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[54] **NOVEL DIAGNOSTIC SYSTEM FOR DIFFERENTIATION OF ENTEROBACTERIACEAE**

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[58] **Field of Search** ..... 195/99–103.5 R

[56] **References Cited**

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[57] **ABSTRACT**

This invention relates to a culture medium for the rapid differentiation and identification of bacteria belonging to the family Enterobacteriaceae.

18 Claims, No Drawings

NOVEL DIAGNOSTIC SYSTEM FOR DIFFERENTIATION OF ENTEROBACTERIACEAE

This invention relates to a culture medium for the rapid differentiation and identification of bacteria of the family Enterobacteriaceae. More specifically, this invention relates to single culture media, the method for their preparation and the utilization of these media for the identification of the bacteria of the family Enterobacteriaceae.

The Enterobacteriaceae are a ubiquitous group of bacteria consisting of frank enteric pathogens (Salmonella and Shigella) and many other opportunistic organisms capable of causing infections in every conceivable body locus. They are defined as gram negative rods that reduce nitrates, are oxidase negative and ferment glucose. Their spectrum of sensitivity to antibiotics varies considerably and in many instances therapy ought be based only upon the identity of the organism. Some members are epidemiologically significant and all require identification for specific diagnosis. Currently, they are the most frequent cause of bacterial infections and account for over 15 million tests per year.

Although certain individual principles and aspects to which this invention relates have been known and used in hospital and field conditions for the determination of bacteria, such factors have generally employed the use of multiple media requiring at least three days and as many as 10–20 separate tests. Although other procedures have been produced to obviate some of the problems involved, such procedures have been complex, awkward and expensive multi-media devices. The media of this invention provide a relatively simply prepared single confluent medium which affords the diagnostician with a large number of differential tests in a single tube. Other advantages and distinguishing characteristics of this invention over the media of the prior art will also be apparent to the skilled artisan as the details of this invention are explored.

In its broad concept this invention relates to media wherein chromogenic β-galactoside substrates are admixed with either (a) a decarboxylase substrate, (b) a deaminase substrate, (c) a urease substrate, (d) a hydrogen sulfide detecting system, or (e) a carbohydrate fermentation system, or the chromogenic β-galactoside substrate is admixed with any combination of such system.

Suitable chromogenic β-galactoside substrates are o-nitrophenyl-β-galactopyranoside (ONPG), 5-bromo-4-chloro-3-indolyl-β-galactoside, and 6-bromo-2-naphthyl-β-D-galactoside, as well as any other well-known agents. Suitable deaminase substrates are such 1-amino acids as phenylalanine, tryptophane, histidine, leucine, norleucine, methionine and norvaline and the like. Urea is used as the substrate for urease. Suitable hydrogen sulfide detecting agents are sodium thiosulfate in the presence of an iron containing salt such as ferric ammonium citrate. Suitable decarboxylase agents are lysine, ornithine and arginine, and the like. Suitable fermentable carbohydrates (or sugar alcohols) are dextrose, mannitol, arabinose, sucrose, dulcitol, rhamnose, and the like. The surprising feature of this invention, as the below described media and color formations indicate, is that for the first time a system has been devised wherein a chromogenic β-galactoside substrate has been combined with the above-mentioned other type substrates without the color reactions of the chromogenic β-galactoside substrate interfering with the efficacy of the tests. Also, as is evident from the herein described media, although it is preferred to strive for as many substrates as possible, it is of course understood that such media can be modified wherein one or more of the above-described a, b, c, d, or e components can be eliminated from the medium.

Essentially the preferred medium of this invention is comprised of such ingredients as bromthymol blue (used as pH indicator), yeast extract (a source of nutrient), dextrose and/or other fermentable carbohydrates, l-lysine (detection of lysine decarboxylase), ferric ammonium citrate and sodium thiosulfate (detection of hydrogen sulfide production), tryptophan, (detection of deaminase and indole), o-nitrophenyl β-galactopyranoside (ONPG) (detection of β-galactosidase activity), trace amounts of lactose to activate the β-galactosidase system, urea for detection of urease and agar as a supporting base, and sodium chloride (for osmotic control). Optionally, starch or carboxymethyl cellulose (or any other cellulose) may be added to enhance gas formation and to prolong the shelf life of the medium.

