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Original Article

A novel culture medium for isolation of rapidly-growing mycobacteria from the sputum of patients with cystic fibrosis

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Abstract

Background: Isolation of mycobacteria from the sputum of patients with cystic fibrosis (CF) is challenging due to the overgrowth of cultures by other bacteria and fungi. In this setting, *Burkholderia cepacia* selective agar (BCSA) has been recommended as a convenient and effective culture medium for the isolation of rapidly-growing, non-tuberculous mycobacteria (NTM). A novel selective culture medium (RGM medium) was evaluated for the isolation of rapidly-growing NTM from the sputum of children and adults with CF.
Methods: A total of 118 isolates of rapidly-growing mycobacteria and 98 other bacteria and fungi were inoculated onto RGM medium. These were assessed for growth at 30 °C over a seven day period. A total of 502 consecutive sputum samples were collected from 210 patients with CF. Each sample was homogenized and cultured onto RGM medium and also onto BCSA. Cultures were incubated for 10 days at 30 °C.
Results: Of 118 isolates of mycobacteria all but one grew well on RGM medium, whereas 94% of other bacteria and fungi were inhibited. A total of 55 sputum samples (from 33 distinct patients) yielded NTM using a combination of both RGM and BCSA (prevalence: 15.7%). NTM were recovered from 54 sputum samples using RGM medium compared with only 17 samples using BCSA (sensitivity 98% vs. 31%; $P \leq 0.0001$). A total of 419 isolates of non-mycobacteria were recovered from sputum samples on BCSA compared with 46 on RGM medium.
Conclusions: RGM medium offers a simple and effective culture method for the isolation of rapidly-growing mycobacteria from sputum samples from patients with CF without decontamination of samples. RGM medium allows for the systematic screening of all sputum samples routinely referred for culture from patients with CF.
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Keywords: Culture media; *Mycobacterium abscessus*; Non-tuberculous mycobacteria

1. Introduction

Non-tuberculous mycobacteria (NTM) are ubiquitous environmental organisms found in soil and water that are increasingly associated with pulmonary infection in patients with cystic fibrosis (CF). Among the NTM, exists a subgroup of so-called rapidly-growing mycobacteria of which there are currently at least 70 distinct species that are defined by their ability to grow on laboratory media within seven days [1]. Infections with NTM can be difficult to recognise and treat [2] with prevalence in sputum estimated at 7 to 24% for patients with CF [3]. The predominant species of NTM within the CF population in the UK, and several other European countries, is

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Mycobacterium abscessus complex (MABSC) [4–6] which comprises three subspecies: *M. abscessus* subsp. *abscessus* [7], *M. abscessus* subsp. *bolletii* [8] and *M. abscessus* subsp. *massiliense* [9,10]. Infection with MABSC is associated with a decline in lung function [4,11] and it may cause severe complications post lung transplantation [12]. There is convincing evidence that the prevalence of infection by MABSC is increasing in the CF population [4,6,13,14], and an association between long term azithromycin therapy and infection with MABSC has been proposed [15], although other studies report conflicting evidence [16,17].

Detection of NTM is routinely achieved by culture of sputum onto both solid media, e.g. Lowenstein–Jensen medium and automated broth-based methods following decontamination of samples [18,19]. Culture in a liquid broth medium can provide more rapid results as well as an increased yield of mycobacteria [20]. However, even after decontamination, cultures may need to be abandoned due to the overgrowth of faster growing microorganisms that frequently colonise the lungs of CF patients such as *Pseudomonas aeruginosa* and species within the *Burkholderia cepacia* complex [19]. Culture on *B. cepacia* selective agar (BCSA) has been shown to be effective for isolation of NTM if incubation is prolonged from five to fourteen days. For example, Esther et al. [21] demonstrated that extended incubation of BCSA afforded an increased recovery rate of NTM from 0.7% to 2.8% using routine culture methods. However, not all NTM will grow on BCSA and overgrowth, particularly by fungi and Gram-negative bacteria, remains a problem [21].

