolated from their corresponding anatomical sources of individual CD patients: UCF3, UCF4, UCF8, 18, and 61a from the ileal; UCF5 and UCF7 from ileocolonic tissue; and 185 from the mesenteric lymph node. These MAP strains originated from surgical tissue samples obtained from CD patients[32-34]. Cow2013 and Cow5 MAP strains were recently isolated from ground beef samples from two cattles with JD. Briefly, tissue samples were ground, homogenized, decontaminated and then inoculated into MGIT culture media with supplements including OADC, *Mycobactin J* and PANTA as described previously[32].

All American type culture collection (ATCC) strains were verified in our laboratory by biochemical and molecular testing, including MAP strain 43 544, *M. avium* subspecies avium 25 291, *M. tuberculosis* strain 25 177, and *M smegmatis* strain 607. All cultures were subcultured in 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) and *Mycoabctin J*. Plates were incubated at 37℃ until visible colonies were observed. Colonies from pure culture verified by Ziehl-Neelsen acid fast stain and IS900-based PCR for MAP were used to inoculate BACTEC 7H9 broth culture supplemented with 500 μL OADC and 2.4 μmol/L *Mycobactin J*. Growth index (GI) was read weekly until optimum growth index was observed. The fresh culture was then used for drug susceptibility testing and for molecular studies.

Minimum inhibitory concentration (MIC) measurement

MIC for RIF and RFB were determined against all micro-organisms used for this study. Starting cultures with a GI range of 500-600 were found optimal for inoculation. The inoculum size for each Bactec bottle used in the drug susceptibility study was approximately 1.0×10^5 CFUs. Serial dilutions of RIF concentration ranging from 0 to 4 mg/L were evaluated against all micro-organisms. MAP strains resistant to RIF > 1.0 mg/L were further tested against RIF concentrations of 10, 20 and 30 mg/L for MIC measurement. These MAP strains were also evaluated against RFB at concentrations ranging from 0.0 to 10 mg/L, excluding UCF4. MAP wild type was included as a control in each batch of analysis. Experiments were repeated for confirmations. Micro-organisms other than MAP were also evaluated for RIF concentrations ranging from 0.0 to 10.0 mg/L. The percentage of RIF or RFB inhibition was used to determine the level of susceptibility for each

Table 1 PCR primers used in this study

Primers	Sequence (5' to 3')	Amplified base pairs (bp)[1]	Amplicon length (bp)
IS900			
P90	GTTCGGGGCCGTCGCTTAGG	22-421	400
P91	GAGGTCGATCGCCCACGTGA		
AV1	ATGTGGTTGCTGTGTTGGATGG	77-384	308
AV2	CCGCCGCAATCAACTCCAG		
rpoB			
Efox1	TTGCCGGCCGAACCGACACA	1-721	721
Fox1r	TGTCGACGTCGAACTCCAGC		
UCF1	TCGATGTCGCTGTCTTTCTC	373-820	448
UCF2	GCTCGGTGATCTGCTCGTTG		
Fox1f	CGGTGTTCATGGGTGACTTC	521-1509	989
DBR	GTAGTGGGACGGGTGCACGTC		
Knight1	ACCACTTCGGCAACCGCCGG	1191-1777	587
Knight2	ACTCGACCTCGCCCGCCTTG		
Ex1a	AAGGTGGTCGACGGCGTGGT	1621-2340	720
Ex2a	GATCTCGTGCTCCTCGATGT		
Ex3	ACGAGGACGCGATCATCCT	2261-2980	720
Ex4	TCGACACGATCTGGTTCGGC		
Ex5	GAACATCGACGGCAATCCCG	2901-3597	697
Hrox1	TCCGTCGAGGACCTGGCTTAA		

[1]Numbers represent nucleotide positions within each gene of MAP.

concentration of antibiotics as follows: % Inhibition = 1 minus (GI of Bactec culture without drug minus GI of Bactec culture with drug)/GI of Bactec culture without drug). The MIC was also determined for each bacterial sample, and was defined as the minimum concentration of antibiotics that induced inhibition of growth by 90%.

