Short Signature rpoB Gene Sequence to Differentiate Species in Mycobacterium abscessus Group (MAG)

Jian Rong Bao, Kileen Shier, Ronald Master, Robert Jones, and Richard Clark

Corresponding Author(s): Jian Rong Bao, Quest Diagnostics

Review Timeline:

| Event                  | Date        |
|------------------------|-------------|
| Submission Date        | December 21, 2021 |
| Editorial Decision     | March 28, 2022 |
| Revision Received      | May 17, 2022 |
| Editorial Decision     | July 5, 2022 |
| Revision Received      | July 8, 2022 |
| Accepted               | July 18, 2022 |

Editor: Digby Warner

Reviewer(s): The reviewers have opted to remain anonymous.

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1128/spectrum.02534-21
Dr. Jian Rong Bao  
Quest Diagnostics  
14225 newbrook dr  
Chantilly, Virginia 20151

Re: Spectrum02534-21 (Short Signature rpoB Gene Sequence to Differentiate Species in Mycobacterium abscessus Group (MAG))

Dear Dr. Jian Rong Bao:

Thank you for submitting your manuscript to Microbiology Spectrum. Your paper has been considered by two expert reviewers who agree that the paper describes a potentially useful approach to differentiating species within the Mycobacterium abscesses complex. However, both reviewers have identified specific revisions which are required to ensure the results are interpreted adequately and that the potential utility of this approach (and its limitations) are fairly presented. In particular, concerns were raised that the potential limitations of the approach are inadequately acknowledged, and that the number of species tested (especially M. massillense and M. bolletii genomes) is small and should be expanded.

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Sincerely,

Digby Warner  
Editor, Microbiology Spectrum  
Journals Department  
American Society for Microbiology  
1752 N St., NW  
Washington, DC 20036  
E-mail: spectrum@asmusa.org

Reviewer comments:

Reviewer #1 (Comments for the Author):

This study was aimed at developing a rapid pyrosequencing assay focusing on one target gene (rpoB) to discriminate between the three subspecies of the Mycobacterium abscessus complex (MABC) in addition to the two main species of the M. abscessus-M. chelonae complex. The authors argue that this assay will improve identification and treatment of infections caused by the members of this complex. Several studies have already been published describing approaches to discriminate between
the different species of M. abscessus including rpoB sequencing, therefore this work is not entirely novel. However, being able to reliably identify the three subspecies of MABC is important mostly for predicting the susceptibility to macrolides that are key drugs for the treatment of infections caused by MABC. Two of the MABC species (abscessus and bollettii) are generally resistant to macrolides whereas the third species (massiliense) is susceptible. In this regard, the work presented in this manuscript is relevant.

The manuscript itself is at time lacking organization and clarity. Details are missing, particularly regarding the in-silico methodology leading to the choice of rpoB as a target and the identification of the target signatures and should be provided in Methods. There are some significant issues with this work. It is unclear how the assay was validated after being developed and before being utilized to test the collection of 111 clinical MCAC isolates. There is a lack of a gold standard identification method to which the results obtained with could be compared to determine the sensitivity and specificity of the pyrosequencing assay. It seems that most if not all of this part of the validation was performed in-silico with an in-house database containing publicly available rpoB sequences extracted from GenBank. No information is provided on what type of QC the authors performed on their database. One of the problems with using Genbank for clinical purpose is that sequences are not curated and might not always be accurate. One perfect example is ATCC strain 700868 that is labeled as Mycobacterium abscessus but was identified as Mycobacterium bolletti with the assay described in this manuscript. Were additional sequence data available for all the extracted rpoB sequences? This absence of a solid reference method is also a potential problem for the retrospective testing on 111 MCAC isolates. All the alternative methods of identification used in this study have some limitations and do not allow full evaluation of the accuracy of the SSS assay. Methods such as WGS, if available or line probe assay would have been preferable.