More specifically, the preferred medium is comprised and prepared as follows:

	Ingredient	Grams/liter
(A)	Bromthymol Blue	0.05
	Yeast Extract	3.0
	Dextrose	0.9
	L-lysine	12.0
	Ferric Ammonium Citrate	0.4
	Sodium Thiosulfate	1.5
	Agar	12.0
	Lactose	0.02
	Sodium Chloride	0.75
(B)	O-nitrophenyl-β-galactopyranoside	0.75
	Tryptophan	3.5
	Urea	1.0

After weighing the components of part A of this medium, sufficient distilled water is added to bring the volume, to 700 ml. The resulting suspension is then heated and with the aid of a magnetic stirrer is brought into solution. The pH of the solution is adjusted to about 6.9, after which it is autoclaved at 15 p.s.i. for 15 minutes. The remaining components of the medium, (B) are brought to about pH 6.9 and sterilized by filtering through a Nalgene filter. Both (A) and (B) components are then admixed under sterild conditions. The final pH is adjusted to about 7.0. The medium is then dispensed in suitable quantities in sterile screw-capped tubes, allowing the agar to cool while the tubes are angled to obtain a butt and slant configuration according to standard techniques. The inside of the screw-cap is fitted with a p-dimethylamino-benzaldehyde-impregnated paper disc previously prepared according to standard techniques suitable for the detection of indole.

Although the foregoing formulation is the most preferred ingredient-concentration, quite naturally modifications may be made to achieve substantial, but varying degrees of success. Thus, it is comtemplated that the foregoing ingredient-concentrations may be modified and still be within the spirit of this invention, as follows: Bromthymol blue (0.025–0.15 g/l) yeast extract (1.5–9 g/l) dextrose (0.5–5 g/l) l-lysine (5.0–20.0 g/l) ferric ammonium citrate (0.1–1.2 g/l) sodium thiosulfate (0.1–1.2 g/l) tryptophan (1.2–10.5 g/l), agar (10–20

gms) urea (0.5–1.5 g/l) with the adjustment to the pH to about 6.7–7.1.

The use of the foregoing media presumes the primary isolation of cultures of pure cultures. When used, the butt is stabbed and the slant streaked with the test culture in the usual manner that is used to inoculate other tubed media having a slant and butt, and the screw cap loosely replaced onto the tube. Tne inoculated culture is permited to grow at 37°C for about 24 hours and read within 24–72 hours from inoculation. Of course, it is possible to permit the inoculated culture at temperatures below 37°C but in such instances the growth period is proportionately longer.

The media provide for possible color differentiation wherein the proteus-providence group of organisms give rise to a brown slant, hydrogen sulfide-producing organisms causing a blackening in the butt, lysine positive organisms giving a green butt, lysine negative organisms giving a yellow butt, urease positive organisms give a blue-green to blue color in the butt or at the butt/slant juction. In the case of *Proteus* species, this bluish color will mask the lysine reaction which is not essential for the identification of this group in the presence of urea. The ONPG reacting organisms give rise to a green color in the slant while ONPG negative organisms turn the slant blue. The p-dimethylaminobenzylaldehyde impregnated paper disc turns red to show the presence of indole.

The expected reactions of the above-exemplified medium are as follows:

Glucose: If lysine is not decarboxylated and glucose is fermented, the pH of the butt will drop below 6.2, resulting in yellow color. However, if the organism is strongly urease positive, an overriding alkaline reaction is produced and the butt will turn blue/green to blue.

Lysine: If the pH drops to about 6.2 as a result of glucose fermentation and lysine is decarboxylated, the pH will then increase to 6.6–6.9 and the butt will turn green.

H_2S: If H_2S is produced a black heavy deposit may appear in the butt, or a trace of black may form at the butt-slant junction.

Tryptophane: If tryptophane is deaminated, it forms indole pyruvic acid (IPA). IPA in turn, complexes with iron to produce a brownish slant.

ONPG: If the organism has an inducible β-galactosidase, galactose is split from ONPG liberating the yellow-collored o-nitrophenol. In the presence of the blue slant that forms in this medium if no deaminase activity is present, the combination of the yellow o-nitrophenol and blue, produce a green colored slant. Blue slants occur if the organism tested is both ONPG and deaminase negative.

Urea: If the organism produces urease, ammonia is formed causing a rise in pH above 7.0. With strong urease producers such as *Proteus* species, the color of the butt will turn blue/green to blue. Due to the deaminase activity combined with urease activity with *Proteus* species, the slant will turn blue/green or green/brown (olive). With weaker urease producers such as Klebsiella, a blue/green color may only be produced at the butt-slant junction.

Indole: If indole is formed as a result of tryptophanase activity, the disc insert in the cap will turn red to violet. It will remain colorless if indole is not produced.

