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Characterization of Ornithine Decarboxylase-Positive, Nonmotile Strains of the *Klebsiella-Enterobacter* Group

JOHN M. MATSEN AND DONNA J. BLAZEVIC

Department of Laboratory Medicine and Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota 55455

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Thirty-seven strains of ornithine decarboxylase-positive, nonmotile *Klebsiella-Enterobacter* organisms isolated from 36 patients were studied by biochemical and serological testing. Five strains gave biochemical reactions which conformed closely to those of *Escherichia coli*; three strains gave positive Quellung reactions to specific *Klebsiella* antisera. (Two of these were thought to be *Enterobacter* in spite of this typing reaction.) The remaining 29 strains were classified as *Enterobacter*. These results demonstrate the necessity of doing both an ornithine decarboxylase test and a motility test to differentiate *Klebsiella* from *Enterobacter*. Had only a motility test been done, they all would have been called *Klebsiella*.

With increasing frequency, diagnostic microbiology laboratories are separating strains of *Klebsiella* and *Enterobacter* into separate genera. The increased effort in separating these enteric organisms by genus is justified on the basis of epidemiological value, differences in antibiotic sensitivity patterns, and the professional satisfaction and academic value of a more complete identification. Once an organism has been determined as belonging to the *Klebsiella-Enterobacter* group on the basis of reactions on Kligler's or triple sugar iron agar and the IMVC reactions, separation into genera may be done by testing for ornithine decarboxylase production and motility (7). By definition, *Klebsiella* is nonmotile, and more than 98% of the strains are ornithine decarboxylase-negative (2, 3, 6). Greater than 95% of *Enterobacter* strains are ornithine decarboxylase-positive and motile (2, 3, 6). Organisms which are characteristic of the *Klebsiella-Enterobacter* group but which are ornithine decarboxylase-positive and nonmotile create a problem in classification. In this study, both biochemical and serological means were used to further characterize such strains in an attempt to define the genus to which they belong.

MATERIALS AND METHODS

The organisms included in this study were isolated from clinical specimens. Primary isolation was made on sheep blood-agar or eosin-methylene blue (EMB) plates. Colonies from these plates were picked to Triple Sugar Iron agar (BBL) slants, and tests for indole production, citrate utilization, ornithine decarboxylase production, and motility were done. Strains which gave indole and citrate results compatible with *Klebsiella* and *Enterobacter* and which were ornithine decarboxylase-positive and nonmotile were re-subcultured on EMB plates to determine whether a single strain was present. Colonies were then subcultured to Trypticase Soy Agar (BBL) slants for further biochemical studies.

Biochemical studies. Indol production was determined by using 1% tryptone (Difco) containing 0.5% NaCl. Cultures were incubated for 48 hr at 37 C, and the presence of indol was detected with Kovac's reagent (2). Ability to utilize citrate was determined on Simmon's citrate agar (Difco). Tests were incubated at 37 C for 4 days before results were considered negative, and all negative citrate tests were repeated at least once. Methyl Red and Voges-Proskauer tests were carried out by using M.R.-V.P. Medium (Difco). After incubation for 2 days at 37 C, the Methyl Red test was performed by the method of Edwards and Ewing (2). For the Voges-Proskauer test, 0.2 ml of 40% KOH and 0.6 ml of 5% α-naphthol in absolute ethyl alcohol was added to 1 ml of the 2-day-old culture; development of a bright red color within 4 hr indicated a positive test.

Decarboxylase tests were determined by both the Falkow method (5) and the Moeller method (8); broth media (Difco) were used with amino acid concentrations of 0.5 and 1.0%, respectively. Control broths without amino acids were also used. After inoculation, the tubes were overlaid with melted paraffin and incubated at 37 C. The Falkow broths were read at 24 hr and the Moeller broths were read at 4 days.