Several studies have clearly suggested that rpoB sequencing should not be used as a stand alone for identification of M. abscessus complex species mainly due to the occurrence of horizontal gene transfer between these different subspecies. This has been observed particularly with M. massiliense that can transfer M. abscessus rpoB and therefore could be misidentified as M. abscessus by the SSS assay while still being susceptible to macrolides. Recommendations are to use a two-tier approach with rpoB sequencing as the primary assay, followed with a secondary assay looking a different target such as erm (41) for example. This potential issue should have been addressed by the authors.

Other issues:

- Line 38: M. abscessus complex species are not phenotypically indistinguishable
- Line 73: rpoB is not a multi copy gene as stated
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More generally, this table is very confusing and of limited interest and therefore should be deleted

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2. There are contradictory statements in the manuscript. Authors state that sequencing of the 16S rRNA lacks differential base-pairs for the MCAC species, but they use 16S sequencing as the reference method, as indicated in Table 3. It is not clear which method the authors used as their reference. Authors cited 16S rRNA in Table 3, 16S-ITS in reference 5 (doi: 10.1128/JCM.42.12.5493-5501.2004), and part of the rpoB in reference 1 and primer sequence (https://doi.org/10.1099/ijs.0.63969-0).
3. Please expand information describing the collection of strains- when and where were the samples isolated? Were they typed at the place of isolation?
4. The manuscript would be more transparent if the authors included how they found the discriminative sequence.
5. Geneious is headquartered in New Zealand. Please mention the particular tools used in this study.
6. Please include information that pyrosequencing technology is discontinued.
7. It would be of value if the authors could expand the test of their method against a larger database of strains M. abscessus complex. It could be done in silico, for example against the collection of strains published in https://doi.org/10.1038/s41598-020-73607-x. Currently, their method was tested for six strains of M. massiliense and four strains of M. bolletii.
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- Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file.
- Manuscript: A .DOC version of the revised manuscript
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The concerns have been addressed in the revised manuscript [Results (page 11) and Discussion (page 14) sections]. More than 90 (including the 5 type strains) reference sequences of the three MAG species were additionally analyzed in-silico and agreed 100% to the rpoB typing method for both ID and sequence homology. The analytical results were added to the manuscript (p14), including Table 4.

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Thanks. The word “novel” was used to describe the method, including the primers, database and procedures, not necessarily a novel methodology or technology.

The manuscript itself is at time lacking organization and clarity. Details are missing, particularly regarding the in-silico methodology leading to the choice of rpoB as a target and the identification of the target signatures and should be provided in Methods.

More descriptions had been added (p6). Additional in-silico analysis was carried out (p8) and results were added to the manuscript (p14).

The in-silico methodology has been described more detailly in the paper (p6), which we think was adequate for this application study. The development that led to the choice of the targeted fragment and the primers was a gradual process when both the preliminary experiments and in-silico analysis were conducted during the study.

There are some significant issues with this work. It is unclear how the assay was validated after being developed and before being utilized to test the collection of 111 clinical MCAC isolates. There is a lack of a gold standard identification method to which the results obtained with could be compared to determine the sensitivity and specificity of the pyrosequencing assay.

We used three standards for the comparisons:

1. Reference strains from authentic resources, such as ATCC, or the survey agencies.
2. Use Sanger sequencing for 40-siolate subset to validate the SSS method identifications.
3. Compare the SSS method \textit{in-silico} results from the type strains or reference strains.

To follow the reviewer’s recommendations, we added extra \textit{in-silico} analysis (88+5 type strains) from well-characterized strains, and they had both 100% agreements with the \textit{rpoB} typing method and their sequence homologies (p8 and p11).

For the comments on sensitivity and specificity calculations, we use “agreement” for identification result category for the better fit we think (Table 4).

