Radiometric studies on the use of selective inhibitors in the identification of *Mycobacterium* spp.

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Summary. Radiometric selective inhibition tests were developed and evaluated for the rapid differentiation of *Mycobacterium* spp. Both a *p*-nitrobenzoic acid (PNB) test and a commercially-prepared *p*-nitro- α -acetylamino- β -hydroxypropionophenone (NAP) test successfully differentiated *M. tuberculosis* and *M. bovis* from “atypical” mycobacteria or mycobacteria other than tubercle bacilli (MOTT). Thiophene-2-carboxylic acid hydrazide (TCH) readily distinguished human *M. tuberculosis* strains from *M. bovis*, irrespective of resistance to isoniazid. Both PNB and TCH tests were utilised in a routine radiometric susceptibility testing scheme over a period of 1 year in which 110 isolates of *M. tuberculosis*, 10 of *M. bovis* and one isolate of BCG were correctly differentiated from 10 isolates of MOTT. The rapidity, sensitivity and specificity of these radiometric tests can play a useful role in mycobacterial identification.

Introduction

The so called “atypical” mycobacteria, or mycobacteria other than tubercle bacilli (MOTT), are well established pathogens of man. They normally require some pre-existing condition such as bronchitis or emphysema before they can cause pulmonary disease and the immunocompromised patient is particularly prone to infection. Predictably, patients with the acquired immune deficiency syndrome are a group in whom infections with MOTT are appearing with increasing frequency (Jenkins, 1987; Lévy-Frébault *et al.*, 1987; Males *et al.*, 1987).

The growth of some of the MOTT on conventional media is slow and it can be many weeks between receipt of the specimen and production of the final report. Whilst this does not necessarily affect the clinical management of the patient, clearly a more rapid method of isolation and identification is desirable. The Bactec 460® (Johnston Laboratories) detects the growth of mycobacteria by measuring the evolution of ^{14}C -labelled CO_2 from a ^{14}C -labelled substrate, the degree of growth being expressed as a growth index (GI). With this system, positive results are obtained in

between 7 and 12 days but the positive GI only indicates the presence of a mycobacterium and further tests are necessary to differentiate between the tuberculosis complex and MOTT.

Conventional mycobacterial identification has for many years been based upon the use of selective inhibitors for the differentiation of *Mycobacterium tuberculosis* and *M. bovis* from the MOTT (Marks, 1976). Resistance of *M. tuberculosis* and *M. bovis* to *p*-nitrobenzoic acid (PNB) and hydroxylamine is well established (Tsukamura and Tsukamura, 1964; Tsukamura, 1965, 1970). The resistance of some strains of *M. kansasii* to PNB has also been noted (Tsukamura, 1984). Hydroxylamine would appear to offer no advantage over PNB as a screening agent (Collins and Yates, 1979).

The differential properties of *p*-nitro- α -acetylamino- β -hydroxypropionophenone (NAP), described by Eidus *et al.* (1960), have rarely been used in conventional screening techniques. However NAP inhibition has been adapted to radiometric techniques and has been found to be rapid and sensitive (Lazslo and Siddiqi, 1984).

Thiophene-2-carboxylic acid hydrazide (TCH) is useful in differentiating the classical, African and Asian variants of *M. tuberculosis* (Harrington and Karlson, 1966). The classical human variant is resistant to TCH irrespective of resistance to isoniazid, whereas all other variants are susceptible, as is *M. bovis* (Yates *et al.*, 1984).

Received 26 April 1988; accepted 1 Sep. 1988.

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The purpose of this investigation was to develop radiometric selective inhibition tests and investigate their value in the routine screening and susceptibility testing of clinical isolates of mycobacterial species.

Materials and methods

Mycobacterial strains

The following were obtained from the National Collection of Type Cultures, Colindale Avenue, London: *M. avium* NCTC 10437, *M. bovis* NCTC 10772, *M. flavescens* NCTC 10271, *M. fortuitum* NCTC 10394, *M. gordonae* NCTC 10267, *M. kansasii* NCTC 10268, *M. scrofulaceum* NCTC 10803 and *M. tuberculosis* NCTC 7416 (H37Rv). Three isoniazid-resistant strains of *M. tuberculosis*, labelled TB1, TB2 and TB3, and three strains of *M. kansasii* (B1, B2 and B3) were received from M. Chadwick, Brompton Hospital, London. Further isolates of *M. kansasii* were received from P. A. Jenkins, PHLS Mycobacterium Reference Unit, Cardiff (strain C1) and from C. Byrne, Peamount Hospital, Dublin (strain P1). Three strains of *M. bovis* from bovine para-tracheal lymph nodes and three strains of *M. tuberculosis* from human sputum, isolated in this laboratory, were also studied.

