JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 2028–2030
0095-1137/94/$04.00+0

Vol. 32, No. 8

Biodegradable, Effective Substitute for Xylene in the Ehrlich Indole Procedure

J. MICHAEL MILLER,[1]* M. DARREN MILLER,[2] PATRICK E. DRISCOLL,[1] AND PHYLLIS MILLER[1]

Centers for Disease Control and Prevention, Atlanta, Georgia 30333,[1] and Providence Christian Academy, Lilburn, Georgia 30247[2]

Received 25 January 1994/Returned for modification 12 April 1994/Accepted 27 April 1994

Three extracting reagents were compared for effectiveness in the Ehrlich indole procedure: xylene (the recommended reagent), Hemo-De (a terpene-based product containing *d*-limonene), and Micro-Clear (an isoparaffinic hydrocarbon). Thirty-three strains representing 12 species of indole-positive aerobes or facultative anaerobes and 50 strains representing 11 species of indole-positive strict anaerobes were tested using the three reagents. Xylene extraction allowed indole detection in all of the isolates tested. Micro-Clear allowed detection in all of the aerobic isolates and in 49 of 50 anaerobes. Hemo-De allowed indole detection in 32 of 33 aerobes and in 49 of 50 anaerobes. There was no significant difference in the results among the reagents. Because Micro-Clear is biodegradable, nonflammable, noncarcinogenic, and odorless, we feel that this product should be considered a safe and effective substitute for xylene in the Ehrlich indole procedure.

Indole is the product of the reaction of tryptophanase with tryptophan found in proteins. The detection of indole in the laboratory is accomplished when certain aldehydes react with the indole to form a colored compound. The Ehrlich indole procedure is the standard method used to detect indole production in gram-negative bacteria which do not belong to the family *Enterobacteriaceae* (3) and in anaerobes (5). In the Ehrlich procedure, xylene is added to a 48-h broth culture of the isolate to be tested. The mixture is shaken to extract the indole from the medium and to concentrate it at the top of the tube in the xylene layer. Ehrlich's reagent is gently added to reveal a red color within the xylene layer in indole-positive organisms. Because of the xylene extraction step, the Ehrlich indole procedure is more sensitive than the Kovács indole procedure routinely used for most *Enterobacteriaceae*.

Xylene is a volatile compound, and disposal is becoming a problem for the laboratories that use it. It cannot be disposed of by being poured down drains because its low flash point of 28.9°C makes it a flammable solvent (1a). It is potentially neurotoxic to humans after prolonged exposure and can cause skin irritation after even mild exposures (14). If xylene is used to clean immersion oil from stained slides, the potential direct skin exposure time increases.

Hemo-De (Fisher Scientific, Pittsburgh, Pa.), a terpene-based product, has been shown to be effective as a substitute for xylene in the Ehrlich indole procedure (6, 7) and in parasitology procedures (1, 8, 12, 13). This product contains *d*-limonene, a terpene, and has a strong citrus odor. Terpenes are natural oils derived from citrus fruits and corn and were thought to pose little danger to users. Early analyses showed an association between citrus oils and experimental epidermal hyperplasia in mice (13). Recently, *d*-limonene was shown to be nephrotoxic and to be associated with tumors in male rats (2, 4). No human corollary has been reported. Hemo-De is considered hazardous waste (material safety data sheet, Fisher Scientific [6a]). Jones et al. (9) reported that both xylene and AmeriClear (another terpene-based product) were considered hazardous waste.

Micro-Clear (Micron Environmental Industries, Fairfax, Va.) is a paraffinic hydrocarbon and is not regulated as a hazardous waste. It has been used effectively as a xylene substitute in histology and pathology procedures (9, 11), apparently without accompanying problems. It, along with Ehrlich's reagent, is a component of the Micro-Clear Indole Test Kit produced by the same manufacturer. Micro-Clear has not been evaluated for its potential use in clinical microbiology procedures.

Ehrlich's reagent contains *p*-dimethylaminobenzaldehyde. Although the material safety data sheet for this product does not list it as carcinogenic, it is somewhat toxic and is an irritant to eyes and skin (4a). State regulations for disposal should be reviewed for all of these products.

This study compared Hemo-De and Micro-Clear as replacements for xylene in the Ehrlich indole procedure.

Organisms tested. The organisms used in this study are listed in Table 1. Most are fastidious organisms that were either slow growers or known to be weak indole producers. Common members of the *Enterobacteriaceae* were not included in the study set because they are usually tested with Kovács reagent, which does not require xylene. Indole-positive aerobic organisms were taken from stock cultures held at −70°C and passed twice on 5% sheep blood agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The plates were incubated at 35°C in CO_2. Indole-positive anaerobes were reconstituted from lyophilized stock cultures. Anaerobes were passed twice on CDC Anaerobic Blood Agar (Carr-Scarborough Microbiologicals, Stone Mountain, Ga.), and the plates were incubated at 35°C in an anaerobic chamber containing 10% H_2, 5% CO_2, and 85% N_2. *Klebsiella pneumoniae* and *Clostridium perfringens* were included in the study as negative controls.

