A simple and rapid method for the detection and identification of mycobacteria using mycobactin

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Summary. A system was developed for the identification of mycobacteria such as *Mycobacterium tuberculosis* and *M. avium*, by thin layer chromatography of ⁵⁵Fe-labelled mycobactin. Approximately 2×10^3 mycobacteria were detected within 24 h and little operator time or skill was required. *M. avium*, *M. intracellulare* and *M. scrofulaceum* were found to have lower requirements for iron than other mycobacteria and this may influence their growth in host organisms.

Introduction

Pathogenic mycobacteria are relatively slow growing in laboratory culture media. Therefore, differential identification cannot be achieved for 3–12 weeks by conventional methods. Identification is important because the sensitivities of mycobacteria to antibiotics depends on the species. This is particularly so with the *Mycobacterium avium* — *intracellulare* — *scrofulaceum* (MAIS) group and the *M. tuberculosis* (tubercle) complex which can cause similar infections but show little similarity in their susceptibilities to antibiotics. Infections with these two groups of mycobacteria have increased in recent years, particularly in patients with AIDS. Infections with other less common mycobacterial pathogens are also important. These infections should be treated as quickly as possible, but there are few rapid methods to identify the causative organisms. Methods that identify mycobacteria by nucleic acid amplification and hybridisation are rapid and sensitive, but they usually require separate processing for each species, with unique probe sequences for each organism to be identified.

However, there are other biochemical methods for the identification of mycobacteria that have not been fully developed. These are based on the principle that mycobacteria produce a number of unique compounds. Whereas some compounds are unique to particular species, others, like the mycobactins which are involved in iron acquisition, have common structures but differ in the components of their side groups.¹ Most species produce up to four molecular variants of mycobactins which differ in the position and length of the five alkyl chain residues.² These molecules can be distinguished by extraction with alcohol followed by thin layer chromatography (TLC).¹ The ability to identify mycobacteria by their mycobactin TLC sep-

aration patterns was used in the chemotaxonomic identification of mycobacteria,^{3–7} but the methods required too many organisms to be of use in clinical identification. The aim of this study was to increase the sensitivity of this approach and thus provide a simple method that could be used routinely for the identification of mycobacteria.

Materials and methods

Bacterial strains

The following strains of mycobacteria were used: *M. avium* NCTC 8559, *M. intracellulare* NCTC 10425 and M12⁶, *M. tuberculosis* H37Rv NCTC 7416, *M. bovis* NCTC 10772, *M. bovis* var BCG NCTC 5692, *M. scrofulaceum* NCTC 10803, *M. smegmatis* NCIB 8548, *M. vaccae* NCTC 10916, *M. fortuitum* NCTC 10394 and 10395, *M. thermoresistibile* NCTC 10409, and *M. neoaurum* NCTC 10818.

Media and growth conditions

All strains were maintained on Lowenstein–Jensen medium after growth at 37°C. For the expression of mycobactins, the organisms were grown on slopes of the following low-iron defined medium containing (/L): glucose 10 g, glycerol 5 ml, asparagine 5 g, 1.66 mM MgSO₄, 1.8 μM MnSO₄, 7 μM ZnSO₄, 0.18 μM FeSO₄ and purified agar 1.5 g. Where appropriate, 0.1 mM desferrioxamine mesylate (Sigma), 1 mM 2,2'-dipyridyl (Sigma) or β-cyclodextrin 0.5% w/v were added to the cooled agar before it set.

Extraction and labelling of the mycobactin

Bacterial colonies were picked with a sterile plastic loop and placed in an Eppendorf centrifuge tube containing 0.5 ml of either methanol or ethanol. The

stem of the loop was removed and the loop plus alcohol was mixed for 15 min with a blood tube rotator. The tube was then centrifuged for 5 min at 13 500 rpm and the supernate was transferred to a new tube. The mycobactin was labelled with $^{55}\text{FeCl}$ (Code IES 3, Amersham International) 0.02 μCi in 20 μl of sterile 20 μM HCl for 5 min on the tube rotator. The labelled mycobactin was extracted with 1 ml of chloroform by gentle mixing for 3 min. Distilled water (100 μl) was added and the tubes centrifuged for 10 s at 13 500 rpm. The methanol was discarded and the chloroform was removed by heating at 80°C.