Over a period of 1 year 131 fresh clinical isolates were studied, comprising strains of *M. tuberculosis* (110 isolates), *M. bovis* (10), BCG (1), *M. kansasii* (2), *M. fortuitum* (2), *M. avium-intracellulare* (3), *M. gordonae* (2) and *M. xenopi* (1). All isolates were identified by conventional methods (Marks, 1976).

During this study strains were maintained at -70°C in 2-ml volumes of Middlebrook 7H9 Medium (Difco).

Media

Media for conventional identification methods were prepared as described by Marks (1976). Lowenstein-Jensen (LJ) slopes containing TCH (Biomérieux) 5 $\mu\text{g}/\text{ml}$ or PNB (Mast Laboratories) 500 $\mu\text{g}/\text{ml}$ were stored at 4°C before use. Bottles containing 4 ml of Bactec 12B Medium, labelled with ^{14}C -palmitic acid, were obtained from Johnston Laboratories Inc., Towson, MD, USA.

Reagents

TCH (Sigma) was dissolved in ethanol 50% v/v to a concentration of 200 $\mu\text{g}/\text{ml}$ and was then diluted with sterile distilled water to a final concentration of 50 $\mu\text{g}/\text{ml}$. Immediately before use, 0.1 ml of this solution was added aseptically to 4 ml of Bactec 12B medium, giving a final concentration of approximately 1.2 $\mu\text{g}/\text{ml}$.

To 1 g of PNB (Sigma) dissolved in 10 ml of 1N aqueous NaOH was added 70 ml of sterile distilled water and a few drops of phenol red 0.1% w/v. This solution was then neutralised by the addition of 1N aqueous HCl, made up to 100 ml with sterile distilled water and autoclaved at

121°C for 10 min. The sterile solution was stored at 4°C before use. From this PNB stock solution 0.2 ml was added to 4-ml volumes of Bactec 12B medium immediately before inoculation, giving a final PNB concentration of c. 500 $\mu\text{g}/\text{ml}$.

Glass vials, each containing a paper disk impregnated with NAP 5 μg , were obtained commercially (Johnston Laboratories).

Radiometric tests

All cultures for radiometric testing were incubated at 37°C in an aerobic atmosphere containing CO_2 10%. The amount of growth was determined daily for 5 days with a Bactec 460TB instrument (Johnston Laboratories). The amount of $^{14}\text{CO}_2$ liberated was directly related to the metabolism of the labelled substrate and to the growth of the mycobacterial culture. The degree of growth was expressed in terms of the growth index (GI).

Cultures of mycobacteria to be treated in both conventional and radiometric selective inhibition tests were grown in 4 ml of Bactec 12B medium until a growth index of 999 or greater was achieved; 0.1-ml volumes were then used to inoculate conventional LJ slopes and test vials containing the inhibitory agents and control vials without inhibitors. The NAP inhibition test was inoculated as described elsewhere (Morgan *et al.*, 1985).

The following criteria were used to interpret growth patterns in selective inhibition tests:

- two consecutive daily decreases in GI after 48 h and a continuing GI decrease = susceptible;
- slight GI increase in the first 48 h and then no increase = susceptible;
- increased daily GI reading to >400 = resistant;
- no increase or slight decrease in GI in the first 48 h and then three consecutive daily increases = resistant.

Results

Reference strains

PNB. Reference strains representing eight mycobacterial species were examined for susceptibility to PNB. *M. avium* NCTC 10437, *M. flavescens* NCTC 10271, *M. fortuitum* NCTC 10394, *M. gordonae* NCTC 10267 and *M. scrofulaceum* NCTC 10803 each showed a rapid increase in GI over a 5-day period (fig. 1). In contrast, *M. bovis* NCTC 10772, *M. tuberculosis* NCTC 7416 and *M. kansasii* NCTC 10268 were markedly inhibited by PNB.

