A BIOCHEMICAL STUDY OF 141 STRAINS OF ENTEROBACTERIACEAE ISOLATED FROM TISSUES AND FLUIDS OF ANIMAL ORIGIN

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SUMMARY.

The following tests were performed on 141 strains of Enterobacteriaceae isolated from a variety of animal tissue and fluid submitted for bacteriological examination: motility, acid production from glucose, mannitol, adonitol, dulcitol, inositol, lactose, salicin and sucrose, gas production from glucose, indole production, gelatin liquefaction, hydrogen sulphide production, methyl red and Voges-Proskauer test, urea hydrolysis, growth in potassium cyanide and Koser's citrate, and deamination of phenylalanine.

The strains were grouped as Salmonellae 15, Arizona 2, Escherichia coli **19, Alkalescens-Dispar 8,** Escherichia freundii **2, Bethesda-ballerup 13,** Klebsiella ozaenae **3,** Klebsiella pneumoniae **19,** Klebsiella **sp. 12, Cloaca 27, Providence 2 and ungrouped 19.**

The importance for further studies and pathogenicity assessment on lesser known strains of Enterobacteriaceae is stressed.

I. INTRODUCTION.

The family Enterobacteriaceae contains genera in which are included bacteria ranging from those with no recognised pathogenicity for animals to those with marked pathogenicity. Many possessing little or no pathogenicity as primary pathogens are important as secondary invaders. However, there are also numerous bacteria in this family whose pathogenicity has not been adequately assessed and as the infections caused by them are not as common as those produced by Salmonellae and Shigellae little attention has been given to their isolation and identification. Examples are bacteria of the Providence and Bethesda-ballerupensis groups.

In veterinary medicine, Salmonellae are recognised as potential pathogens and most veterinary diagnostic laboratories have long since adopted techniques for the selective isolation of these bacteria from animal tissues and fluids which are usually grossly contaminated with bacteria of intestinal or environmental origin. Considerable attention has also been given to the pathogenicity of biochemical and antigenic types of *Escherichia coli,* especially in the pathogenesis of infectious scours in calves.

The publication in 1956 (Cowan 1956) of a scheme separating the members af the Enterobacteriaceae into groups on biochemical characteristics suggested that it may be possible to pay more attention to the lesser known pathogens using methods consistent with the speed and economy necessary

in a diagnostic laboratory. Cowan's scheme is based to a large extent on the results obtained in the study of strains of human origin. It was considered desirable therefore to determine whether the tests described would be of value when employed for the identification of organisms isolated from specimens of animal tissues and fluids submitted to this Institute, especially those belonging to the groups Providence, Klebsiella, Bethesda-ballerupensis and Arizona.

This note describes the application of a limited range of biochemical tests to 141 strains belonging to the family Enterobacteriaceae.

II. METHODS.

(1) Source of Strains.

Strains were isolated on blood, MacConkey and Salmonella-Shigella agar used during the routine examination of specimens submitted to this laboratory. Special attention was given to strains of Enterobacteriaceae in samples of milk from cases of bovine mastitis, to strains that preliminary tests suggested were Salmonellae but did not agglutinate Salmonella group B, C, D or E antisera, and to strains suspected to be species of Klebsiella.

The strains were considered to belong to the family if they were gram-negative, non-sporing rods, which grew readily in media containing only meat extract or peptone, and fermented glucose.

(2) Examination of Strains.

The tests employed were those considered to be adaptable to facilities in this laboratory, and to enable biochemical characterisation of strains in the manner described by Cowan (1956), viz:—

Motility.—This was determined by microscopic examination of 6-and 24-hour broth cultures by the dark field method of illumination. In a few instances the semisolid medium method (Edwards and Ewing 1955) was used to check results obtained by the microscopic method.

Fermentation Tests.—Acid production from glucose, mannitol, adonitol, dulcitol, inositol, lactose, salicin and sucrose was determined using peptone broth containing 1 per cent. of the test substance with Andrade's solution as indicator. Durham tubes were included to detect gas production in tubes containing glucose. Media were incubated up to 21 days before being discarded as negative.

