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COMPARATIVE EVALUATION OF THE NITRATE REDUCTASE ASSAY AND THE RESAZURIN MICROTITRE ASSAY FOR DRUG SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* AGAINST FIRST LINE ANTI-TUBERCULOSIS DRUGS

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ABSTRACT

Tuberculosis remains as a serious infection disease of worldwide distribution, with high morbidity and mortality, mainly in low socio-economic condition countries. The state of emergency of tuberculosis caused by the resistant and multidrug-resistant (MDR) strains, became the main threat to the tuberculosis treatment and control programs. A fast detection method for the resistant strains will allow the implementation of an adequate treatment and contribute for controlling the dissemination of these resistant strains. This study evaluated the performance of the nitrate reductase assay in solid (NRA-LJ) and liquid (NRA-7H9) media, to determine the susceptibility to first line anti-tuberculosis drugs: isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and streptomycin (SMR). Both methods NRA-LJ and NRA-7H9 were evaluated among 18 strains with a known susceptibility profile. The resazurin microtiter assay (REMA) was performed as a reference method. One hundred percent of accordance was observed between NRA-7H9 and REMA for the four tested drugs. When the NRA-LJ method was compared to REMA, the sensitivity and the specificity to INH, RMP, EMB and SMR were 100%, 100 %, 85.7%, 76.9% and 80%, 100%, 75% and 80%, respectively. From the 57 clinical isolates of *M. tuberculosis* evaluated by NRA-7H9 and REMA, 56 (98.2%) were sensitive to all antibiotics tested (INH, RMP, EMB and SMR) by the NRA-7H9 method, while three of these strains were resistant to INH by REMA. One strain showed resistance to INH and RMP for both methods, and MIC of 1.0 µg/ml to INH for both methods, while MIC of 1.0 and 2.0 µg/ml to RMP for REMA and NRA-7H9, respectively. The three assays showed a high level of agreement for rapid detection of rifampicin and isoniazid resistance. Regarding rapidness, the detection of color change in the NRA method is within instants as compared to the overnight incubation required for the REMA test. NRA might represent an inexpensive and alternative assay for rapid detection of resistance in low-income countries.

Key words: Tuberculosis, *Mycobacterium tuberculosis*, Nitrate reductase assay, susceptibility tests, drug resistance.

INTRODUCTION

Tuberculosis (TB) remains a serious infections disease of worldwide distribution, with high morbidity and mortality, mainly in low socio-economic condition countries. The increase in the number of TB cases caused by resistant and multidrug-resistant (MDR-TB) strains, or resistant to at least rifampicin (RMP) and isoniazid (INH), became a threat to TB control programs (18). The early identification of patients with TB resistant strains to first line drugs: INH, RMP, pirazinamid (PZA),

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ethambutol (EMB) and streptomycin (SMR), allows an adequate prescription and contributes for controlling the spreading of these strains (9,17).

The proportion method (4) is the most used for determining susceptibility to drugs. This method requires up to two months for obtaining the results, which is a limitation. In addition, the result is qualitative, and not quantitative. Several alternative commercial or in house methods have been developed. The BACTEC TB-460, MGIT (*Mycobacteria Growth Indicator Tubes*) and the E-test are simple and fast, but require radioactive or expensive products, making it hampering their use in developing countries (7,12).

Recently, fast, reproductive and low cost methods for determining susceptibility to drugs have been described (1,14). These methods are colorimetric, they are based on the use of oxidation-reduction indicators such as resazurin and 3-[4,5,dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and they have been used for determining the minimal inhibitory concentration (MIC) of first and second line anti-tuberculosis drugs (10,14). With similar characteristics to colorimetric methods, the nitrate reductase assay (NRA) has been described, which is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite. The use of specific reagents produce a change of color in the presence of nitrites indicating a positive result (2,13).The aim of this study was to evaluate nitrate reductase assay in 7H9 and in LJ media as an instrument for determining susceptibility profiles of *M. tuberculosis* clinical isolates to first-line drugs, comparing with the rezasurin microplate assay (REMA), which has been showed high agreement with the Proportion Method, moreover it allows to determine the MIC which establish values to resistance, assigning this way, a qualitative and quantitative evaluation of results.

MATERIAL AND METHODS

M. tuberculosis strains

A panel of 18 *M. tuberculosis* strains with known susceptibility profile, of the Laboratório de Micobactérias from the Fundação Universidade Federal de Rio Grande (FURG) was initially evaluated. These strains were used to standardize the nitrate reductase assay in Middlebrook 7H9 (NRA-7H9) (supplemented with 0.5% of glycerol and 10% oleic acid, albumin, dextrose and catalase (OADC)) and NRA in Löwenstein-Jensen (NRA-LJ) solid medium while REMA was used as the gold standard.