We report the evaluation of a novel agar-based culture medium (RGM medium) designed for the isolation of rapidly-growing mycobacteria from the sputa of patients with CF. RGM medium is based on Middlebrook agar and contains a proprietary mix of supplementary growth factors and selective agents. In a preliminary study, the medium was challenged with pure cultures of mycobacteria and other bacteria and fungi. RGM medium was then compared to BCSA for the isolation of NTM from 502 sputum samples from patients with CF.

2. Materials and methods

2.1. Culture media

Pre-poured plates of *B. cepacia* selective agar (BCSA; Product Ref: 44347) were purchased from bioMérieux, Basingstoke, UK. The formulation of BCSA is based on that described by Gilligan et al. [22] and it incorporates a cocktail of selective agents including ticarcillin, polymyxin B, crystal violet and bile salts. Blood agar was prepared from Columbia agar powder (Oxoid, Basingstoke, UK) and supplemented with 5% defibrinated horse blood (TSC Biosciences, Buckingham, UK). Unless stated, all other named materials were obtained from Sigma-Aldrich, Poole, UK. The preparation of RGM medium: Middlebrook 7H9 broth was prepared in-house from its basic ingredients and supplemented with 1% bacteriological agar (Oxoid) and various proprietary growth factors before sterilisation at 116 °C for 20 min. After cooling to 50 °C, the medium was supplemented with OADC supplement (oleic acid, bovine serum albumin, dextrose, catalase) and further supplemented with a proprietary mix of four antimicrobial agents. The agar was then dispensed into 90 mm Petri dishes.

2.2. Bacterial strains

A collection of 118 isolates of rapidly-growing mycobacteria previously isolated from CF sputum samples by standard methods was used for evaluation of RGM medium. These included *M. abscessus* subsp. *abscessus* (n = 66), *Mycobacterium chelonae* (n = 35), *M. abscessus* subsp. *massiliense* (n = 8), *M. abscessus* subsp. *bolletii* (n = 3), *Mycobacterium fortuitum* (n = 3), *Mycobacterium salmoniphilum* (n = 2) and *Mycobacterium immunogenum* (n = 1). Forty four isolates were obtained from the Microbiology Department, Freeman Hospital, Newcastle upon Tyne, UK and all were from distinct patients. Seventeen were kindly provided by St. Vincent's University Hospital, Dublin, Ireland and were also from distinct patients. The remaining 57 were consecutive clinical isolates kindly supplied by Public Health England, Newcastle upon Tyne, UK. The species and subspecies identity of all strains had been previously confirmed by sequencing of at least two of three housekeeping genes (*rpoB*, *hsp*65 and *sodA*) as described by Blauwendraat et al., 2012 [7].

Non-mycobacterial strains (n = 98) were obtained from national culture collections (n = 67) or from the culture collection of the Microbiology Department, Freeman Hospital, Newcastle upon Tyne (n = 31). The collection was chosen to include a range of species commonly recovered from the sputa of patients with CF and included an international *P. aeruginosa* reference panel [23]. The non-mycobacteria comprised: *P. aeruginosa* (n = 54), *Enterobacteriaceae* (n = 7), *B. cepacia* complex (n =5), *Streptococcus* spp. (n = 4), *Stenotrophomonas maltophilia* (n = 4), *Staphylococcus* spp. (n = 3), *Enterococcus* spp. (n = 2), *Achromobacter* spp. (n = 2), *Acinetobacter* spp. (n = 2), *Pandoraea* spp. (n = 2), *Bacillus subtilis* (n = 1), *Elizabethkingia miricola* (n = 1), *Haemophilus influenza*e (n =1), *Moraxella catarrhalis* (n = 1), *Neisseria flavescens* (n = 1) *Aspergillus* spp. (n = 3), *Candida* spp. (n = 2), *Scedosporium* spp. (n = 2) and *Geosmithia argillacea* (n = 1).