Thin layer chromatography

The radiolabelled sample was resuspended in 20 μl of methanol and spotted on to a 10 $\times$ 10 cm silica gel 60 high performance thin layer chromatography (HPTLC) plate with concentrating zone (Merck, Germany), or a 20 $\times$ 20 cm silica gel TLC plate without concentrating zone (Merck). The samples were separated with petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v. At the completion of a run, the solvent front was marked with a spot of $^{55}\text{FeCl}$, and the plates were scanned with an AMBIS MK2, (AMBIS, USA), radio-analytic system^{8,9} for up to 18 h for the presence of radioactive spots.

Results

Detection of fast-growing mycobacteria

To test the feasibility of radiolabelling mycobactins to identify mycobacteria, three fast-growing mycobacterial strains, *M. smegmatis*, *M. fortuitum* NCTC 10394 and *M. neoaurum*, were grown on slopes of the defined medium at 37°C for 5 days. Sets of one, two, five or 10 colonies were picked. The cells were extracted with ethanol, labelled with $^{55}\text{FeCl}$ 0.02 μCi and separated on TLC plates without concentrating zones. Scans of these samples revealed that the method was only just sensitive enough to detect a single colony of *M. neoaurum*, but could easily detect single colonies of the other mycobacteria, which had formed bigger colonies. The overall resolution was poor, and as the sample size increased there were problems with lipids that bound iron and caused streaking of the plates.

Detection of slow-growing mycobacteria

M. tuberculosis H37Rv, *M. tuberculosis* H37Ra, *M. avium*, *M. intracellulare* NCTC 10425, *M. bovis* NCTC 10772 and *M. scrofulaceum* were incubated at 37°C for 14 days on the defined medium and treated like the rapidly growing bacteria described above. The scans of these samples (fig. 1) showed that whilst diffuse spots could be detected for the tubercle bacilli at the expected Rf positions,⁷ no spots were detected for any members of the MAIS group. Therefore, these organisms were able to grow under conditions of lower

iron concentration than the other species examined without showing the synthesis of mycobactin. *M. bovis* grew very poorly on this medium, presumably because of the presence of large amounts of glycerol, and failed to produce a detectable spot.

Fig. 1. Identification of slow-growing mycobacteria by radio-TLC. *M. bovis* (Bov), *M. tuberculosis* H37Rv (Rv), *M. tuberculosis* H37Ra (Ra), *M. avium* (Av), *M. scrofulaceum* (Sc) and *M. intracellulare* (Int) were grown on iron-limiting medium for 14 days at 37°C. Mycobactin extracts from two colonies of these cultures were labelled with ^{55}Fe and separated by TLC on silica gel 60 with petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v. The migration distance of the solvent is indicated with ^{55}Fe spots.

Fig. 2. Improved detection method. *M. smegmatis* (Sm), *M. fortuitum* (For), *M. vaccae* (Vac), *M. thermoresistible* (Th) and *M. neoaurum* (Neo) were grown overnight, and *M. intracellulare* strain M12 (M12), *M. tuberculosis* H37Rv (Rv) and *M. tuberculosis* H37Ra (Ra) were grown for 4 days at 37°C. *M. vaccae* was grown in the presence of 0.1 mM desferrioxamine mesylate. Mycobactin extracts from a single colony of these cultures were labelled with ^{55}Fe and separated by TLC on a silica gel 60 plate with concentrating zone with petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v. The base of the plate and the solvent front are indicated with ^{55}Fe spots; < indicates the end of the concentrating zone.

