APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1981, p. 1310-1315 Vol. 41, No. 6
0099-2240/81/061310-06$02.00/0

Detection of *Escherichia coli* in Foods: Indole Staining Methods for Cellulosic and Polysulfone Membrane Filters

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Received 18 November 1980/Accepted 23 March 1981

Optimized procedures for staining *Escherichia coli* colonies on cellulosic and polysulfone membrane filters are described. An explanation for the behavior of the Ehrlich reaction on membrane filters is suggested.

A recent ICMSF (International Commission on the Microbiological Specifications for Foods) study (4) recommends use of the direct membrane filter (MF) plating method (2) of Anderson and Baird-Parker (ABP) for enumeration of *Escherichia coli* in foods. The technique calls for incubating MF at 44.5°C for 24 h on a tryptophan-rich medium. Indole in colonies is detected by its reaction with 4-dimethylaminobenzaldehyde (DAB) in acid to produce a red color. The stain produces diffuse red zones instead of sharply defined colonies; at the same time, a general browning of indole-negative colonies weakens the differentiation. Because of this, an upper counting limit of 150 colonies on 85-mm membranes was recommended in the preliminary protocol to the ICMSF study (4).

We recently described a promising modification to this method, using hydrophobic grid-membrane filters (HGMF) (6). However, two problems arose when the new technique was used in other laboratories: (i) on cellulosic MF (Oxoid or Millipore), the above-mentioned problem caused difficulties in scoring HGMF which were densely packed with mixed flora; and (ii) on polysulfone MF (Gelman Tuffryn), indole-positive colonies did not stain.

It quickly became evident that the accumulation and retention of indole were very different for cellulosic and polysulfone MF and that it would be necessary to develop staining procedures specific for each material. After preliminary experiments with a number of the complex color reactions of indole (7), we concluded that the acidic DAB (Ehrlich) reaction remained the best basis for demonstrating indole.

MATERIALS AND METHODS

HGMF. Cellulose ester HGMF (47-mm diameter, 0.45-μm pore size, 1,984 grid cells) were printed in our laboratory from HAWP 04700 MF (Millipore Ltd., Mississauga, Ontario, Canada), using a cross-country ski wax as the hydrophobic material (6). Polysulfone HGMF (60 by 60 mm, 0.45-μm pore size, 2,500 grid cells) were purchased from QA Laboratories Ltd. (Etobicoke, Ontario, Canada). The base material for these was Tuffryn HT-450 (Gelman Instrument Co., Montreal, Canada).

Indole staining procedures. The following two staining procedures were used, together with the ABP staining method (2). For method A, cellulosic HGMF were placed on filter paper wetted with 0.5% DAB in 1 N HCl and then (still on the filter paper) irradiated under an ultraviolet (UV) lamp (254 nm, 10,000 μW/cm^2 at 6 cm) for 30 min. For method B, polysulfone HGMF were placed on a filter paper wetted with a reagent prepared from equal volumes of 2.5% DAB in 1 N HCl-90% ethanol (solution 1) and 1% potassium persulfate (solution 2) mixed just before use. Still on the filter paper, they were heated under an infrared lamp or in an 80°C oven for 5 min. After color development by both methods, HGMF were returned to the agar surface for counting.

Microbial counts. For the first experiment, dilute inocula of *E. coli* biotype 1 cells from an 18-h culture in tryptic soy broth (Difco Laboratories, Detroit, Mich.) were blended with 10 g of food in 90 ml of diluent, using a Stomacher. For the second experiment, chicken and beef naturally contaminated with *E. coli* were used. For the ABP direct plating method, the procedure of Rayman et al. (4) was used. Counts on HGMF were carried out as previously described (5, 6). All filters were incubated on Tryptone bile agar for 24 h at 44.5°C and stained for indole by the appropriate procedure as described above. Indole-negative colonies were provided by the natural flora of the foods.