The following tables illustrate the identification of the organisms of the Enterobacteriaciae.

Table I

Group I: Hydrogen Sulfide Positive	H_2S	Tryptophan	Indole	Lysine	ONPG	UREA
Arizona	+	−	−	+	+	−
Edwardsiella	+	−	+	+	−	−
Salmonella	+	−	−	+	−	−
P. mirabilis	+	+	−	−	−	+
P. vulgaris	+	+	+	−	−	+
Citrobacter freundii	+	−	−	−	+	d
Group II: Tryptophan Positive (excluding Group I organisms)	H_2S	Tryptophan	Indole	Lysine	ONPG	UREA
P. morganii	−	+	+	−	−	+
P. rettgeri	−	+	+	−	−	+
Providencia	−	+	+	−	−	+
Group III: Indole Positive (excluding Groups I and II organisms)	H_2S	Tryptophan	Indole	Lysine	ONPG	UREA
E. coli	−	−	+	d[1]	+	−
Shigella[2]	−	−	− or +	−	−	−
Klebsiella	−	−	− or +	+	+	d
Group IV: Indole Negative	H_2S	Tryptophan	Indole	Lysine	ONPG	Urea
Shigella	−	−	− or +[3]	−	−[2]	−
Salmonella	−	−	−	+	−	−
Citrobacter	−	−	−	−	+	d
Klebsiella	−	−	− or +	+	+	d
E. cloacae	−	−	−	−	+	d
E. aerogenes	−	−	−	+	+	−
E. hafniae	−	−	−	+	+ or 31	−
E. liquefaciens	−	−	−	+ or −[4]	+ or −	d
Serratoa	−	−	−	+	+	d

[1]Different biochemical types.
[2]With exception of *Shigella sonnei*
[3]− or + = majority positive.
[4]− or + = majority negative.

COLOR REACTIONS OF ENTEROBACTERIACEAE WITH EXAMPLE I MEDIUM

Organisms	Slant	Butt	Indole
*(1) *Providencia*	Brown	Yellow	+
(2) *P. rettgeri*	Brown/Blue-Olive	Blue	+
(3) P. morganii	Brown/Blue-Olive	Blue	+
(4) P. mirabilis	Brown/Blue-Olive	Blue/Black	−
(5) *P. vulgaris*	Brown/Blue-Olive	Blue/Black	+
(6) *Klebsiella*	Green	Blue/Yellow	±
(7) *Citrobacter freundii*	Green	Black/Yellow	−
(8) *Arizona*	Green	Black/Green	−
(9) *Edwardsiella*	Blue	Black/Green	+
(10) *E. coli*	Green	Green or Yellow	+
(11) *Serratia*	Green	Green	−
(12) *E. liquefaciens*	Blue or Green	Green or Yellow	−
(13) *E. aerogenes*	Green	Green	−
(14) *E. cloacea*	Green	Yellow (gas)	−
(15) *S. sonnei*	Green	Yellow (no gas)	−
(16) *Salmonella*	Blue	Black	−
(17) *Shigella Blue*	Yellow	− or +	
(18) *E. hafniae*	Blue or Green	Green	−

/= Two color in the butt.
*Numbers cross-correlate with FIGS. 1 and 2.

We claim:

1. A culture medium having a pH in the range of about 6.7–7.2 suitable for determining the identification of bacteria of the family Enterobacteriaceae which comprises a chromogenic β-galactosidase substrate in combination with a member of the group consisting of (a) a decarboxylase substrate, (b) a deaminase substrate, (c) a urease substrate, (d) a hydrogen sulfide detecting system, or (e) a carbohydrate fermentation system.

2. A culture medium of claim **1** wherein the chromogenic β-galactosidase substrate is chosen from the group consisting of o-nitrophenyl-β-galactopyranoside, 5-bromo-6-chloro-3-indolyl-β-D-galactoside and 6-bromo-2-naphthyl-β-D-galactoside, the decarboxylase agents are lysine, ornithine and arginine, the urease substrate is urea and a carbohydrate fermentation system selected from the group consisting of dextrose, mannitol, arabinose, sucrose, dulcitol, rhamnose, the deaminase substrate is selected from the group consisting or l-aminoacids, preferably tryptophane, phenylalanine, and histidine, and the hydrogen sulfide detection system containing an iron salt in combination with hydrogen sulfide detecting agent preferably in the form of a thiosulfate.