Indole Production.—This was detected by the addition of Ehrlich's reagent after the organisms had grown in tryptone broth for 24 hours at 37 deg. C.

Gelatin Liquefaction.—Nutrient gelatin stabs were incubated at 37 deg. C. for 21 days then cooled to about 4 deg. C. before noting whether liquefaction had occurred.

Hydrogen sulphide (H_2S).—Lead acetate impregnated filter papers were used to detect hydrogen sulphide production from cultures grown on nutrient agar slopes at 37 deg. C. for seven days.

Methyl Red Test (MR).—Methyl red solution was added to buffered glucose phosphate broth cultures after incubation at 37 deg. C. for three days.

Voges Proskauer Test (VP).—This was done on 3-day cultures of buffered glucose phosphate broth using Barritt's (1936) method.

Urease.—Urea hydrolysis was detected by Christensen's agar method (Christensen 1946).

Potassium cyanide Growth Test (KCN).—Braun's KCN medium as put forward by Moeller and described by Edwards and Ewing (1955) was used. The medium was deep frozen at —25 deg. C. and thawed immediately before use.

Growth in Koser's Citrate Medium.—The medium used was that described in Standard Methods for the Examination of Water and Sewerage (American Public Health Association 1946).

Phenylalanine Test.—The method used was that described by Henricksen (1950).

III. RESULTS.

One hundred and sixty-three isolates were collected for examination, but 22 subsequently proved either not to belong to the Enterobacteriaceae or gave positive tests for urea hydrolysis and phenylalanine deamination and were considered to be strains of Proteus. Proteus strains were not further considered.

Table 1.

SOURCE OF STRAINS.

Biochemical Grouping.	Animal Species.							
	Fowls.	Ducks.	Cockatoos.	Pigs.	Horses.	Cattle.	Dogs.	Sheep.
Salmonella	6	..	..	2	..	6	..	..
S. pullorum	1	..	..	..	..	..	..	..
Arizona	1	..	..	..	..	1	..	..
Escherichia coli	3	3	..	7	2	4	..	..
A–D group	7	..	..	1	..	..	..	..
E. freundii	..	..	..	..	..	2	..	..
Bethesda-ballerup	5	..	..	5	1	2	..	..
K. ozaenae	2	..	..	..	..	1	..	..
K. pneumoniae	5	..	2	1	..	9	1	1
Cloaca	7	..	..	4	..	16	..	..
Providence	..	..	..	..	..	2	..	..
Klebsiella sp.	..	..	1	..	..	11	..	..
Ungrouped	4	1	..	9	..	4	1	..
Total	41	4	3	29	3	58	2	1

Table 1 gives details of the number studied in each group and the animal species from which they were isolated. The biochemical characteristics of the strains are given in Tables 2, 3 and 4

Details of strains in individual groups other than Proteus are considered in the order of groups given by Cowan (1956). No isolates were placed in the categories of *Salmonella typhi, Shigella sonnei,* other Shigellae, *Klebsiella rhinocleromatis* or Hafnia.

(*a*) *Salmonella.*

Strains 28, 29, 30, 37, 40, 59, 60, 61, 62, 123, 124, 130, 137, 138 were Salmonellae.

These fifteen strains were only part of those isolated at this laboratory during the period in which this work was done. In many instances they were included as they did not agglutinate any of the antisera to Salmonella groups B, C, D, or E, although they conformed to the pattern of Salmonella in the biochemical tests used routinely* at this Institute.

Fourteen strains gave the results listed by Cowan (1956), except that Strains 28, 29, 37, 40, 59, 60, 61, 62, 123, 127 did not produce hydrogen sulphide. Strains 130 and 138 produced only slight blackening of the lead acetate paper and Strain 124 produced this only after three days' incubation.