Demonstration of relative affinities for indole by cellulose ester and polysulfone MF. Indole (0.7 ml of 0.2 mM solution in 50% ethanol) was pipetted onto a Tuffryn HT MF, and a Millipore HAWP MF was placed on top. Similar pairs were made by using 0.7 ml of 1 and 5 mM indole solutions, and then a reversed set was made in which indole solutions were first added to Millipore MF. The MF pairs were equilibrated overnight at 20°C in petri dishes in a moist atmosphere, separated, dried in a current of warm air, and stained by method B. Diffuse reflectances of the dried disks were compared with that of an untreated dry disk by using a reflectance photometer and a green (5,000 nm) interference filter.

Adsorption of Ehrlich reaction products to cellulose ester and polysulfone MF. A mixture of 1 mM indole, 0.5% DAB, and 1 N HCl in 50% ethanol was stirred for 30 min at 20°C. Millipore and Tuffryn MF were prewetted (1 N HCl in 50% ethanol) and added to the reaction mixture. At intervals, disks were removed, blotted, rinsed for 1 min in 50% ethanol, and then irradiated as in method A. Reflectances of the wet and dry disks at various stages were compared (as above) with a dry white control disk. In a second experiment, prewetted MF were stirred for 10 min in an equilibrated mixture of various DAB concentrations with 1 mM indole and 1 N HCl in 50% ethanol. In a third experiment, HCl concentrations were varied in a mixture of 1 mM indole and 0.5% DAB in 50% ethanol.

RESULTS

Differentiation of *E. coli* colonies on MF. Figures 1 and 2 show cellulose ester HGMF stained by the ABP method and method A, respectively. All carried similar concentrations of *E. coli* colonies. However, in Fig. 1a and 2a

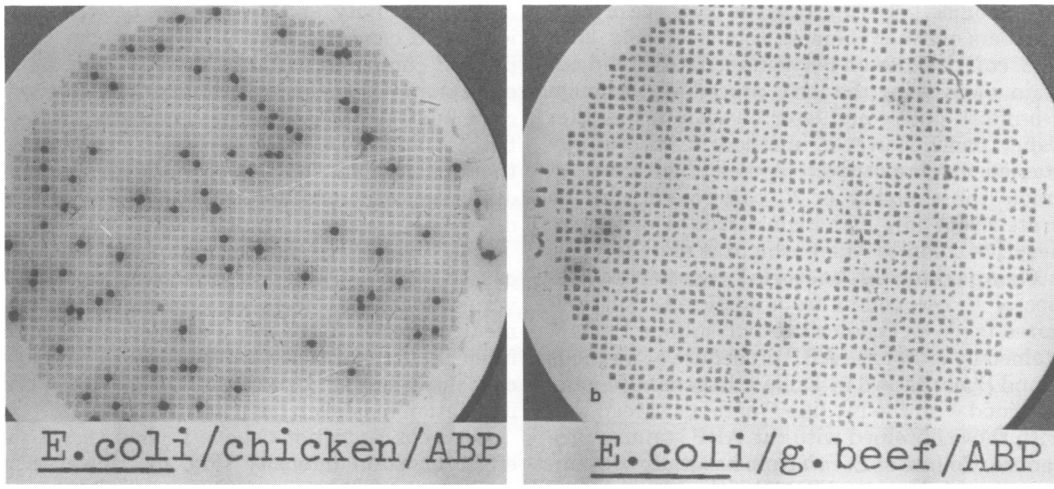

FIG. 1. *Cellulose ester HGMF carrying uniform loads of E. coli colonies, stained by the ABP method. (a) Few indole-negative colonies present; (b) many indole-negative colonies present.*

