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Spot Indole Test: Evaluation of Four Reagents

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Kovacs indole reagent, *p*-dimethylaminobenzaldehyde, Ehrlich indole reagent, and *p*-dimethylaminocinnamaldehyde were used as spot indole reagents to test 359 strains of gram-negative rods growing on 5% sheep blood agar, Trypticase soy agar (BBL Microbiology Systems), and MacConkey agar. The *p*-dimethylaminocinnamaldehyde reagent was the most sensitive of those tested and provided results that were easiest to interpret. The *p*-dimethylaminocinnamaldehyde reagent was able to detect *Providencia alcalifaciens* indole production because of the red-violet color unique to that organism. All reagents tested were accurate in detecting indole produced by members of the *Enterobacteriaceae* family, with the exception of *P. alcalifaciens*.

Rapid, accurate, and clinically significant laboratory results are important to clinicians. In recent years, microbiologists have begun to benefit from automated laboratory technology and have come to utilize more frequently those tests and procedures that provide rapid laboratory results. Of the many spot tests available, the spot indole test should be a frequently used one; it is certainly valuable in formulating reportable data.

Arnold and Weaver (1) modified the routine 24-h indole test procedure for *Enterobacteriaceae* by heavily inoculating 1.0 ml of tryptone broth, incubating the broth at 37°C for 2 to 4 h, and testing with Kovacs reagent (prepared with isoamyl alcohol). Vracko and Sherris (6) showed that the detection of indole production by certain members of the *Enterobacteriaceae* family could be done almost instantly by using filter paper moistened with 5% *p*-dimethylaminobenzaldehyde (DMABA) in 10% aqueous hydrochloric acid. Lowrance et al. (4) determined quantitatively that another spot indole reagent, *p*-dimethylaminocinnamaldehyde (DMACA), was four times more sensitive than DMABA. Their results indicated that DMACA could detect 3 μg of indole per ml and that DMABA measured 6 to 12 μg/ml.

The DMACA reagent is especially useful in detecting indole produced by certain anaerobic bacteria (2, 5). Lombard and Stargel (G. L. Lombard and M. D. Stargel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C174, p. 64) described studies with DMACA which would detect indole and indole derivatives. A lavender to red-violet color indicated a positive reaction for indole derivatives, and blue or blue-green color indicated a positive reaction for indole with the DMACA reagent.

The spot indole procedure has received some attention as a valuable tool in the repertoire of rapid tests available for identification of rapid lactose fermenters of the *Enterobacteriaceae* (4). The DMABA spot indole test, when used with other minimal criteria, has been effective in differentiating *Escherichia coli* from the *Klebsiella-Enterobacter* group. The effectiveness of DMACA as a spot test for aerobic bacteria has received little attention in the literature.

In this report we compare four potentially useful spot indole test reagents for their ability to detect indole production by colonies grown on Trypticase soy agar (BBL Microbiology Sytems, Cockeysville, Md.) containing 5% sheep blood (SBA), on MacConkey agar (MAC), and on Trypticase soy agar (TSA). We also document some of the potential errors of the test noted in earlier studies (6; M. J. Bale, C. Peterson, D. C. Hale, and J. M. Matsen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C245, p. 303).

MATERIALS AND METHODS

Organisms. The cultures used in this study were kindly supplied by Dwane Rhoden and by the Diagnostic Laboratories of the Enteric Diseases Branch, Centers for Disease Control. Some of the cultures were isolates submitted for confirmation or for serotyping. Stock cultures were grown overnight in broth and then inoculated into the test media.