Motility was determined by using a semisolid

TABLE 1. *Biochemical and serological reactions of ornithine decarboxylase-positive, nonmotile strains*[a]

Strain	Indol	Methyl Red	Voges-Proskauer	Citrate	Lysine	Arginine	Malonate	Mucate	Urease	Gelatin	Lactose	Adonitol (gas)	Inositol (gas)	Glycerol (gas)	Cellobiose (gas)	Arabinose	Raffinose	Rhamnose	Salicin	Sorbitol	Quellung reaction with *Klebsiella* antiserum	Final Identification
OM 1	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	Type 8	*Klebsiella*
OM 2, 33	−	+	−	−	−	−	−	+	−	−	+	−	−	(+)	−	+	+	+	+	−	Negative	*Escherichia coli* (OM 33 was KCN+)
OM 3	−	−	+	−	−	+	+	+	+	(+)	+	−	−	−	+	+	+	+	(+)	+	Negative	*Enterobacter cloacae*
OM 4, 5, 25	−	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	Type 26, #4,	*E. aerogenes*
OM 11, 13, 26, 30, 31, 34	−	−	+	+	+	−	+	+	−	(+)	+	+	+	+	+	+	+	+	+	+	Negative	*E. aerogenes*
OM 6	+	+	−	+	−	+	−	+	+	(+)	+	−	−	(+)	+	+	+	+	(+)	+	Negative	*E. cloacae*
OM 7	−	−	−	+	−	+	−	+	+	(+)	+	−	−	(+)	+	+	+	+	+	+	Negative	*E. cloacae*
OM 8	+	+	−	+	−	−	−	+	−	−	+	−	−	(+)	−	+	+	+	−	+	Negative	*Escherichia coli* (or *Enterobacter cloacae*)
OM 9	−	−	+	+	−	+	−	+	+	(+)	+	−	−	−	+	+	+	+	+	+	Negative	*Enterobacter cloacae*
OM 10	−	−	+	−	+	−	+	+	−	(+)	−	+	+	+	+	+	+	+	+	+	Negative	*E. aerogenes*
OM 12	−	−	+	(+)	+	−	−	+	−	(+)	+	+	+	+	+	+	+	+	+	+	Negative	*E. aerogenes*
OM 14	+	+	−	+	−	+	+	+	+	−	+	+	−	+	+	+	+	+	(+)	+	Negative	*E. cloacae*
OM 15	−	+	+	+	+	−	−	−	−	(+)	−	−	(+)	+	+	+	+	−	+	−	Negative	*E. cloacae*
OM 16, 20	−	−	+	+	−	+	+	+	+	(+)	−	−	−	−	+	+	−	+	(+)	+	Negative	*E. cloacae*
OM 17, 21	−	−	+	+	+	+	+	+	+	(+)	+	−	−	−	+	+	+	+	+	+	Negative	*E. cloacae*
OM 18, 35	+	+	−	+	+	−	−	+	−	−	+	−	−	(+)	−	+	+	+	(+)	+	Negative	*Escherichia coli* (or *Enterobacter cloacae*)
OM 19	−	+	−	+	−	+	+	−	+	(+)	+	−	−	(+)	+	+	+	+	+	+	Type 39	*Enterobacter cloacae*
OM 22	+	+	−	+	+	+	−	+	−	−	−	−	−	(+)	−	+	+	+	+	+	Negative	*E. cloacae*
OM 23, 37	−	−	+	+	−	−	+	+	+	(+)	+	−	−	−	+	+	+	+	−	+	Negative	*E. cloacae*
OM 24	−	−	+	+	−	−	+	+	+	(+)	+	−	−	−	+	+	+	+	(+)	+	Negative	*E. cloacae*
OM 27	−	+	+	+	+	+	+	+	+	(+)	−	−	−	−	+	+	+	+	(+)	+	Negative	*E. cloacae*
OM 28	−	+	+	+	+	−	+	+	+	(+)	+	−	−	+	+	+	−	+	+	+	Negative	*E. cloacae*
OM 29	−	−	+	−	+	−	+	−	+	(+)	+	−	−	+	−	+	−	+	+	+	Negative	*E. aerogenes*
OM 32	+	+	−	+	−	+	+	+	−	−	+	+	−	+	+	+	+	+	(+)	−	Negative	*E. cloacae*
OM 36	+	+	−	+	−	+	+	+	+	−	−	+	−	+	+	+	+	+	−	−	Negative	*E. cloacae*

[a] All of the strains produced gas from glucose and reduced nitrate; none of the strains produced oxidase, deoxyribonuclease, phenylalanine deaminase, and none liquified gelatin within 18 to 24 hr. Symbols: +, positive in 1 to 2 days; +, positive in 3 or more days; −, negative.

medium containing nutrient broth, 0.5% 2-3-5-triphenyl-tetrazolium, and 0.5% agar; motility was also determined on S I M medium (BBL). The tests were incubated at 37 C for 2 days; positive motility was indicated by growth occurring away from the line of inoculation. Those cultures appearing nonmotile were held at 25 C for 5 more days before being recorded as negative.

