Esculin Hydrolysis by *Enterobacteriaceae*

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Literature reports disagree concerning esculin hydrolysis in the family *Enterobacteriaceae*. A total of 2,490 strains of the family were investigated for esculin hydrolysis by two methods, the esculin spot test and the PathoTec incubation strip, which measures constitutive enzyme, and five growth-supporting methods, which determine both constitutive and inducible enzymes. The five growth-supporting media studied were: Vaughn-Levine, the standard esculin hydrolysis medium (P. R. Edwards and W. H. Ewing, *Identification of Enterobacteriaceae*, 3rd ed., 1972); Vaughn-Levine without iron; Vaughn-Levine without Andrade's indicator; and bile-esculin medium. Growth media were incubated at 35°C and checked every 24 h for 120 h. On growth media, 0.3% of *Escherichia coli* were positive in 24 h, 34% in 48 h, and 61% in 120 h. No strains were positive on the "nongrowth" tests. It appeared that the esculin hydrolysis enzyme(s) of *E. coli* was inducible rather than constitutive. All esculin hydrolyzers, which yielded positive tests on "constitutive tests" and 24-h tests, were limited to the genera *Klebsiella*, *Enterobacter*, and *Serratia* and species of *Proteus vulgaris*, *Proteus rettgeri*, and *Citrobacter diversus*. When used with standardized inoculum size and incubation time, the esculin hydrolysis test is very useful for differentiation within the family *Enterobacteriaceae*.

Hydrolysis of esculin to glucose and esculetin (6,7-dihydroxycoumarin) and detection of esculetin by its reaction with iron were first described for bacteria by Meulen in 1907 (10). Harrison and van der Leek (4) used this reaction and incorporated sodium taurocholate and ferric citrate into esculin medium to examine water for fecal coliforms. Rochaix (8) expanded this work and described a test for the differentiation of streptococci, enterococci, and pneumococci. Meyer and Schöenfeld (7) reported the addition of ox bile to the medium, which then inhibited non-enterococcal streptococci. In the 1930s, Levine and Vaughn et al. (5, 11) rediscovered the value of esculin hydrolysis for differentiation in the family *Enterobacteriaceae*. They described a medium containing peptone, esculin, ferric citrate, K_2HPO_4 , and Andrade's indicator that remains the standard esculin hydrolysis medium used in the enteric section at the Center for Disease Control (2, 3). Although esculin hydrolysis is used by the Center and included in most diagnostic charts about the family *Enterobacteriaceae*, it was rarely used clinically. In 1971, Wasilauskas (12) reported that bile-esculin agar, widely used for the identification of enterococci, could also be helpful for differentiation in *Enterobacteriaceae*. Of 232 strains of *Enterobacteriaceae* tested, he found

esculin hydrolysis at 24 h was limited to the *Klebsiella-Enterobacter-Serratia* group. He also reported that hydrolysis appeared to be both time and inoculum-size related. Lindell and Quinn (6) studied the problem by using bile-esculin slants and a heavy inoculum. They found 2% of *Escherichia coli* positive in 4 h and 18% positive in 24 h. Edwards and Ewing's (2, 3) data are based on 48 h of incubation, making comparisons after 24 h of incubation impossible. Edberg et al. (1), in studying a rapid esculin hydrolysis test based on fluorescence loss, found that all 510 strains of *E. coli* tested were negative at both 4 and 24 h.

Because studies on the hydrolysis of esculin by *Enterobacteriaceae* differ in the length of incubation, media, and inoculum size (Table 1), a study was undertaken to determine this test's taxonomic value for this family. Growth- and nongrowth-supporting media, different indicators, and extended periods of incubation were tested.

MATERIALS AND METHODS

Bacterial strains. A total of 2,490 strains of *Enterobacteriaceae* were studied (Table 2). All organisms except two strains of *Arizona hinshawii*, which were obtained from the New York City Department of Health, were clinical isolates from the General Bac-

TABLE 1. Reported esculin hydrolysis reactions by the family Enterobacteriaceae^a

