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Biochemical Differentiation of the *Enterobacteriaceae* with the Aid of Lysine-Iron-Agar

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ABSTRACT

JOHNSON, JANE G. (Harvard Medical School, Boston, Mass.), LAWRENCE J. KUNZ, WINIFRED BARRON, AND W. H. EWING. Biochemical differentiation of the *Enterobacteriaceae* with the aid of lysine-iron-agar. Appl. Microbiol. **14**:212–217. 1966.—A procedure is described for identifying members of the family *Enterobacteriaceae* isolated from clinical specimens. The methods are based on primary differentiation of the various groups of bacteria by the use of Kligler Iron Agar and lysine-iron-agar. For identification of *Salmonella*, *Shigella*, and Arizona group organisms from stools, Triple Sugar Iron Agar and lysine-iron-agar are employed. The usefulness of this schema for diagnostic bacteriology laboratories is discussed. It is not intended to replace methods used in reference or research laboratories.

A taxonomic system for the *Enterobacteriaceae* was described by Ewing and Edwards in 1960 (3). This schema seems to clarify the positions of various members in the family and, by resolving the so-called paracolons into discrete taxonomic groups, removes much of the confusion regarding family relationships.

Because of increasing interest in infections associated with gram-negative enteric bacteria, it seemed particularly desirable to adapt this system to the exigencies of routine diagnostic bacteriological practice. The use of lysine-iron-agar (2) facilitated the development of the methodology described in this report.

MATERIALS AND METHODS

Media. All of the media for isolation and preliminary differentiation of bacteria were prepared from commercially available dehydrated stocks. Other differential media were prepared and tests were performed according to methods described by Edwards and Ewing (1), with the following exceptions.

Ornithine decarboxylase test medium consisted of Decarboxylase Medium (Difco) with 1% ornithine and 0.3% agar. This semisolid medium was inoculated by a single stab and was incubated no longer than 24 hr; it did not require a paraffin oil seal. Results of decarboxylase tests with this medium compared favorably with those obtained with Moeller's medium.

Acetate agar, used to test the ability of an organism to use acetate as the sole source of carbon, was prepared and used according to Trabulsi and Ewing (6).

Indole tests. In addition to Kovacs' test for indole, a screening test was used in which a paper strip impregnated with reagent (7) was suspended from the cotton plug inside the tube of lysine-iron-agar. Because occasional paper strips gave false negative reactions, Kovacs' method was also used whenever a test for indole was pertinent to the identification of cultures which were negative by the paper strip method.

Bacteria. Known cultures, used for testing media and reagents, were from the collection of the Enteric Bacteriology Unit, Communicable Disease Center. Unknown cultures to which identification procedures were applied were isolated in the hospital's diagnostic bacteriology laboratory from all types of clinical specimens.

Taxonomy, nomenclature, and criteria for identification. The taxonomic system of Ewing and Edwards (3) was employed, and the nomenclature used by them was followed for the most part. Criteria for identification of cultures were taken from the various publications of Ewing and co-workers. These were summarized by Edwards and Ewing (1).

Preliminary examination of cultures. Whenever practical, cultures growing on an enteric differential medium (either MacConkey Agar or Shigella-Salmonella agar) were chosen for study so that the presence of more than one type of organism might be more easily recognized. Colonies to be identified were transferred to lysine-iron-agar (LIA) and then directly to either Kligler Iron Agar (KIA) or Triple Sugar Iron Agar (TSIA) without going back to the colonies. TSIA slants were inoculated with organisms isolated from stool cultures which were being examined for the presence of salmonellae and shigellae only; KIA was employed for organisms isolated from cultures of

other types of specimens in which identification of all the organisms present was required. Colonies were transferred to these media without intermediate purification. The risk of thus obtaining mixed cultures was appreciated, and occasionally such mixtures were observed. Cultures were rarely examined microscopically, and selection of organisms was made primarily on the basis of colonial morphology.

Reactions on LIA. The reactions produced on LIA by the growth of various members of the *Enterobacteriaceae* were described by Edwards and Fife (2). Although the medium was designed to detect Arizona group bacteria, its usefulness is much broader, and it is frequently of value in the recognition of other members of the family. This is especially true when it is employed in conjunction with KIA, as will be shown presently.

Of particular usefulness is the distinctive color reaction (red slant over an acid butt) resulting from the deamination of lysine, because, of the entire family, only members of the *Proteus* and Providence groups give positive reactions on LIA medium. Occasionally strains of *Proteus morganii* fail to produce evidence of lysine deamination because of weak or delayed reactions; such strains are easily recognized by their strong urease activity.