Gelatin liquefaction was determined by using 12% gelatin in nutrient broth. After inoculation, gelatin tubes were incubated at 22 C and observed for liquefaction up to 30 days. An uninoculated control tube was also incubated for the same period.

Deoxyribonuclease production was determined using deoxyribonuclease test base agar (Difco). Plates were inoculated heavily in an area about the size of a dime. After incubation for 18 to 24 hr at 37 C, the plates were flooded with 1 N HCl. A clear zone around the area of organism growth indicated deoxyribonuclease production.

Malonate Broth (modified; Difco) was used to detect utilization of malonate. Cultures were read at 24 and 48 hr; a positive result was indicated by a blue color. All negative malonate tests were repeated.

Mucate utilization was determined using Organic Acid Base KP (Difco). Mucic acid was added by the procedure of Edwards and Ewing (2). Tests were incubated for 14 days before calling them negative.

Urease production was determined by using Christensen's urea agar slants (1). The tests were incubated at 37 C for four days; a positive reaction was indicated by a red color on the slant.

For oxidase production, the cytochrome oxidase test as modified by Ewing and Johnson (4) was used. Cultures were tested after incubation for 18 to 24 hr at 37 C on nutrient agar slants; a blue color was evidence of oxidase production.

Tests for phenylalanine deaminase production were done by using phenylalanine agar slants (Difco). After incubation at 37 C for 18 to 24 hr, a few drops of 10% (w/v) ferric chloride were allowed to run down the slant. The formation of a green color on the slant indicated that phenylalanine deaminase was produced.

Carbohydrate fermentation studies were made in Purple Broth Base (Difco); Durham tubes were used to detect gas formation. Lactose was added in a concentration of 1%; all other sugars were used at a concentration of 0.5%. Lactose solutions were sterilized by autoclaving at 118 C for 10 min. Arabinose and sorbitol were sterilized by filtration and were added aseptically to the broth base. All other sugar broths were autoclaved at 121 C for 10 min. After inoculation, the fermentation broths were incubated at 37 C for 10 days before recording the test as negative.

For nitrate reduction, organisms were inoculated into Nitrate Broth (Difco) and were incubated at 37 C for 18 to 24 hr. After this time, a few drops of 0.8% sulfanilic acid in 5 N acetic acid and a few drops of 0.5% alphanaphthylamine in 5 N acetic acid were added to the culture. Development of a red color within a few minutes indicated the reduction of nitrate to nitrites.

Selected strains were tested for ability to grow in the presence of KCN. The medium was prepared using KCN broth base (Difco); the KCN was added according to the manufacturer's directions. A tube consisting of the base without KCN was used as a growth control. The tubes were inoculated lightly and incubated at 37 C for 2 days.

Serological studies. For completeness of characterization, all strains were tested serologically by using type-specific *Klebsiella* antisera. The majority of the strains reported here were typed in the *Klebsiella* laboratory of the National Communicable Disease Center through the courtesy of William E. Ewing and with the assistance of Mary Alyce Asbury. A number of strains were tested at the University of Minnesota with typing sera produced there to the 72 types of *Klebsiella*. These typing sera were checked for specificity at the NCDC by J. M. Matsen, again with the assistance of Mary Alyce Asbury and through the courtesy of Dr. Ewing. The method used for typing was described by Edwards and Ewing (2). Strains were grown overnight on Worfel-Ferguson agar (Difco). A heavy suspension of organisms was then made in 0.5% phenolized saline for doing preliminary agglutination reactions, and a very light suspension was made from the heavy suspension for purposes of testing for Quellung reactions.

RESULTS

From September 1966 to September 1968, a total of 2,137 strains of *Klebsiella-Enterobacter* (1,682 *Klebsiella* and 455 *Enterobacter*) were isolated from pediatric patients at the University of Minnesota hospitals. Of these, 27 were ornithine decarboxylase-positive and nonmotile, for an incidence of 1.3% overall and an incidence of 5.9% of *Enterobacter* strains. Ten additional strains from adult patients demonstrated similar characteristics and are included in this study. The 37 strains studied represent isolates from 36 patients. The one patient who furnished two strains had two biochemically different isolates.

