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THE DIFFERENTIATION OF COLIFORM ORGANISMS INFECTING THE URINARY TRACT

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In the study of the bacteriology of water it is customary to divide the coliform organisms into several species and subspecies, and the practical value of this subdivision is well proved. In the clinical laboratory the detailed investigation of such strains from urinary infections is not generally practicable. The work reported here suggests that differentiation into species in this manner is both practicable and productive of valuable clinical information. A high incidence of *Bact. aerogenes* strains in infections of paralysed bladders of paraplegic patients was found by Warner (1948) and a similar incidence in obstructive conditions of the urinary tract was reported by Coleman and Taylor (1949): the *Bact. aerogenes* strains in each series were more resistant to penicillin and sulphonamides than were the *Bact. coli* strains.

While it is easy to distinguish between the typical *Bacterium coli commune* of Escherich and the *Bacterium lactis aerogenes* of Escherich there are a number of strains with varying characters which cannot properly be classified without extensive study for which time is not generally available. The problem of naming these organisms is also considerable since the rejection of the generic name *Bacterium* (Judicial Commission, 1954). Kauffmann (1951) has put a strong case for the differentiation of these organisms into two genera, *Escherichia* and *Klebsiella*, and has indicated the value of the fermentation of adonitol and inositol in making this differentiation. In considering which nomenclature to follow we have been guided by the report of the *Coli-aerogenes* Subcommittee of the Society for Applied Bacteriology (1956) which avoids the problem of the location of the intermediate strains by placing them in a separate genus, *Citrobacter*; we have therefore used the generic terms *Escherichia*, *Citrobacter*, and *Klebsiella* as defined in this report. The major points of distinction are shown in Table I.

For some years in this laboratory coliform organisms have been roughly classified as *Escherichia* or *Klebsiella* according to the 44° C. MacConkey test. This survey was undertaken (*a*) to assess the value of the 44° C. test and fermentation of adonitol and inositol in the rapid identification of urinary coliforms, and (*b*) to confirm the clinical value of such differentiation in diagnosis and treatment.

Materials and Methods

The strains examined were Gram-negative, lactose-fermenting coliform bacilli recovered from specimens of urine sent for examination at the laboratories of King's College Hospital and Lewisham Hospital. A centrifuged deposit of each specimen was examined and only organisms associated with clinical and cytological evidence of urinary infection were included in the survey. A higher proportion of citrate-positive strains was retained for study so as to obtain approximately equal numbers of citrate-positive and citrate-negative strains.

Biochemical Tests

(a) MacConkey Broth at 44° C. (Ministry of Health, 1939). This was used in a modified Eijkman test.

(b) Methyl Red (M.R.) Test.—Glucose phosphate broth was incubated at 37° C. for 48 hours before the addition of 4 or 5 drops of 0.04% methyl red solution.

(c) Voges Proskauer (V.P.) Test.—The production of acetyl-methyl-carbinol was tested for in glucose phosphate broth by O'Meara's method (Wilson and Miles, 1955).

(d) Production of Indole.—A 48-hour peptone water culture was extracted with xylol, and tested with Ehrlich's rosindole reagent.

(e) Citrate Utilization.—If growth was obtained in Koser's citrate medium a second tube of medium was inoculated from the first; the test was not considered positive unless growth occurred on the second occasion.

(f) Fermentation of Adonitol and Inositol.—This quality was investigated in peptone water with Andrade's indicator and 1% carbohydrate.

(g) Tests of Antibiotic and Sulphonamide Sensitivity.—A ditch plate was used for testing sensitivity to penicillin, the ditch containing 100 units/ml. Sensitivity to streptomycin, chlor- and oxy-tetracycline, and chloramphenicol

was tested by the filter paper strip method, the filter paper being soaked in solutions containing 1,000 μg./ml. of the antibiotic. A number of strains were tested also by a tube dilution technique. Of these, strains assessed as "resistant" by the diffusion method grew at concentrations of 1,000 μg./ml. of streptomycin, of 100 μg./ml. of chloramphenicol, and of 10 μg./ml. of the tetracyclines, while "sensitive" strains were inhibited by 5 μg./ml. of streptomycin, by 10 μg./ml. of chloramphenicol, and by 5 ug./ml. of the tetracyclines. For this reason the strip method was considered adequate for routine use.

