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Biochemical and Antibiotic Susceptibility Studies of H_2S-Negative *Citrobacter*

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Ninety-four strains of H_2S-negative *Citrobacter* were biochemically characterized and their antibiograms were determined. The antibiograms demonstrated not only a difference from *Enterobacter cloacae* but also a difference within the *Citrobacter* group between the indole-negative and indole-positive strains. These differences were statistically significant and emphasize the importance of the indole reaction as an aid to speciation of the H_2S-negative *Citrobacter*.

The H_2S-negative *Citrobacter* have caused a taxonomic problem for some time as is readily apparent from the excellent reviews of the literature published by Ewing and Davis (3, 4). A number of recent studies dealing solely with indole-positive strains of atypical *Enterobacteriaceae* have been published. Macierewicz (8) proposed the genus *Padlewskia* for these organisms. Washington et al. (10) studied the atypical *Enterobacter cloacae*. Fredricksen (6) proposed the name *Citrobacter koseri* for the group of atypical indole-positive organisms. Young et al. (11) would place the organisms they studied in a new genus, *Levinea*. Booth and McDonald (2) described a new indole-positive *Citrobacter* species to which they give no specific epithet. According to the descriptions of Ewing (4), most of the aforementioned H_2S-negative organisms would be included in the genus *Citrobacter*.

Because antibiograms may serve as an additional useful taxonomic tool, we determined the antibiotic susceptibility patterns of 94 strains of H_2S-negative *Citrobacter*. These patterns, as well as the biochemical characterizations, readily separated this group of bacteria from *E. cloacae* with which they have been confused in the past (4). In addition, the antibiograms reinforce the importance of the indole reaction in defining the group of bacteria as the above studies suggest.

MATERIALS AND METHODS

Seventy-five strains of H_2S-negative *Citrobacter* were isolated from clinical specimens submitted to the diagnostic microbiology laboratory of the University of Minnesota Hospitals between March 1971 and March 1972. The isolates, upon preliminary screening with triple-sugar-iron agar, motility-indole-ornithine medium, Simmons citrate, phenylalanine-urea medium, and Kovac's oxidase, appeared to be atypical members of the *Enterobacter* group of bacteria. They were submitted to further biochemical testing by the standard methods described by Ewing and Davis (5). In addition, all isolates were tested by the single, high-content disk method of Bauer et al. (1) for susceptibility to the following antibiotics: ampicillin, carbenicillin, cephalothin, chloramphenicol, colistin, gentamicin, kanamycin, naladixic acid, nitrofurantoin, streptomycin, sulfisoxazole, and tetracycline. For comparative purposes, the antibiotic susceptibilities of 231 typical *Enterobacter* species isolated during the same time period were determined for 11 of the 12 antibiotics.

An additional 19 strains of H_2S-negative *Citrobacter freundii*, kindly provided by W. H. Ewing, were similarly characterized biochemically and tested for antibiotic susceptibilities.

RESULTS

The sources of the clinical isolates are given in Table 1. The greatest number of both species of H_2S-negative *Citrobacter* were recovered from the urinary tract. *C. diversus* was isolated from the respiratory tract almost as often as from the urinary tract. The two spinal fluid isolates were from pediatric patients.

The biochemical characteristics of the 75 clinical isolates of H_2S-negative *Citrobacter* are presented in Table 2. Twenty-four of the isolates were *C. freundii* and 51 were *C. diversus*. The organisms were readily distinguished from the *Enterobacter* group of bacteria by their reactions in the indole, methyl red, Voges-Proskauer, and lysine decarboxylase tests. The reactions in adonitol and KCN served to speciate the H_2S-negative *Citrobacter*. *C. diversus* was inhibited by KCN and fermented adonitol, whereas *C. freundii* showed the opposite pattern of reactions.

The difference between the susceptibility patterns of the H_2S-negative *Citrobacter* species and those of the *Enterobacter* group is shown in Table 3. These data also include the results of those isolates received from Ewing because the overall results were not significantly different from those derived from our laboratory alone. For 10 of the 12 antibiotics tested, no significant difference was seen. All the organisms were relatively resistant to ampicillin and susceptible to most other antibiotics. However, with carbenicillin and cephalothin the *Enterobacter* group and H_2S-negative *Citrobacter* revealed differing susceptibilities. The *Enterobacter* group was susceptible to carbenicillin and resistant to cephalothin, whereas the H_2S-negative *Citrobacter* had the opposite pattern of susceptibility. The differing rates of susceptibility to these two antibiotics were statistically significant ($P < 0.001$). The scattergram of zone sizes of inhibition for the carbenicillin-cephalothin combination presented in Fig. 1 illustrates the difference between *Enterobacter* and *Citrobacter* more vividly.