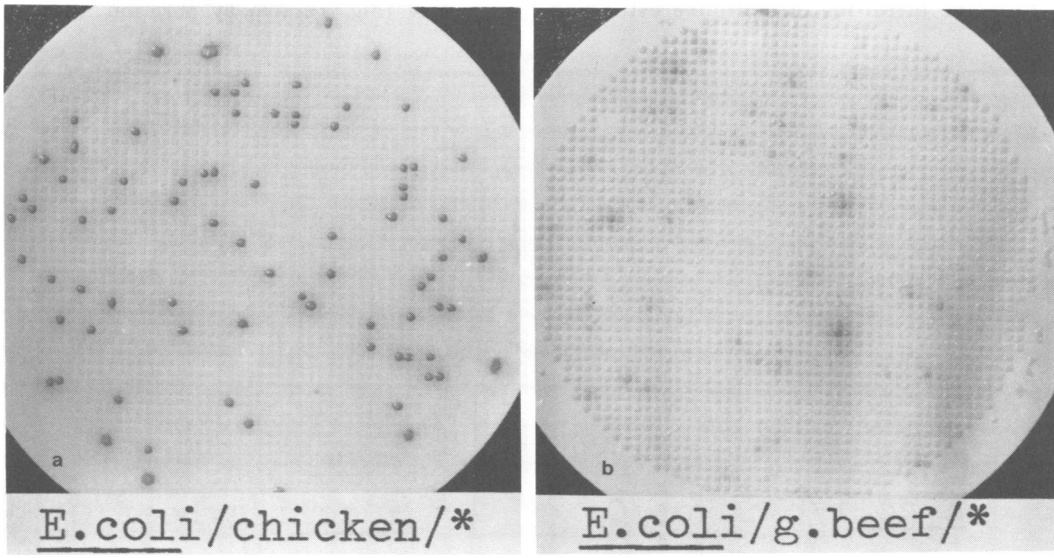

FIG. 2. *Cellulose ester HGMF carrying uniform loads of E. coli colonies, stained by method A. (a) Few indole-negative colonies present; (b) many indole-negative colonies present.*

there were few indole-negative colonies, whereas in Fig. 1b and 2b the HGMF were almost saturated with indole-negative colonies. The difficulty of counting *E. coli* in the presence of large numbers of other colonies when using the ABP stain (Fig. 1b) is apparent.

The reduced DAB concentration in method A provided adequate color for recognizing indole-positive colones; however, surrounding zones were too faint to be seen. This resulted in sharper demarcation of *E. coli* colonies (Fig. 2). Browning of indole-negative colonies was also greatly reduced. The improved visibility of *E. coli* colonies, particularly in the presence of large numbers of other colonies, is obvious (Fig. 2b).

E. coli colonies on polysulfone HGMF did not stain when using the ABP procedure, although a uniform pink could later be seen in the filter paper pads. However, method B successfully stained indole-positive colonies. Figure 3 illustrates the visibility of *E. coli* colonies in the presence of low and high densities of indole-negative colonies when using this method.

Comparison of bacterial recoveries. Ratios of *E. coli* counts from artificially contaminated foods on cellulosic or polysulfone HGMF, stained by either the ABP procedure or methods A and B, are shown in Table 1. In general, counts obtained with the modified methods agreed well with those obtained with the ABP staining procedure. Counts of *E. coli* from naturally contaminated foods by using HGMF with either method A or method B compared favorably with those from the conventional ABP direct plating method (Table 2).

Relative affinities of cellulose ester and polysulfone MF for indole or Ehrlich reaction products. Indole reached an identical final distribution between the two MF types, regardless of whether it was added first to Millipore or to Tuffryn. Reflectances of stained, dried MF (Fig. 4) show that indole migrated almost completely into the Millipore filter. Red reaction products were not bound by Tuffryn MF (Fig. 5). They were quickly adsorbed by Millipore MF, however, and desorbed extremely slowly when MF were later rinsed with 50% ethanol.

Chemical and physical effects in stain intensity and contrast. Wet MF had markedly lower reflectivity, and the changes between various stain concentrations were somewhat compressed compared with the dry MF (Fig. 5). If one assumes that the eye's ability to resolve two close tints depends roughly on relative reflectance changes [i.e., $\Delta R/(1 - R)$], it can be seen that the apparent contrast between colonies and other areas increased considerably as the MF dried.

The magnitude of the intensity changes caused by UV irradiation for a given set of MF varied somewhat from experiment to experiment; however, there was a marked increase in intensity only for those stains prepared with low concentrations of DAB (Fig. 6). At 5% DAB, UV irradiation may even have had a negative effect.