Sensitivity to sulphathiazole, sulphadiazine, and sulphanilamide was tested on the medium of Jewell and Pearmain (1954), using Evans "sentest" tablets. Since with very few exceptions an organism was either sensitive to all these sulphonamides or to none, sensitivity has been recorded as "sensitive or resistant to sulphonamide," the few strains which gave conflicting answers to tests with one or more sulphonamides being assessed as "sensitive."

(h) Virulence Tests.—A limited number of strains were tested for virulence to mice; 0.2 ml. of tenfold dilutions in distilled water of an overnight 20% horse serum broth culture was mixed with an equal quantity of mucilage of tragacanth B.P. and inoculated intraperitoneally into each of three mice; these were observed for a week, unless death occurred earlier. In most instances the number of viable organisms in the inoculum was estimated by the method of Miles and Misra (1938).

Results

Seventy-three citrate-negative and 63 citrate-positive strains were examined by the tests recommended by the Ministry of Health (1939) for the classification of coliform bacilli in water supplies. The results are shown in Table I.

TABLE I

CHARACTERISTICS AND NOMENCLATURE OF COLIFORM ORGANISMS

Citrate	M..R	V.P.	Indole	44° C. Test	Gelatin Liquefaction	Wilson *et al.* (1935)	Nomenclature Cttee. (1956)	No. of Strains
−	+	−	+	+	−	*Bact. coli* Type I	*Esch. coli* I	66
−	+	−	−	−	−	*Bact. coli* Type II	*Esch. coli* II	0
−	+	−	+	−	−	Irregular I Coli-like I	*Esch. coli* III	7
+	+	−	−	−	−	Intermediate type I	*Citrobacter freundii* I	8
+	+	−	+	−	−	Intermediate type II	*Citrobacter freundii* II	12
+	−	+	−	−	−	*Bact. aerogenes* Type I	*K. aerogenes* I	33
+	−	+	+	−	−	*Bact. aerogenes* Type II	*K. aerogenes* II	10
+	−	+	−	−	+	*Bact. cloacae*	*K. cloacae*	0
+	−	+	−	−	+	−	*Erwinia carotovora**	0
							Total:	136

* Characterized by pectin liquefaction.

Of the citrate-negative, methyl-red-positive strains, 66 out of 73 were of the classical *Escherichia coli* I; though *Klebsiella aerogenes* I predominates in the citrate-positive group, about a third were methyl-red-positive intermediate strains or *Citrobacter freundii*.

The fermentation reactions of a second series of 74 citrate-negative and 41 citrate-positive organisms to adonitol and inositol are given in Table II; the finding that the majority of citrate-positive strains ferment both adonitol and inositol is in agreement with that of Kauffmann.

TABLE II

ADONITOL AND INOSITOL FERMENTATION

	Adonitol		Inositol	
	Positive	Negative	Positive	Negative
Citrate-positive ..	38 (92·7%)	3 (7·3%)	38 (92·7%)	3 (7·3%)
Citrate-negative ..	3 (4·1%)	71 (95·9%)	2 (2·7%)	72 (97·3%)

The sensitivities to five antibiotics and to sulphonamide are shown in Table III. The sensitivities of the two groups of citrate-positive strains (*Klebsiella* and *Citrobacter*) are very similar and differ markedly from those of the citrate-negative *Escherichia* with respect to streptomycin, chloramphenicol, and sulphonamide.