It seems that most if not all of this part of the validation was performed in-silico with an in-house database containing publicly available \textit{rpoB} sequences extracted from GenBank. No information is provided on what type of QC the authors performed on their database. One of the problems with using Genbank for clinical purpose is that sequences are not curated and might not always be accurate. One perfect example is ATCC strain 700868 that is labeled as Mycobacterium abscessus but was identified as Mycobacterium bolletii with the assay described in this manuscript. Were additional sequence data available for all the extracted \textit{rpoB} sequences? This absence of a solid reference method is also a potential problem for the retrospective testing on 111 MCAC isolates. All the alternative methods of identification used in this study have some limitations and do not allow full evaluation of the accuracy of the SSS assay. Methods such as WGS, if available or line probe assay would have been preferable.

Our in-house database was established from the type strains and reference strains, not randomly from the GenBank. The database has been validated according to the results of identification and sequence homology from reference methods (Sanger sequencing, \textit{rpoB} typing).

The ATCC 700868 strain identification as \textit{M. bolletii} was confirmed from the multi-gene-based Line-probe method as well as Sanger sequencing (p10).

Several studies have clearly suggested that \textit{rpoB} sequencing should not be used as a stand alone for identification of \textit{M. abscessus} complex species mainly due to the occurrence of horizontal gene transfer between these different subspecies. This has been observed particularly with \textit{M. massiliense} that can transfer \textit{M. abscessus} \textit{rpoB} and therefore could be misidentified as \textit{M. abscessus} by the SSS assay while still being susceptible to macrolides. Recommendations are to use a two-tier approach with \textit{rpoB} sequencing as the primary assay, followed with a secondary assay looking a different target such as \textit{erm} (41) for example. This potential issue should have been addressed by the authors.

A discussion was added for the potential identification discrepancies (p14).

Other issues:
Line 38: \textit{M. abscessus} complex species are not phenotypically indistinguishable

Modified (line 56).

Line 73: \textit{rpoB} is not a multi copy gene as stated

Removed.
Line 95: It seems that the hypervariable site that is targeted is within the rpoB gene coding sequence and not in the promoter region as stated. Please clarify

Modifies (p6).

Line 113: Should this read Table 3 instead of Table 2? In addition, in Table 3, should it read rpoB instead of 16SrRNA?

Corrected (Table 3). The last column is 16S rRNA gene for the complex ID (MCAC).

More generally, this table is very confusing and of limited interest and therefore should be deleted

The Table 3 has been re-formatted and re-organized, which should make it easier to understand. We would like to keep it as we think the information is useful.

Reviewer #2 (Comments for the Author):

The article presents a new method of identification of species of Mycobacterium abscessus complex. The authors indicated a 35 bp sequence and validated its usefulness for differentiation of 111 strains of M. abscessus-chelonae complex with pyrosequencing. While the method could be valuable for a broader public, the manuscript requires substantial corrections.

1. Please mention Hain Lifescience test GenoType NTM-DR for differentiation of M. abscessus complex. Please remove a comment that signature sequence hybridization was tested with a limited number of samples, as the method was tested in silico with over 1500 strains.

Removed.

2. There are contradictory statements in the manuscript. Authors state that sequencing of the 16S rRNA lacks differential base-pairs for the MCAC species, but they use 16S sequencing as the reference method, as indicated in Table 3. It is not clear which method the authors used as their reference. Authors cited 16S rRNA in Table 3, 16S-ITS in reference 5 (doi: 10.1128/JCM.42.12.5493-5501.2004), and part of the rpoB in reference 1 and primer sequence (https://doi.org/10.1099/ijs.0.63969-0).

The 16S rRNA gene was not used as the reference method but was used to identify the isolates to MCAC when the isolates were collected for the study.

We used the reference #1 & #5 so that the type strains (M. bolletii & M. massiliense) were addressed.

The reference 1 is where the new taxa M. bolletii was described. We used the PCR primers described in the reference for our Sanger sequencing for the method comparison, not for this SSS method itself.