After the conclusion of the first experiment, 57 *M. tuberculosis* clinical isolates obtained from patients of the Tisiology Department of the Prefeitura Municipal de Rio Grande - Rio Grande do Sul, Brazil, from December 2005 to August 2006 were tested to MIC determination by NRA-7H9 and using the REMA as reference method. The reference strain H37Rv (ATCC 27294) was used as a control. All the isolates and the control strain were maintained in Ogawa-Kudoh culture medium at 37ºC, until they were tested.

Antibiotics

INH (Sigma® lot:91K1467 Switzerland), RMP (Sigma® lot:78H0773 Switzerland) and SM (Sigma® lot:61K119926 Switzerland) were diluted to 1 mg/ml and EMB (Sigma® lot:10K0774 Belgium) to 10 mg/ml. For preparation of the stock solution, sterile distilled water was used for INH, EMB and SMR and methanol for RMP. All the antibiotic stock solutions were sterilized by filtering through a 0.22 µm membrane and stored at -20ºC until use.

Reagents

Sodium resazurin (Applied Research Institute - USA) stock solution was prepared at 0.22 mg/ml in sterile distilled water and stored at 4ºC. The nitrate reductase developer was prepared with one part of concentrated hydrochloric acid 50% (vol/vol), two parts of sulphanilamide 0.2% (w/vol) and two parts of n-1-naphtylamine 0.1% (w/vol). All reagents were manufactured by Synth (Brazil).

REMA

The REMA was performed adapting the technique proposed by Palomino *et al*. (14). Briefly, the inocula were prepared from a culture of approximately 15 days in Ogawa-Kudoh medium. The culture was suspended in Middlebrook 7H9 broth supplemented with 10% OADC, (Becton Dickinson - UK), adjusted to the tube number one of the McFarland scale and diluted 1:25 in water. Antibiotic stock solutions were diluted in 7H9 just before use to a concentration four times the initial concentration to be tested. A volume of 75 µl of 7H9 was placed in each well of a sterile 96 wells microtiter plate, with the exception of the external wells which were filled with 200 µl of water. A two-fold serial dilution of each drug was prepared directly on the plate. The concentrations used were from 4 to 0.007 µg/ml for INH and RMP and from 16 to 0.03 µg/ml for EMB and SMR. The cutoff to define susceptibility/resistance was 0.25, 0.5, 5 and 8 µg/ml for INH, RMP, EMB and SMR, respectively (16). A volume of 75 µl of the inoculum was added to each well. Controls for growth of each isolate and medium sterility were used in each plate. The plates were closed, sealed and incubated at 37ºC. After seven days of incubation, 25 µl of resazurin solution were added to each well and the plate returned to the incubator for two more days. Color alteration from blue (oxidized state) to pink (reduced state) indicates bacterial growth. MIC was defined as the lowest drug concentration that inhibited bacterial growth, characterized by the lack of color changing.

NRA-7H9

The assay was adapted from that proposed by Kumar *et al*. (8). Briefly, the inocula were prepared from a 15-days culture in Ogawa-Kudoh medium. The culture was resuspended in

Middlebrook 7H9 broth with 1 mg/ml of sodium nitrate ($NaNO_3$) supplemented with 10% OADC, adjusted to the tube number one of the McFarland scale, and diluted in water in the proportion of 1:50. Antibiotic stock solution was thawed and diluted in 7H9 without $NaNO_3$ to a concentration four times the initial concentration to be tested. Preparation of the plate, addition of antibiotic and inoculum was identical to the REMA assay. After 7 days of incubation, 26 µl of NRA developer were added to the positive control wells. If a pink color was observed, then the developer was added to the remaining wells. If the color of the positive control well remained unchanged, the plate was returned to the incubator and this procedure was repeated on the 10th and 14th days. The results were considered negative when the color of the developer was unchanged, and positive, when a pink color was observed.

NRA-LJ

The standard NRA-LJ medium was prepared according to the procedure previously described (2), with the potassium nitrate (KNO_3) replaced by sodium nitrate in the concentration of 1 mg/ml. The $NaNO_3$ was added to the tubes with and without antibiotics. In the tubes with antibiotics, the following concentrations were used: 0.2 µg/ml for INH, 40 µg/ml for RMP, 2 µg/ml for EMB and 4 µg/ml for SMR (13). The inoculum was adjusted to a turbidity equivalent to the tube number one of the McFarland scale. A volume of 200 µl of the suspension was added to the tubes with antibiotics. A volume of 200 µl of the suspension diluted 1:10 in phosphate buffer saline solution (PBS) was used to inoculate tubes without antibiotics as a growth control. The tubes were incubated at 37ºC and after 7 days, 500 µl of NRA developer were added to the control tubes (without antibiotics). If color change was observed in the controls, the developer was also added to the remaining tubes. However, if there was no color change in the control tubes, they were returned to the incubator and the procedure was repeated on the 10th day and, if necessary on the 14th day. The isolate was considered resistant if there was no alteration from pink to red or purple with color intensity higher than the one observed in the control tube.