Closer examination of Fig. 1 reveals a group of H_2S-negative *Citrobacter* susceptible to carbenicillin and not readily distinguishable from *Enterobacter* on the basis of zone size distribution. Reexamination of these isolates showed the majority of them (15 of 18) to be indole-negative, adonitol-negative and, therefore, *C.*

TABLE 1. *Sources of 75 clinical isolates of H_2S-negative Citrobacter*

Source	No. of strains isolated	
	C. diversus	*C. freundii*
Cerebral spinal fluid	1	1
Blood	3	1
Urinary tract	22	11
Respiratory tract	19	3
Wound or abscess	1	0
Stool	0	2
Miscellaneous	5	6
Total	51	24

TABLE 3. *Antibiotic susceptibilities of H_2S-negative Citrobacter and Enterobacter group*

Antibiotic	% Susceptible[a]		
	C. freundii	*C. diversus*	*Enterobacter*
Ampicillin	30.2	3.9	16.3
Carbenicillin	31.0	2.0	90.7[b]
Cephalothin	58.1	90.2	7.0
Chloramphenicol	100	96.1	97.7
Colistin	100	100	NT[c]
Gentamicin	100	100	100
Kanamycin	95.3	100	93
Naladixic acid	97.7	98.0	97.7
Nitrofurantoin	83.7	94.1	83.7
Streptomycin	88.4	96.1	95.3
Sulfisoxazole	81.4	98.0	93.0
Tetracycline	90.7	90.2	90.7

[a] *C. freundii*, 43 strains; *C. diversus*, 51 strains; *Enterobacter*, 231 strains.
[b] Forty-three strains.
[c] NT, Not tested.

TABLE 2. *Biochemical characteristics of 75 clinical isolates of H_2S-negative Citrobacter*

Test or substrate	*C. freundii* (24 strains)		*C. diversus* (51 strains)	
	No. positive	% Positive	No. positive	% Positive
H_2S (TSI)	0	0	0	0
Indole	15	62.5	51	100
Methyl red	24	100	51	100
Voges-Proskauer	0	0	0	0
Citrate, Simmons	21	87.5	50	98.0
Urease, Christensen	20	83.3	48	94.1
KCN	14[a]	73.7	0	0
Cytochrome oxidase	0	0	0	0
Phenylalanine deaminase	0	0	0	0
Arginine dihydrolase	23	95.8	48	94.1
Ornithine decarboxylase	15	62.5	51	100
Lysine decarboxylase	0	0	0	0
Motility	23	95.8	47	92.2
Malonate	6	25.0	42	82.4
Adonitol	0	0	51	100

[a] Only 19 strains were tested.

freundii. The indole-positive, H_2S-negative *C. freundii* biotype was readily distinguishable not only from the *Enterobacter* group but also from the indole-negative biotype of *C. freundii*.

When the indole reaction was considered in relation to the antibiotic susceptibility pattern seen with the H_2S-negative *Citrobacter*, a significant difference was seen (Table 4). More of the indole-negative strains were susceptible to ampicillin and carbenicillin than were the indole-positive strains (ampicillin: 75.0 versus 3.8%, $P < 0.001$; carbenicillin: 73.3 versus 3.8%, $P < 0.01$). On the other hand, no significant difference was demonstrated between the rate of susceptibility of *C. diversus* and the indole-positive biotype of *C. freundii* for any of the 12 antibiotics. Zone size distribution for all 12 antibiotics also failed to distinguish between the indole-positive strains.

Table 5 presents the biochemical characteristics of 94 H_2S-negative *Citrobacter* separated on the basis of their indole reactions. In general, the characteristics were little different from the breakdown previously presented (Table 2). However, three major differences in reactions —adonitol, KCN, and ornithine—were seen. The change in percent positive reactions with adonitol and KCN is to be expected since the indole-positive group of organisms includes members from both *C. diversus* and *C. freundii*.

