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Evaluation of a rapid differentiation test for the *Mycobacterium tuberculosis* complex by selective inhibition with ρ-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide

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SUMMARY

SETTING: Mycobacterial growth in media to which inhibitory substances are added has been used in species identification. Growth of the *Mycobacterium tuberculosis* complex (MTC) is inhibited by ρ-nitrobenzoic acid (PNB), whereas non-tuberculous mycobacteria (NTM) are resistant. Thiophene-2-carboxylic acid hydrazide (TCH) is useful in the differentiation of MTC when performed together with other tests.

OBJECTIVE: To develop a test using PNB or TCH added to culture medium, and to evaluate its usefulness in the screening of mycobacteria isolates.

DESIGN: In 2001, PNB testing was performed in 109 *M. tuberculosis* strains identified by Instituto Adolfo Lutz (IAL) and 52 NTM strains from the institute's culture collection. The drugs were added to Löwenstein-Jensen (LJ) medium and to BBL-MGIT.

RESULTS: Species differentiation of MTC with the MGIT/TCH method was similar to that observed using the conventional LJ/TCH method. The accuracy of the MGIT/PNB method to differentiate NTM and MTC strains was 99.4%. The BBL-MGIT system allowed presumptive identification in 3–11 days, compared to ⩾12 days with LJ medium.

CONCLUSION: A simple, low-cost test using growth inhibitors may be incorporated into a modern, safe and quick methodology enabling differentiation of MTC and NTM.

KEY WORDS: *M. tuberculosis*; identification; PNB; TCH

THE RE-EMERGENCE of tuberculosis in many countries in different regions of the world is a common public health concern. Rapid and precise diagnosis of each case is necessary for appropriate control of the disease. Isolation, identification and susceptibility testing are essential procedures that should be performed as quickly as possible, so that adequate treatment can be prescribed.

The use of liquid media has been suggested as the most efficient and quickest procedure for the isolation of mycobacteria and susceptibility testing. However, as well as being isolated, these microorganisms should be promptly identified. Although *Mycobacterium tuberculosis* infection is most common, infection due to mycobacteria other than *M. tuberculosis*, or non-tuberculous mycobacteria (NTM), is on the increase in many countries.[1] It is important to establish *M. tuberculosis* infection at an early stage for the establishment of adequate treatment of tuberculosis patients who follow treatment regimens different from patients infected with other mycobacteria.

The BACTEC 460TB (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) method enables differentiation between the *M. tuberculosis* complex (MTC) and other mycobacteria by means of the NAP test (ρ-nitro-α-acetylamino-β-hydroxypropriophenone), which produces results in 4–6 days, but uses radioactive media.[2] Other methods, such as molecular probes and high performance liquid chromatography (HPLC), have been proposed in the differentiation of mycobacterial species. These methods, however, require procedures that are technically complex, laborious and costly. Regions that have scarce resources may not be able to perform these procedures and thus require simple but rapid tests.[3]

A method that uses liquid medium in a tube with an indicator for monitoring mycobacterial growth (BD BBL™ MGIT™, Becton Dickinson) was recently introduced.[4] The system is efficient in the rapid isolation and detection of drug resistance in these microorganisms.[5] It is safe, as it does not use radioactive material. The objectives of the present study were to develop a test adding ρ-nitrobenzoic acid (PNB) to the medium to evaluate its use in differentiating MTC from other mycobacteria. This may reduce the time required for species differentiation.

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The ability of mycobacteria to grow in the presence of inhibitory substances in a suitable medium has been widely used in the identification of different species.[2,6] It has been reported that growth of the MTC is inhibited by PNB 500 μg/ml, whereas NTM are resistant to this concentration. Although a small percentage of these bacteria may be susceptible to the substance, as suggested by Rastogi et al.[7] and Tsukamura et al.,[6] the study developed by Martins et al.[8] showed that mycobacterial growth in PNB-containing medium may be used as a presumptive test for NTM.

TCH is also used for differentiation within the MTC when performed together with other tests.[9] African and Asian strains, as well as *M. bovis*, are susceptible to TCH, whereas most *M. tuberculosis* isolates from humans were resistant to 5 μg/ml of the compound added to the culture medium.[10]

MATERIAL AND METHODS

Microorganisms

The following strains were used in the trial: 109 *M. tuberculosis* strains received by the Mycobacterial Department at Instituto Adolfo Lutz for identification and susceptibility testing in March, May and November 2001; and 52 NTM strains, including *M. fortuitum, M. peregrinum, M. chelonae, M. abscessus, M. terrae, M. avium, M. intracellulare, M. kansasii, M. xenopi, M gordonae, M. simiae, M. malmoense* and *M. nonchromogenicum*, from the Instituto Adolfo Lutz culture collection.

Inoculum preparation

Inocula were prepared from cultures on Löwenstein-Jensen (LJ) medium. A few colonies were emulsified in flasks containing glass beads and 2 ml of sterile distilled water, allowing the turbidity to be greater than McFarland 1 standard. The resulting suspension was left to stand for 15 min to allow larger clumps to settle; 1 ml of the supernatant was then transferred to another tube, where the turbidity level was adjusted to McFarland scale No 1. This bacterial suspension was used as the work suspension.