The reference 5 was where the new taxa M. massiliense was described and multi-loci sequencing method, including partial rpoB sequencing, was used.
3. Please expand information describing the collection of strains—when and where were the samples isolated? Were they typed at the place of isolation?

Additional information has been added to the manuscript (p5). The isolates were identified as MCAC at the collection.

4. The manuscript would be more transparent if the authors included how they found the discriminative sequence.

Additional information was added in the manuscript (p6, as discussed above).

5. Geneious is headquartered in New Zealand. Please mention the particular tools used in this study.

Geneious Prime was used. The headquarter has been corrected (Line 115).

6. Please include information that pyrosequencing technology is discontinued.

Pyrosequencing technology is not discontinued, but PyroID has been replaced with Q48 or Q24 instruments. This was listed in line 252-255.

7. It would be of value if the authors could expand the test of their method against a larger database of strains M. abscessus complex. It could be done in silico, for example against the collection of strains published in https://doi.org/10.1038/s41598-020-73607-x. Currently, their method was tested for six strains of M. massiliense and four strains of M. bolletii.

Additional in-silico analysis was added as suggested and all of the SSS method has 100% agreement to the rpoB typing results (p11).

8. English needs to be corrected, as there are a few poorly structured sentences (line 39-41, line 42-44 etc.). Line 52 "not" instead of "nor". Line 73- Did you perhaps mean that there are several alleles circulating in the population?

Revised.

Staff Comments:

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Done.

• Upload a compare copy of the manuscript (without figures) as a "Marked-Up Manuscript" file.

Done.

• Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file.

Done.

• Manuscript: A .DOC version of the revised manuscript

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• Figures: Editable, high-resolution, individual figure files are required at revision, TIFF or EPS files are preferred

NA.

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July 5, 2022

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Quest Diagnostics  
14225 newbrook dr  
Chantilly, Virginia 20151

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Dear Dr. Jian Rong Bao:

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(i) including a reference to the NTM Hain Life Sciences test;
(ii) restructuring the indicated sentence regarding NTM infections; and
(iii) citing ANI as reference method.

As these revisions are quite minor, I expect that you should be able to turn in the revised paper in less than 30 days, if not sooner.

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Sincerely,

Digby Warner

Editor, Microbiology Spectrum

Reviewer comments:

Reviewer #2 (Comments for the Author):

The Authors strengthened their research by including a reference set of well-characterized strains. The authors generally responded to comments and left minor issues of the presentation unanswered. For example, I do feel that mentioning a popular NTM Hain Life Sciences test in the introduction section would add credibility to the manuscript. Restructuring the sentence in previous line 52, to clearly define the subject to infections and not (possibly) to humans, would make the manuscript more elegant. It would probably be better to refer to ANI as a reference method than to rpoB typing from studies by Minias et al. RpoB typing showed issues due to HGT in the original research. ANI, based on WGS, is a more credible method of differentiation.

Preparing Revision Guidelines

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(i) including a reference to the NTM Hain Life Sciences test;
   Two references (#19 & 20) were added in the introduction (Lines 82-84).

Accordingly, all the reference #s after the #18 have been moved 2 digits up in the manuscript.

(ii) restructuring the indicated sentence regarding NTM infections; and
   The sentences have been restructured (Lines 50-54).

(iii) citing ANI as reference method.
   The ANI was cited as the reference method for the in-silico analysis (Lines 159-163).

Accordingly, the following changes were based on the reference method:
   1. A few statement modifications (Lines 237-239, & 297-299).
   2. A few data in Table 4 were re-arranged or re-calculated and checked (Page 26).

As these revisions are quite minor, I expect that you should be able to turn in the revised paper in less than 30 days, if not sooner.
July 18, 2022

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Quest Diagnostics
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Re: Spectrum02534-21R2 (Short Signature rpoB Gene Sequence to Differentiate Species in Mycobacterium abscessus Group (MAG))

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