Strains	Reported reactions											
	Wasilauskas (12)			Lindell and Quinn (6)			Edwards and Ewing (2)		Edberg et al. (1)			
	BEM (4 h)	BEM ^b (18 h)	NT ^c	BEM (4 h)	BEM ^b (24 h)	NT	VL (48 h)	VL ^b (72 h)	Rapid	BEM ^b (24 h)	VL (24 h)	NT
<i>E. coli</i>	0	0	69	2	18	126	31	51	0	0	0	510
<i>Klebsiella</i>	100	100	57	NA ^d	NA		NA	NA	NA	NA	NA	21
<i>K. pneumoniae</i>	X ^e	X		100	100	149	99	100	99	99	99	210
<i>K. ozaenae</i>	X	X		Y ^f	Y		75	75	79	79	86	14
<i>K. rhinoscleromatis</i>	X	X		Y	Y		15	15	Y	Y	Y	
<i>Enterobacter</i> species	100	100	34	NA	NA		NA	NA	NA	NA	NA	
<i>E. cloacae</i>	X	X		36	80	126	29	29	67	67	72	21
<i>E. aerogenes</i>	X	X		100	100	39	98	98	100	100	100	45
<i>E. hafniae</i>	X	X		24	28	25	6	8	0	0	0	11
<i>E. agglomerans</i>	X	X		46	78	46	Y	Y	Y	Y	Y	
<i>Serratia</i> species	100	100	25	NA	NA		NA	NA	NA	NA	NA	
<i>S. marcescens</i>	X	X		100	100	55	Y	Y	100	100	100	2
<i>S. rhubidaea</i>	X	X		100	100	4	Y	Y	100	100	100	2
<i>S. liquefaciens</i>	X	X		100	100	7	75	2	86	72	86	7
<i>Proteus</i> species	0	0	30	NA	NA		NA	NA	NA	NA	NA	
<i>P. mirabilis</i>	X	X		0	0	52	0	1	0	0	0	180
<i>P. vulgaris</i>	X	X		15	15	27	59	62	67	80	80	10
<i>P. rettgeri</i>	X	X		33	55	9	30	39	40	40	40	30
<i>P. morgani</i>	X	X		0	0	31	0	0	0	0	0	22
<i>Providencia</i> species	Y	Y		NA	NA		NA	NA	NA	NA	NA	
<i>P. stuartii</i>	Y	Y		0	0	10	0	0	0	0	0	8
<i>P. alcalifaciens</i>	Y	Y		0	0	14	0	0	0	0	0	10
<i>Citrobacter</i> species	0	0	2	NA	NA		NA	NA	NA	NA	NA	
<i>C. freundii</i>	X	X		3	3	38	1	3	0	0	0	9
<i>C. diversus</i>	X	X		0	20	15	1	9	0	0	0	6
<i>Salmonella</i>	0	0	11	0	0	172	0	0	0	0	0	16
<i>Shigella</i>	0	0	2	0	0	118	0	0	0	0	0	12
<i>A. hinshawii</i>	0	0	2	0	0	0	0	0	0	0	0	1

^a Numbers rounded to nearest whole percent.^b Cumulative percent positive with time is given.^c NT, Number tested.^d NA, Not applicable.^e Not identified to species.^f Not reported.

teriology Section of the Microbiology Division of Montefiore Hospital and Medical Center. *Enterobacteriaceae* were identified according to the scheme of Edwards and Ewing (2, 3) by their reactions on triple sugar and motility agars, their ability to produce indole, acetoin, urease, H₂S, and deoxyribonuclease, to utilize malonate, to decarboxylate ornithine and lysine, to deaminate phenylalanine, and to hydrolyze *o*-nitrophenyl- β -D-galactopyranoside. When necessary, additional biochemical and appropriate serological tests were performed (2, 3).

Nomenclature. In this manuscript we prefer to use the nomenclature of Edwards and Ewing rather than that of *Bergey's Manual of Determinative Bacteriology*, 8th ed.; the following names are affected: (the *Bergey's Manual* name is given in parentheses):

Citrobacter diversus (*C. intermedius*), *Arizona hinshawii* (*Salmonella arizonae*), *Enterobacter hafniae* (*Hafnia alvei*), *Enterobacter agglomerans* (*Erwinia herbicola*), *Providencia alcalifaciens* (*Proteus inconstans*), and *Providencia stuartii* (*Proteus inconstans*). *Serratia liquefaciens* and *Serratia marcescens* are not listed in the 8th edition of *Bergey's Manual*.