Tests

Tests were performed using conventional LJ medium and the BBL-manual MGIT system (Becton Dickinson), which consists of modified Middlebrook 7H9 broth medium enriched with OADC (albumin, dextrose and catalase).

PNB was added to both media with a final concentration of 500 μg/ml of medium. For TCH, a final concentration equal to 5 μg/ml was used. Control media were LJ medium and BBL-MGIT medium with no addition of inhibitory substances. The final concentrations of PNB and TCH were chosen because the same concentrations have been reported in other systems using liquid media such as BACTEC 460.[11] To establish that PNB concentration would also work in MGIT, we conducted preliminary experiments testing different concentrations of PNB (125 μg/ml, 250 μg/ml and 500 μg/ml) in BBL-MGIT media. The results showed that all 17 *M. tuberculosis* strains were susceptible to PNB at concentrations of 250 μg/ml and 500 μg/ml, and that four strains were resistant to PNB at 125 μg/ml but susceptible at 500 μg/ml. Based on these results it was concluded that the optimal concentration was 500 μg/ml.

Culture

LJ medium was inoculated with 5 μl of the work suspension and BBL-MGIT system was inoculated with 500 μl of 1:5 dilution of the work suspension. Both media were then incubated at 37°C.

Reading

Reading of the tubes in the BBL-MGIT system began on the third day after inoculation, using BACTEC Micro MGIT (Becton Dickinson) fluorescence reader calibrated according to the manufacturer's instructions.[4]

Tubes containing the inhibitory drugs were then read after the detection of fluorescence in the control tube. A strain was considered susceptible when the tube containing the inhibitor did not show fluorescence 2 days after positive results were observed in the control tube, whereas if fluorescence occurred the strain was considered resistant.

Reading of LJ tubes was started after the 12th day of inoculation. The strain was considered to be resistant when tubes containing the inhibitory substances presented growth patterns similar to that of the control tube.

RESULTS

The results of PNB growth inhibition tests in LJ and BBL-MGIT system using mycobacterial strains are presented in the Table. All the MTC strains, which included one *M. tuberculosis* Beijing and one *M. bovis* BCG strain, were inhibited by PNB on both LJ and MGIT. Of the 52 NTM strains, all were resistant to PNB on LJ medium except for one *M. peregrinum* and one *M. xenopi* strain. The *M. peregrinum* strain susceptible on LJ was also susceptible in MGIT, while the *M. xenopi* strain susceptible on LJ was resistant in MGIT. Growth in medium with TCH produced the following results: of the 109 MTC strains, nine did not grow in the presence of TCH, among these one *M. tuberculosis* Beijing and one *M. bovis* BCG strain.

DISCUSSION

Species differentiation of MTC with the MGIT/TCH method was similar to that observed with the conventional LJ/TCH method.

Table Evaluation of ρ-nitrobenzoic acid (PNB) added to Löwenstein-Jensen (LJ) and BBL-MGIT media in the differentiation between *M. tuberculosis* complex and non-tuberculous mycobacteria

Species	Strains *n*	PNB 500 μg/ml BBL-MGIT S	BBL-MGIT R	LJ S	LJ R
M. tuberculosis	107	107	0	107	0
M. tuberculosis Beijing	1	1	0	1	0
M. bovis BCG	1	1	0	1	0
M. fortuitum	6	0	6	0	6
M. peregrinum	4	1	3	1	3
M. chelonae	4	0	4	0	4
M. abscessus	4	0	4	0	4
M. terrae	4	0	4	0	4
M. avium	6	0	6	0	6
M. intracellulare	3	0	3	0	3
M. kansasii	6	0	6	0	6
M. xenopi	5	0	5	1	4
M. gordonae	3	0	3	0	3
M. simiae	2	0	2	0	2
M. malmoense	2	0	2	0	2
M. nonchromogenicum	3	0	3	0	3
Total	161	110	51	111	50

S = susceptible R = resistant.

The results of growth inhibition of the 109 *M. tuberculosis* strains in BBL-MGIT system with 500 μg/ml PNB were in agreement with previous reports[6,11,12] and with the results obtained in LJ medium. While results in solid medium were mostly obtained after a minimum incubation of 12–20 days, BBL-MGIT allowed presumptive identification of MTC in 3–11 days; 54% of the strains were identified within 5 days.

The test described in the present study was a quick, low cost method that eliminated the need for molecular tests, which are costly and cumbersome. Once a strain grows in MGIT medium with PNB it can be reported as NTM, as it has been already reported in the literature for LJ medium.[9] The accuracy of the MGIT/PNB method to differentiate NTM strains from MTC was 99.4%.

In settings with a high prevalence of tuberculosis and low resources there is a need of rapid tests for isolation, identification and susceptibility testing against anti-tuberculosis drugs. Species identification using methodologies like Gen-Probe, polymerase chain reaction or HPLC can be too expensive and cumbersome. BBL manual MGIT offers rapid mycobacterial isolation from clinical specimens and susceptibility testing. Based on this study we suggest a simple test using inhibitory substances to differentiate between MTC and NTM. This test could be an important addition to this modern, safe and quick methodology.

