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New Diagnostic System for the Identification of Lactose-fermenting Gram-negative Rods[1]

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The identification of prompt lactose-fermenting gram-negative rods has generally relied heavily upon colonial morphology coupled with one or more indole, methyl red, Voges-Proskauer, citrate (IMViC) parameters, hydrogen sulfide, and motility. Studies were undertaken to compare diagnoses dependent solely upon the more orthodox criteria to a system for identification based upon hydrogen sulfide, ornithine decarboxylase, and citrate utilization (HOC). The results suggest that the IMViC scheme of identification is neither consistent nor applicable when applied to the current nomenclature of the above group of organisms and should be discarded, whereas the HOC system may prove to be of significant value to clinical microbiologists.

Indole, methyl red, Voges-Proskauer, citrate (IMViC) has been the standard scheme employed by clinical laboratories for the identification of prompt, lactose-fermenting gram-negative rods. The varied response patterns of this group of organisms within the IMViC outline is currently taught to the uninitiated in microbiology as dogma. In practice, the diagnosis of these organisms has become somewhat arbitrary in that clinical microbiologists do not adhere strictly to the scheme, but rather, rely heavily upon one or more IMViC criteria and couple it with colonial morphology or completely bypass the biochemical data in favor of a judgment based solely upon macroscopic observations on a selective medium.

An alternative system, which we propose in this paper for the identification of gram-negative lactose-positive rods, arose out of a study undertaken to evaluate ornithine decarboxylase in conjunction with IMViC parameters for relating the standard to the more recent nomenclature advocating the transfer of the genus *Aerobacter* to *Enterobacter* (6). Because of the variability of IMViC data, more consistent criteria were required with pertinent bearing to the proposal of Hormaeche and Edwards (6). Because uniform results were obtained with only three criteria, namely, hydrogen sulfide production, ornithine decarboxylation, and citrate utilization, we have entitled this identifying scheme "HOC." This system receives substantial support from the data of Ewing, Davis, and Edwards (3), who employed the decarboxylation of ornithine as a means of distinguishing *Enterobacter* from *Klebsiella*. They found that 99.1% of *Klebsiella* strains were ornithine decarboxylase-negative, and 94.9% of *Enterobacter* strains were ornithine decarboxylase-positive.

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MATERIALS AND METHODS

Prompt lactose-fermenting gram-negative rods (402) isolated from routine clinical specimens received in this laboratory were used in two studies: 246 strains were included in an initial study, A, and 156 comprised a later study, B. Specimens were cultured, and individual colonies selected from MacConkey Agar (MAC) were concomitantly subcultured to another MAC to ascertain purity and to a tryptic soy agar plate to serve as a source of inoculum.

Colonies were tested for the presence of deoxyribonuclease (BBL) and cytochrome oxidase (Path-O-Tec CO strips) to preclude the presence of any nonpigmented *Serratia* organisms or members of the *Aeromonas* group.

Colonies from the tryptic soy agar plate were inoculated into S I M medium (Difco) for the detection of hydrogen sulfide and indole. Simmons' formulation was employed to determine citrate utilization and methyl red (MR) and Voges-Proskauer (VP) peptone broth (Albimi Laboratories, Inc., Flushing, N.Y.) for acidification of substrate. Ornithine decarboxylase was assayed with either Falkow's (4) or Møller's (9) formulation, or both; each was overlayered with 5 mm of mineral oil to preserve anaerobiosis and was incubated 24 hr at 37 C.

RESULTS

The distribution of organisms possessing ornithine decarboxylase among IMViC-identified gram-negative lactose-positive rods is given in Table 1. A comparison of the two studies indicates that results were not consistent, and a wide range of values were obtained, with the possible exception of *Escherichia coli*. Data for *Citrobacter* in the second study should not be considered significant because the sample contained only two of the species. Reactions for *Aerobacter* ranged from 41 to 100%, whereas those for *Klebsiella* spp. were from 0 to 33%. This serves to underscore the irrelevance of ornithine decarboxylase when applied to the older nomenclature resulting from the IMViC scheme of classification.