Microbial genomic DNA extraction, nested PCR and nucleotide sequencing

Extraction of genomic DNA from mycobacterial isolates was performed for verification of the presence of the IS900 gene and for analysis of rpoB gene sequence. The DNA extraction, purification and quantification were performed as described previously[11]. Nested PCR for IS900 amplification was performed using the primers p90/91 and AV1/AV2 as listed in Table 1[11]. The size of amplified product was determined on 2% agarose gel. Appropriate negative controls for PCR consisting of sterile TE buffer or sterile water in place of the DNA template were used in parallel with each round of PCR preparation. Positive MAP DNA from strain ATCC 43015 was prepared independently and added to PCR tubes at a different facility using separate supplies.

Unlike the nested IS900-based PCR assay, only one round of PCR assays was developed for amplification of two different regions of the rpoB gene. Therefore, one PCR reaction contained the UCF1/UCF2 primers for amplification of 448 bp whereas the second PCR reaction contained the Knight1/Knight2 primers for amplification of 587 bp. The ingredients and protocol conditions of the rpoB-based PCR assays were as described previously[11]. Table 2 lists the nucleotide sequence for all primers used in this study. Each PCR product was purified from agarose using the Purelink Quick Gel Extraction Kit following the procedure described by the manufacturer (Invitrogen). Purified DNA was then quantitated and subjected to nucleotide sequencing. The latter was performed using the GenomeLab DTCS-Quick Start Kit following the manufacturer's instructions (Beckman Coulter). Both DNA strands were sequenced for each PCR product using appropriate nucleotide primers at the Biomolecular Science Center DNA Sequencing Core Facility at the University of Central Florida. BLAST analysis was performed using the Pubmed.gov database, and the rpoB sequence from MAP strain K-10[35] was used as our reference wildtype control sequence for comparison purposes.

In vitro selected RIF-MAP mutant

An RIF resistant MAP mutant (UCF5-RIF16r) was selected through the generation of rpoB mutation *via* adaptive resistance in the presence of antibiotics. This was performed by exposing our wild-type MAP strain UCF5 to increase the concentrations of RIF ranging from 1 to 16 mg/L. Initially, MAP strain UCF5 was inoculated into a BACTEC bottle containing 1 mg/L RIF. Following incubation, the surviving MAP cells were sub-cultured into a new BACTEC bottle with double RIF concentrations. The process was repeated several times until a new MAP strain (UCF5-RIF16r) was selected that was able to survive in the presence of 16 mg/L of RIF. This new resistant strain was then tested for MIC against RIF and RFB and investigated for possible rpoB mutations as described earlier.

RESULTS

Identification of MAP

Genomic DNA from all bacterial isolates was subjected to IS900-based PCR in order to confirm the identity of all MAP strains. This procedure involved two rounds of PCR, using p90/91 primers in the first round to amplify a 400 bp sequence. The use of AV1/AV2 in the second round of PCR amplified a 308 bp sequence and provided exceptional sensitivity and enhanced specificity for the confirmation of MAP. As expected, all 11 MAP isolates were confirmed for the presence of IS900. In addition, *M. avium* subspecies avium, *M. smegmatis* and *M tuberculosis* displayed negative results for the presence of the IS900.

rpoB amplification and sequence analysis

Following the IS900 PCR analysis, genomic DNA from each identified MAP strain was used as template for PCR employing rpoB primers (Table 1). Amplification of rpoB sequences enabled the possibility for amplicon purification, sequencing, and subsequently BLAST analysis. The use of primers UCF1/UCF2 and Knight1/Knight2 enabled the amplification of two regions of the rpoB gene of MAP. These regions overlapped similar sequences in the rpoB gene of *M. tuberculosis* previously associated with rifamycin resistance. Moreover, PCR with Knight1/Knight2 primers amplified a sequence that harbored the 81 bp variable site 1363-1443, a highly probable determinant region for rifamycin resistance in closely related bacteria. Amplicons from both regions of the rpoB gene were obtained by successful PCR.

Nucleotide sequencing of both rpoB regions for all MAP strains was performed for both forward and reverse primer reactions to exclude any possible errors in the data.