Presumptive identification can be made by observation of cording formation on Ziehl-Neelsen (ZN) stain[13,14] from the positive culture; however, this is not a definitive differentiation as some NTM also produce cording. A definitive identification can be obtained using conventional methods, such as niacin test, nitrate reduction and catalase production, but these tests are time consuming. The PNB test, which gave the best result, requires two additional MGIT tubes for a definitive identification. This test has many advantages: it usually takes 5 instead of 30 days using conventional biochemical methods, it will improve the overall protocol of isolation, identification and susceptibility testing using the MGIT system, and the cost of combined niacin strips, nitrate and catalase reagents is similar to that of the two extra tubes of MGIT.

With this study we would like to suggest the following procedure:

1 Establish presumptive identification of the *M. tuberculosis* complex by growth characteristics (rough and cream colonies) and by microscopic examination of ZN smear from the positive culture (cording).
2 On strains presumptively identified as MTC, perform combined susceptibility and PNB testing. Set up one control tube, one tube with PNB, two tubes containing the drugs, one with isoniazid and one with rifampicin. If ethambutol or another drug is also to be tested, include an additional tube with that drug.
3 Pyrazinamide (PZA) susceptibility testing may be performed by pyrazinamidase testing.[15]
4 In cases where PNB is negative and PZA is resistant, *M. bovis* can be suspected; TCH should be tested, and a negative result means a strong suggestion of *M. bovis*.

This procedure will enable the laboratory to perform quick combined susceptibility testing and differentiation of MTC from NTM species. Laboratories using the automated BACTEC MGIT 960 system may also use the PNB differentiation test, but the procedure needs to be developed.

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RÉSUMÉ

CONTEXTE : Pour l'identification des espèces, on a recouru largement à la capacité de développement des mycobactéries dans des milieux auxquels des substances inhibitrices avaient été ajoutées. La croissance du complexe *Mycobacterium tuberculosis* (CMT) est inhibée par l'acide ρ-nitrobenzoique (PNB) auquel les mycobactéries non tuberculeuses (NTM) sont résistantes. L'hydrazide de l'acide thiophène-2-carboxylique (TCH) est utile pour la différentiation du CMT en complément d'autres tests.
OBJECTIF : Développer un test utilisant l'addition au milieu de culture de PNB ou de TCH et évaluer son utilité pour le dépistage des isolats de mycobactéries.
SCHÉMA : Le test PNB a été pratiqué pour 109 souches de *M. tuberculosis* analysées par l'Instituto Adolfo Lutz (IAL) en 2001 et 52 souches NTM qui appartenaient à la collection de cultures de l'Institut. Les produits ont été ajoutés au milieu de Löwenstein-Jensen (LJ) et dans le système BBL-MGIT.
RÉSULTATS : La différentiation des espèces du CMT grâce à la méthode MGIT-TCH a été similaire à celle observée avec la méthode conventionnelle LJ/TCH. La précision de la méthode MGIT/PNB pour différentier les souches NTM d'avec le CMT a été de 99,4%. Le système BBL-MGIT a permis une identification probable après 3 à 11 jours, alors que le milieu LJ ne fournissait de résultats visibles qu'après le 12ème jour d'incubation.
CONCLUSION : Un test simple et de faible coût, utilisant des inhibiteurs de croissance, peut être incorporé à une méthodologie moderne sure et rapide et permet une différentiation exacte entre le CMT et les NTM.

RESUMEN

MARCO DE REFERENCIA: El crecimiento de las micobacterias en medios complementados con sustancias inhibidoras se ha utilizado para identificar las diferentes especies. El ácido ρ-nitrobenzoico (PNB) inhibe el crecimiento del complejo *M. tuberculosis* (CMT), pero no tiene efecto sobre las micobacterias no tuberculosas (MNT). La prueba de la hidracida del ácido tiofeno-2-carboxílico (TCH) es útil para diferenciar el CMT cuando se asocia con otras pruebas.
OBJETIVO: Desarrollar una prueba utilizando un medio de cultivo complementado con PNB y TCH y evaluar su utilidad en la identificación sistemática de los aislados de micobacterias.
MÉTODOS: Se practicó la prueba del PNB a 109 cepas de *M. tuberculosis* identificadas en el Instituto Adolfo Lutz (IAL) en 2001 y a 52 cepas de MNT de la colección de cultivos del Instituto. Los medicamentos se añadieron al medio Löwenstein-Jensen (LJ) y al sistema BBL-MGIT.
RESULTADOS: La diferenciación de las especies del CMT con el método MGIT/TCH fue análoga a la obtenida con el método convencional LJ/TCH. La precisión del método MGIT/PNB para diferenciar las cepas de MNT de las del CMT fue del 99,4%. El sistema BBL-MGIT permitió la identificación provisional entre 3 y 11 días y el medio LJ sólo después del 12ª día.
CONCLUSIÓN: La incorporación de una prueba sencilla, de bajo coste y que utiliza inhibidores del crecimiento, a un método moderno, seguro y rápido permite diferenciar el CMT de las MNT.