The susceptibility of another five *M. kansasii* isolates and strain NCTC 10268 to PNB was studied further (fig. 2). NCTC 10268 was again inhibited by PNB in the radiometric test as was strain B2; both yielded only very light growth on LJ-PNB slopes. One isolate (B3) grew slowly in the radiometric medium and produced a light growth on the LJ-PNB slope. The remaining isolates

Fig. 1. Susceptibility of mycobacteria to *p*-nitrobenzoic acid (PNB): (a) *M. avium* NCTC 10437; (b) *M. bovis* NCTC 10772; (c) *M. flavescens* NCTC 10271; (d) *M. fortuitum* NCTC 10394; (e) *M. gordonae* NCTC 10267; (f) *M. kansasii* NCTC 10268; (g) *M. scrofulaceum* NCTC 10803; (h) *M. tuberculosis* NCTC 7416; control (●) and PNB 500 µg/ml (○).

Fig. 2. Growth of six *M. kansasii* strains in the presence of *p*-nitrobenzoic acid (PNB) (○) or *p*-nitro- α -acetylamino- β -hydroxypropiophenone (NAP) (△) and control (●): (a) NCTC 10268; (b) B1; (c) B2; (d) B3; (e) C1; (f) P1.

Fig. 3. Susceptibility of mycobacteria to *p*-nitro- α -acetyl-amino- β -hydroxypropionophenone (NAP): (a) *M. avium* NCTC 10437; (b) *M. bovis* NCTC 10772; (c) *M. scrofulaceum* NCTC 10803; (d) *M. tuberculosis* NCTC 7416; (e) *M. tuberculosis* TB1 (isoniazid resistant); (f) *M. tuberculosis* TB2 (isoniazid and streptomycin resistant); control (●) and NAP (○).

produced heavy growth in both conventional and radiometric media containing PNB.

NAP. *M. tuberculosis* NCTC 7416 and *M. bovis* NCTC 10772 were inhibited by NAP, whereas *M. avium* NCTC 10437 and *M. scrofulaceum* NCTC 10803 were resistant (fig. 3). In contrast to the results obtained in the PNB test, all isolates of *M. kansasii* grew rapidly in the presence of NAP (fig. 2).

TCH. *M. tuberculosis* NCTC 7416, three clinical isolates of *M. tuberculosis* and two of the three isoniazid-resistant strains of *M. tuberculosis* were resistant to TCH (fig. 4). In contrast, *M. bovis* NCTC 10772 and three *M. bovis* isolates from veterinary sources were all inhibited by TCH. *M. avium* NCTC 10437 and *M. scrofulaceum* NCTC 10803 were not inhibited by TCH.

Experience with clinical isolates

During a 1-year period, 131 isolates of mycobacteria identified by conventional methods were tested by radiometric methods. The results obtained with the radiometric methods are given in the table. All isolates of the *M. tuberculosis-bovis* complex were inhibited by both PNB and NAP, whereas the 10 isolates of MOTT were uniformly resistant to both agents.

Of the strains identified as *M. tuberculosis* or *M. bovis*, 110 were resistant to TCH and were identified as *M. tuberculosis*; the remainder were inhibited by TCH and were identified as *M. bovis*, with the exception of one isolate of BCG. There was complete agreement between the radiometric and conventional methods of identification.

Two isolates of *M. bovis* and one isolate of *M. tuberculosis* were resistant to isoniazid. One other isolate of *M. tuberculosis* was resistant to streptomycin and isoniazid. Cross-resistance between isoniazid and TCH was not evident.

Discussion

Clinically significant isolates and reference strains of mycobacteria were successfully differentiated in this study by use of PNB and TCH as selective inhibitors in a radiometric technique. Since the observation by Tsukamura and Tsukamura (1964) that PNB was a useful differentiating agent for mycobacteria, several workers have used susceptibility to 500 μ g/ml in LJ medium as a routine screening method. This investigation has demonstrated that a radiometric PNB test performs as well as the conventional PNB test and a commercially produced radiometric NAP test.

Fig. 4. Susceptibility of mycobacteria to thiophene-2-carboxylic acid hydrazide (TCH): (a) *M. bovis* NCTC 10772; (b-d) *M. bovis* (recent veterinary isolates); (e) *M. tuberculosis* NCTC 7416; (f-h) *M. tuberculosis* (recent human isolates); (i) *M. tuberculosis* TB1 (isoniazid resistant); (j) *M. tuberculosis* TB2 (isoniazid and streptomycin resistant); (k) *M. scrofulaceum* NCTC 10803; (l) *M. avium* NCTC 10437; control (●) and TCH 1.2 $\mu\text{g/ml}$ (○).