Nongrowth-supporting media: esculin spot test. The determination of esculin hydrolysis by fluorescence loss was measured with a 366-nm ultraviolet light generator (1). Briefly, 0.02% esculin solution was pipetted onto filter paper on microscope slides. A bacterial colony was rubbed on the test paper, and, after 30 min at 35°C, esculin hydrolysis was measured by observing under ultraviolet light.

TABLE 2. *Esculin hydrolysis by Enterobacteriaceae in growth and nongrowth media*^a

Strain	No. tested	Test and media																
		Rapid		BEM ^a				VL ^b				VL-A ^b						
		Spot test	PathoTec strip	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	90 h	120 h
<i>E. coli</i>	974	0	0	0.3	35	52	56	61	0.8	38	54	59	61	0.6	34	50	58	61
<i>K. pneumoniae</i>	410	99	99	99	100	100	100	100	99	100	100	100	100	99	100	100	100	100
<i>K. ozaenae</i>	20	70	75	75	75	75	75	75	75	80	80	80	80	75	75	75	75	75
<i>K. rhinoscleromatis</i>	3	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33
<i>E. cloacae</i>	28	54	54	50	54	57	61	61	54	57	61	61	65	54	54	58	61	61
<i>E. aerogenes</i>	42	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
<i>E. hafniae</i>	14	7	7	0	7	7	7	7	7	7	7	7	7	7	7	7	7	7
<i>E. agglomerans</i>	6	33	33	33	33	50	50	50	33	50	50	50	50	33	50	50	50	50
<i>S. marcescens</i>	12	80 ^c	90	84	92	92	92	92	92	92	92	92	92	92	92	92	92	92
<i>S. rhubidaea</i>	5	100 ^c	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
<i>S. liquefaciens</i>	13	85	85	77	85	85	85	92	85	92	92	92	92	85	85	85	85	92
<i>P. mirabilis</i>	720	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>P. vulgaris</i>	40	53	57	53	55	55	57	60	62	57	60	62	65	55	55	60	60	60
<i>P. rettgeri</i>	45	33	36	33	36	36	38	38	36	38	38	40	40	36	36	38	40	40
<i>P. morganii</i>	47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>P. stuartii</i>	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>P. alcalifaciens</i>	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. freundii</i>	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. diversus</i>	15	0	0	0	0	7	14	14	0	7	14	14	14	0	0	7	14	14
<i>Salmonella</i> species	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Shigella</i> species	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. hinshawii</i>	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Y. enterocolitica</i>	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

^a Numbers rounded to nearest whole percent.^b The cumulative percent positive with time is given.^c Not all tested because of pigment production.

Darkening indicated esculin hydrolysis; no loss indicated no hydrolysis.

PathoTec esculin. An experimental (not available commercially) 4-h esculin hydrolysis PathoTec strip (W13482-4, General Diagnostics, Morris Plains, N.J.) was used. One bacterial colony was emulsified in 0.5 ml of distilled water in a screw-cap test tube (100 by 13 mm). The strip was dropped in the inoculum and incubated at 35°C for 4 h. A positive reaction occurred when the liquid turned black; a negative reaction had no color change. As has been reported (1), this strip is more sensitive than the previous rub-in type of strip, and, as such, results between the two are not comparable (9).

Growth-supporting media (all incubations at 35°C): bile-esculin medium. Commercial bile-esculin medium (BEM; Difco Laboratories, Detroit, Mich.), at a pH of 6.6, containing 0.1% esculin, 4% oxgall, and 0.05% ferric citrate, made as per instructions from the manufacturer, was utilized as selective, differential medium. Agar slants in test tubes (100 by 13 mm) were poured, so that the slant was approximately equal in length to the butt, and inoculated by rubbing the slant and stabbing the butt with a straight wire lightly touched to the surface of a colony. Incubation was for 120 h. A reaction was considered positive if the slant turned blue-black and negative if no change from the original color occurred. Some *E. coli* strains produced two to five dark colonies on a lawn of colorless colonies at 24 h of incubation. Because these dark colonies represented, based on visual observation, less than 0.0001% of the population on the slant, this "stippled effect" was considered negative.

