JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1983, p. 609–613
0095-1137/83/090609-05$02.00/0

Vol. 18, No. 3

Comparison of Three Reagents for Detecting Indole Production by Anaerobic Bacteria in Microtest Systems

GEORGE L. LOMBARD AND V. R. DOWELL, JR.*

Centers for Disease Control, Center for Infectious Diseases, Hospital Infections Program, Atlanta, Georgia 30333

Received 29 November 1982/Accepted 12 May 1983

Three reagents for detecting indole, Kovac, Ehrlich, and *p*-dimethylaminocinnamaldehyde (DMCA), were evaluated with commercial microtest systems for characterizing and identifying anaerobic bacteria. The DMCA reagent, the most sensitive of the three reagents, gave a positive reaction with 445 of 449 strains of various indole-producing anaerobic bacteria. There was 99.6% agreement between the results obtained with the DMCA in the microtest systems and results using the conventional tube test to detect indole by using xylene extraction and Ehrlich reagent. Ehrlich reagent detected indole in 163 of 176 (92.6%) indole-positive strains when the inoculum was overlaid with mineral oil before incubation. Kovac reagent was the least sensitive of the reagents tested. When the inoculum was overlaid with mineral oil, Kovac reagent detected only 80 of 108 (74.0%) of indole-positive strains. In addition to being the most sensitive reagent for detection indole, DMCA also allowed detection of indole derivatives (skatole, 3-indolepropionic acid, and 3-indolebutyric acid) produced by some clostridia.

The ability of certain bacteria to form indole from the amino acid tryptophan is an important phenotypic characteristic. Demonstration of indole production is a useful test for characterizing, and is sometimes required for accurately identifying, some species of the genera *Bacteroides*, *Fusobacterium*, *Clostridium*, *Propionibacterium*, and *Peptococcus*. Included in each of these genera are some species that produce indole and others with very similar phenotypic characteristics that do not produce indole.

Recently, several commercial microtest systems have been marketed for characterizing and identifying anaerobic bacteria (3, 9–12). However, several investigators have reported erroneous identification of certain anaerobes because indole production was not detected. Starr et al. (12) reported that 7 of 86 anaerobe strains tested by them were erroneously identified with the API Anaerobe-20 microtest system because of negative reactions for indole with Kovac reagent. Similarly, Stargel et al. (11) found that 89.6% of the indole-producing strains they tested with the BBL Minitek microtest system were positive with Kovac reagent and that the results were only slightly improved when Ehrlich reagent was used. In this study, we compared the use of Ehrlich reagent (7), Kovac reagent (7), and *p*-dimethylaminocinnamaldehyde (DMCA) reagent for detecting indole in commercial microtest systems.

MATERIALS AND METHODS

Microorganisms. The bacterial strains used in this study were either clinical isolates recently identified by the Anaerobe Reference Laboratory of the Centers for Disease Control or lyophilized strains from the stock culture collection maintained in the Anaerobic Bacteria Branch of the Centers for Disease Control. The purity of the cultures and their cultural and biochemical characteristics were determined as described by Dowell and Hawkins (2).

Preparation of bacterial cell suspensions. Each culture was processed as described by Stargel et al. (11) with minor modifications. After growth was noted on the anaerobe blood agar plates, the plates were removed from the anaerobic glove box, and colonies were examined for purity with a dissecting microscope. The bacterial growth on blood agar was harvested with a sterile polyester fiber swab and was transferred to a tube containing 15 ml of Lombard-Dowell (LD) broth (4). The turbidity was adjusted to that of a McFarland no. 5 nephelometer standard.

Reagents. Kovac and Ehrlich reagents for indole testing were prepared as outlined by MacFaddin (7). The DMCA reagent was prepared as described by Sutter and Carter (13). A 1-g portion of DMCA was dissolved in 99 ml of 10% (vol/vol) aqueous hydrochloric acid and stored in a brown bottle in the refrigerator at approximately 4°C.

Sensitivity of the indole reagents. The sensitivity of the DMCA, Kovac, and Ehrlich reagents in detecting indole and related compounds was tested with LD broth containing various amounts of indole, 3-indoleacetic acid, 3-indolebutyric acid, 3-indolepropionic acid, 3-indolepyruvic acid, and 3-indolemethyl (ska-

tole). An aqueous stock solution (1 mmol/ml) of each compound was prepared, and serial 10-fold dilutions of the solutions were made in LD broth. Samples (1 ml) of each dilution were tested in tubes by the conventional procedure for indole detection with Kovac and Ehrlich reagents (2, 7), and 0.1-ml samples of each dilution were placed on Whatman no. 1 filter paper saturated with the DMCA reagent and observed up to 2 min for a change in color.

