Author’s response to reviews

Title: An evaluation of methods for the isolation of nontuberculous mycobacteria from patients with cystic fibrosis, bronchiectasis and patients assessed for lung transplantation

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Responses to Reviewer reports:
Tavs Qvist (Reviewer 1): Stephenson et al. have performed a large study of RGM medium finding it suited to isolate pathogenic NTM species, the most important being M. abscessus. They collected respiratory samples from 676 patients and compared Löwenstein-Jensen medium and MGIT to RGM medium at two temperatures. RGM at 30 degrees proved more sensitive than AFB culture, but several other NTM species of questionable importance were also isolated. The method seemed to miss M. simiae and to a lesser degree M. xenopi.

The study is large and well thought-out. The clinical question is important and NTM do indeed pose a threat to these vulnerable patient populations. A faster and easier isolation method for particularly M. abscessus is in demand. The high isolation rate of other NTMs is a manageable problem. The authors make a compelling case for exploring a medium like RGM. It is a shame that Burkholderia cepacia plates are not also included, but they are mentioned and the context is properly presented. It is a weakness of the study that not all MGIT samples were incubated for 6 weeks as recommended. The manuscript is well written and will interest readers.

Author response: Thank you for your positive review. The reason for not including Burkholderia cepacia plates was that RGM has been shown conclusively to be far superior for isolation of NTM including M. abscessus (see studies by Preece et al. [18] and Plongla et al. [20]). This may not have been clear to readers so we have added a sentence to emphasise data from one of these studies (see lines 95-97).

I only have minor comments

1. Page 10 line 189 Any isolate of NTM that was only recovered by RGM at one incubation temperature (or was only isolated using AFB culture) was re-inoculated onto two plates of RGM medium and two plates of RGM medium without antibiotic supplement that were incubated at 30°C and 37°C for up to 28 days. How did these count in the overall test of diagnostic sens. and spec. (table 5). Were only the initial culture result counted?

Author response: Only initial culture results were used for calculation of sensitivity and specificity. We have added a sentence (line 203) to state that: “Information
obtained from these subcultures was not used in the calculation of specificity or sensitivity for the various culture methods”.

2. Page 12, line 219: "Overall, the data suggested little added benefit of using RGM at 37°C and that incubation temperature of 30°C should be recommended for routine use, as originally proposed [18]." This belongs under Discussion, not results.

Author response: We accept this point. The sentence has been cropped to state merely that “Overall, the data suggested little added benefit of using RGM at 37°C” without drawing further conclusions. (line 230). The poor performance at 37°C is unambiguous and we do not feel that it requires further elaboration in the discussion.

3. Page 14 The sensitivity for detection of MAC was also higher than that of AFB culture (sensitivity 83% vs. 70.2%; P = 0.21) - I would recommend reformulating without the use of the word sensitivity here, as the difference is not statistically significant: for example more MAC was detected with RGM30 than with AFB, but the difference was not statistically significant.

Author response: Agreed. The sentence now reads: “The number of MAC isolates recovered on RGM was also higher than the number recovered by AFB culture, but this was not statistically significant” (line 245).

4. Page 15, line 224: Comment: This demonstrates that if you know what you are looking for, NTM are much easier to find. This is among other things related to having an experienced lab tech, who can spot and scoop single colonies. This could be a challenge when implementing a system like RGM in the clinical routine outside of specialized labs. The skills of the lab tech should not be underestimated.

Author response: We very much agree with this general point. However, we believe it applies to all culture methods for isolation of NTM. No changes are suggested by the reviewer.
5. Page 15, line 255 Colony counts are important as stated here - a bit more elaboration on this issue in the discussion would be welcome.

Author response: We have devoted 22 lines of text in the discussion to the issue of colony counts (lines 430-451). Although we agree on the importance of this issue, we feel that further speculation on the relevance of the colony count would not be supported by the available evidence and can only be addressed by longitudinal studies over a number of months, or even years, that examine the microbiology of future samples, the development of disease and its subsequent progression.

6. Page 26, line 479 colonization is mentioned. In the case of M. gordonae possible lab contamination should be considered.