Improved detection method

The method was made more sensitive by using radioactive iron with a high specific activity (Cat IES 3, Amersham International), which enabled shorter growth times for the bacteria. Thus, the fast-growing organisms could be detected from overnight growth and the slow growing organisms could be detected after incubation for 4 days (fig. 2). The TLC plates were replaced with HPTLC plates which improved the resolution and shortened the separation times, but this increased the cost of the system. The presence of interfering waxes was overcome by an initial extraction with methanol in place of ethanol (fig. 2).

Nearly all of the organisms produced bands with the expected R_f values.^{3,6,7} The exception was *M. intracellulare* strain M12 which seemed to have adapted to produce a previously uncharacterised mycobactin pattern. This organism showed its original culture and biochemical properties and produced chloroform-soluble exochelins as expected from *M. intracellulare*, and thus was not considered to be a contaminant. Changes have been observed in the mycobactin profiles of *M. paratuberculosis* NADC 18 and a strain of *M. scrofulaceum*.² These changes, which are stable, have never been observed for mycobacteria other than those belonging to the MAIS group, and have been identified only in MAIS group organisms that have been grown for many subcultures in iron-deficient media. Thus, the expected profile for a freshly isolated strain would be that of a spot with an R_f of 0.89 with the solvent system as described.

By slightly modifying the solvent system to give ratios of petroleum spirit:ethyl acetate:butanol of 20:25:30 v:v:v, the separation patterns remained unchanged but differentiation of the single strain of *M. bovis* var BCG tested from tubercle bacilli was possible (fig. 3). The disadvantage of this method is that the R_f values obtained for all of the mycobactins tested differed from those already published.

Mycobactin detection of MAIS organisms

Mycobactin was occasionally produced by the MAIS group of organisms grown on the defined medium, and sometimes when grown on Lowenstein—Jensen medium, but the results were inconsistent. To improve the production of mycobactin, the medium was made more iron-limiting by the inclusion of the non-utilisable iron-binding compounds desferrioxamine mesylate (0.1 mM or 1 mM) or 2,2'-dipyridyl (0.1 mM or 1 mM), or by adding β -cyclodextrin. The cells grew poorly in the presence of 2,2'-dipyridyl, taking *c.* twice as long to grow as the iron-sufficient controls. The cells grown in the presence of 0.1 mM desferrioxamine mesylate or β -cyclodextrin grew to form usable microcolonies within 4–6 days. The cells grown in these iron-limiting conditions consistently produced detectable amounts of mycobactins from single colonies (fig. 3). The amount of mycobactin produced by each was measured with the AMBIS

Fig. 3. Mycobactin production by MAIS group organisms. *M. thermoresistible* (Th) was grown overnight, and *M. intracellulare* (Int), *M. avium* (AV), *M. scrofulaceum* (Sc), *M. tuberculosis* H37Rv (Rv) and *M. bovis* var BCG (BCG) were grown for 4 days at 37°C on iron-limiting medium containing 0.1 mM desferrioxamine mesylate. The *M. avium* (Av) and *M. scrofulaceum* (SC) samples were from cells grown on iron-limiting medium containing β -cyclodextrin 0.5% w/v. Mycobactin extracts from a single colony of these cultures were labelled with ⁵⁵Fe and separated by TLC on a silica gel 60 plate with concentrating zone with petroleum spirit:butanol:ethyl acetate 20:25:30 v:v:v; < indicates the end of the concentrating zone.

scanner as cpm. From this it was calculated that the cells grown in the presence of β -cyclodextrin yielded 31–64% of the mycobactin produced by growth in the presence of desferrioxamine mesylate. In all cases the decreased growth of these organisms was overcome by the increase in sensitivity resulting from the increase in mycobactin production. The addition of desferrioxamine mesylate in amounts greater than 0.1 mM had no additional effect on the amounts of mycobactin produced by these organisms, but the growth of the bacteria was considerably reduced, and was completely inhibited, by a concentration of 5 mM.