Xylene substitutes. Micro-Clear and Hemo-De are marketed for use as solvents and clearing agents in histology and cytology (9, 11).

Ehrlich's reagent. Ehrlich's reagent consists of 95 ml of 95% ethanol, 1 g of *para*-dimethylaminobenzaldehyde, and 20 ml of concentrated HCl. The aldehyde is dissolved in the alcohol,

* Corresponding author. Mailing address: Diagnostic Microbiology Section, Bldg. 1, Rm. B250, C16, Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-3029. Fax: (404) 639-1381. Electronic mail address: jmm8@cidhip1.em.cdc.gov.

TABLE 1. Results from the Ehrlich indole procedure using three extracting reagents

Organism	No. of positive strains/no. tested		
	Xylene	Micro-Clear	Hemo-De
Fastidious aerobes			
Aeromonas hydrophila	3/3	3/3	3/3
Flavobacterium indologenes	2/2	2/2	2/2
Flavobacterium meningosepticum	3/3	3/3	3/3
HB-5	1/1	1/1	1/1
Kingella indologenes	3/3	3/3	2/3
Pasteurella multocida	3/3	3/3	3/3
Plesiomonas shigelloides	2/2	2/2	3/3
Vibrio vulnificus	7/7	7/7	6/6
Vibrio parahaemolyticus	3/3	3/3	3/3
Weeksella zoohelcum	3/3	3/3	3/3
Weeksella virosa	3/3	3/3	3/3
Anaerobes			
Bacteroides thetaiotaomicron	4/4	4/4	4/4
Bacteroides uniformis	5/5	5/5	5/5
Bacteroides ovatus	4/4	4/4	4/4
Clostridium bifermentans	6/6	6/6	6/6
Clostridium sordellii	5/5	5/5	5/5
Fusobacterium necrophorum	5/5	5/5	5/5
Fusobacterium nucleatum	5/5	5/5	5/5
Peptostreptococcus asaccharolyticus	5/5	5/5	5/5
Prevotella intermedia	5/5	4/5	4/5
Prevotella nigrescens	1/1	1/1	1/1
Propionibacterium acnes	5/5	5/5	5/5

and then the acid is slowly added. The reagent is prepared in small quantities and stored at 4°C (3).

The Ehrlich indole procedure (3). Each aerobe was taken from a 5% sheep blood agar plate and inoculated into three tubes of brain heart infusion broth (Becton Dickinson), and the tubes were incubated for 48 h. Anaerobes were taken from CDC Anaerobic Blood Agar plates and inoculated into three tubes of Lombard-Dowell indol-nitrate broth (Carr-Scarborough), and the tubes were incubated for 72 h (5). Slowly growing isolates were held for 4 days, as necessary, to attain good growth.

After incubation, all of the tubes (three for each organism) were tested for the presence of indole. One milliliter of either xylene (American Scientific Products, McGaw Park, Ill.), Micro-Clear, or Hemo-De was added to each broth culture. The mixture was shaken vigorously to extract the indole and was allowed to stand until the extractant formed a layer on top of the aqueous phase. Then 0.5 ml of Ehrlich's reagent was added down the side of the tube without shaking. The presence of indole is indicated by the immediate development of a pink ring below the extractant layer. Weak reactions are usually detected within 5 min of the addition of Ehrlich's reagent.

Table 1 shows the results of testing for indole production using organisms that are fastidious or are weak indole producers. Xylene, the reference reagent, allowed the detection of indole in all of the indole-positive strains tested. Micro-Clear detected indole in all of the aerobic organisms tested and in 49 of 50 anaerobes tested. One strain of *Prevotella intermedia* was indole negative with Micro-Clear. Hemo-De allowed indole detection in 32 of 33 aerobes and in 49 of 50 anaerobes tested. One strain each of *Kingella indologenes* and *P. intermedia* was indole negative with Hemo-De. The same strain of *P. intermedia* was missed by both alternate reagents. All strains producing discrepant results were retested to confirm the error. The errors in indole detection for both Micro-Clear and Hemo-De occurred with organisms exhibiting very weak indole-positive responses with xylene. The negative controls reacted appropriately.

When the results were analyzed by chi-square analysis, there was no significant difference between the results obtained with Micro-Clear, Hemo-De, and xylene ($P > 0.5$).

Micro-Clear and Hemo-De proved to be equally effective as substitutes for xylene in the Ehrlich indole procedure (Table 1). The only problems occurred with *K. indologenes* and *P. intermedia*, organisms exhibiting very weak indole-positive reactions in this study. It has been suggested that because of its natural origin, Hemo-De may exhibit lot-to-lot variability (11), although we did not test for variability in either compound. Hemo-De has a strong citrus odor that quickly became a nuisance when an open container of it was nearby. Micro-Clear would be a logical choice; it is odorless and is advertised as nontoxic and noncarcinogenic.