Author response: This is an interesting and useful point. As well as laboratory contamination, the specimen may also be contaminated from the environment (in one example, we found out that a patient generated specimens while in the shower). We have edited the sentence to state that “the recovery of such species that transiently colonize the respiratory tract as commensals, or arise from environmental contamination, may be regarded as more of a hindrance than a benefit when using RGM medium). (line 452).

7. Page 33, line 613 Competing interests: The authors should consider whether any patents, past present or pending could be relevant to declare here.

Author response: The reference number for the relevant patent application has been added (line 614).

Maeve Smith (Reviewer 2):

Thank you for asking me to review this large study comparing culture methods for nontuberculous Mycobacteria in a varied pulmonary patient population. It is an interesting study and provides good support for RGM medium as a diagnostic tool.
Author response: Thank you for your positive review.

Comments

1. The abbreviation RGM should be explained as the start (Rapidly Growing Mycobacteria medium)

   Author response: This has been added to the introduction (line 91).

2. The introduction should provide some explanation or background as to why the authors decided to evaluate RGM at two different temperatures; there is no rationale given for this or for why 37C was chosen; furthermore the studies referenced for RGM (Preece, Plongla and Eltringham) all used 30C.

   Author response: We have added the following text to the introduction: It is well recognised that different species of NTM have different temperature preferences and that duplicate sets of media at two incubation temperatures are frequently recommended for optimal recovery of a wide range of species [14]. We therefore inoculated duplicate plates of RGM medium and compared incubation at 30°C with incubation at 37°C. (line 109).

3. Description of patients and samples (Table one) belongs in results

   Author response: It would be usual for the methods to describe the patient population who were sampled and this is the purpose of Table 1. We would therefore respectfully maintain that Table 1 is best placed in the methods.

4. More detail regarding sample collection/storage prior to culture should be provided; for examples, if samples were transferred from specialty clinics what was the time allowed between collection and culture? How were samples stored prior to culture if
any time delays- room temp/refrigerated? Was this uniform for all samples as it has the potential to affect culture?

Author response: We have added the following text (line 124): Most samples were cultured on the day of sample collection and the remainder after storage for up to 48 h at 4°C.

5. Clinical inclusion/exclusion criteria should be clarified- were patients with a previous history or current known history of NTGM infection included? Were any patients already on treatment for NTM included etc? Or were all patients included those with no prior history of NTM, clinically stable and routine surveillance samples? Needs clarified.

Author response: We have added a line (line 123) to state that “We cultured all consecutively submitted samples from these patient groups with no other selection criteria”.

6. The results section contains a lot of text that perhaps isn't necessary/in the correct section- for example, line 219-221 is probably more appropriate for the discussion and lines 272-274 the methods, line 314 etc.

Author response: We feel that these are mostly valid points. The comment about line 219-221 has been dealt with in our response to reviewer 1 / point 2. The text in line 314 has been relocated to the discussion (line 419). Lines 272-274 serve to factually define what is included in the M. chelonae complex. We cannot see how this would easily fit into the method section and we believe this could be reasonably retained in the results.

7. The section in the discussion regarding the clinical significance of additional isolates of NTM could be expanded. Whilst obviously I appreciate that the answer to this question is outwith the scope of this study, it is of real importance when developing and advocating a diagnostic tool. For example, would the correlation between colony count and the likelihood of detection on AFB perhaps indicate that AFB culture techniques might be more clinically relevant.
Author response: I understand the desire of the reviewer for us to further address the question of clinical relevance – as it is undoubtedly of critical importance. We have devoted 22 lines of text to addressing this issue (lines 430-451) but, as the reviewer has recognised, the answer to this question can only be answered by further (longitudinal) studies and correlation of culture results with detailed clinical data. We have resisted the temptation to speculate further in the discussion as we do not believe we have the evidence to support further speculation regarding the clinical significance of isolates that are only detected using RGM medium.

Additional minor changes:

A missing item of data was added regarding re-inoculation of M. xenopi (line 342).

An important paper that is highly relevant has been published since the submission of our R2 version and this reference has been added so that our final text is up to date (Ranvholt et al.). An additional line of text has been added to refer to this study (line 365).