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Evaluation of a Microtiter System for Identification of Anaerobic Bacteria

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The Anaerobe Combo Panel (American MicroScan, Mahwah, N.J.) was evaluated for its ability to identify anaerobic bacteria. The frozen, 96-well panel utilizes 24 biochemical reactions and four antimicrobial agents for species identification. The Anaerobe Combo Panel was used to test 114 clinical isolates of strict anaerobes. Reactions were read after 48 h, and the results were compared with those obtained with the PRAS II system (Scott Laboratories, Inc., Fiskeville, R.I.). Discrepancies between the two systems were resolved by gas-liquid chromatography. With the Anaerobe Combo Panel, 84% of the organisms were able to grow, and 89% of these were correctly identified to genus level and 78% to species level. The Anaerobe Combo Panel was easy to inoculate and read, but some of the reactions were difficult to interpret, and not all of the derived codes were found in the code book.

Broth microdilution systems have proven to be reliable methods for the identification and susceptibility testing of clinical bacterial isolates. A number of systems are commercially available for members of the family *Enterobacteriaceae*, nonfermenters, gram-positive organisms, and recently for gram-negative anaerobic bacilli (5). This report describes the results obtained with the Anaerobe Combo Panel (American MicroScan, Mahwah, N.J.), which is designed for the identification and susceptibility testing of anaerobic bacteria. The panel was tested on clinical isolates of obligate anaerobes that also were identified by means of the PRAS II system (Scott Laboratories, Inc., Fiskeville, R.I.).

MATERIALS AND METHODS

All of the organisms tested were fresh clinical isolates obtained from patients at The Presbyterian Hospital, New York. Appropriate specimens suitable for anaerobic culture were collected and transported in the syringe used for aspiration or in an Anaerobic Specimen Collector (BBL Microbiology Systems, Cockeysville, Md.). Primary media for anaerobes included reducible anaerobic Columbia agar with 5% sheep blood, cysteine, dithiothreitol, and palladium chloride (anBAP) (2); kanamycin-vancomycin-laked blood agar with menadione and hemin; colistin-nalidixic acid blood agar; neomycin blood agar; and prereduced, anaerobically sterilized, chopped-meat glucose broth (CMG; Scott Laboratories, Inc.). All plates were prereduced in a GasPak (BBL) jar at room temperature for 24 h before inoculation. After inoculation, plates were incubated at 35°C in plastic bags equipped with hydrogen generators and redox indicators (Bio-Bag; Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.).

Colonies from primary plates were subcultured both aerobically and anaerobically to determine purity and aerotolerance. Gram stains were performed on strict anaerobes. Gram-negative cocci were presumed to be *Veillonella* isolates and were not tested further. Colonies were examined under long-wave UV light; each showing red fluorescence was presumptively identified as a black-pigmented *Bacteroides* species. Gram-positive nonbranching bacilli were tested by catalase and spot indole tests; those giving positive reactions were presumptively identified as *Propionibacterium acnes*. Other gram-positive rods and gram-negative bacilli were inoculated into the Anaerobe Combo Plate and the PRAS II systems as described below. Gas-liquid chromatography (GLC) was performed on catalase-negative or branching gram-positive rods and on all gram-negative rods that were not clearly identified by the system being evaluated, and GLC was also performed when necessary to help resolve discrepancies in identification between the two systems.

Pure cultures of the organisms to be tested were inoculated into 5 ml of reduced thioglycolate 135C broth containing vitamin K and hemin (American MicroScan) and incubated for 24 h at 35°C. This culture was used to inoculate the Anaerobe Combo Plates and the PRAS II system.

Anaerobe Combo Panel. Premarket samples of the Anaerobe Combo Panel were obtained from the manufacturer (American MicroScan). These frozen, 96-well trays utilize 24 biochemical reactions and four single-concentration antimicrobial agents (vancomycin, colistin, kanamycin, and rifampin) for species identification. The remaining wells are used to determine the MICs of nine antibiotics. The biochemical substrates included arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, starch, sucrose, trehalose, xylan, bile-esculin, bile, esculin, nitrate, formate-fumarate, Tween, arginine, and sodium polyanetholesulfonate.