DAB concentrations above 1% had very little effect on stain intensity (Fig. 6). However, a pronounced yellowing could occur as DAB concentrations increased further. The red intensity increased with increasing acidity until the HCl concentration approached 3 N and decreased after 4 N (Fig. 7). (On evaporation at room

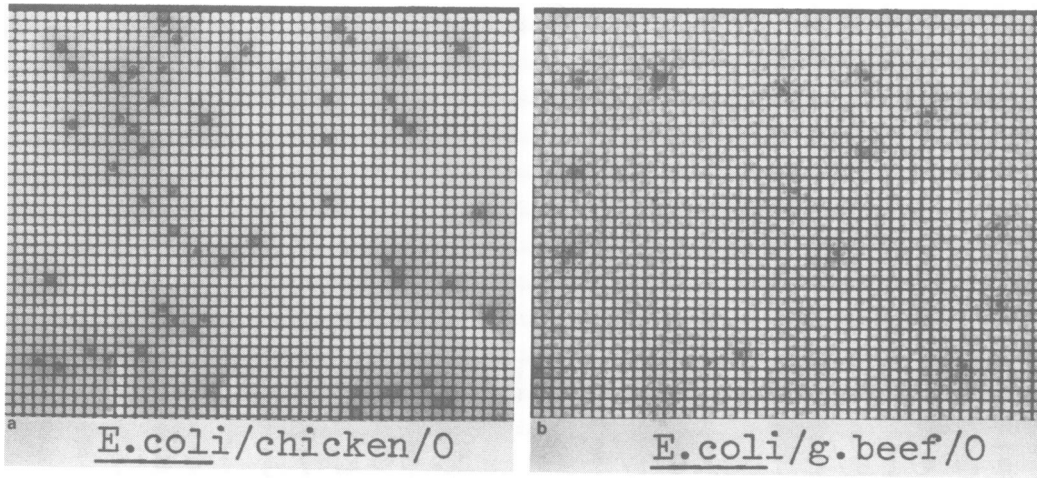

FIG. 3. *Polysulfone HGMF carrying uniform loads of E. coli colonies, stained by method B. (a) Few indole-negative colonies; (b) many indole-negative colonies.*

TABLE 1. *Ratios[a] of E. coli counts on HGMF: modified procedures versus ABP staining procedure*

Food	Cellulose ester,[b] method A/ABP		Polysulfone,[c] method B/ABP	
	Mean	Standard deviation	Mean	Standard deviation
Beans	0.72	0.03	1.63	0.25
Cheese	0.96	0.03	0.77	0.14
Chicken	0.93	0.07	1.05	0.19
Potatoes	1.04	0.09	2.77	1.70
Beef chunks	0.99	0.04	1.18	0.43

[a] All ratios obtained from counts averaged over three subsamples.
[b] Millipore HAWP.
[c] Gelman Tuffryn HT-450.

TABLE 2. *Ratios[a] of E. coli counts: HGMF versus ABP direct-plating (DP) method*

Food	Cellulose ester HGMF[b]/DP		Polysulfone HGMF[c]/DP	
	Mean	Standard deviation	Mean	Standard deviation
Beef (deboned)	1.06	0.43	1.07	0.32
Chicken emulsion	0.92	0.48	0.85	0.41
Chicken (deboned)	1.12	1.11	1.40	1.24

[a] All ratios obtained from counts averaged over seven subsamples.
[b] HGMF stained by method A.
[c] HGMF stained by method B.

temperature, 1 N HCl gradually concentrated to about 7 N before disappearing altogether.)

DISCUSSION

The need to formulate two procedures when confirming *E. coli* points up the very different behaviors of polysulfone and cellulose ester as substrates in this test. It seems that whereas polysulfone MF provide a relatively inert matrix for colony growth and staining reactions, cellulose ester MF may participate significantly; the result may or may not be beneficial. The complexity of the Ehrlich reaction on MF seems at times to have led to confusion over procedural points, and a comment is appropriate.