Cultures which decarboxylate lysine produce an alkaline (purple) reaction throughout the medium; lack of decarboxylase activity results in an alkaline (purple) slant and acid (yellow) butt. Intragroup differentiation, based on decarboxylation of lysine, is of practical importance in distinguishing between *Shigella* (negative) and *Escherichia coli* (usually positive); *Salmonella*, Arizona group (both positive), and *Citrobacter* (negative); and *Klebsiella* (positive) and *Aerobacter cloacae* (negative).

Identification procedures. Presumptive identification of certain members of the family can be made directly from the reactions observed on the primary differential media (KIA, LIA, and indole), sometimes with the additional aid of tests for urease activity.

For the identification of those cultures remaining unidentified by these reactions, several secondary series of differential tests were designed. The selection of the components of these secondary series was based on: (i) knowledge of the identities of the organ-

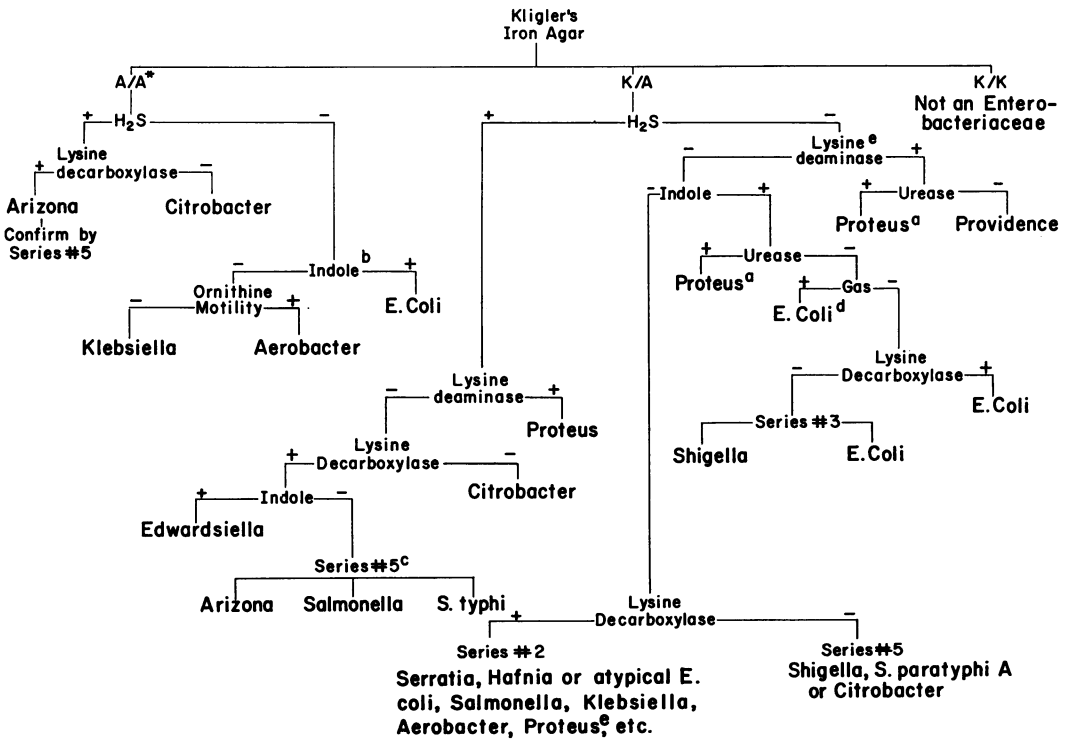

FIG. 1. *Schema for differentiation and identification of Enterobacteriaceae. *A = acid; K = alkaline; letter over oblique line = reaction on slant; letter under line = reaction in butt. [a] Use series 4 to identify Proteus to species. [b] Because Klebsiella may be indole+ and Escherichia coli may give false-negative test for indole by paper strip method, lac+ H_2S^- strains should be tested in series 1 which will also detect H_2S^- Citrobacter. [c] Strains of Citrobacter which may simulate Salmonella or Arizona on LIA may be detected by characteristic odor and biochemical series 5. [d] May also be lac− strains of Klebsiella or Aerobacter which will be recognized by series 1. (See "b" above). [e] Occasional strains of P. morganii may give weak or delayed reactions for lysine deaminase and negative or weakly positive reactions for indole on the paper test strip. Also, rare cultures of Proteus may yield equivocal reactions in LIA. Such strains will be recognized in subsequent tests as indicated.*

isms giving various patterns of reactions on the preliminary series, (ii) estimates of the frequency of occurrence of the various groups in our clinical material, and (iii) desire to keep the number of tests to a minimum without sacrificing accuracy.