2.3. Evaluation of RGM Medium with pure cultures

Prior to being tested, all strains were retrieved from storage in glycerol at −20 °C and subcultured onto Columbia agar plus 5% horse blood for seven days. Each isolate was suspended in 1 ml of saline (0.85%) to a turbidity equivalent to a McFarland 0.5 standard (approximately 1.5×10^8 CFU/ml) using a densitometer. For 'rough' colony types of mycobacteria, where clumping occurred, vortexing with three sterile 3 mm glass beads for 10 min effectively dispersed all clumps. A 1 μl aliquot of each suspension of mycobacteria was inoculated onto the medium and spread with a loop to obtain isolated colonies. Filamentous fungi were inoculated in the same way. Suspensions of all other bacterial isolates and *Candida* spp. were inoculated onto test media using a multipoint inoculator to deliver inocula of approximately 1 μl per spot (i.e. approximately 1.5×10^5 CFU).

All plates were incubated at 30 °C and growth was recorded daily for up to seven days. For non-mycobacteria, Columbia blood agar (for bacterial isolates) or Sabouraud agar (for fungal isolates) were used as controls to demonstrate viability of isolates. For mycobacteria, RGM medium without antimicrobials was used for this purpose.

2.4. Inoculation of sputum samples onto RGM medium and BCSA

Consecutive sputum samples (n = 502) obtained from 210 adults and children with CF were processed between February and September 2014. The age range was < 1 year to 77 years. All samples were routine samples submitted to the Microbiology Department, Freeman Hospital, UK for culture. No additional samples were taken for the purposes of this study. Sputum samples received from patients with CF were digested (1:1) with sputasol (Oxoid) and 10 μl aliquots cultured onto whole plates of RGM medium and BCSA. The inoculum was spread using a loop to obtain isolated colonies. Both media were incubated for 10 days at 30 °C and growth was recorded after four, seven and ten days.

2.5. Species identification of isolates from clinical samples

All isolates recovered on either medium were identified using matrix-assisted laser-desorption–ionization time-of-flight mass spectrometry (Bruker, Coventry, UK). For suspected isolates of mycobacteria, full protein extractions were required for mycobacterial speciation and, after numerous protocols were evaluated, the method recommended by Saleeb et al. [24] was adopted. Species and subspecies identity was further confirmed by sequencing at least two of three housekeeping genes (*rpoB*, *hsp*65 and *sodA*) as previously described [7].

2.6. Statistical analysis

Any difference in performance of the two media was investigated for statistical significance using McNemar's test with the continuity correction applied. Statistical significance was assigned to a probability (P) value of ≤ 0.05.

2.7. Evaluation of the stability of RGM medium

Sixty plates of RGM medium were prepared and stored at 4 °C. These were inoculated as described above on a weekly basis over a period of 12 weeks with four isolates of mycobacteria (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, *M. abscessus* subsp. *massiliense* and *M. chelonae*) and seven other isolates (*P. aeruginosa* (n = 4); *Burkholderia multivorans*, *Candida albicans* and *Enterobacter cloacae*). The seven non-mycobacteria were carefully selected as indicators of deterioration of each of the selective agents in the medium. Media were incubated at 30 °C and examined daily for up to 14 days.

3. Results

3.1. Evaluation of RGM medium with pure cultures

A total of 117 out of 118 mycobacteria grew on RGM medium within seven days of incubation, and only one isolate of *M. abscessus* subsp. *abscessus* failed to grow. Of the 117 that grew, 84% showed evidence of growth after only two days of incubation and 99% showed growth within four days; one isolate of *M. salmoniphilum* was only visible after seven days of incubation. Of 98 non-mycobacteria inoculated onto RGM medium, 92/98 (94%) were completely inhibited including all Gram-positive and fungal isolates. Only *B. cepacia* complex (2/5), *Pandoraea* spp. (2/2) and *Achromobacter* sp. (1/2) were able to grow within seven days of incubation. All 216 isolates grew well on control media.