Table 2 rpoB and susceptibility data for all micro-organization

Microorganism	Strain	RIF MIC (mg/L)	RFB MIC (mg/L)[1]	Inhib. at 1 mg/L of RIF (%)[2]	rpoB BLAST result[3]	Amino acid change[4]
MAP	ATCC43544	≤ 1.0	≤ 1.0	91 ± 0.71	WT	NC
MAP	UCF3	≤ 1.0		94 ± 2.83	WT	NC
MAP	UCF4	≤ 2.5		79 ± 7.07	A2284C	N762H
MAP	UCF5	≤ 1.0		92.5 ± 0.71	WT	NC
MAP	UCF7	≤ 1.0		88 ± 5.66	WT	NC
MAP	UCF8	≤ 1.0		90 ± 1.41	WT	NC
MAP	18	≥ 30	≤ 5.0	42 ± 1.00	C1367T	T456I
MAP	185	≤ 4.0	≤ 1.0	61 ± 7.07	Silent	NC
MAP	Cow2013	≤ 1.0		95 ± 1.41	WT	NC
MAP	Cow5	≤ 1.0		96.5 ± 0.71	WT	NC
MAP	61a	≤ 1.0		90 ± 3.54	WT	NC
M avium subspecies. avium[5]	ATCC25291	≤ 1.0		90 ± 1.41		
M tuberculosis[5]	ATCC25177	≤ 1.0		92 ± 1.41		
M smegmatis[5]	ATCC607	≥ 9.0		6.6 ± 1.7		
UCF5-RIF16r	Modified UCF5	≥ 30	≥ 10	32 ± 1.41	T1442C	L481P

[1]Limited investigation involving MAP18, MAP185, and UCF5-RIF16r; [2]Values are expressed as mean ± SD; [3]Wild type (WT) indicates identical investigated rpoB sequence compared to MAP K-10 strain, and differentially expressed rpoB mutations are indicated; [4]Amino acid positions correspond to MAP strain K-10 numbering system from Li *et al* 2005, and the alteration is indicated with single letter amino acid codes and the corresponding codon number. NC corresponds to no change in beta subunit sequence based on investigated rpoB regions; [5]rpoB sequence was not investigated.

Furthermore, sequence data was reported as completely accurate upon the confirmation of error-free reactions. In addition, rpoB sequences for all MAP strains were compared through BLAST analysis using MAP strain K-10 as a reference strain, and amino acid positions were numbered based on MAP strain K-10. The numbering system for *E. coli* provided by Ramaswamy *et al* in 1998 was also used in order to avoid discrimination of our observed results, and to enable a more general comparison of previously published data relevant to other mycobacterial species.

Sequence analysis found identical, or wild-type (WT), and non-identical sequences. MAP strains consisting of the latter were further characterized for silent mutations with no effect on amino acid expression, or mutations that differentially expressed the amino acid sequence of the beta subunit. Of the 11 MAP clinical isolates, 9 consisted of no rpoB mutation in the two regions initially investigated (Table 2). However, two MAP strains possessed rpoB mutations, and these consisted of MAP strain 18 and 185. The rpoB mutations for MAP strain 18 included C1367T and T1375C. The C1367T mutation had significant amino acid changes from Thr456 to Ile456 in the beta subunit of RNA polymerase, which corresponded to a Ser508 to Ile508 change according to the *E. coli* numbering system. To our knowledge, this amino acid change was considered novel as well as indicative of RIF and RFB resistance. The T1375C mutation had no effect on the beta subunit, and was, therefore, characterized as being silent (Table 2). Alternatively, MAP strain 185, which was also isolated in our laboratory from the surgical mesenteric lymph node tissues of a CD patient, showed a total of five rpoB mutations including T534C, T795C, C1335A, T1375C, and C1578T (Table 2). None of the five mutations altered the amino acid sequence in the beta subunit, and consequently were also termed silent. The effects of all rpoB mutations on RIF and RFB susceptibility were investigated following sequence analysis.