After experimentation, several secondary series of biochemical tests appeared to be satisfactory.

RESULTS

Figure 1 depicts the schema for differentiation and identification of *Enterobacteriaceae* by means of their respective patterns of reaction on KIA and LIA and in the paper strip test for indole and Christensen's rapid test for urease activity.

Tables 1 through 5 list the series of biochemical tests used for identification of those organisms not fully identified by the primary differential tubes. From the reaction patterns observed in the latter, the possible identities of the unknown culture can be predicted, and the appropriate secondary series of biochemical tests can be selected for differentiating between them. The appropriate series of tests to be performed in given instances are indicated on the flow chart in Fig. 1.

TABLE 1. *Biochemical series 1**

Test or substrate	*Escherichia coli*	*Klebsiella*	*Aerobacter*	*Citrobacter*
Indole	+	−, +	−	−
Methyl red	+	−	−	+
Voges-Proskauer	−	+	+	−
Citrate	−	+	+	+
Ornithine	+, −	−	+	−, +
Motility	+	−	+	+

* Symbols: + = positive reaction; − = negative reaction; +, − = may be either + or − (the sign that appears first indicates the reaction given by the majority of strains).

TABLE 2. *Biochemical series 2**

Test or substrate	*Salmonella*	*Serratia*	*Hafnia*	*Klebsiella*	*Aerobacter*
Arabinose	+	−	+	+	+
Raffinose	−	−	−	+	+
Rhamnose	+	−	+	+	+
Sorbitol	+	+	−	+	+
Indole	−	−	−	−, +	−
Voges-Proskauer	−	+	+, −	+	+
Ornithine	+	+	+	−	+
Motility	+	+	+	−	+

* Symbols: + = positive reaction; − = negative reaction; +, − = may be either + or − (the sign that appears first indicates the reaction given by the majority of strains).

TABLE 3. *Biochemical series 3**

Test or substrate	*Shigella*	*Escherichia coli*
Acetate	−	+ (rare −)
Mucate	−	+ (rare −)
Xylose	−†	+ (rare −)
Salicin	−	+, −
Motility	−	+, −
Citrate	−	− (very rare +)

* Symbols: − = negative reaction; +, − = may be either positive or negative; usually two or more of the first four reactions are positive with *E. coli*.

† Some shigellae ferment xylose. However, the most commonly occurring types do not.

TABLE 4. *Biochemical series 4**

Test or substrate	H_2S^+		H_2S^-	
	Proteus vulgaris	*P. mirabilis*	*P. morganii*	*P. rettgeri*
Indole	+	−		
Ornithine	−	+	+	−
Citrate			−	+
Mannitol			−	+

* Symbols: + = positive reaction; − = negative reaction.

Series 1 is useful for differentiating among lactose fermenters, both typical as well as aberrant, lactose-negative (lac$^-$) forms. It separates *E. coli* from the *Klebsiella* and *Aerobacter* groups, and permits separation of members of the two latter groups on the basis of tests for ornithine decarboxylase and motility, which are included in the series.

Series 2 is useful in identifying *Serratia*, *Hafnia*, aberrant forms of *Salmonella* and *Citrobacter*, and members of the *Klebsiella* and *Aerobacter* groups. In our experience, the organisms most frequently identified by this series belong to the *Klebsiella*, *Aerobacter*, and *Serratia* groups.

Series 3 is designed principally to differentiate between *Shigella* and aberrant (nonmotile, lac$^-$, anaerogenic) *E. coli*.

Series 4 is designed for speciation of *Proteus* cultures. If the H_2S-producing capacity of the organism is known from either KIA or LIA, only the appropriate portion of the series need be employed.