Indole diffuses readily away from polysulfone MF, leaving detectable amounts only in the producing colonies. In contrast, cellulose ester adsorbs indole strongly, so that large quantities accumulate in zones around *E. coli* colonies. In addition, some Ehrlich reaction products bind very strongly to cellulose ester. Although the ABP procedure reveals indole producers splendidly on Millipore, the large diffuse zones make counting difficult at moderate to high colony densities. Reducing overall staining intensity until these zones pale from visibility, as in method A, gives better colony definition.

Colors appear quickly but diffusely when cellulose ester MF are placed on the ABP reagent. After a 30-min "development" colony differentiation appears sharper. The improvement is variously attributed to the effect of UV or infrared illumination, normal laboratory lights, or sunlight behind glass (3, 4). These apparently contradictory opinions may each be correct under suitable circumstances.

Indole has been reported to react reversibly (7) with DAB in dilute acid to produce an unstable red 3-substituted benzylidene-indolenine (I). In stronger acid (when MF dry out during development, for example) increasing 2-substitution (II) occurs. The primary products reportedly change to colorless diindylmethanes, which may then dehydrogenate to red or blue diindolomethenes. In addition, indole may first polymerize (1) or oxidize to indigo. Fortunately, the product mix seems to be red or purple, regardless of the actual reactions prompted by the staining procedure.

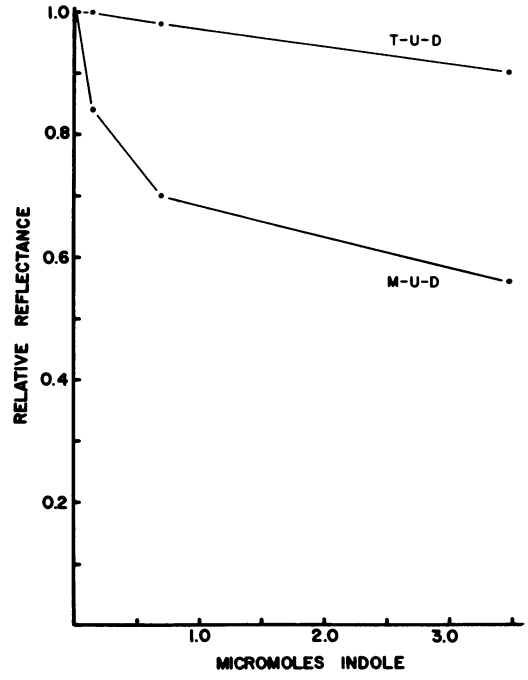

FIG. 4. *Reflectances of the separated, Ehrlich-stained MF after allowing indole to distribute itself between contacting pairs. Abbreviations: T, Tuffryn HT-450; M, Millipore HAWP; U, not irradiated with UV; I, irradiated with UV; D, dry; W, wet.*

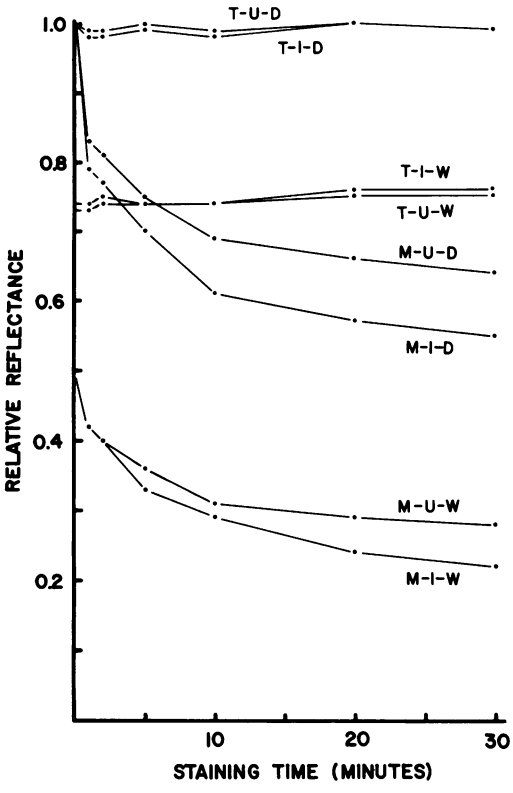

FIG. 5. *Reflectances of wet and dry MF, with and without irradiation, after staining various times in mixtures of 1 mM indole, 0.5% DAB, and 1 N HCl in 50% ethanol. See Fig. 4 for explanation of abbreviations.*

procedure, drying is more important than the wavelength of the illumination; improved contrast attributed to various illuminations results from the combined effects of increasing acid concentration (Fig. 7) and the increased "covering power" of the white MF material as it dries (Fig. 5). Any illumination will warm the MF and promote drying, but the local relative humidity determines its actual extent. In this respect also, a nonvolatile acid such as H_2SO_4 is less desirable