3.2. Evaluation of RGM medium and BCSA with sputum samples

Results with sputum samples showed that out of 502 samples tested from 210 distinct patients, 55 samples from 33 distinct patients yielded NTM giving an overall prevalence of 15.7%. Twenty one of the 210 patients were colonised with MABSC (prevalence: 10%). The mycobacteria isolated from 55 samples comprised *M. abscessus* subsp. *abscessus* (56.4%), *M. abscessus* subsp. *massiliense* (20%), *M. chelonae* (10.9%) *Mycobacterium avium* (3.6%), *Mycobacterium llatzerense* (3.6%), *M. salmoniphilum* (3.6%) and *Mycobacterium mucogenicum* (1.8%). Table 1 shows the numbers of each species recovered by the two media. The calculation of sensitivity in Table 1 is for comparative purposes only and assumes that all mycobacteria were recovered by a combination of the two methods. Clearly this cannot be proven and moreover might be considered highly unlikely for slower growing species such as *M. avium*. RGM medium enabled the detection of NTM from 54 of 55 positive samples whereas BCSA recovered NTM from 17 of 55 positive samples (sensitivity: 98% vs. 31%; $P \leq 0.0001$).

Table 1
Mycobacteria recovered from culture of 502 sputum samples on *Burkholderia cepacia* selective agar (BCSA) and RGM medium.

Species	Total (either medium)	BCSA		RGM	
	n	*n*	Sensitivity (%)	*n*	Sensitivity (%)
M. abscessus subsp. *abscessus*	31	13	42	31	100
M. abscessus subsp. *massiliense*	11	3	33	11	100
M. chelonae	6	1	17	5	83
M. avium	2	0	0	2	100
M. llatzerense	2	0	0	2	100
M. salmoniphilum	2	0	0	2	100
M. mucogenicum	1	0	0	1	100
Total mycobacteria	55	17	31	54	98

For patients who had NTM found in their sputum (n = 33), 23 were detected using RGM only, one was detected using BCSA only (*M. chelonae*) and nine were detected using both media ($P \leq 0.0001$). Fifty nine percent of mycobacteria recovered on RGM medium were isolated after four days of incubation compared with 35% of mycobacteria that were recovered on BCSA. No further isolates of NTM were isolated after seven days of incubation using BCSA.

A notable feature of RGM medium was its ability to prevent the growth of fungi and Gram-positive bacteria and no isolates of either of these groups were recovered from 502 sputum samples during ten days of incubation (Table 2). Also, although both of these selective media are designed to inhibit the growth of *Pseudomonas*, RGM medium was much more effective with only two isolates recovered, compared with 32 isolates able to grow on BCSA. Some isolates of Gram-negative bacteria, most notably *B. cepacia* complex and *Achromobacter* spp., could not be completely inhibited on RGM medium but growth was frequently restricted. Furthermore, mycobacteria produced bright white colonies on RGM medium whereas Gram-negative species that were able to grow generated blue, or occasionally pink, colonies. The high selectivity of RGM medium enabled the recovery of NTM in pure culture from 52 samples whereas BCSA enabled the recovery of a pure culture of NTM from only two samples.

Table 2
Other species recovered from culture of 502 sputum samples on *Burkholderia cepacia* selective agar (BCSA) and RGM medium.

	Number of isolates (n)	
	BCSA	RGM
Fungi and Yeasts	226	0
Arthrographis kalrae	2	0
Aspergillus fumigatus	69	0
Aspergillus terreus	6	0
Candida spp.	121	0
Exophiala dermatitidis	23	0
Scedosporium apiospermum	5	0
Gram-negative bacteria	136	46
Pseudomonas spp.	32	2
Burkholderia cepacia complex	30	18
Stenotrophomonas maltophilia	24	0
Achromobacter spp.	21	18
Enterobacteriaceae	14	2
Inquilinus limosus	4	2
Ochrobactrum spp.	4	0
Pandoraea spp.	3	3
Acinetobacter lwoffii	1	0
Methylobacterium radiotolerans	1	0
Rhizobium radiobacter	1	0
Sphingomonas sp.	1	0
Delftia acidovorans	0	1
Gram-positive bacteria	57	0
Staphylococcus spp.	29	0
Enterococcus spp.	14	0
Streptococcus spp.	8	0
Granulicatella adiacens	2	0
Lactobacillus paracasei	2	0
Micrococcus luteus	1	0
Nocardia cyriacigeorgica	1	0
Total non-mycobacteria	419	46

3.3. Evaluation of stability of prototype medium

There was no reduction in the quality of growth of the four species of *Mycobacterium* after storage of media at 4 °C for 12 weeks and RGM medium maintained complete inhibition of the seven non-mycobacteria tested.