Detection of indole in microtest systems with a modified spot test procedure. Each of the commercially prepared microtest systems used in the study was inoculated according to the instructions provided by the manufacturer and incubated in an anaerobic glove box containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 for 48 h at 35°C.

Two drops of the culture from the esculin well were removed with a sterile Pasteur pipette and spotted on a Whatman no. 1 filter paper saturated with DMCA reagent. The spot was observed up to 2 min, and the change in color was recorded. The culture suspension from the esculin well was used in this part of the study because this substrate was the only common substrate included in the microtest systems used (API Lactobacillus-50, API Anaerobe-20, and BBL Minitek) that did not contain a pH indicator.

Tube test. To serve as a control for indole production, 3 to 5 ml of the remaining LD broth cell suspension of each strain was incubated in an anaerobic glove box at 35°C for 48 h and tested for indole with Ehrlich reagent after xylene extraction as described by Dowell and Hawkins (2).

Comparison of three reagents for detecting indole in the Minitek microtest system. Various anaerobic bacteria were tested with Kovac, Ehrlich, and DMCA reagents with samples from the same cultures used in the BBL Minitek system. A cell suspension of each strain was made in LD broth as previously described, and 2 drops of each were placed in each of five wells of the Minitek system. Two of the five wells containing the cell suspension were overlaid with 2 drops of sterile mineral oil, and a plain 6-mm sterile filter paper disk was added to one of the remaining wells. After the plates were incubated in an anaerobic glove box for 48 h at 35°C, 2 drops of xylene were added to each well except for the well containing the paper disk. The xylene and culture in each well were mixed with a separate sterile Pasteur pipette. The xylene was then allowed to separate from the cell suspension in the wells. Two drops of Kovac reagent were added to two of the wells, 2 drops of Ehrlich reagent were added to two other wells (one well overlaid with mineral oil and one well not overlaid with mineral oil with each reagent), and 3 drops of the DMCA reagent were added to the fifth well containing the filter paper disk. A change in color within 2 min was recorded.

As a control for indole production, approximately 6 ml of the remaining LD broth cell suspension of each culture was incubated in an anaerobic glove box at 35°C for 48 h and tested for indole with Ehrlich reagent by the conventional Centers for Disease Control technique (2).

RESULTS AND DISCUSSION

Sensitivity of reagents. The sensitivity of the three reagents in detecting indole and indole derivatives added to LD broth is shown in Table 1. The DMCA reagent was 100 times more sensitive than Kovac reagent and 10 times more sensitive than Ehrlich reagent in detecting indole. The color formed with the solutions spotted on the DMCA-saturated filter paper ranged from dark blue with high concentrations of indole to blue-green with intermediate concentrations and light blue or blue-gray with the lowest concentration of indole detected. All three reagents produced a color change with some of the indole-derivative compounds (3-indolemethyl, 3-indolepropionic acid, and 3-indolebutyric acid). The color (bluish red) produced in the xylene extracts of higher concentrations of indole derivatives with Kovac and Ehrlich reagents was distinctly different from the bright red produced with indole. However, a light red or pink color was produced with low concentrations of the three indole derivatives when both Kovac and Ehrlich reagents were added. This color was very similar to that formed with these reagents when the medium contained low concentrations of indole. The color of DMCA-saturated filter paper with indole derivatives varied from magenta with higher concentrations (100 μmol/ml) to violet with the lowest concentration detected (0.1 μmol/ml). No color change occurred when uninoculated LD broth was tested with Kovac, Ehrlich, or DMCA reagent.

TABLE 1. Sensitivity of three reagents in detecting indole and indole derivatives in LD broth[a]

Compound	Lowest concn (μmol/ml) detected with reagent:		
	Kovac	Ehrlich	DMCA
Indole	10	1.0	0.1
3-Indolebutyric acid	100	100	1.0
3-Indolemethyl	100	10	1.0
3-Indolepropionic acid	100	100	1.0

[a] No change in color occurred in LD broth containing 1.0, 10, or 100 μmol/ml of 3-indoleacetic acid or 3-indolepyruvic acid.