Data analysis

Data analysis was performed by the Statistical Package for Social Sciences (SPSS) version 10.0 for Windows. The Receiver Operating Characteristic (ROC) curve analysis was also carried out the same software. In this study, the term sensitivity represents the ability to detect the strain truly susceptible to the drug, whereas specificity represents the ability to detect the clinical isolate truly resistant to the drugs.

RESULTS

Evaluation of NRA-7H9 e NRA-LJ

Initially, the results obtained by the NRA-7H9, NRA-LJ and REMA assays were compared to determine the susceptibility profile of the 18 *M. tuberculosis* strains from the mycobacteria collection of the Laboratório de Micobactérias of FURG. The **NRA-7H9** identified 10 resistant and 8 susceptible strains for INH and RMP; 4 resistant and 14 susceptible strains for EMB; 5 resistant and 13 susceptible strains for SMR, in perfect agreement (100%) with the REMA (Table 1).

The NRA-LJ presented 100% agreement for RMP and some disagreement for the INH, EMB and SMR antibiotics when compared to REMA. Sixteen strains agreed for INH (eight resistant and eight susceptible) and two diverged, and both were considered susceptible by NRA-LJ, but resistant by the REMA (MIC = 1.0 µg/ml and 0.5 µg/ml). To EMB, twelve strains were susceptible and three resistant, however, three strains presented different results, with two susceptible by REMA (MIC = 1.0 µg/ml and 0.5 µg/ml), but resistant by NRA-LJ and one strain resistant by REMA (MIC = 16 µg/ml) and susceptible by NRA-LJ. To SMR, four strains were resistant and eleven susceptible by both methods, however three strains were susceptible by REMA (MIC of 4.0 µg/ml, 2.0 µg/ml and 2.0 µg/ml) and resistant by NRA-LJ (Table 1). The strains with diverging results were re-evaluated twice by the three methods. The values of the ROC curve for INH, RMP, EMB and SMR were, 0.9, 1.0, 0.8 and 0.78 respectively, by NRA-LJ. In NRA-7H9, ROC of the four antibiotics was 1.0.

Table 1. Susceptibility test results of 18 strains of *M. tuberculosis* by NRA - 7H9 and NRA-LJ compared to REMA.

	REMA							
	INH		RMP		EMB		SM	
	S (%)	E (%)	S (%)	E (%)	S (%)	E (%)	S (%)	E (%)
NRA 7H9	100	100	100	100	100	100	100	100
NRA LJ	100	80	100	100	85.7	75	76.9	80

INH=isoniazid; RMP=rifampicin; EMB=ethambutol; SM=streptomycin; NRA=nitrate reductase; REMA=resazurin microplate assay; LJ=Löwenstein-Jensen; S=sensitivity; E=specificity.

Evaluation of NRA-7H9 assay for clinical isolates susceptibility determination

NRA-7H9 was used to the analyses of 57 *M. tuberculosis* clinical isolates and compared to the REMA method. One strain was resistant to INH and RMP with MIC of 1.0 µg/ml for INH by both methods and MIC of 1.0 and 2.0 ìg/ml for RMP by NRA-7H9 and REMA, respectively. From the 57 isolates, 56 (98.2%) were susceptible to all antibiotics tested (INH, RMP, EMB and SMR) by the NRA-7H9 method, whereas three strains were resistant to INH by REMA.

DISCUSSION

Strains of *M. tuberculosis* resistant to anti-TB drugs constitute an important public health problem (18). Efficient, fast, simple and low cost tests for detection of resistant strains are necessary for the qualification of TB control, allowing an adequate treatment and interruption of the bacillus transmission (11). Colorimetric methodologies have been suggested as an important tool for detection of resistant strains in developing countries. Methods like 3-[4,5,dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), REMA and NRA take approximately ten days in order to obtain the susceptibility result after isolation of the bacterium (11). This period is similar to that taken by BACTEC TB-460 or MGIT methods, which despite being fast they present the disadvantage of necessitating high cost equipment and radioactive substances (13).

The internal validation of NRA-7H9 and NRA-LJ was performed with 18 strains previously known susceptibility profile. It showed 100% of sensitivity and specificity for REMA and NRA-7H9 methods for the four studied antibiotics (INH, RMP, EMB, SMR). The agreement between NRA-7H9 and REMA methods may have been influenced by the similarity of the two methods, both carried out in liquid media (8).