Effect of rpoB mutations on MIC

Correlation between rpoB sequence analysis and inhibitory growth rates of RIF was assessed for all MAP strains. Initially, we tested all MAP strains in the presence of 1 mg/L RIF for comparison purposes (Table 2). This concentration was found to be the MIC of our wild-type control. Hence, our initial susceptibility test was to screen for suspicious growth characteristics against this concentration of RIF. Of the bacterial isolates studied, 8 MAP strains including ATCC43544 (control), UCF3, UCF5, UCF7-8, 61a, Cow2013, Cow5, and two non-MAP controls including *M. tuberculosis* and *M. avium* subspecies avium had an MIC ≤ 1.0 mg/L RIF (Table 2). In addition, there were no observed rpoB mutations in these 8 RIF susceptible MAP strains. The MIC for RIF of the remaining MAP strains 18, 185, UCF4, and *M. smegmatis* was determined as ≥ 30, ≤ 4.0, ≤ 2.5 and ≥ 9.0 mg/L, respectively (Table 2). Furthermore, MAP strains 18 and 185 had rpoB mutations in the 81 bp variable region as discussed earlier, and MAP strain UCF4 had no rpoB mutations within the two regions. Despite the lack of rpoB mutations, *M. smegmatis* had MIC of ≥ 9 mg/L RIF. RIF-resistance MAP strains were also evaluated for susceptibility against RFB, a more potent rifamycin antibiotic. Interestingly, the MIC for RFB against MAP strain 18 and 185 was ≤ 5.0 mg/L and ≤ 1.0mg/L, respectively (Table 2). The MIC values for the drugs against all microorganisms are listed in Table 2.

Correlation between selected rpoB resistant mutation and MIC in UCF5-RIF16r

An RIF resistant MAP strain, termed UCF5-RIF16r, was selected via culturing wild-type parent strain UCF5 in increasing concentrations of RIF over an extended time period. Specifically, this was accomplished by exposing parent strain UCF5 to a five-fold increase in RIF concentrations over approximately 2 mo. UCF5-RIF16r was then maintained in cultures with > 16.0 mg/L RIF. Genomic DNA was then extracted and followed by IS900-nested PCR and rpoB-based PCR analysis. Consequently, the newly selected resistant strain was confirmed for the

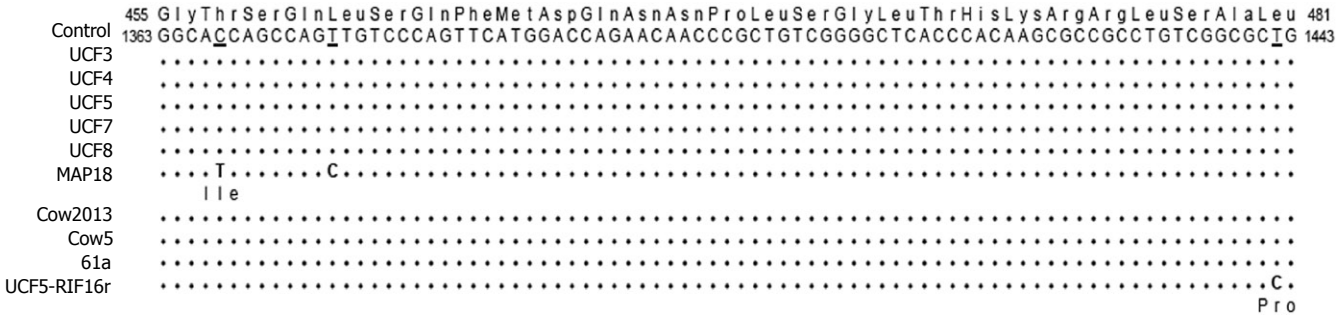

Figure 1 Sequence alignment of 81 bp region in rpoB gene of MAP. The region overlaps the 81 bp rifamycin resistant determinant region within *M. tuberculosis*. For MAP, this sequence is harbored in the region amplified by primers Knight1/Knight2. Differentially expressed rpoB mutations are indicated for MAP strains 18 and UCF5-RIF16r. Base pairs and amino acids range from 1363-1443 and 456-481. Numbering of amino acids correspond to MAP strain K-10 beta subunit. Dots correspond to homologous bases and specific nucleotides are underlined in the control sequence. MAP strain 185 is excluded from diagram.

presence of IS900, and both regions of the rpoB gene were analyzed. We detected a single nucleotide rpoB mutation T1442C, which caused a differentially expressed amino acid from Leu481 to Pro481 in the beta subunit of RNA polymerase, which corresponded to a Leu533 to Pro533 change according to the *E. coli* numbering system (Table 2). Furthermore, the rpoB mutation was located within the 81 bp variable region, and the effect of the selected mutation on susceptibility to RIF was then investigated. The MIC for UCF5-RIF16r against RIF and RFB was $\geqslant$ 30 mg/L and $\geqslant$ 10 mg/L, respectively.