The unusually large percentage of ornithine decarboxylase-positive *E. coli* strains (Table 1) prompted us to compare our own data with those of other investigators (Table 2). Techniques differ among the authors (3, 5, 7–9) regarding temperature, length of incubation, and assay medium; the results are a reflection of these differences. In general, our data from both studies (A and B) resulted in a higher incidence of ornithine decarboxylase-positivity for *E. coli*. Moreover, Møller's (9) and Falkow's (4) media yielded similar results, whereas Ewing, Davis, and Edwards (3) obtained a greater discrepancy.

The reactions of organisms of the newer nomenclature within the proposed HOC system are tabulated in Table 3. An obvious advantage of this organization is that fewer methods are required to complete an identification within the group. Hydrogen sulfide production was included specifically for elucidation of *Citrobacter*, whereas the absence of citrate utilization was the most crucial characteristic in the HOC scheme for *E. coli*. Ornithine decarboxylase, nonessential for the identification of the above organisms, was most applicable for separating *Klebsiella* from *Enterobacter*.

In study A, 246 strains, initially identified according to IMViC parameters, were subjected to HOC criteria and then were recatalogued (Table 4). *E. coli* and *E. freundii* remained unchanged, but the *E. freundii* strains were simply transposed to the *Citrobacter* group. Of those strains identified via IMViC, 35% (18 of 51) of *E. intermedia* strains proved to be *Klebsiella*; 12 of 55 (21%) *A. aerogenes* strains became *Klebsiella* in the HOC code. *Klebsiella* and *A. cloacae* diagnosed with IMViC criteria appeared to be in perfect agreement with tests of the new scheme; the later study (B) revealed that only 45% of the *A. cloacae* became *Enterobacter* and 66% of IMViC-identified *Klebsiella* strains retained the same appellation.

TABLE 1. *Incidence of ornithine decarboxylase among IMViC-identified gram-negative, lactose-positive rods*[a]

Organism	No. tested	No. ornithine decarboxylase-positive	Per cent ornithine decarboxylase-positive
E. coli	105 (70)	102 (59)	97 (84)
E. freundii (*Citrobacter*)	17 (2)	15 (0)	88 (0)
E. intermedia	51 (25)	33 (14)	64 (56)
A. aerogenes	55 (29)	43 (12)	78 (41)
A. cloacae	8 (24)	8 (11)	100 (45)
Klebsiella	10 (6)	0 (2)	0 (33)

[a] Numbers within parentheses indicate values of study B; other values are from study A.

TABLE 2. *Comparison of ornithine decarboxylase results for E. coli from various studies*

Reference	Medium employed	Assay method	Time of incubation	Temp of incubation	Per cent of *E. coli* strains ornithine decarboxylase-positive
Gale (5)	A[a]	CO_2 production		30 C[b]	90
Møller (7)	A[a]	CO_2 production		30 C[c]	57
Møller (8)	[d]	*p*H change	18 hr	22 C	46
Møller (9)	Møller	*p*H change	1–10 days	37 C	71
Ewing et al. (3)	Møller	*p*H change	1–4 days	37 C	51
This paper (A)	Falkow	*p*H change	1–4 days	37 C	78
	Møller	*p*H change	24 hr	37 C	97
This paper (B)	Møller	*p*H change	24 hr	37 C	84
	Falkow	*p*H change	24 hr	37 C	71

[a] Chemically defined medium containing specific amino acid substrate.
[b] Assay performed at 30 C; strains grown at 25 C for 18 hr.
[c] Assay performed at 30 C; strains grown at 25 C for 22 hr.
[d] Buffered specific amino acid substrate.

The frequency of indole-positive cultures among IMViC-characterized organisms is listed in Table 5. For *E. coli*, all strains were positive, indicating 100% correlation. However, for other genera, agreement was less than might be expected. Although there was complete absence of indole among *Klebsiella* strains in group II (organisms identified according to IMViC reactions), 7% of the *Klebsiella* strains subsequently diagnosed with HOC tests (group III) were indole-positive.

TABLE 3. *Reactions of gram-negative, lactose-positive rods utilizing the HOC outline*[a]

Organism	H	O	C
E. coli.....	0	V	0
Citrobacter.....	+	V	+
Enterobacter....	0	+	+
Klebsiella.....	0	0	+

[a] H = hydrogen sulfide; O = ornithine decarboxylase; C = citrate; + = positive; V = variable; and 0 = negative.