Detection of indole in microtest systems with a spot test procedure. Table 2 shows the results from testing 516 strains of anaerobes for indole with the spot test by using growth in the esculin well of the API *Lactobacillus* 50 system. The overall agreement between results from the spot test with DMCA reagent and results from the tube test with xylene extraction and Ehrlich reagent was 99.5%.

In a study of indole production by 68 strains of *Propionibacterium acnes* isolated from the conjunctival sacs of healthy eyes (8), the positive spot test results agreed 100% (67 positive, 1 negative) with the tube test results for indole, whereas results when Ehrlich reagent was added to the API Anaerobe 20 microtest system agreed

TABLE 2. Detection of indole production by anaerobic bacteria in the API *Lactobacillus* 50 microtest system with DMCA reagent compared with a tube test using xylene extraction and Ehrlich reagent

Species	No. of strains examined	No. of strains positive for indole		Agreement (%)[a]
		Spot test with DMCA reagent	Tube test	
Arachnia propionica	1	0	0	100
Bacteroides asaccharolyticus	6	6	6	100
Bacteroides distasonis	11	0	0	100
Bacteroides fragilis	109	0	0	100
Bacteroides thetaiotaomicron	28	27	28	96
Bacteroides ovatus	4	4	4	100
Bacteroides vulgatus	15	0	0	100
Bifidobacterium adolescentis	4	0	0	100
Bifidobacterium eriksonii	7	0	0	100
Clostridium bifermentans	4	4	4	100
Clostridium cadaveris	4	4	4	100
Clostridium indolis	2	2	2	100
Clostridium sordellii	16	16	16	100
Clostridium sporogenes[b]	12	0	0	100
Clostridium tetani	7	3	3	100
Clostridium spp. (18 other species)[c]	86	0	0	100
Fusobacterium mortiferum	15	0	0	100
Fusobacterium necrogenes	1	0	0	100
Fusobacterium necrophorum	29	29	29	100
Fusobacterium nucleatum	18	18	18	100
Fusobacterium russii	2	0	0	100
Fusobacterium varium	2	0	0	100
Lactobacillus spp.	4	0	0	100
Peptococcus asaccharolyticus	8	8	8	100
Peptococcus magnus	2	0	0	100
Peptococcus prevotii	2	0	0	100
Peptostreptococcus anaerobius	4	0	0	100
Propionibacterium acnes	94	93	93	100
Propionibacterium avidum	3	0	0	100
Propionibacterium granulosum	8	0	0	100
Streptococcus intermedius	3	0	0	100
Veillonella parvula	5	0	0	100

[a] Agreement for all strains tested, 99.5%.

[b] All 12 strains of *C. sporogenes* produced a violet color reaction indicative of an indole-derivative compound (1).

[c] The *Clostridium* strains were 3 *C. barati*, 11 *C. butyricum*, 1 *C. carnis*, 1 *C. chauvoei*, 2 *C. clostridiiforme*, 4 *C. difficile*, 1 *C. glycolicum*, 2 *C. histolyticum*, 3 *C. innocuum*, 3 *C. limosum*, 2 *C. novyi* type A, 8 *C. paraputrificum*, 10 *C. perfringens*, 6 *C. ramosum*, 14 *C. septicum*, 3 *C. subterminale*, 2 *C. symbiosum*, and 10 strains of *C. tertium*.

91% (61 positive versus 67 positive) with results from the tube test.

In a study of 152 strains of various *Bacteroidaceae* (Table 3), the DMCA spot test detected indole production by 57 strains versus 40 strains detected by Ehrlich reagent added to the Minitek system. There was good agreement between the spot test and the conventional tube test, but the agreement between the Minitek system with Ehrlich reagent and the tube test was less satisfactory with certain *Bacteroidaceae* species. The greatest discrepancies between the two tests were found with weak indole-producing strains of *Bacteroides asaccharolyticus, Bacteroides ovatus*, and *Bacteroides thetaiotaomicron*. Excellent agreement (100%) was found between the two tests in the examination of *Fusobacterium* species (Table 3).