Media and reagents. TSA slants, MAC plates, and SBA plates were purchased from BBL Microbiology Systems, stored at 4°C, and warmed to room temperature before they were used. Peptone water (2%) was prepared by suspending 20 g of peptone (Difco Laboratories, Detroit, Mich.) and 5 g of sodium chloride in

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TABLE 1. Organisms used in the spot indole test

Organism	No. of strains tested
Enterobacteriaceae	
Arizona hinshawii	8
Citrobacter freundii	10
Edwardsiella tarda	7
Enterobacter aerogenes	6
E. agglomerans	10
E. cloacae	10
Escherichia coli	35
Enteric group 10	1
Hafnia alvei	9
Klebsiella oxytoca	11
K. ozaenae	5
K. pneumoniae	12
K. rhinoscleromatis	3
Kluyvera sp.	2
Morganella morganii	18
Proteus mirabilis	10
P. vulgaris	19
Providencia alcalifaciens	32
P. rettgeri	13
P. stuartii	28
Salmonella typhi	2
Typical *salmonellae*	39
Serratia liquefaciens	7
S. marcescens	8
S. rubidaea	2
Shigella sonnei	6
Other shigellae	14
Yersinia enterocolitica	11
Y. pseudotuberculosis	4
Y. kristensenii	2
Yersinia sp.	2
Other	
Flavobacterium sp.	8
Cardiobacterium hominis	2
HB-5	3

1 liter of distilled water and then sterilizing at 121°C for 15 min.

Kovacs reagent was prepared by adding 10 g of DMABA to 150 ml of isoamyl alcohol and then slowly adding 50 ml of concentrated pure HCl.

The modified Kovacs reagent (DMABA) was 5% DMABA in 10% aqueous HCl (3, 4, 6).

Ehrlich indole reagent was prepared by adding 1 g of DMABA to 95 ml of ethyl alcohol and then slowly adding 20 ml of concentrated HCl.

The DMACA reagent was prepared by adding 1 g of DMACA (Aldrich Chemical Co., Milwaukee, Wis.) to 100 ml of 10% (vol/vol) HCl.

Indole, if present, combines with the aldehydes present in Kovacs, DMABA, or Ehrlich reagent to produce red on the saturated filter paper. The DMACA reagent reacts by producing blue to blue-green in the presence of indole. All spot test reactions were read within 10 s after the organisms were applied to the moistened paper. The control procedure, a routine Kovacs indole test, was performed by adding approximately 0.5 ml of Kovacs reagent to a 24-h culture grown in 2% peptone water. Tests on indole-negative organisms were repeated at 48 h. A positive control indole test was noted by the presence of a red ring at the surface of the broth after Kovacs reagent was added, whereas a negative control test exhibited a yellow ring.

Whatman no. 1 filter paper was placed into open, clean plastic petri dishes and moistened with each of the four reagents. Organisms were scraped from the surface of SBA, MAC, and TSA with clean applicator sticks and were gently rubbed onto the saturated filter paper.

For a test of the influence of indole-positive strains on adjacent indole-negative strains, *Escherichia coli* and *Klebsiella oxytoca* (both indole positive) were each streaked onto separate SBA plates in a single streak. Strains of *Serratia marcescens, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis,* and *Providencia alcalifaciens* were streaked perpendicularly to the indole-positive strains and tested at 3, 8, and 12 mm from the indole-positive strain.

RESULTS

Table 1 lists the strains that were tested with the spot indole procedure. At least 32 different species were tested (some organisms were grouped, as in the "typical salmonellae" and "other shigellae"). Results from the spot indole procedure paralleled the control Kovacs indole test results when the spot test was performed with colonies taken from either SBA or TSA. As others have shown, we found that spot indole test results on colonies from MAC were not dependable (6).

With the exception of *P. alcalifaciens,* expected positive color reactions developed with each reagent when colonies were taken from SBA and TSA, but not when they were taken from MAC. Kovacs, DMABA, and Ehrlich reagents exhibited red on moistened filter paper with indole-positive strains and either no color or a slightly yellow color with indole-negative strains. The DMACA reagent elicited blue to blue-green with the indole-positive organisms and no color with indole-negative strains. Although *P. alcalifaciens* is an indole-positive organism, it exhibited indole-negative reactions in the Kovacs, DMABA, and Ehrlich spot tests but a strong lavender or red-violet color with the DMACA reagent. Therefore, a red or blue reaction was considered a positive spot indole test with DMACA.

Table 2 shows the overall reactivity of the spot indole procedure with 183 known indole-positive control strains. The SBA and TSA were much more appropriate for testing the organisms used in the spot test procedure than was MAC, since 85, 92, and 22%, respectively, of the indole-positive strains could be detected with Kovacs, DMACA, and Ehrlich spot test reagents.