Series 5 tests are used to confirm presumptive identification of *Salmonella*, *Shigella*, or Arizona group organisms on which agglutination tests with the appropriate antisera are also performed. In addition, this series helps to distinguish be-

TABLE 5. *Biochemical series 5*[a]

Test or substrate	*Salmonella* (majority)	*S. paratyphi A*	*S. typhi*	Arizona group	*Edwardsiella*	*Citrobacter*	*Shigella*[b]
Gas from glucose	+	+	−	+	+	+	−[c]
Lactose	−	−	−	D	−	D	−[d]
Sucrose	−	−	−	−	−	D	−[d]
Mannitol	+	+	+	+	−	+	D
Salicin	−	−	−	D	−	D	−
Dulcitol	+	+	− or (+)	−	−	D	D
Lysine decarboxylase	+	−	+	+	+	−	−
Motility	+	+	+	+	+	+	−
Gelatin	−	−	−	(+)	−	−	−
Urea	−	−	−	−	−	D	−
H_2S	+	−	+W	+	+	+	−
Indole	−	−	−	−	+	−	D
Methyl red	+	+	+	+	+	+	+
Voges-Proskauer	−	−	−	−	−	−	−
Citrate (Simmons')	+	+	−	+	−	+	−
Malonate	−	−	−	+	−	D	−

[a] Symbols: + = positive; − = negative; (+) = delayed; D = different biochemical types; +W = weak.

[b] Use biochemical series 3 to differentiate between *Shigella* and anaerogenic, lac⁻ nonmotile strains of *Escherichia coli* (A-D biotypes).

[c] Certain biotypes of *S. flexneri* 6 produce gas (these are indole⁻).

[d] *S. sonnei* ferments lactose and sucrose slowly.

tween *S. typhi* and other salmonellae and between the Arizona group and *Salmonella*.

Figure 2 delineates the schema used for the recognition and identification of the Arizona group, *Salmonella*, and *Shigella* from feces. Instead of KIA, TSIA is used because of the additional screening value of the third sugar, sucrose, not present in KIA. For better understanding of the flow chart, the probable identities of the organisms to be discarded as "nonpathogens" are listed in Fig. 2.

When there is high probability that a selected organism is a *Salmonella* or *Shigella*, agglutination tests and a confirmatory series of biochemical tests (series 5) are performed. In other instances, when the probability is low that a suspicious organism is an enteric pathogen, additional screening tests are used, such as indole and Voges-Proskauer tests or biochemical series 3. While this procedure occasionally delays the recognition of a rarely occurring pathogenic biotype, it eliminates extensive use of biochemical tests and antisera which would be needlessly consumed in the majority of cases.

The data in Table 6 provide a basis for estimating the relative frequencies of isolation of various members of the *Enterobacteriaceae* in a hospital laboratory. These data are comparable to other tabulations made at intervals over a 2-year period, and reflect the incidence in this hospital of the respective organisms in various types of clinical specimens other than stools. No Arizona group bacteria have been isolated from any specimens during this period. Only 1 strain of *Edwardsiella* (4) and 10 to 15 strains of *Hafnia* were recognized during the same period.

Although members of the *Aerobacter* group are not differentiated from each other routinely in this hospital, the majority of isolates of *Aerobacter*, when identified, conformed to the *Aerobacter* A subgroup (*A. cloacae*). It is noteworthy that *P. vulgaris* and *P. morganii*, said to be the most common pathogenic *Proteus* species (5), were much less frequently encountered in our clinical material than were the other two members of this group.

DISCUSSION

The taxonomic system proposed by Ewing and Edwards (3) is particularly satisfying in that it disposes of the catch-all grouping of only poorly related bacteria which have been accumulating under the designation "paracolon." With clarification of the separate status of each of the several groups of so-called paracolons, it seems timely to attempt a large-scale appraisal of the clinical significance of the respective groups of *Enterobacteriaceae* in human infections. Such an evalua-