A total of 348 strains of anaerobes were tested for production of indole in the Minitek microtest system with the three different reagents (Table 4). Kovac reagent was the least sensitive of the three reagents in detecting indole, and significant differences were noted between the wells that were overlaid with mineral oil and those that were not. Eighty (74%) of the 108 indole-positive strains were positive when the cell suspension was overlaid with mineral oil and tested with Kovac reagent; however, only 57 (53%) of the 108 strains were positive for indole with Kovac reagent when the cell suspension was not overlaid. One hundred and two (94%) of

TABLE 3. Detection of indole production by *Bacteroides* and *Fusobacterium* species in the BBL Minitek microtest system with DMCA and Ehrlich reagents and in a tube test with xylene extraction and Ehrlich reagent

Species	No. of strains examined	No. (%) of strains positive for indole[a]		
		Minitek		Tube test
		Ehrlich reagent	DMCA reagent	
B. asaccharolyticus	6	2 (33)	6 (100)	6 (100)
B. ovatus	2	1 (50)	2 (100)	2 (100)
B. thetaiotaomicron	26	13 (50)	25 (96)	26 (100)
F. necrophorum	16	16 (100)	16 (100)	16 (100)
F. nucleatum	8	8 (100)	8 (100)	8 (100)

[a] A total of 94 strains gave negative reactions with all three tests. These included 11 *B. distasonis*, 53 *B. fragilis*, 17 *B. vulgatus*, 6 *Bacteroides* spp., 5 *F. mortiferum*, and 2 *F. russii*. Overall percentages of strains positive for indole were: Minitek with Ehrlich reagent, 69%; Minitek with DMCA reagent, 98%; tube test, 100%.

the 108 indole producers gave a positive result with the Ehrlich reagent when overlaid with mineral oil, and 69 (64%) gave a positive result when not overlaid. The test with the DMCA reagent and the conventional tube test each detected 107 (99%) of the 108 indole-positive strains. One strain of *B. thetaiotaomicron* was positive by the tube procedure and negative with the DMCA reagent, and another strain of *B. thetaiotaomicron* was negative with the tube test but positive with the DMCA reagent. It was not necessary to overlay the cell suspension containing the filter paper disk, probably because indole, a volatile substance, became entrapped in the fibers of the paper disk in sufficient quantity to be detected by the DMCA reagent.

From the results of this study, it appears that Kovac reagent is not sufficiently sensitive for use with the microtest systems for detection of indole, regardless of whether the wells are overlaid with mineral oil or not. Failure to detect indole could result in misidentification of 20 to 25% of anaerobe isolates.

Harley-Mason and Archer (5) first described the use of the DMCA reagent for detecting indole and indole intermediate compounds. They concluded that the DMCA reagent was about 10 times more sensitive than Ehrlich reagent in detecting indole but was less selective than Ehrlich reagent because the DMCA reagent produced a color change with certain indole intermediate compounds. Lowrance et al. (6) compared the DMCA reagent with Kovac reagent and with *p*-dimethylaminobenzaldehyde

TABLE 4. Detection of indole production by various anaerobic bacteria in the BBL Minitek microtest system with Kovac, Ehrlich, and DMCA reagents, and in a conventional tube test with xylene extraction and Ehrlich reagent

Species	No. of strains examined	No. of strains positive for indole[a]					
		Minitek test					
		Kovac reagent		Ehrlich reagent		DMCA reagent	Tube test
		Overlaid	Not overlaid	Overlaid	Not overlaid		
B. asaccharolyticus	4	2	2	4	3	4	4
B. ovatus	10	5	4	9	6	10	10
B. thetaiotaomicron	39	21	16	34	18	38	38
C. bifermentans	13	12	7	13	8	13	13
C. sordellii	17	16	8	17	10	17	17
C. sporogenes[b]	19	0	0	0	0	0	0
Fusobacterium gonidiaformans	4	3	2	4	3	4	4
F. necrophorum	15	15	13	15	15	15	15
F. nucleatum	6	6	5	6	6	6	6

[a] A total of 221 strains gave negative reactions in all of the tests. These included 2 *Bacteroides capillosus*, 12 *B. distasonis*, 79 *B. fragilis*, 8 *Bacteroides melaninogenicus* subsp. *melaninogenicus*, 16 *B. vulgatus*, 5 *Bifidobacterium adolescentis*, 2 *Bifidobacterium breve*, 8 *Bifidobacterium eriksonii*, 19 *C. difficile*, 14 *C. histolyticum*, 15 *C. innocuum*, 12 *C. perfringens*, 6 *C. ramosum*, 7 *C. septicum*, 2 *C. subterminale*, 2 *C. symbiosum*, 4 *C. tertium*, 4 *C. tetani*, 3 *F. mortiferum*, and 1 *F. varium*.