TABLE 2. Results of spot indole tests

Reagent	No. of positive detected/no. of positive controls (% positive)		
	Blood agar	TSA	MAC
Kovacs	156/183 (85)	168/183 (92)	41/183 (22)
DMABA	156/183 (85)	168/183 (92)	41/183 (22)
Ehrlich	155/183 (85)	168/183 (92)	41/183 (22)
DMACA	183/183 (100)	183/183 (100)	42/183 (23)

The DMACA reagent was able to detect the weakly indole-positive *P. alcalifaciens* strains missed by the other spot indole reagents. Three of the *P. alcalifaciens* strains were negative in the 24-h control Kovacs test but were weakly positive after 3 days of incubation. With the DMACA spot test reagent, these weakly positive strains were easily detected by the characteristic lavender color.

Of all indole-positive organisms tested, only *P. alcalifaciens* produced a lavender color with the DMACA reagent. Twenty-six strains of *Providencia stuartii* (formerly biogroup 4 of *P. alcalifaciens*) tested with the DMACA reagent produced blue (positive); two produced lavender.

Thirteen indole-positive, nonfermentative gram-negative rods were tested. DMACA was more efficient in detecting indole production by the spot test than were the other three reagents (Table 3). Indole-positive strains of HB-5 were detected only by the DMACA reagent and not by the other three reagents.

Indole-positive *Enterobacteriaceae* colonies may render adjacent indole-negative colonies falsely positive (6). Indole-positive *K. oxytoca* and *E. coli* were shown to confer falsely positive results onto neighboring indole-negative *S. marcescens, E. cloacae, K. pneumoniae,* and *P. mirabilis*. The falsely positive characteristic was lost when the colony was transferred in pure culture to an uninoculated plate and retested after 24 h. Indole-negative strains were rendered falsely positive if they grew within 5 mm of the positive strain. The red reaction of *P. alcalifaciens* with the DMACA reagent was unchanged when the organism was grown next to *E. coli* or *K. oxytoca*.

DISCUSSION

The spot indole procedure with Kovacs, DMABA, Ehrlich, or DMACA reagent was an accurate detector of indole production by members of the *Enterobacteriaceae* when organisms were taken from blood agar or TSA, but not from MAC (Table 2). MAC could not be used as a source of colony growth for the spot indole test because it consistently led to false-negative tests, an observation reported by others (6). The red positive indole reactions exhibited by Kovacs, DMABA, and Ehrlich reagents and the blue or blue-green positive indole reactions exhibited by DMACA appeared within 2 to 10 s after colony growth was applied to filter paper moistened with the reagent. The correlation between the control indole tests and the DMACA spot tests performed on members of the *Enterobacteriaceae* was virtually 100%.

P. alcalifaciens exhibited unique lavender or red-violet reactions with the DMACA reagent, reminiscent of the red-violet observed with certain anaerobic organisms and considered to be "indole derivative" in these anaerobes (2). This unique color response from DMACA was not altered when cultures were held for up to 4 weeks at room temperature on TSA and retested.

Each of the four reagents tested was shown to be an effective indicator of indole production, but the DMACA reagent was more sensitive and reacted earlier and more strongly than did the other three reagents.

We believe that the spot indole test can be used effectively in clinical laboratories to aid in the early recognition of some commonly isolated organisms (4, 6; Bale et al., Abstr. Annu. Meet.

TABLE 3. Spot indole reactions of three species of nonfermentative indole-positive, gram-negative rods taken from SBA

Organism	No. positive/no. tested			
	Kovacs	DMABA	Ehrlich	DMACA
Flavobacterium sp.	8/8	8/8	8/8	8/8
Cardiobacterium hominis	2/2	2/2	2/2	2/2
HB-5	0/3	0/3	0/3	3/3

Am. Soc. Microbiol. 1981, C245, p. 303) and that DMACA is at least as sensitive as other reagents, and perhaps more efficient, in detecting indole production by these isolates.

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