TABLE 4. *Distribution of IMViC-identified organisms within the HOC system*

Organism	HOC				
	No. of strains	*E. coli*	*Citrobacter*	*Enterobacter*	*Klebsiella*
E. coli.....	105	105			
E. freundii.....	17		17		
E. intermedia...	51			33	18
A. aerogenes...	55			43	12
A. cloacae.....	8			8	0
Klebsiella.....	10			0	10

All organisms other than *E. coli* demonstrated utilization of citrate.

DISCUSSION

The biochemical characterization of gram-negative rods, titled IMViC (10), originally compiled by British water microbiologists (*The Bacteriological Examination of Water Supplies*, Report 71, H.M. Stationery Office, 1934) and adapted by clinical microbiologists, has been one of the tenets underlying bacteriological practice. However, in our judgment and in the judgment of Steel (11), this device is no longer suitable nor applicable for current diagnostic microbiology and should be discarded.

Generally, the principles governing the use for classification of particular biochemical reaction(s) are its practical value within the group studied, ease with which the test can be performed, and reliability of the test. The criteria incorporated within HOC appear to be more applicable to the organisms under study.

Gale (5) reported that the production of the amino acid decarboxylases by *E. coli* is inhibited by growth temperatures of 37 C, and that optimal formation of these enzymes occurs between 20 to 26 C. Yet, Møller (7, 8) found that 46 and 57% of the strains (in another study of *E. coli*) were ornithine decarboxylase-positive when cultivated in his medium at 22 and 30 C, respectively. In direct contrast were the results he obtained with organisms grown at 37 C when 71% of *E. coli* strains were ornithine decarboxylase-positive (9). No explanation was offered for the discrepancy.

The higher incidence of ornithine decarboxylase-positive *E. coli* strains reported here could be traced to our use of clinical isolates rather than

TABLE 5. *Incidence of indole- and citrate-positive strains identified with various criteria*

Group[a]	Organism	No. tested	No. indole-positive	Per cent indole-positive	No. citrate-positive	Per cent citrate-positive
I	*E. coli*	105	105	100	0	0
	Citrobacter	17	5	29	17	100
II	*A. aerogenes*	55	6	11	55	100
	A. cloacae	8	1	12	8	100
	E. intermedia	51	14	27	51	100
	Klebsiella	10	0	0	10	100
III	*Enterobacter*	84	19	22	84	100
	Klebsiella	40	3	7	40	100

[a] (I) Organisms that retained their appellation regardless of characters employed for their identification; (II) organisms that have derived their nomenclature from IMViC reactions; and (III) those organisms identified via HOC and tested for indole.

stock cultures. Similarly, Falkow (4) found that the percentage of strains positive for lysine decarboxylase increased with the use of fresh clinical isolates. Time, temperature, and substrate medium are possible variables in the detection of ornithine decarboxylase; however, we limited the incubation period to 37 C for 24 hr. There were no essential changes in the ornithine decarboxylase reaction for *Klebsiella* and *Aerobacter* (now *Enterobacter*) after 24 hr of incubation (3). For whatever reason ornithine decarboxylase values might fluctuate among *E. coli* strains, ornithine decarboxylase is not a significant character for identification of this species in the HOC system, nor do we think it should be.

Reactions incorporated within the IMViC scheme are subject to doubt, especially when applied to organisms characterized by the more recent nomenclature. Møller (8) found that indole was present in 19% of *Klebsiella* strains, and in this study we showed that 7% of *Klebsiella* and 22% of *Enterobacter* strains were indole-positive (Table 5). When indole is related to the same strains and identified according to their biochemical reactions using the older nomenclature, 11% of *A. aerogenes*, 12% of *A. cloacae*, and 27% of *E. intermedia* strains were indole-positive. As for the reliability of MR, it has been shown that *Enterobacter* subgroup C is MR-positive, a reaction previously reserved solely for *Escherichia* (1). Also, Cowan and Steel (2) reported that one-third of *Enterobacter* strains are VP-positive at 37 C, and two-thirds are positive at 25 C.

Because of the excellent correlation of citrate results (Table 5), the reaction should be included in any diagnostic framework of gram-negative lactose-positive rods.

Thus, the criteria of IMViC should not be preserved for historic reasons, but rather, new means should be sought and enlisted for rapid diagnoses.

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