The early experiments with desferrioxamine and β -cyclodextrin were done with *M. vaccae* as a faster growing model organism for the MAIS group. *M. vaccae* is usually regarded as lacking the ability to produce mycobactin, although one mycobactin-positive strain has been reported.³ In agreement with these workers we were unable to demonstrate convincingly that mycobactin was produced by the type strain when grown on the iron-limiting medium alone. However, we found that if it was grown in the presence of desferrioxamine or β -cyclodextrin at 0.1 mM, a band with an R_f similar to that reported previously for strain NCTC 10916, which produced trace amounts, was obtained (fig. 2). This suggests that mycobactin synthesis by *M. vaccae* might be increased or induced under some conditions of iron limitation.

Sensitivity

To calculate the sensitivity of the method, bacterial numbers were calculated by direct microscopical

counting in tests with *M. smegmatis*. A calibration curve was constructed of cell numbers against radioactive cpm detected by the scanner. Desferrioxamine mesylate was included in the agar, and radioactive iron with a high specific activity was used. The sensitivity of the method was calculated to be 2.87×10^3 bacteria. In theory this should be adequate for the direct detection of bacteria in sputum as most sputum samples contain more than 10^3 cells/ml.

Discussion

We have refined the identification method originally proposed by Snow and White¹ and developed for taxonomic purposes³ for use in the routine identification of mycobacteria, based on the published Rf values of a large number of representative species.²⁻⁷ The method requires the calculation of the Rf values of the mycobactins, which is not difficult but could be eliminated by comparing the patterns obtained with a set of known mycobactins run on a track alongside the samples, as size markers are used in SDS-PAGE of proteins. Rapidly growing mycobacteria can be identified within 24 h, and slow growing ones within 5 days. Most mycobacterial species can be identified within a few hours if sufficient organisms are available. A scanner was used in these experiments to detect the radioactivity but X-ray film could be used if a scanner was not available although the exposure times would be longer.

The limitation of this approach is that mycobacteria that do not produce mycobactins cannot be identified. Three species do not produce mycobactins in substantial quantities—*M. paratuberculosis*, *M. vaccae* and *M. leprae*. The first two have the potential to produce mycobactins^{3,6,10} and the small amounts produced normally can be increased by growing the organisms on iron-limiting medium containing desferrioxamine mesylate. However, *M. paratuberculosis* grows so slowly under iron-limited conditions (some strains take 18 months to grow) that the technique is probably impractical for this organism. Although *M. leprae* has not been shown to produce mycobactin, it

has not as yet been tested by this highly sensitive method.

The results of the study have highlighted two atypical properties of mycobactin production, and thus iron-acquisition, by the MAIS group. The first is that they grow in conditions that would be iron-limiting to the other *Mycobacterium* spp. tested without producing mycobactin. This indicates that the MAIS group have a lower requirement for iron than the other mycobacteria, or that they use their exochelin-mediated iron uptake systems more effectively. The lower requirement for iron by the MAIS group would explain why *M. avium* has been observed¹¹ to grow transiently under iron-sufficient conditions in man. Transient changes clearly create problems in using this identification method directly on clinical specimens, as MAIS strains might not always synthesise detectable mycobactins. Thus, for the guaranteed detection of the MAIS group the cell would need to be grown on an iron-limiting medium, such as that used in this study.

The second unusual property of the MAIS group is the change in the profiles obtained after many subcultures under low iron conditions. The properties of these organisms are almost identical to those of the original isolates. The differences are in the mycobactin profiles, and in a 2–3-fold shortening of the growth time of the altered cells. This indicates that mutants of the MAIS group are being selected during growth in the iron-limiting medium, and could explain the discrepancies observed previously for mycobactin “J” from *M. paratuberculosis* NADC 18,² with which two different mycobactin profiles were obtained.

The most common *Mycobacteria* found in clinical situations are *M. tuberculosis* and the MAIS group of organisms, but there is also the risk of serious infection by organisms such as *M. fortuitum* and *M. chelonae* and others from hospital water supplies, operating theatres and haemodialysis units. Our main concern has been the identification of mycobacteria that cause disease, but the monitoring of mycobacteria in the environment is also important. The use of radio-labelled mycobactin provides a simple system for the identification of mycobacteria under all of these circumstances.

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