TABLE III

SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS

Drug	Citrate-positive						Citrate-negative		
	Klebsiella Sensitive			*Citrobacter* Sensitive			*Escherichia* Sensitive		
	Total	No.	%	Total	No.	%	Total	No.	%
Penicillin ..	22	0	—	19	0	—	53	10	18·9
Streptomycin	40	5	12·5	19	1	5·2	122	107	87·8
Chloramphenicol	40	16	40	19	6	31·6	122	119	97·5
Aureomycin ..	40	40	100	19	19	100	122	116	95·1
Terramycin ..	31	31	100	5	5	100	122	122	100
Sulphonamide	18	1	5·6	17	3	17·6	57	51	89·5

Mouse Virulence

Ten strains of *K. aerogenes* 1 and 12 strains of *Esch. coli* I were tested. Viable counts were only made on the inocula of eight strains with the results shown in Table IV. A median lethal dose for each strain was calculated by the method of Irwin and Cheeseman (1939). The lethal dose of the *Esch. coli* strains is more uniform than that of the *K. aerogenes* strains, which varies very widely.

In spite of the variation it would appear that under these conditions *Esch. coli* strains are more virulent

for mice than *K. aerogenes* strains. This was confirmed by an additional experiment in which at a 10^{-2} dilution of an 18-hour broth culture 70% of 30 mice inoculated with 10 strains of *Esch. coli* were killed whereas all mice injected with 12 strains of *K. aerogenes* survived.

TABLE IV
AVERAGE MEDIAN LETHAL DOSES

Organism	Viable Count (Organism ml.)	Death Rate					L.D. 50 (Organism ml.)
		Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
E3810 ..	$15{\cdot}0 \times 10^8$	—	—	3 3	0 3	0 3	$4{\cdot}7 \times 10^7$
E3814 ..	$3{\cdot}0 \times 10^8$	3 3	2 3	0 3	—	—	$1{\cdot}9 \times 10^7$
E3857 ..	$6{\cdot}9 \times 10^8$	3 3	2 3	1 3	—	—	$2{\cdot}2 \times 10^7$
E4191 ..	$8{\cdot}5 \times 10^8$	—	3 3	2 3	1 3	—	$5{\cdot}8 \times 10^7$
						Average L.D. 50	$3{\cdot}65 \times 10^7$
K3726 ..	$12{\cdot}5 \times 10^8$	3 3	0 3	0 3	—	—	$394{\cdot}5 \times 10^7$
K4042 ..	$2{\cdot}6 \times 10^8$	3 3	3 3	0 3	—	—	$8{\cdot}5 \times 10^7$
K4003 ..	$2{\cdot}4 \times 10^8$	3 3	0 3	0 3	—	—	$75{\cdot}0 \times 10^7$
K. Cherry	$3{\cdot}1 \times 10^8$	3 3	0 3	0 3	—	—	$98{\cdot}8 \times 10^7$
						Average L.D. 50	$144{\cdot}2 \times 10^7$

The sign — signifies no test done at this dilution.

Clinical Findings

The clinical histories of an approximately equal number of patients infected with citrate-positive and citrate-negative strains were examined and the results are shown in Table V.

TABLE V
RELATION TO UROLOGICAL DISEASE OF GENUS OF INFECTING ORGANISM

Diagnosis	Citrate-positive	Citrate-negative
Enlarged prostate	25	3
Neurological bladder	3	1
Post-operative	11	12
Surgical urological disease ..	12	11
Medical conditions	8	30
Diagnosis unknown	16	18
Total	79	76

The term "enlarged prostate" is applied to benign and malignant enlargements and refers to infections occurring both before and after prostatectomy, and this point is further discussed below. "Post-operative" infections were mostly gynaecological, but included all except those occurring after urological surgery, while "medical conditions" comprised pyelitis, pyelonephritis, and cystitis not included under the other headings. There was no apparent difference between the incidence of *Citrobacter* and *Klebsiella* strains. In approximately 80% of cases these strains occurred as the sole infecting organism.

Discussion

Two points are obvious from these results; citrate-positive and citrate-negative urinary strains differ not only in their range of susceptibility to chemotherapeutic agents, but also in their association with certain types of clinical condition.