The Anaerobe Combo Panels were thawed and reduced by placing them into a GasPak jar for 4 h at room temperature. A standardized suspension was prepared by adding a sufficient volume of the thioglycolate broth culture to 5 ml of reduced sterile water (American MicroScan) to produce a turbidity matching that of a McFarland no. 1 standard. This standardized suspension (2.5 ml) was added to a tube containing 22.5 ml of reduced sterile water with 0.02% Tween 80. After being mixed by inversion several times, the entire contents of the tube were poured into the inoculation tray, and the inoculator was replaced. The inoculator was then lowered onto an Anaerobe Combo Panel so that the prongs entered all of the wells except the sterility control. The inoculator was removed and discarded, the panel cover was replaced, and the panels were incubated for 48 h at 35°C in GasPak jars.

After incubation, 0.02% bromcresol purple indicator at pH

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6.8 was added to the carbohydrate wells; 1% ferric ammonium citrate was added to the esculin and bile-esculin wells; and 0.8% sulfanilic acid, 0.5% *N,N*-dimethyl alpha-naphthylamine, and zinc dust were added to the nitrate well.

Acid production in the carbohydrate wells was indicated by a yellow or gray color after the addition of the bromcresol purple. A black precipitate in the esculin wells indicated a positive reaction. A red color in the nitrate well, after the addition of the reagents, signified reduction. The effect on growth of sodium polyanetholesulfonate, formate-fumarate, Tween, arginine, vancomycin, kanamycin, colistin, rifampin, and bile was determined by comparing growth in those wells with that in the peptone-yeast glucose control well.

A six-digit code (seven digits for gram-negative bacilli) was derived on the basis of the various reactions. The identity of an isolate was determined by comparing this code to those in a code book provided by the manufacturer.

PRAS II system. The components of the PRAS II system included various prereduced, anaerobically sterilized biochemical substrates in tubes containing 5 ml and sealed with rubber septa. The septa were wiped with 70% alcohol before inoculation. Thioglycolate broth culture (0.5 ml) was injected into a tube of CMG, which was then incubated for 24 h. Tubes of biochemical substrates were each inoculated with 0.05 ml of the CMG culture by using a special inoculator provided by the manufacturer. The substrates utilized were the same as those in the Anaerobe Combo Panel, except that xylose was used instead of xylan. Peptone-yeast gelatin was added for gram-positive bacilli, and PRAS II semisolid indole motility medium was included for gram-negative rods. The CMG culture was also used to inoculate anBAP plates on which were placed disks containing vancomycin (5 μg/ml), colistin (10 μg/ml), kanamycin (1,000 μg/ml), or rifampin (15 μg/ml) (6).

Tubes and plates were incubated for 48 h. The anBAP plate was examined for zones of inhibition around the disks; zones of 10 mm or more were considered to indicate inhibition. Growth from the anBAP plate was also used to perform spot indole and catalase tests. Fermentation of the carbohydrates was determined by removing the rubber septum from each tube and measuring the pH with a pencil electrode. Values of 6.0 or less were interpreted as acid. Nitrate reduction and esculin hydrolysis were determined with the same reagents that were employed with the Anaerobe Combo Panel. A black precipitate in semisolid indole medium indicated H_2S production, and failure of the peptone-yeast gelatin to gel after 15 min at 5°C indicated hydrolysis.

GLC. GLC for volatile fatty acids was performed by acidifying a portion of the CMG culture with H_2SO_4 and adding a one-half volume of ether. After vortexing, centrifugation, and freezing in an alcohol-dry ice bath, 20 μl of the ether extract was injected into the port of a Dohrman Envirotech Anabac unit. Fatty acids were assayed on the same unit by using a methylated extract of the CMG.

RESULTS

A total of 114 isolates of strict anaerobes in thioglycolate broth were inoculated into the Anaerobe Combo Panel and the PRAS II system. Of these organisms, 96 grew in both systems; an additional 5 organisms grew in the PRAS II system but failed to grow in the Anaerobe Combo Panel. Of these five organisms, two were identified by the PRAS II system as *Bacteroides fragilis*, and the other three were *Bacteroides melaninogenicus*, *Bacteroides intermedius*, and *Fusobacterium* sp. Thirteen organisms failed to grow in either system. Of these, six were gram-positive cocci and seven were gram-negative bacilli, including an isolate presumptively identified as *B. melaninogenicus* by red fluorescence. An isolate identified as *Bacteroides disiens* by the Anaerobe Combo Panel failed to grow in the PRAS II system.