These 37 strains produced ornithine decarboxylase with both of the methods used; all were nonmotile, produced acid and gas from dextrose, and reduced nitrates. None of the organisms produced deoxyribonuclease, oxidase, or phenylalanine deaminase and none liquified gelatin in 18 to 24 hr. Most of those which did liquify gelatin did so after 21 days. The results of these and other biochemical tests are given in Table 1. Strains giving similar reactions are grouped together. These results were compared to the results obtained with the tribe *Klebsielleae* by Ewing (3), and our interpretation of organism identification is given in Table 1. Biochemically, one strain resembled *Klebsiella*, 31 appeared to be *Enterobacter*, and five of the strains had biochemical reactions which conformed more closely to those of *Escherichia coli* (2, 3) than to the tribe (division) *Klebsielleae*. When these five strains were tested for growth in KCN broth, one strain was

positive, but it was our interpretation that the overall biochemical results of this strain still most closely resembled *E. coli*.

Of the 37 strains tested, only three (8.1%) gave positive Quellung reactions with the *Klebsiella* antisera. One of these, OM #1, gave a strong Quellung reaction with type 8 *Klebsiella* antiserum and was also thought to be *Klebsiella* biochemically. OM #4 was thought to be *Enterobacter aerogenes* biochemically, but it gave positive Quellung reactions with absorbed type 26 *Klebsiella* antiserum. Strain OM #19, thought biochemically to be an *E. cloacae*, gave a positive Quellung with type 39 *Klebsiella* antiserum.

The remainder of strains were minimally capsulated or noncapsulated, gave nonspecific "O" aggutination, and were nonreactors by Quellung testing to the battery of *Klebsiella* antisera.

DISCUSSION

It is apparent from the data presented here and also from those of others (3, 6) that some organisms of the *Klebisella-Enterobacter* group cannot be classified easily on the basis of the few biochemical tests usually done in the diagnostic microbiology laboratory. However, it would appear that the great majority of strains of this group which are ornithine decarboxylase-positive and nonmotile should be classified in the genus *Enterobacter*. This is substantiated by the fact that only one of the 37 strains studied by extensive biochemical and serological testing was thought to be a *Klebsiella*. This would also be somewhat in agreement with the work of Ewing (3), who found no *K. pneumoniae* that were ornithine decarboxylase-positive. According to his data, the only *Klebsiella* which might be positive for this characteristic is *K. ozaenae*, which includes strains of *Klebsiella* serotypes 4, 5, and 6. Ewing (2) reports 4% of *K. ozaenae* to be ornithine decarboxylase-positive.

By biochemical testing alone, we would have called one of the study strains *Klebsiella*. This strain was subsequently shown to have a positive Quellung reaction to type 8. One strain which typed with type 26 antisera and one strain which typed with type 39 antisera would have been called *Enterobacter* by biochemical testing alone. In this regard, Edwards and Fife (*unpublished data* as reported in reference 2) found biochemically typical motile cultures of *E. aerogenes* and *E. cloacae* which possessed prominent capsules. The majority of their strains gave positive agglutination and Quellung tests with types 8, 11, 21, 26, and 69 *Klebsiella* antisera. Therefore, it is probable that the strain which biochemically resembled *Enterobacter*, even though nonmotile, but which gave a positive Quellung to type 26 antiserum is in reality *Enterobacter*, as well as the type 39 strain. Even though certain strains typing with type 8 antiserum were thought by Edwards to be *Enterobacter* on the basis of biochemical testing, we consider the strain typing with type 8 in our study to be truly a *Klebsiella*.

It is perhaps also worth noting here that Edwards and Ewing (2) report 0.3% of *Escherichia coli* as being both indole- and citrate positive and 52% of *E. coli* as being ornithine decarboxylase-positive. When these same investigators tested *E. coli* for growth in KCN broth, they found 1% to be positive. The three citrate-positive strains classified here as *E. coli* represent much less than 0.1% of the total number of *E. coli* isolated in our laboratory during the 2-year period of this study. After concluding that an organism is of the *Klebsiella-Enterobacter* group, it would seem, in light of the results of this study and the fact that about 5% of *Enterobacter* species may be ornithine decarboxylase-negative (2, 3, 6), that it is necessary to do the ornithine decarboxylase and motility tests in tandem when attempting to most accurately differentiate *Klebsiella* and *Enterobacter* in the diagnostic laboratory. The great majority of the nonmotile, ornithine-decarboxylase-positive strains did turn out to be *Enterobacter* species; if a motility alone had been used to identify these strains initially, they would have been called *Klebsiella*.

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