Our results confirm those of Warner and of Coleman and Taylor that the citrate-positive group most commonly appears in association with lesions which impede urinary outflow and permit a state of stagnation to be set up in the bladder.

It may well be that conditions such as these must exist before a less virulent organism can establish itself. Our mouse virulence tests suggest that *Klebsiella* are less virulent than *Escherichia*. On the other hand, it is difficult to understand why the opportunities for cross-infection with *Klebsiella* and *Citrobacter* should be greater than those with *Escherichia*; the reservoir of *Escherichia* in the gut of all healthy adults is incomparably greater than that of the citrate-positive organisms, which are found in a much lower proportion of human faeces, and are almost invariably greatly outnumbered by the citrate-negative coliforms. One reason may well be the greater resistance of the citrate-positive group to antibiotics; protective chemotherapy with penicillin and streptomycin, for instance, is now commonly used at prostatectomy. The greater ability of *Klebsiella* to survive desiccation and exposure to sunlight has been postulated by Ørskov, Ørskov, and Paerregaard (1956) to explain their finding of a high instance of cross-infection with citrate-positive strains amongst infants and young children in the wards of two Danish hospitals in spite of the predominance of *Escherichia* in the faecal flora, though they state that they have no direct evidence for this hypothesis.

However these infections occurred, the distinction between citrate-positive and citrate-negative organisms is valuable from a clinical standpoint. The complete classification is time-consuming, but the close correspondence of *Citrobacter* to *Aerogenes* in our findings permits the broad division into groups, *Escherichia* and *Klebsiella-Citrobacter*. The citrate test using Koser's medium may take at least three days to perform; Simmons' citrate agar is generally considered a less critical test of citrate utilization. The 44° C. test gives a more rapid result and is theoretically excellent, especially if put up directly from the original specimen as suggested by Enticknap and Stephens (1951). Our experience with it, however, has been unsatisfactory on several grounds. One disadvantage is that it has been designed for detecting *Coli* I and will only identify

a proportion of the *Escherichia* strains; also little margin is allowable in the temperature of the water-bath, and minor faults of the thermostat or stirring mechanism may give rise to false results. In a more recent survey as many as 24% of citrate-positive strains gave apparently positive 44° C. tests and only 61 (80.3%) of 76 of citrate-negative strains were positive to this test. The methyl red and Voges Proskauer tests will not distinguish between *Citrobacter* and *Escherichia* and, in any case, are also very prodigal of time.

TABLE VI

RESULTS OF INOSITOL AND ADONITOL FERMENTATION WITH 74 CITRATE-NEGATIVE AND 40 CITRATE-POSITIVE STRAINS

Inositol and Adonitol	Citrate-negative	Citrate-positive
Both +	1/74 (1·4%)	37/40 (92·5%)
+/− or −/+	3/74 (4·1%)	0/40 —
Both −	70/74 (94·6%)	3/40 (7·5%)

A test employing the fermentation of adonitol and inositol is free from these disadvantages, and at 24 hours detected accurately 92.5% of aerogenes and 98.7% of coli strains, as may be seen from a consideration of Table VI. A strain which ferments one sugar only may be presumed to be an *Escherichia*.

This test has been used satisfactorily in routine work in this laboratory for the last six months; it seems to be the most rapid and reliable test available until rapid methods for testing citrate utilization are proved reliable; Simmons' citrate agar is in course of study.

Summary

The methods available for classifying coliform bacilli are not particularly suitable for routine use in a clinical laboratory. The value of accurate classification has been examined, with particular reference to the pathological conditions of the urinary tract in which the various types of coliform bacilli are found, and to the sensitivity of the organisms to chemotherapeutic agents, and a differential test employing the fermentation of adonitol and inositol has been investigated. It is concluded that there is value in distinguishing between coliform types, as they frequently occur under different conditions and have a widely differing susceptibility to drugs; the fermentation test combines the advantages of speed and considerable reliability and would appear to be the method of choice until rapid methods of testing citrate utilization are available.

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