3. A culture medium of claim **1** wherein the chromogenic β-galactasidase substrate is in combination with a decarboxylase substrate.

4. A culture medium of claim **1** wherein the chromogenic β-galactosidase substrate is in combination with a deaminase substrate.

5. A culture medium of claim **1** wherein the chromogenic β-galactosidase substrate is in combination with a hydrogen sulfide detecting system.

6. A culture medium of claim **1** wherein the chromogenic β-galactosidase substrate is in combination with a urease substrate.

7. A culture medium of claim **1** wherein the chromogenic β-galactosidase substrate is in combination with a carbohydrate fermentation system.

8. A culture medium having a pH in the range of about 6.7–7.2 suitable for determining the identification of bacteria of the Enterobacteriaceae which comprises the following ingredients, said ingredients being present in proportions indicated:

Ingredients	Grams/liter
Bromthymol Blue	0.025–0.158
Yeast Extract	15.–9
Dextrose	0.5–5
L-lysine	2.5–40
Ferric Ammonium Citrate	0.1–1.2
Sodium Thiosulfate	0.1–1.2
Agar	10–20
Lactose	0.05–3.0
Sodium Chloride	0.5–1.0
O-nitrophenyl-β-galactopyranoside	0.5–4.5
Tryptophan	1.0–10
Urea	0.5–1.5
Water q.s. to make	1000 cc.

9. A process for preparing compositions of claim **8** which comprises admixing the bromthymol blue, yeast extract, dextrose, lysine, ferric ammonium citrate, sodium thiosulfate, agar, lactose and sodium chloride ingredients in suitable quantities of water, adjusting the pH to about 7.0, autoclaving the resulting solution followed by adding sterile o-nitrophenyl-η-galactopyranoside, urea and tryptophan, with q.s. water to form the defined concentrations.

10. The process for the identification of bacteria of the Enterobacteriaceae which comprises inoculating the media defined by claim **8** allowing the inoculated media to grow for at least about 24 hours at 37°C, followed by the identification of the bacteria according to the herein described changes for lysine, hydrogen sulfide, tryptophan, ONPG, urea and indole.

11. The process for the identification of bacteria of the Enterobacteriaceae which comprises inoculating the media defined by claim **8** allowing the inoculated media to grow for at least about 24 hours at 37°C, followed b the idenification of the bacteria according to

the herein described color changed for glucose, lysine, hydrogen sulfide, tryptophan, o-nitrophenyl β-galactopyranoside urea and indole.

12. A sterile culure medium having a pH of about 6.7–7.2 comprising:

Ingredient	Grams/liter
Bromthymol Blue	0.05
Yeast Extract	3.0
Dextrose	0.9
L-lysine	12.0
Ferric Ammonium Citrate	0.4
Sodium Thiosulfate	1.5
Agar	12.0
Lactose	0.02
Sodium Chloride	0.75
O-nitrophenyl-β-galactopyranoside	0.75
Tryptophan	3.75
Urea	1.0
Water q.s. to make	1000 cc.

13. A process for preparing composition of claim **12,** which comprises admixing the bromthymol blue, yeast extract, dextrose, lysine, ferric ammonium citrate, sodium thiosulfate, agar, lactose and sodium chloride ingredients in suitable quantities of water, adjusting the pH to about 7.0, autoclaving the resulting solution followed by adding sterile o-nitrophenyl-β-galactopyranoside urea and tryptophan, with q.s. water to form the defined concentrations.

14. The process for the identification of bacteria of the Enterobacteriaceae which comprises inoculating the media defined by claim **12,** allowing the inoculated media to grow for at least about 24 hours at 37°C, followed by the identification of the bacteria according to the herein described changes for lysine, hydrogen sulfide, tryptophan, ONPG, urea and indole.

15. The process of claim **14** wherein the color change results are read according to the following table:

Table

Group I: Hydrogen Sulfide Positive

	H_2S	Tryptophan	Indole	Lysine	NPG	Urea
Arizona	+	−	−	m+	+	−
Edwardsiella	+	−	+	+	−	−
Salmonella	+	−	−	+	−	−
P. mirabilis	+	+	−	−	−	+
P. vulgaris	+	+	+	−	−	+
Citrobacter freundii	+	−	−	−	+	d

Group II: Tryptophan Positive (excluding Group I organisms)

	H_2S	Tryptophan	Indole	Lysine	NPG	Urea
P. morganii	−	+	+	−	−	+
P. rettgeri	−	+	+	−	−	+
Providencia	−	+	+	−	−	+

Group III: Indole Positive (excluding Groups I and II organisms)