Table. Identification of 131 isolates of *Mycobacterium* spp. by radiometric PNB, NAP and TCH inhibition tests

Species	Number of isolates	Results of inhibition tests with					
		PNB		NAP		TCH	
		S	R	S	R	S	R
<i>M. tuberculosis</i>	110	110	0	110	0	0	110
<i>M. bovis</i>	10	10	0	10	0	10	0
BCG	1	1	0	1	0	1	0
<i>M. kansasii</i>	2	0	2	0	2	0	2
<i>M. fortuitum</i>	2	0	2	0	2	0	2
<i>M. avium/intracellulare</i>	3	0	3	0	3	0	3
<i>M. gordonae</i>	2	0	2	0	2	0	2
<i>M. xenopi</i>	1	0	1	0	1	0	1

S=sensitive; R=resistant.

A small proportion of isolates of *M. kansasii* and *M. marinum* will not grow in the presence of PNB (Tsukamura, 1970, 1981). In the present study, two strains of *M. kansasii* failed to grow in the radiometric PNB test and produced a scanty growth on conventional media containing PNB after incubation for 3 weeks. If sensitivity was interpreted as the complete absence of growth on LJ slopes, the conventional test would suggest that these strains were resistant to PNB. However, these strains would be considered susceptible if resistance was indicated only when growth of 20 or more colonies was evident, and the 19 colony = susceptible: 20 colony = resistant endpoint is commonly used in sensitivity tests on mycobacteria.

The PNB-susceptible *M. kansasii* strains were not inhibited by NAP; from the results presented above it would appear that NAP is less inhibitory than PNB for these strains. However, they grew slowly in the presence of NAP and high GI values were not attained. Difficulty in interpretation of NAP sensitivity results for strains of *M. kansasii* has been encountered by other workers, necessitating a longer incubation period of 5–7 days (Morgan *et al.*, 1985). Although the NAP test would appear to be more sensitive than the PNB test for the identification of *M. kansasii*, it has been shown that not all strains of *M. marinum*, *M. chelonae* subsp. *chelonae* and *M. xenopi* are resistant to NAP in the radiometric test (Laszlo and Siddiqi, 1984). Similar difficulties are encountered with the conventional NAP test if conditions are not carefully standardised, inoculum size and incubation temperature being particularly important (Laszlo and Eidus, 1978).

When 131 clinical isolates were examined, the radiometric PNB test was found to be as accurate as the NAP test for the differentiation of MOTT from the *M. tuberculosis* complex. PNB is less expensive than NAP, is more easily obtained and has for many years been a reliable selective agent

in conventional media. The additional dilution of the inoculum for the NAP test (Morgan *et al.*, 1985) is unnecessary for the PNB test, as the method is that used for sensitivity testing with other anti-mycobacterial agents.

The results obtained with the radiometric TCH test were similar to those obtained with the conventional LJ slope method. Preliminary work during the development of this test emphasised that it was important to use an actively metabolising inoculum with a GI of at least 999.

Although the number of isoniazid-resistant isolates of *M. tuberculosis* in this study was small, any cross-resistance with TCH did not interfere with the sensitivity of either the conventional or the radiometric test. In a comprehensive study of the relationship between resistance of *M. tuberculosis* to isoniazid and TCH, Yates *et al.* (1984) concluded that cross-resistance occurred infrequently. In conventional TCH tests, a TCH concentration of 5 µg/ml is used, whereas in the radiometric test sensitivity was greater with TCH 1.2 µg/ml, allowing earlier detection of growth.

While the incidence of bovine tuberculosis is low in Ireland, the possibility of zoonotic transmission exists and will remain until eradication of tuberculosis from cattle is achieved. The epidemiological monitoring of bovine strains is essential for the mycobacteriology laboratory (Collins and Grange, 1987).

Marks (1976) noted that basic classification of mycobacteria is enhanced when drug susceptibility tests are combined with other screening methods. This study has demonstrated that radiometric methods of detection and susceptibility testing can be further supplemented with rapid, sensitive and accurate differential tests for presumptive identification.

We thank Professor J. Flynn for his support and encouragement and Kilian McDaid for drawing the figures.

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