[b] All 19 strains of *C. sporogenes* produced a violet color reaction with the DMCA reagent indicative of an indole-derivative compound (1).

reagent. They reported that the most sensitive of the reagents tested, DMCA, regularly detected 0.1 μg of indole per ml and sometimes as little as 0.05 μg of indole per ml.

Our results were similar to those of the above investigators (5, 6), with some exceptions. We also found that the DMCA reagent was the most sensitive, followed by the Ehrlich and then by the Kovac reagents. Our results with Kovac reagent differed from the results of Lowrance et al. (6), who reported they were able to detect 0.1 μg of indole per ml with the reagent.

We consider the ability of the DMCA reagent to produce a color with indole derivative compounds a distinct advantage. Detection of compounds such as 3-indolemethyl (skatole), 3-indolepropionic acid, and 3-indolebutyric acid is useful in identifying certain *Clostridium* species (1).

All of the *Clostridium sporogenes* strains examined produced a deep violet color with the DMCA reagent, which indicated production of one or more indole derivatives. We have also noted this same color with type A, proteolytic type B, proteolytic type F, and type G *Clostridium botulinum* strains (1; M. Dezfulian, G. L. Lombard, and V. R. Dowell, Jr., unpublished data).

Because of the sensitivity of the DMCA reagent in detecting low levels of indole and because of its ability to detect certain indole intermediate compounds, use of the reagent with commercial microtest systems could significantly improve the usefulness of the systems in identifying anaerobic bacteria.

LITERATURE CITED

1. **Dezfulian, M., and V. R. Dowell, Jr.** 1980. Cultural and physiological characteristics and antimicrobial susceptibility of *Clostridium botulinum* isolated from foodborne and infant botulism cases. J. Clin. Microbiol. **11**:604–609.
2. **Dowell, V. R., Jr., and T. M. Hawkins.** 1974. Laboratory methods in anaerobic bacteriology. Centers for Disease Control laboratory manual. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.
3. **Dowell, V. R., Jr., G. L. Lombard, M. D. Stargel, S. D. Allen, F. S. Thompson, and A. Y. Armfield.** 1978. Biotyping of anaerobic bacteria associated with human disease, 47–67. *In* A. Balows and H. D. Isenberg (ed.), Biotyping in the clinical microbiology laboratory. Charles Thomas, Publishers, Springfield, Ill.
4. **Dowell, V. R., Jr., G. L. Lombard, F. S. Thompson, and A. Y. Armfield.** 1977. Media for isolation, characterization, and identification of obligately anaerobic bacteria. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.
5. **Harley-Mason, J., and A. A. P. G. Archer.** 1958. Use of *p*-dimethylaminocinnamaldehyde as a spray reagent for indole derivatives on paper chromatograms. Biochem. J. **60**:60P.
6. **Lowrance, B. L., P. Reich, and W. H. Traub.** 1969. Evaluation of two spot-indole reagents. Appl. Microbiol. **17**:923–924.
7. **MacFaddin, J. F.** 1976. Biochemical tests for identification of medical bacteria, p. 99–108. The Williams & Wilkins Co., Baltimore.
8. **McNutt, F., S. D. Allen, L. A. Wilson, and V. R. Dowell, Jr.** 1978. Anaerobic flora of the normal conjunctival sac. Arch Opthalmol. **96**:1448–1450.
9. **Moore, H. B., V. L. Sutter, and S. M. Finegold.** 1975. Comparison of three procedures for biochemical testing of anaerobic bacteria. J. Clin. Microbiol. **1**:15–24.
10. **Stargel, M. D., G. L. Lombard, and V. R. Dowell, Jr.** 1978. Alternative procedures for identification of anaerobic bacteria. Am. J. Med. Technol. **44**:709–722.
11. **Stargel, M. D., F. S. Thompson, S. E. Phillips, G. L. Lombard, and V. R. Dowell, Jr.** 1976. Modification of the Minitek Minaturized Differentiation system for characterization of anaerobic bacteria. J. Clin. Microbiol. **3**:291–301.
12. **Starr, S. E., F. S. Thompson, V. R. Dowell, Jr., and A. Balows.** 1973. Microtest system for identification of anaerobic bacteria. Appl. Microbiol. **25**:713–717.
13. **Sutter, V. L., and W. T. Carter.** 1972. Evaluation of media and reagents for indole-spot tests in anaerobic bacteria. Am. J. Clin. Pathol. **58**:335–338.