Complete Vaughn-Levine medium. Complete Vaughn-Levine esculin hydrolysis broth medium (VL) was made from individual ingredients (11) and included 0.5% peptone, 0.3% esculin (I.C.U. Pharmaceuticals, Cleveland, Ohio), 0.05% ferric citrate, 0.1% K_2HPO_4 , and Andrade's indicator and had a final pH of 7.4. It was autoclaved for 15 min at 121°C. Five milliliters of the solution was dispensed into screw-cap tubes (100 by 13 mm). Tubes were inoculated by touching the top of a bacterial colony with a bacteriological wire and immersing it in the broth. Incubation was for 120 h. The reaction was considered positive when the tube became blue-black; a negative reaction was denoted by no color change.

VL without iron. VL without iron (VL-F) was prepared as complete VL without adding ferric citrate. The reaction was considered positive when the Andrade's indicator turned from colorless to red or pink.

VL without Andrade's indicator. VL without Andrade's indicator (VL-A) was made as complete VL without the inclusion of Andrade's indicator. The reaction was considered positive when the tube became blue-black; a negative reaction had no color change.

Esculin hydrolysis was recorded for BEM and all VL media every day for 5 days. Before the tubes were discarded (5 days), a subculture was made to MacConkey and to blood agars to detect possible contamination.

RESULTS

E. coli. Of the 974 strains of *E. coli* tested, none were positive by either the spot test or PathoTec strip. A total of 0.3% were positive in 24 h on BEM, whereas 0.8% were positive at the same time on VL and VL-A. At 48 h postinoculation, 34% of *E. coli* became positive on BEM, whereas 37% became positive on VL and 34% on VL-A. The only other species demonstrating negative hydrolysis at 24 h and positive results at 48 h was *C. diversus*, with 0% positive in 24 h and 7% positive at 48 to 72 h. At each 24-h interval, additional strains of *E. coli* became positive.

At 24 h, approximately 3% of *E. coli* showed two to five moderately dark colonies in a lawn of negative bacteria on the BEM slant. These slants invariably became black at 48 h. Slants showing this slightly stippled pattern at 24 h were considered negative. *E. coli* generally darkened the slant only 2 to 5 mm into the butt at 48 h when positive, compared with *Klebsiella*, which consistently darkened the entire butt in 24 h. At 72 h, however, *E. coli* darkened the entire butt. At 24 h, *E. coli* was negative in VL or VL-A media.

Klebsiella. A total of 99% of *K. pneumoniae* yielded strong spot tests, and 99% produced positive reactions with PathoTec strip tests. Similarly, 99% were positive in 24 h on BEM, increasing to 100% positivity at 72 h of incubation. Unlike *E. coli*, the entire slant and butt became black in 24 h. In VL and VL-A broths, 99.0% and 98.8%, respectively, were positive in 24 h, increasing to 100% at 48 h.

Of 20 strains of *K. ozaenae*, 75% were positive on BEM and VL-A, whereas 80% were positive on VL. Seventy and 75% were positive by the esculin spot and PathoTec strip tests, respectively. Only one of three *K. rhinoscleromatis* was positive. The overall positivity rate (as defined by the total number of strains positive divided by the total number tested) for the genus *Klebsiella* was 99.4%.

Serratia. All *S. rubidaea* and 92% of *S. marcescens* hydrolyzed esculin. Spot tests were not possible with pigmented *Serratia*. *S. liquefaciens* demonstrated an increase in hydrolysis over time. The overall positivity rate for the *Serratia* was 94%.

Enterobacter. All *E. aerogenes* were positive by all tests at all incubation periods. Like *K. pneumoniae*, the entire BEM slant and butt become dark at 24 h. One of 14 strains of *E. hafniae* was positive on growth and nongrowth media, whereas 3 of 6 strains of *E. agglomerans* were positive. *E. cloacae* was variable. *E.*

cloacae consistently produced weaker hydrolysis by both rapid and growth medium testing than all other organisms. At 24 h, only 2 to 5 mm of the slant was darkened by esculinase-producing strains. In no case was a stippled pattern on the slant noticed. The butt gradually became darker as incubation progressed.

***Proteus* and *Providencia*.** All *Proteus mirabilis*, *Proteus morganii*, *P. stuartii*, and *P. alcalifaciens* were negative by all tests. *Proteus vulgaris* and *Proteus rettgeri* were variable on all media tested.

***Citrobacter*.** All 16 strains of *C. freundii* were negative. All *C. diversus* strains were negative by rapid testing, but 7% were positive at 48 h of incubation on growth media.