	H_2S	Tryptophan	Indole	Lysine	NPG	Urea
E. coli	−	−	+	d[1]	+	−
Shigella[2]	−	−	− or +	−	−	−
Klebsiella	−	−	− or +	+	+	d

Group IV: Indole Negative

	H_2S	Tryptophan	Indole	Lysine	NPG	Urea
Shigella	−	−	− or +[3]	−	−[2]	−

Table-Continued

Group I: Hydrogen Sulfide Positive

	H_2S	Tryptophan	Indole	Lysine	NPG	Urea
Salmonella	−	−	−	+	−	−
Citrobacter	−	−	−	−	+	d
Klebsiella	−	−	− or +	+	+	d
E. cloacae	−	−	−	−	+	d
E. aerogenes	−	−	−	+	+	−
E. hafniae	−	−	−	+	+ or −	−
E. liquefaciens	−	−	−	+−[4]	+ or −	d
Serratia	−	−	−	+	+	d.

[1]Different biochemical types.
[2]With exception of *Shigella sonei.*
[3]− or + = majority positive.
[4]+ or − = majority negative.

16. The process of claim **14** wherein the color change results are read according to the following chart:

COLOR REACTIONS OF *ENTEROBACTERIACEAE* WITH EXAMPLE

ORGANISMS	Medium SLANT	Medium BUTT	INDOLE
(1) *Providencia*	Brown	Yellow	+
(2) *P. rettgeri*	Blue-brown to Olive	Blue	+
(3) *P. morganii*	do.	Blue	+
(4) *P. mirabilis*	do.	Blue/Black	−
(5) *P. vulgaris*	do.	Blue/Black	+
(6) *Klebsiella*	Green	Blue/Green	±
(7) *Citrobacter freundii*	Green	Black/Yellow	−
(8) *Arizona*	Green	Black/Green	−
(9) *Edwardsiella*	Blue	Black/Green	+
(10 *E. coli*	Green	Green or Yellow	+
(11) *Serratia*	Green	Green	−
(12) *E. liquefaciens*	Blue or Green	Green or Yellow	−
(13) *E. aerogenes*	Green	Green	−
(14) *E. cloacae*	Green	Yellow (gas)	−
(15) *S. sonnei*	Green	Yellow (no gas)	−
(16) *Salmonella*	Blue	Black	−
(17) *Shigella*	Blue	Yellow	− or +
(18) *E. hafniae*	Blue or Green	Green	−.

/= Two colors in the butt.
* Numbers cross-correlate with FIGS. 1 and 2.

17. The process for the identification of bacteria of the Enterobacteriaceae which comprises inoculating the media defined by claim **12,** allowing the inoculated media to grow for at least about 24 hours at 37°C, followed by the identification of the bacteria according to the herein described color changes for glucose, lysine, hydrogen sulfide, tryptophan, o-nitrophenyl β-galactopyranoside urea and indole.

18. The process of claim **17** wherein the color change results are read according to the following chart:

COLOR REACTIONS OF ENTEROBACTERIACEAE WITH EXAMPLE I

Organisms	MEDIUM Slant	MEDIUM Butt	Indole
*(1) *Providencia*	Brown	Yellow	+

-Continued

COLOR REACTIONS OF ENTEROBACTERIACEAE WITH EXAMPLE I

Organisms	MEDIUM Slant	Butt	Indole
(2) *P. rettgeri*	Blue-brown to Olive	Blue	+
(3) *P. morganii*	do.	Blue	+
(4) *P. mirabilis*	do.	Blue/Black	−
(5) *P. vulgaris*	do.	Blue/Black	+
(6) *Klebsiella*	Green	Blue/Green	±
(7) *Citrobacter freundii*	Green	Black/Yellow	−
(8) *Arizona*	Green	Black/Green	−
(9) *Edwardsiella*	Blue	Black/Green	+
(10) *E. coli*	Green	Green or Yellow	+
(11) *Serratia*	Green	Green	−
(12) *E. liquefaciens*	Blue or Green	Green or Yellow	−
(13) *E. aerogenes*	Green	Green	−
(14) *E. cloacae*	Green	Yellow (gas)	−
(15) *S. sonnei*	Green	Yellow (no gas)	−
(16) *Salmonella*	Blue	Black	−
(71) *Shigella*	Blue	Yellow	− or +
(18) *E. hafniae*	Blue or	Green	−.

/ = Two colors in the butt.

* Numbers cross-correlate with FIGS. 1 and 2.

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