Sequencing rpoB genes in MAP strains 18, UCF4 and UCF5-RIF16r

We attempted to sequence the entire 3.6 kb rpoB gene in order to determine the source of the variable increase in MIC for RIF against MAP strain UCF4, and to detect any other possible rpoB mutations leading to amino acid alterations in strains 18 and UCF5-RIF16r. We designed additional primers for PCR amplification of sequences outside that amplified by primers UCF1/UCF2 and Knight1/Knight2 (Table 1). After successful PCR amplification of these regions, nucleotide sequencing was performed. Through BLAST analysis, we found a significant rpoB mutation further downstream of the 81 bp variable region in MAP strain UCF4. Specifically, an A2284C mutation occurred within the region amplified by primers Ex1a/Ex2a. This mutation resulted in an Asn762 to His762 amino acid change, leading to a possible explanation for the higher MIC for RIF compared to our wild-type control strain 43544. No other rpoB mutations leading to amino acid changes were observed in MAP strains 18, UCF5-RIF16r, and UCF4. After obtaining collective sequence information for all MAP isolates, we aligned the 81 bp variable region to display the location of each mutation amino acid change that correlated with a high level of RIF resistance (Figure 1).

Detection of rpoB in PBMNC infected with MAP

To show the potential for sequence analysis in the rpoB gene of MAP clinical isolates in correspondence with RIF and RFB resistance, we contaminated human PBMNC with 1.0×10^3 CFU of MAP strain 18. Prokaryotic genomic DNA was then extracted from the blood mixture, followed by IS900-PCR and rpoB-based PCR analysis. As expected, our protocol detected MAP in the blood sample and successfully amplified both regions of the rpoB gene of MAP (data not shown). The nucleotide sequence was then analyzed for the possible prediction of susceptibility to RIF or RFB.

DISCUSSION

The main purpose of our study was (1) to characterize MAP's potential for developing RFB and RIF resistance, (2) associate RFB and RIF resistance with mutations in the rpoB gene of MAP, and (3) provide an effective protocol for detecting resistant mutations in MAP strains linked to CD. Despite the fact that *M. smegmatis* is known to be naturally resistant to rifamycins without rpoB mutations[36], microorganisms such as *M. avium* spp. avium, *E. coli*, *H pylori, S. aureus and M. kansasii* have laid the foundation for this association[14,16,18,19,21,37]. Moreover, various strains of closely related *M. tuberculosis* have set a fine trend for rifamycin resistance, as seen through rpoB mutations[6,17,21,38-46]. Our goal was to discover this trend in MAP, and develop an effective method for analyzing RFB and RIF resistance in the bacterium through rpoB-based PCR analysis.

Unlike *M. tuberculosis* and members of the *M. avium* complex (MAC), MAP is a fastidious micro-organism and requires prolonged incubation time. Consequently, conventional drug susceptibility testing against MAP strains is ineffective, thus leading us to develop an alternative method for determining drug resistance in MAP.

The UCF1/UCF2 and Knight1/Knight2 rpoB primers enabled the successful amplification and sequencing of two probable RFB and RIF resistant determinant regions (Table 1). These regions overlap similar sequences in the rpoB gene of *M. tuberculosis* that have displayed mutations upon rifamycin resistance, including the 81 bp region 1276-1356 within cluster I, and a region further located in the upstream of the beginning of rpoB[6,15,20]. Consequently, our determinant regions of interest in the rpoB gene of MAP were concluded based on the available information for closely related bacteria.

All MAP isolates were identified through an IS900 nested PCR reaction and each was investigated for rpoB

sequences and susceptibility tests against RIF. Those isolates suspected to be RIF resistant based on high MIC results were subjected to susceptibility tests against RFB. Consequently, the control, MAP strain 18 and UCF5-RIF16r were included in a RFB inhibition test. MAP strain 185 was also included in the RFB test due to the strain's suspicious susceptibility against RIF. Unlike RIF, RFB is not commercially available for laboratory use. Hence, information based on antibiotic resistance in our study was subjective mainly for RIF.