One of the many factors contributing to the biohazardous nature of compounds is the flash point, which could pose a problem if the product is disposed of into the sewer system. Xylene has a flash point of 28.9°C and Hemo-De and Micro-Clear have flash points of 49.4°C and 74°C, respectively, making Micro-Clear less flammable than xylene or Hemo-De.

Nontoxic Micro-Clear was also effective in our laboratory for cleaning immersion oil from stained slides; it could be used instead of xylene for this purpose, eliminating the potentially toxic skin exposure to xylene. More than 100 slides were cleaned with Micro-Clear. Xylene dried somewhat faster but was no more effective than Micro-Clear in cleaning the slides. Both Hemo-De and Micro-Clear are at least 25% less expensive than xylene.

The results of this study indicate that Micro-Clear is a biodegradable, nonhazardous, cost-effective substitute for xylene in the Ehrlich indole procedure. The decision to replace xylene with Micro-Clear would support virtually all microbiology laboratory safety policies that encourage the substitution of nonhazardous substances for hazardous ones. Micro-Clear is also useful for removing immersion oil from stained slides.

REFERENCES

1. **Aldeen, W. E., and D. Hale.** 1992. Use of Hemo-De to eliminate toxic agents used for concentration and trichrome staining of intestinal parasites. J. Clin. Microbiol. **30**:1893–1895.

1a. **J. T. Baker, Inc.** 1989. Xylenes. Material safety data sheet. J. T. Baker, Inc., Phillipsburg, N.J.

2. **Borghoff, S. J., M. E. Andersen, and R. B. Conolly.** 1991. Protein nephropathy and kidney cancer in male rats: qualitative and quantitative issues and human relevance. Chemical Industry Institute of Toxicology Activities **11**:1–4.

3. **Clark, W. A., D. G. Hollis, R. E. Weaver, and P. Riley.** 1984. Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.

4. **Dietrich, D. R., and J. A. Swenberg.** 1991. The presence of alpha globulin is necessary for d-limonene promotion of male rat kidney tumors. Cancer Res. **51**:3512–3521.

4a. **Difco Laboratories, Inc.** 1986. MUG PLUS test Ehrlich's reagent. Difco Laboratories, Inc., Detroit, Mich.

5. **Dowell, V. R., and T. M. Hawkins.** 1975. Laboratory methods in anaerobic bacteriology. Center for Disease Control laboratory manual. U.S. Department of Health and Human Services, Public Health Service, Center for Disease Control, Atlanta.

6. **Egleton, J. H., G. P. Fraser, B. Gerber, A. L. Logan, C. J. Rinehardt, S. M. Reynolds, and R. Neimeister.** 1986. Evaluation of a xylene substitute in Ehrlich indole test. J. Clin. Microbiol. **24:**859.
6a. **Fisher Scientific.** Hemo-De. Material safety data sheet. Fisher Scientific, Fair Lawn, N.J.
7. **Gubash, S. M., and E. E. Bennett.** 1989. Use of terpene-based solvents (Hemo-De, Histoclear, and Shandon and BDH xylene substitutes) in place of xylene in the Ehrlich indole test. J. Clin. Microbiol. **27:**2136–2137.
8. **Gubash, S. M., and L. Milburn.** 1990. Use of terpene-based products, with and without anhydrous alcohol, as clearing agents in trichrome staining technique for intestinal protozoa. J. Clin. Microbiol. **28:**610–611.
9. **Jones, R. T., A. W. Morton, and A. A. Moghissi.** 1993. Comparison of deparaffinization agents for an automated immunostainer. J. Histotechnol. **16:**367.
10. **Karlberg, A. T., A. Boman, and B. Melin.** 1991. Animal experiments on the allergenicity of d-limonene—the citrus solvent. Ann. Occup. Hyg. **35:**419–426.
11. **Moghissi, A. A.** 1990. Toxicity and environmental assessment of tissue clearing agents used in histology. Presented at the National Society of Histotechnologists' Annual Region II Seminar.
12. **Neimeister, R., A. L. Logan, and J. H. Egleton.** 1985. Modified trichrome staining technique with a xylene substitute. J. Clin. Microbiol. **22:**306–307.
13. **Neimeister, R., A. L. Logan, B. Gerber, J. H. Egleton, and B. Kleger.** 1987. Hemo-De as substitute for ethyl acetate in formalin-ethyl acetate concentration technique. J. Clin. Microbiol. **25:**425–426.
14. **Roe, F. J. C., and W. E. H. Peirce.** 1960. Tumor promotion by citrus oils: tumors of the skin and urethral orifice in mice. J. Natl. Cancer Inst. **24:**1389–1403.