The use of microtiter plates has the advantage of lower cost, fast and quantitative results. The disadvantage is aerosol production, making it necessary the use of biological safety cabinets (11) not readily available in some developing countries.

When the results of NRA-LJ and REMA were compared, it was observed 100% agreement for RMP, however there were disagreements in relation to the antibiotics INH, EMB e SMR. The factors which led to these differences can be associated to the fact that NRA-LJ is carried out in solid medium and REMA in liquid medium. These differences were also found in previous studies (13). Another possible explanation for the lower specificity and sensitivity values would be degradation of the antibiotics EMB and SMR in LJ medium. The accuracy for RMP, easiness of execution and low cost of NRA-LJ method is attractive to be used as a screening method for detection of MDR strains, as resistance to RMP is a marker for MDR detection (5,15).

An advantage of the NRA-7H9 in relation to REMA is that the result can be read immediately after addition of the developer. REMA needs one or two extra days after addition of resazurin before the result can be read.

NRA-7H9 and REMA were used to determine the susceptibility of 57 clinical isolates. Three isolates were resistant to INH by REMA and susceptible by NRA-7H9. One isolate was resistant to INH and to RMP by both methods. This MDR isolate was obtained from the sputum of a TB patient diagnosed in 2004 and previously treated for TB, but failed to complete the treatment, which is in agreement with studies that show that previous treatments are risk factors for the selection of resistant strains (3,6). The identification of a MDR strain highlights the need for implementation of susceptibility tests in the routine tuberculosis diagnose. The NRA-7H9 assay presented advantages to rapid and inexpensive determination of susceptibility profiles in clinical isolates. Detection of color change in the NRA is faster (immediately after addition of the reagents) than redox methods and more cost-effective for developing countries. Moreover, as nitrate reductase activity is used as one of the discriminatory tests for the identification of *M. tuberculosis*, this method provides specificity to the test unlike the general metabolic activity detected by other dyes. NRA might represent an inexpensive and alternative assay for rapid detection of resistance in low-income countries.

RESUMO

Avaliação comparative dos métodos Nitrato Redutase e Microdiluição com Resazurina para testar a sensibilidade do Mycobacterium tuberculosis frente aos anti-tuberculosos de primeira linha

A tuberculose permanece como uma séria doença infecciosa, com distribuição mundial, alta morbidade e mortalidade, ocorrendo principalmente em paises com baixa condição econômica. O estado de emergência da tuberculose causada por cepas resistentes e multirresistentes tornou-se uma importante ameaça para o tratamento e programas de controle da tuberculose. Uma rápida detecção de cepas resistentes permitirá a implantação de um tratamento adequado e contribuirá para controlar a disseminação destas cepas. Este estudo avaliou a performace do ensaio nitrato redutase em meio sólido (NRA-LJ) e meio líquido (NRA-7H9), para determinar a sensibilidade frente aos fármacos antituberculosos de primeira linha: isoniazida (INH), rifampicina (RMP), etambutol (EMB) and estreptomicina (SMR). Ambos os métodos, NRA-LJ e NRA-7H9, foram avaliados com 18 cepas com conhecido perfil de sensibilidade. O ensaio de microplaca com resazurina (REMA) foi utilizado como método de referência. A concordância observada entre NRA-7H9 and REMA foi de 100% para os quatro fármacos testados. Quando o método NRA-LJ foi comparado com o REMA, a sensibilidade e especificidade

para INH RMP e SMR foram de 100%, 100%, 85,7%, 76,9% e 80%, 100%, 75% and 80%, respectivamente. Dos 57 isolados clinicos de *M. tuberculosis* avaliados por NRA-7H9 e REMA, 56 (98.2%) foram sensíveis a todos antibióticos testados (INH, RMP, EMB e SMR) pelo método NRA-7H9, enquanto três destas cepas foram resistentes para INH pelo REMA. Uma cepa mostrou resistência para INH e RMP por ambos os métodos, e CMI de 1,0 µg/ml para INH para ambos os métodos, enquanto CMI de 1,0 e 2,0 µg/ml para RMP pelo REMA e NRA-7H9, respectivamente. Os três ensaios mostraram um alto nível ded concordância para uma rápida detecção de resistência a rifampicina e isoniazida. Com relação à rapidez na obtenção dos resultados, a detecção na mudança de cor nos métodos NRA é imediata enquanto para o método REMA é necessário incubar *overnight*. Os métodos NRA podem representar uma alternativa, de baixo custo e rápida detecção de resistência, em países com poucos recursos.

Palavras-chave: Tuberculose, *Mycobacterium tuberculosis*, Nitrato redutase ensaio, teste de sensibilidade, fármaco-resistência.

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