Our significant findings included the discovery of rpoB mutations within MAP strains 18, UCF5-RIF16r, and UCF4. Both strains 18 and UCF5-RIF16r showed a significant increase in the level of resistance due to amino acid alterations within the binding site of RIF. Moreover, upon our additional sequencing of the entire 3.6 kb rpoB gene for all three MAP strains along with a control, a significant rpoB mutation A2284C was uncovered leading to an Asn762 to His762 amino acid change in the beta subunit. These results justified the minor increase in MIC against RIF for strain UCF4. In addition, the complete sequencing of the rpoB in MAP strains 18 and UCF5-RIF16 proved that no additional rpoB mutations leading to an amino acid change were present, confirming the correlation between detected mutations and increased MIC in these micro-organisms. The finding illustrates that a single amino acid change in the rpoB gene of MAP may result in an increased level of resistance to RIF, which may have a significant clinical impact, specifically when the amino acid alterations occur within the drug's binding site.

Overall, our results display a novel rpoB mutation, C1367T, in MAP strain 18 not previously reported in the literature including rpoB studies in *E. coli* and *M. tuberculosis*. In addition, the A2284C rpoB mutation discovered in MAP strain UCF4 was also novel to our knowledge; however this mutation did not influence RIF resistance significantly compared to MAP strains 18 and UCF5-RIF16r. A possible explanation for this observation is that the mutation in strain UCF4 was located distant from the drug binding site. The selected rpoB mutation in strain UCF5-RIF16r has previously been reported to occur in *M. tuberculosis* as well as in *E. coli*[13]. More significantly, Leu481 in the beta subunit of MAP overlaps that of Leu413 in *Thermus aquaticus*, and this amino acid has been reported to make direct contacts with RIF in its bound state through Vanderwaals interactions[13]. Hence, the selected change from Leu481 to Pro481 in MAP strain UCF5-RIF16r may have affected the direct interaction between the beta subunit and the drug.

The rationale in selecting the RIF resistant mutant UCF5-RIF16r was to determine the potential for MAP to evolve adaptive resistance to RIF, and the location of the rpoB mutation that accounted for the resistance. This data, combined with our analysis of MAP strain 18, showed the potential for MAP to evolve resistance to RFB and RIF *in vivo* and *in vitro* through mutations in the 81 bp variable region. Collectively, after analyzing the resistance characteristics for MAP strain 18 and UCF5-RIF16r, we considered the rpoB 81 bp variable regions to be the most significant for determining resistant mutations for RFB and RIF.

MAP strain 185 consisting of five silent mutations within rpoB was not considered significant because they did not alter the beta subunit sequence. Moreover, we encountered unusual PCR results and difficulties in extracting genomic DNA from strain 185; therefore, we were unable to sequence its entire rpoB gene and account for the slight resistance to RIF. Hence, the mechanism of the partial resistance against RIF for this strain remained undetermined.

Our rpoB-based protocol on human blood mixed with MAP was accomplished through contaminating normal human blood with MAP strain 18, and extracting bacterial DNA directly from the blood mixture. As a result, a sufficient amount of bacterial genomic DNA was obtained for successful amplification of our regions of interest within rpoB (data not shown). The purpose of this approach was to assess the effectiveness of our protocol on CD patient blood.

Information regarding patient history may explain a rationale for the observed results. Ironically, the patients' medical history indicated that none of them was on anti-MAP treatment. Instead these patients had received a variety of anti-inflammatory and immunosuppressants. However, alarming new reports suggest that some of these drugs may contain antimicrobial activity, especially when tested against few strains of MAP[47]. Hence, it is inconclusive to rule out the possibility of antibiotic resistance development *via* alternative drugs.

In conclusion, through the application of our protocol on CD patient samples, we may assist in the determination of RFB and RIF susceptible MAP strains for the treatment of CD. The inconclusive results from the recent Australian clinical trial using RFB, calrithromycin and clofazamine for treatment of patients with CD[9] suggested either the absence of MAP or the presence of drug resistance MAP in non-responders. Therefore, detection of drug resistance in pathogens like MAP is now necessary. Our protocol may address the rationale for MAP resistance to RFB in these patients. Based upon the *in vitro* selection of a RFB and RIF resistant MAP strain (UCF5-RIF16r), it is likely that this trend occurs *in vivo*, as supported by the data from MAP strain 18. Furthermore, as RFB is applied more towards the treatment of CD, our protocol will be of utmost significance for the optimization of CD treatment with related antibiotics.

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