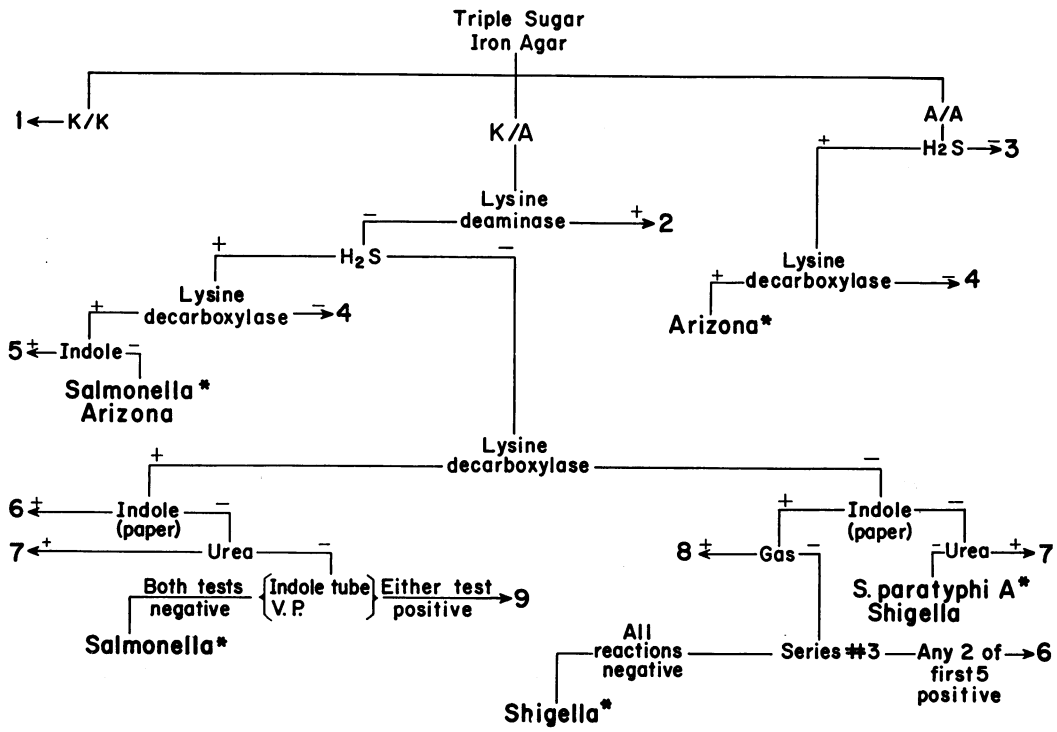

FIG. 2. *Schema for differentiation and recognition of Shigella, Salmonella, and Arizona group in stool cultures. * Confirm by agglutination test with appropriate antisera and by biochemical series 5 and, in the case of Shigella, biochemical series 3; ← or → = discard. Probable identities of discards are: (1) not an Enterobacteriaceae; (2) Proteus or Providence group; (3) Escherichia coli, Klebisella, Aerobacter, or H_2S^- Citrobacter; (4) Citrobacter; (5) Edwardsiella; (6) E. coli; (7) Proteus (lysine decarboxylase reaction may be equivocal) (8) E. coli, Proteus, Providence group, etc.; (9) E. coli, Klebsiella, Hafnia, Serratia, etc.*

TABLE 6. *Frequency of isolation of Enterobacteriaceae from urine specimens, July 1963*

Organism	Lac+	Lac−	Total
Shigella			0
Escherichia coli	282*	25	307
Salmonella			0
Arizona group			0
Citrobacter	11	3	14
Klebsiella	157	0	157
Aerobacter	17	7	24
Hafnia			0
Serratia	0	6	6
Proteus vulgaris	0	1	1
P. mirabilis	0	119	119
P. morganii	0	21	21
P. rettgeri	0	40	40
Providence group	0	14	14

* Figures refer to number of specimens from which respective organisms were isolated in significant quantities (>30 colonies).

tion is being completed, and will be the subject of a separate communication.

To what extent a system of classification such as has been proposed may be implemented at the level of clinical bacteriological practice will depend on the simplicity of media, tests, and methodology required for identification of the various members of the family. The methodology described in this report has been used in our laboratory in essentially the present form for more than 2 years. It has been found useful in identifying the large majority of cultures of *Enterobacteriaceae* isolated from clinical specimens during this period. Occasionally aberrant forms are recognized which do not fit into the schema, but usually these are easily identified by additional tests. Only rarely is there encountered an isolate which cannot be identified. It is obvious, of course, that the schema is not useful for nonfermentative gram-negative rods which do not belong to the family.

This system has been adopted with some modifications by other laboratories with apparent ease and success. From a consideration of the flow sheets and tables, the methodology may be adapted to a level of complexity consistent with the resources of individual laboratories. Consideration of the relative frequency of occurrence of the various groups and species in our experience may be helpful in influencing decisions to omit or include certain differential steps.

The degree of possible error involved at any level of modification may be determined to some extent from the tables of reactions of large numbers of the various groups and species which have been compiled and tabulated by Edwards and Ewing (1). For example, they show that 3% of Providence cultures may not be immediately identified because they do not deaminate phenylalanine (and presumably, therefore, lysine).

Similarly, atypical strains in other groups may not be readily recognized or may be incorrectly identified. Such errors of identification may be excluded by the application of more rigid criteria which are, however, impractical for most clinical bacteriological laboratories. While the methodology described here sacrifices a small degree of accuracy for savings in time and laboratory resources, it seems to provide a realistic approach to the handling of *Enterobacteriaceae* cultures in the clinical laboratory. It must be emphasized that the procedures described are intended for use in clinical or hospital laboratories only.

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