ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1981, p. 556-557
0066-4804/81/100556-02$02.00/0

Vol. 20, No. 4

Use of Pyrazinamidase Activity in *Mycobacterium tuberculosis* as a Rapid Method for Determination of Pyrazinamide Susceptibility

J. KENNETH McCLATCHY,* ANNA Y. TSANG, AND MARK S. CERNICH

Department of Clinical Laboratories, National Jewish Hospital and Research Center, National Asthma Center, Denver, Colorado 80206

Received 8 May 1981/Accepted 20 July 1981

Pyrazinamidase activity in clinical isolates of *Mycobacterium tuberculosis* has been previously found to correlate with susceptibility to the antituberculosis drug pyrazinamide. The Wayne method for determining pyrazinamidase activity, a technique also utilized as an aid in the identification of mycobacteria, and a thin-layer chromatography method were found to be useful screening methods for susceptibility testing, since resistant strains are pyrazinamidase negative. These simple methods overcome the difficulty in growing *M. tuberculosis* at pH 5.5, as is required in the conventional method of susceptibility testing.

The clinical use of pyrazinamide (PZA), an effective antituberculosis drug, is hampered by the difficulty in performing reliable susceptibility tests. The drug has maximal activity in vitro at pH 5.5 (2) and, unfortunately, many strains of *Mycobacterium tuberculosis* grow poorly or not at all in an acidic medium. For example, PZA susceptibility was conventionally determined by precisely following the method of Stottmeier et al. (3). This method tests the ability of *M. tuberculosis* strains to grow on 7H10 agar adjusted to pH 5.5 and containing 100 μg of PZA per ml. Resistance in this study was defined as growth on drug-containing media greater than 1%, as compared with the control. It was found that 46 of 232 clinical isolates tested would not grow satisfactorily when subcultured to the acidic 7H10 medium. Most of the 46 strains were resistant to several other antituberculosis drugs.

It was previously reported that PZA-susceptible tubercle bacilli have a pyrazinamidase (PZase) that metabolizes PZA to pyrazinoic acid (POA) and that PZA-resistant strains have lost PZase activity (1). We have confirmed this observation. Thirty-one strains were tested for PZA susceptibility by the pH 5.5 7H10 agar method and for POA production by using a thin-layer chromatography method. The strains of *M. tuberculosis* to be tested for POA production were grown in 7H11 broth to an optical density of 0.3 at 600 nm (Coleman Jr. Spectrophotometer). PZA was added to a concentration of 100 μg/ml in 5 ml of 7H11 broth in screw-cap tubes, and the resulting mixture was incubated at 37°C. Samples (0.5 ml) were removed both before and 48 h after incubation and placed in tubes in an 80°C water bath for 1 h. Bacteria were then removed by centrifugation at 2,000 × *g* for 3 min. Supernatant (20 μl) was spotted onto a silica gel thin-layer chromatography plate with fluorescent indicator (Uniplate AGBF; Analtech, Inc., Newark, Del.). Chromatograms were developed in a solvent of isopropanol and 2 N ammonium hydroxide (70:30) for about 7 h. After they were air dried, the plates were examined under ultraviolet illumination. PZA and POA spots were readily detected; the R_f value was 0.95 for PZA and 0.65 for POA. Standards were spotted on each plate and included (in 7H11 broth) 20 μl of a 100-μg/ml solution of PZA, 20 μl of a 100-μg/ml solution of POA, and a mixture of 10 μl each of a 100-μg/ml solution of PZA and a 100-μg/ml solution of POA. Seventeen strains found to be susceptible by the agar method were positive for PZase activity. Thirteen isolates found to be resistant by the agar method did not have PZase activity. One strain which was found to be resistant by the agar method was PZase positive. However, it was an isolate that was 50% resistant and was a mixture of susceptible and resistant organisms.

One-hundred twenty-three strains were then tested for PZase activity by thin-layer chromatography. Histories were obtained for 86 patients. Of 52 patients claiming no previous PZA treatment, 48 had organisms which were positive for PZase activity. Of those 34 patients with a history of PZA treatment, 31 had organisms which were PZase negative. The remaining three patients had received PZA treatment sporadically or for only a short period of time. Histories on the remaining 37 patients were inadequate

for determining whether or not they had had previous PZA treatment. Of the 37 isolates, 23 were found to be PZase positive (susceptible) and 14 were PZase negative (resistant). This is not surprising, since all of the patients under study were being treated for infections with strains of *M. tuberculosis* that were resistant to several of the other commonly used antituberculosis drugs.

PZase activity was also determined by the method described by Wayne (4). This test has been previously used in the identification of nontuberculous mycobacteria. Several loopfuls (5 to 10 mg wet weight) of each strain of *M. tuberculosis* were obtained from 7H11 agar, placed onto the surface of Wayne PZA medium, and incubated at 37°C. After 4 days, 1.0 ml of freshly prepared 1% ferrous ammonium sulfate was added to each tube. The tubes were refrigerated for 4 h and then examined. The presence of a pink band indicates PZase activity. Strains (262) of *M. tuberculosis* were tested for POA production by this method, and the results were compared with those of the thin-layer chromatography method. Of 199 strains determined to be positive for POA by the thin-layer chromatography method, 194 were also determined to be positive by the Wayne method. Of 63 strains determined to be negative by the Wayne method, 55 were also determined to be negative by the thin-layer chromatography method, which seems to be somewhat more sensitive in detecting small quantities of POA.

The Wayne method requires that large inoculum sizes be used or false-negative readings may result. Any degree of pink color is read as a positive result. Cultures containing a mixture of susceptible and resistant organisms will, therefore, usually be read as PZA susceptible.

We conclude that determination of PZase activity by either the Wayne or the thin-layer chromatography method is suitable for use in clinical microbiology laboratories. The Wayne method, however, is technically easier to perform and is already in use in many mycobacteriology laboratories as a taxonomic aid. We therefore propose this new application of the Wayne method as a qualitative screening method for determining susceptibility to PZA. It must be noted that this PZase test is not useful for testing nontuberculous mycobacteria for susceptibility, because many of these species give positive PZase test results, even though these species are resistant to the drug.

This work was supported by Public Health Service grant AI-02641 from the National Institute of Allergy and Infectious Diseases.

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