4. Discussion

The CF Foundation and the European Cystic Fibrosis Society have jointly produced evidence-based draft guidelines on the Management of Nontuberculous Mycobacteria in Individuals with Cystic Fibrosis that have been disseminated for consultation (2014 Unpublished). Annual screening for NTM is recommended in patients who spontaneously produce sputum. The recommended methodology involves decontamination of sputum samples using N-acetyl-L-cysteine (0.5%)-NaOH (2%), staining of sputum smears for acid fast bacilli and culture on both solid and liquid media for a minimum of six weeks. The limitations of these methods are highlighted in the guideline and these include a substantial reduction in the viability of mycobacteria due to the decontamination process or incomplete elimination of non-mycobacteria necessitating further rounds of decontamination [25]. The method is both expensive and labour intensive. The potential use of BCSA is highlighted but not specifically recommended.

Although the routine use of a dedicated culture medium for isolation of *B. cepacia* complex from the sputa of CF patients is accepted practice, this is not yet the case for rapidly-growing mycobacteria despite the fact that the prevalence of the latter may be much higher, as exemplified by this study where 54 isolates of mycobacteria were recovered on RGM medium compared with 30 isolates of *B. cepacia* complex recovered using BCSA. One reason for this is likely to be the lack of a convenient and effective method for isolation of rapidly-growing NTM. RGM medium offers a simple and convenient tool that can be applied to the culture of all routinely submitted sputum samples from patients with CF. Such systematic screening will ensure that diagnosis of a significant infection with NTM is not unduly delayed. For example, a positive sputum culture for MABSC is more likely to indicate the presence of NTM-mediated lung damage rather than asymptomatic colonisation [5,26] and multiple positive cultures despite treatment is associated with a poor outcome [4,11]. Furthermore, routine screening of all submitted sputum samples from CF patients may assist in prompt identification of risk factors leading to acquisition, subsequent infection and transmission of NTM [27]. Any screening method may lead to the detection of transient colonisation with mycobacteria that may have no clinical significance. This can be problematic, as patients may be assumed to be infected and may be segregated from other patients unnecessarily. This problem can be alleviated to a large extent by prompt species identification of the isolate [7,28] to assess likely

pathogenicity and, wherever possible, prompt submission of further sputum samples.

Under proposed and existing guidelines [29], NTM may be undetected for up to a year (or longer if successive conventional cultures are compromised by contamination events). It was impractical to perform a direct comparison of RGM medium with standard AFB culture as specimens were submitted for standard AFB culture infrequently. For example, for the 33 patients who were found to harbour mycobacteria in this study (i.e. using RGM plus BCSA), specimens were only submitted for standard AFB culture for ten of these patients (30%) during the 8-month trial period and only six of these yielded mycobacteria (18%). Conversely, for the 177 patients that were found not to harbour mycobacteria, only 22 patients (12%) had specimens referred for standard AFB culture during the 8-month trial period. Of these 22 patients, mycobacteria (of any variety) were only recovered from one patient (*M. abscessus* subsp. *abscessus*). A future study that compares RGM medium with standard AFB culture is however warranted as the latter is more sensitive than BCSA for detection of rapidly-growing mycobacteria [21]. It is essential that further external validation is performed in different centres so that the true value of RGM medium can be properly assessed. It is anticipated that RGM medium will be made commercially available in due course but until then, the authors are committed to making the culture medium freely available to clinical laboratories who wish to independently verify the findings of this study.

In conclusion, we propose that routine systematic use of RGM medium could facilitate a greater understanding of the true prevalence and clinical significance of rapidly growing mycobacteria in patients with CF, however it will not preclude the need to perform annual screening using conventional mycobacterial culture methods as slow growing mycobacteria (e.g. *M. avium*) will not be reliably detected. A multi-centre study that includes laboratories in diverse geographical locations is warranted to assess the true value of this new method.

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