In 69 instances, the genus and species identifications provided by the Anaerobe Combo Panel coincided with the identifications by the PRAS II system, and no further studies were undertaken with these isolates. In 18 cases, discrepancies between the Anaerobe Combo Panel results and the PRAS II results occurred, and GLC was performed. Of these organisms, six were confirmed by GLC as correctly identified to species level, and 10 were confirmed as correctly identified to genus level by the Anaerobe Combo Panel. One organism was *Bifidobacterium magnum*, misidentified by the Anaerobe Combo Panel as *Actinomyces odontolyticus*. The remaining isolate, identified as *Fusobacterium nucleatum* by the Anaerobe Combo Panel and as *Bacteroides ruminicola* by the PRAS II system, was probably *Bacteroides oralis* or *Bacteroides buccae*.

The 85 organisms correctly identified by the Anaerobe Combo Panel included *B. fragilis*, 20; *Bacteroides distasonis*, 2; *Bacteroides vulgatus*, 4; *B. oralis*, 2; *B. intermedius*, *Bacteroides thetaiotaomicron*, *Bacteroides asaccharolyticus*, *Bacteroides uniformis*, and *Bacteroides ovatus*, 1 each; *Bacteroides* species, 6; *Clostridium perfringens*, 12; *Clostridium tertium*, 2; *Clostridium innocuum*, 1; *Peptostreptococcus parvulus*, 3; *Peptostreptococcus anaerobius*, 2; *Peptococcus asaccharolyticus*, 2; *Peptostreptococcus* sp., 2; *A. odontolyticus*, 2; *Actinomyces viscosus*, 1; *Actinomyces "meyeri,"* 2; *F. nucleatum*, *Fusobacterium* sp., 3; *Bifidobacterium dentium*, 3; *Eubacterium lentum*, 2; *Eubacterium alactolyticum*, 1; and *Propionibacterium acnes*, 7.

In nine instances, the code generated was not found in the code book, and, consequently, no identification could be provided by the Anaerobe Combo Panel. These organisms were identified by the PRAS II system and GLC as *Fusobacterium mortiferum*, *B. disiens*, *B. fragilis* (two isolates), *B. intermedius*, *Bifidobacterium* sp., *Eubacterium* sp., *Clostridium mangenoti*, and *Propionibacterium acnes*.

Of the 101 identified organisms, the PRAS II system correctly identified 81 to species level and 94 to genus level, as confirmed by GLC or identical results with the Anaerobe Combo Panel. In seven instances, the PRAS II system required GLC for identifying the isolates. These organisms included *E. lentum* (two isolates), *Eubacterium* sp., *C. mangenoti*, *Propionibacterium acnes*, *Fusobacterium necrogenes*, and *Bifidobacterium* sp.

DISCUSSION

Microtiter systems for the identification and susceptibility testing of bacterial isolates have been shown to be convenient, accurate, and in many instances, cost effective. It is therefore neither inappropriate nor unexpected that this technology would be applied to anaerobic bacteria.

An initial concern was that the geometry of the system, i.e., the surface/volume ratio in the wells, would permit exposure to air and a rapid loss of the low oxidation-reduction potential required for the growth of anaerobes. However, 84% of the organisms grew in the Anaerobe Combo Panel, a recovery rate only slightly less than the 89% obtained with the PRAS II system and somewhat better than that reported for other systems (4). It should be noted that some of the most fastidious and oxygen-susceptible organisms, such as *Veillonella* sp. and the majority of the *B.*

melaninogenicus isolates, were removed during the preliminary screen and were not tested in either system. With the Anaerobe Combo Panel, 89% of the isolates were correctly identified to genus level, and 78% were correctly identified to species level. The panels were simple to inoculate and could be read quickly. The panels must be stored in the frozen state and prereduced before use. Anaerobiosis must be provided by the user. In this regard, the panels were too large to fit into the commercial plastic bags for anaerobic incubation. At times the reactions were difficult to interpret, and all of the derived codes were not always found in the code book. Expansion of the data base and a chart listing the various biochemical reactions would be very helpful.

In this study, the PRAS II system was considered the standard by which the Anaerobe Combo Panel was evaluated. The PRAS II system utilizes the same type of medium as does the VPI system (3) and has been shown to be an accurate identification system for obligate anaerobes (1). In our study, the PRAS II system correctly identified 80% of the organisms to species level and 93% to genus level.

Although not evaluated in this study, MICs of the nine antimicrobial agents most frequently utilized for the treatment of anaerobic infections are also identified with the Anaerobe Combo Panel.

The Anaerobe Combo Panel has a number of features that would recommend it to the clinical laboratory, not the least of which is the potential for automated reading and interpretation by laboratories that already own the TouchScan or autoSCAN-3 (American Scientific Products, Div. American Hospital Supply Corp., Biomedical Marketing Communications, McGaw Park, Ill.) hardware.

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