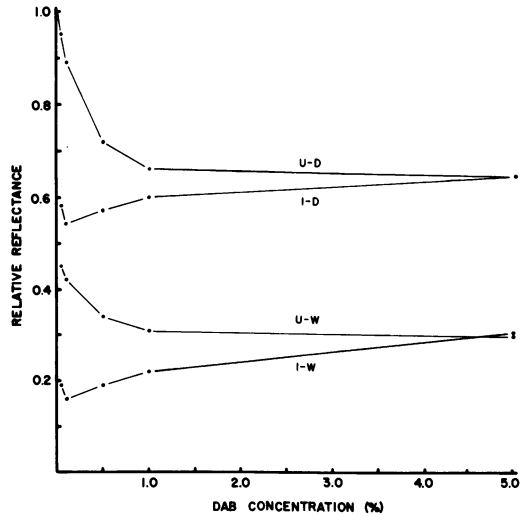

FIG. 6. *Effect of DAB concentration, with or without irradiation by UV, on Ehrlich stain intensity in Millipore MF. See Fig. 4 for explanation of abbreviations.*

In strongly acid solutions, the monovalent primary products are removed as colorless multivalent cations. Therefore, although drying during development tends to drive the initial equilibria completely over, it is not conducive to the formation of I or II and subsequent reaction products. The effect may be seen if polysulfone MF are allowed to dry during development. On rewetting, intense red-purple colonies appear briefly, presumably through the release of copious quantities of monovalent ions such as I or II by the sudden decrease in acidity. Cellulose ester adds further complications by irreversibly adsorbing one or more of the red reaction products, thus itself driving and stabilizing the reaction. One may demonstrate this by adding HAWP material to an indole-DAB mixture too dilute for visible reaction; the MF will slowly but continually turn pink.

UV light markedly assists color development on cellulose ester only when DAB is at low concentration (e.g., <1%). Thus, in the ABP

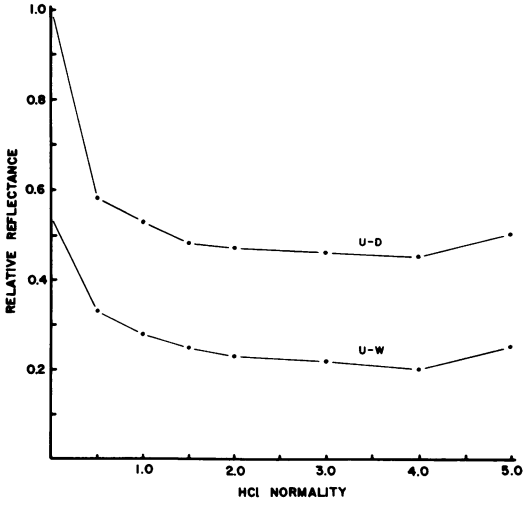

FIG. 7. *Effect of HCl concentration on Ehrlich stain intensity in Millipore MF. See Fig. 4 for explanation of abbreviations.*

than HCl, which, by eventually evaporating, stops the reaction.

Finally, since irreversible reaction with the base material cannot stabilize color development on Tuffryn, oxidation is definitely needed. Oxidation by heated persulfate, as originally advocated by Delaney et al. (3), is very rapid compared with irradiation under normal laboratory UV sources and is to be preferred.

ACKNOWLEDGMENTS

We are grateful to the late B. Aris for the ABP direct-plating method counts and to P. Entis (QA Laboratories Ltd.) for a recommendation to avoid drying during irradiation of Tuffryn HGMF.

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