***Salmonella*, *Shigella*, *Arizona*, *Yersinia*, *Edwardsiella*.** All strains tested were negative by all methods. Strains of *Y. pestis* and *Y. pseudotuberculosis* were not included in this series.

DISCUSSION

The literature reports esculin hydrolysis by *E. coli* to vary from 0 to 20% positive in 24 h. Lindell and Quinn (6), who used a heavy inoculum and reported any darkening of the BEM slant as positive, found 2% *E. coli* positive at 4 h and 20% positive at 24 h. In contrast, using a light inoculum, we found approximately 3% *E. coli* produced two to five moderately dark colonies in a lawn of negative hydrolyzers and only 0.3% true positives. Using a heavy inoculum, and counting any degree of darkening as positive, as Lindell and Quinn (6) did, we would undoubtedly have increased our positivity rate. We felt justified in reporting such strains as negative because the two to five dark colonies in a lawn of negative esculinase producers did not accurately reflect the status or ability of the culture at that time. Edwards and Ewing (2, 3) did not report hydrolysis at 24 h, but they reported results at 48 and 72 h or more. Their 48 and 72 h of incubation data are in accord with ours.

A discrepancy seemed to exist between our report of 0.3 to 0.8% positive *E. coli* in 24 h, Wasilauskas' (12) 0% positive in 24 h, and Lindell and Quinn's (6) 2% positive in 4 h and 20% positive in 24 h. A possible mechanism to explain these discrepancies was provided by Edberg et al. (1). They found that no *E. coli* was positive by either of two rapid tests or at 24 h on growth media. These results were confirmed in this study. This implied that the enzyme for esculin hydrolysis produced by *E. coli* may be inducible rather than constitutive. Edwards and Ewing's data (2, 3) added credence to this

hypothesis by reporting 30.9% positive at 48 h and 50.1% positive at 72 h of incubation.

The glucosidase responsible for esculin hydrolysis of all *Enterobacteriaceae* other than *E. coli* appeared constitutive and readily detectable by both rapid and growth tests.

The incubation data reported here are in general agreement with those reported by Edwards and Ewing (2, 3). It should be noted that the great majority of strains we used were tested within one or two subcultures after patient isolation, whereas Edwards and Ewing (2, 3) reported on strains held as stock cultures for considerable periods of time. Whether fresh clinical isolates behave differently from the same cultures tested after long periods of storage, or whether storage affects the ability of bacteria to perform in rapid tests, is not known. BEM appeared quite comparable to standard VL, but, with several strains, glucosidase detection on BEM required 24 h longer than either VL or VL-A. These instances were too infrequently encountered for statistical analysis, but in no case did a BEM slant become positive before a VL broth. In no case was the difference between BEM and VL enough to cause a taxonomic alteration.

VL-F was not useful in differentiating within the *Enterobacteriaceae* and is not included in Table 2. Andrade's indicator was originally added to VL (5, 11) to detect acid production from glucose, one of the hydrolysis products of esculin. We found the medium with ferric citrate (which detects esculetin) better than that with Andrade's indicator (which detects acid from glucose). Often VL-A tubes were positive while VL-F tubes remained negative. In no case was a VL-A tube negative when a VL-F tube was positive. Our data indicate that the addition of Andrade's indicator to VL can be discontinued.

For the rapid determination of esculin hydrolysis by the family *Enterobacteriaceae*, either the spot test or the PathoTec can be used, with only *Klebsiella-Enterobacter-Serratia*, *P. rettgeri*, and *P. vulgaris* positive. Within this group some *K. ozaenae*, *K. rhinoscleromatis*, *E. cloacae*, *E. agglomerans*, and *S. liquefaciens* were negative (Table 2).

On growth media inoculum size is critical. After 24 h on BEM, a light inoculum, produced by touching the top of a colony with a bacteriological wire, is optimum. With an inoculum consisting of a full loopful of bacteria from solid media, such as that used by Lindell and Quinn (6), a higher positivity rate may be expected. One should also read the BEM slant as close to 18 h as possible since *E. coli* glucosidase is

inducible. At 24-h incubation, with media inoculated with a bacteriological wire, results from BEM, VL, and VL-A were not statistically different (Table 2). Because BEM is also used for the identification of enterococci, we recommend it as the growth medium of choice for routine testing of *Enterobacteriaceae* in the clinical laboratory.

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