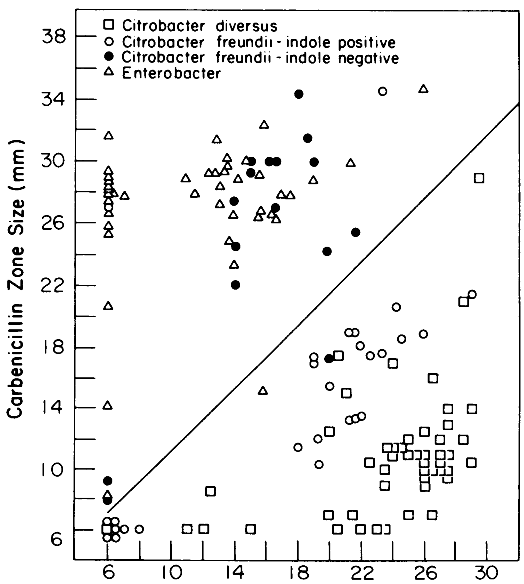

FIG. 1. *Comparison of inhibition zone sizes obtained with carbenicillin and cephalothin for H_2S-negative Citrobacter and Enterobacter.*

TABLE 4. *Relationship of susceptibility to ampicillin and carbenicillin to the indole reaction of H_2S-negative Citrobacter*

Organisms	No. of strains	% Susceptible	
		Ampicillin	Carbenicillin
Indole-positive	78	3.8	3.8
C. diversus	51	3.9	2.0
C. freundii	27	3.7	7.4
Indole-negative (*C. freundii*)	16	75.0	73.3

The third major difference was in the ornithine decarboxylase reaction which correlates closely with the indole reaction (4).

DISCUSSION

Three reports have been published on the antibiotic susceptibilities of the H_2S-negative *Citrobacter*, two on *C. diversus* (7, 10) and one on *C. freundii* (9). Because these have dealt with the two species separately, it is not surprising that the difference in antibiotic susceptibilities between the indole-negative and indole-positive biotypes has not been noted. A fourth report on indole-positive strains also gives some limited information on antibiotic susceptibilities (2). The two reports by Washington et al. (9, 10) and that by Booth and McDonald (2) appeared in the literature before Ewing and Davis's first detailed biochemical characterization was published in August 1971 (4).

Our data for *C. diversus* agrees well with that already published, showing the same resistance to ampicillin and susceptibility to cephalothin reported by Washington et al. (10) and Jones et al. (7). This pattern extends readily to include the indole-positive biotype of *C. freundii* and those indole-positive stains described by Booth and McDonald. Unfortunately, no data for carbenicillin is available for comparison with the high rate of resistance seen in our laboratory for indole-positive strains.

The increased rate of susceptibility to ampicillin seen in our group of indole-negative, H_2S-negative *C. freundii* does not agree with the data of Washington et al. (9). However, it should be noted that this report does include some indole-positive *C. freundii* (20%) which may have influenced their results significantly. The results for carbenicillin from the report of Washington et al. (80% susceptible) agree well with those presented here. It would be interesting to know if the 20% of strains in their report

TABLE 5. *Biochemical characterization of indole-positive and indole-negative strains of H_2S-negative Citrobacter*

Test or substrate	Indole-positive strains (78 strains)		Indole-negative strains (16 strains)	
	No. positive	% Positive	No. positive	% Positive
H_2S (TSI)	0	0	0	0
Indole	78	100	0	0
Methyl red	78	100	16	100
Voges-Proskauer	0	0	0	0
Citrate, Simmons	77	98.7	12	75.0
Urease, Christensen	75	96.2	10	62.5
KCN	55[a]	84.6	6[b]	85.7
Cytochrome oxidase	0	0	0	0
Phenylalanine deaminase	0	0	0	0
Arginine dihydrolase	74	94.9	14	87.5
Ornithine decarboxylase	75	96.2	0	0
Lysine decarboxylase	0	0	0	0
Motility	73	93.6	14	87.5
Malonate	58	74.4	3	18.8
Adonitol	51	65.4	0	0

[a] Sixty-five tested.
[b] Seven tested.

which showed resistance were the indole-positive strains.

The indole reaction appears to have predictive value in relation to antibiotic susceptibilities for H_2S-negative *Citrobacter*. The ornithine decarboxylase reaction has a similar predictive value because it correlates closely with the indole reaction. Perhaps these two reactions rather than the reactions in adonitol and KCN should bear more weight in the final designation of these organisms as to genus and species.

The nature of the resistance to the penicillins exhibited by these *Citrobacter* has not been investigated. A β-lactamase activity seems most probable since it is by far the most common form of bacterial attack on the penicillins. Whatever the mechanism of resistance, it would be interesting to know if it is completely lacking in the sensitive strains of the indole-negative *C. freundii* biotype. Another problem which merits further study is the resistance to ampicillin and carbenicillin seen in approximately 25% of the indole-negative biotype. Whether or not the information for this capacity is carried episomally needs to be investigated. Both conjugation and "curing" experiments would help shed light on the problem. Loss of resistance after treatment with agents such as ethidium bromide or acridine orange would lend credence to our contention that the indole and ornithine reactions may be more reliable guides to speciation than are the reactions with KCN and adonitol.

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