Strain 146 was *S. pullorum* with characteristics as given in Table 2.

(*b*) *Arizona.*

Strains 43 and 45 gave reactions as listed by Cowan (1956) for this group. Strain 45 liquefied gelatin only after seven days' incubation. Strain 43 was isolated from a bovine liver and strain 45 from a chicken lung. The clinical and post-mortem results in both cases did not indicate that these strains were the cause of sickness.

(*c*) *Escherichia coli.*

Strains 3, 27, 32, 42, 44, 46, 56, 57, 70, 71, 73, 74, 76, 103, 120, 128, 129, 132, 134 were considered to be *E. coli.* Included are three strains, Nos. 132, 133 and 134, isolated from the heart, bone marrow and liver respectively, of a duck. These strains were non-motile and encapsulated. Another non-motile strain, No. 120, was isolated from the uterus of a pig. Strain 71 fermented adonitol within 24 hours. Considerable variation occurred in the fermentation of dulcitol, salicin and sucrose, and the ability to produce hydrogen sulphide.

(*d*) *Alkalescens-Dispar Group.*

Strains 4, 5, 6, 7, 8, 9, 14 and 16 belonged to the alkalescens-dispar group. Strain 16 produced acid from both lactose and dulcitol and strain 14 produced acid from lactose. Strains 4, 5, 6, 7, 8, 9 and 14 were isolated from one batch of chickens submitted by one owner.

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* In routine tests, any gram negative bacillus producing acid and gas from glucose, fermenting mannitol and maltose, but not fermenting sucrose or lactose or producing indole is considered to be Salmonella and additional proof is sought by doing the slide agglutination test with Groups B, C, D, and E Salmonella antisera.

Table 2.

BIOCHEMICAL REACTIONS OF STRAINS PLACED IN COWAN'S GROUPS.

Test.	Salmonellae.	*S.pullorum.*	Arizona.	*E. coli.*	A–D Group.	*E. freundii.*	Bethesda-ballerup.	*K. ozaenae.*	*K. pneumoniae.*	Cloaca.	Providence.
	14	1	2	21	8	2	13	3	19	27	2
Motility	+*	−	+	17+4−	−	+	+	−	−	+	−
Gas from glucose	+	+	+	+	−	+	+	+	+	+	−
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+
Acid from mannitol	+	+	+	+	+	+	+	+	+	+	−
Acid from adonitol	−	−	−	1+20−	−	−	−	−	+	10+17−	−
Acid from dulcitol	+	−	−	14+7−	1+7−	−	6+7−	−	8+11−	4+23−	−
Acid from inositol	6+8−	−	−	−	−	+	−	+	+	7+20−	+
Acid from lactose	−	−	−	+	2+6−	+	10+3−	+	+	25+2−	−
Acid from salicin	−	−	−	10+5−6NT	−	+	6+3−4NT	2+1NT	7+12NT	NT	+
Acid from sucrose	−	−	−	9+12−	−	+	10+3−	2+1NT	+	26+1−	+
Indole	−	−	−	+	+	−	−	−	−	−	+
Gelatin liquefaction	−	−	1+1−	−	−	−	−	−	−	+	−
H_2S production	4+10−	−	+	5+16−	−	+	12+1−	2+1−	−	13+14−	−
Voges-Proskauer test	−	−	−	−	−	−	−	−	+	+	−
Methyl red test	+	−	+	+	−	+	+	+	−	−	+
Urea hydrolysis	−	−	−	−	−	+	5+8−	+	+	+	−
Growth in KCN	−	−	−	−	−	+	+	+	+	+	+
Growth in Koser's citrate	+	−	+	−	−	+	+	+	+	+	+
Phenylalanine test	−	−	−	−	−	−	−	−	−	−	+

* Unless otherwise stated, all strains in the category showed the result indicated.
NT = Not tested.

(*e*) *Escherichia freundii.*

Strains 101 and 102 were both isolated from bovine milk samples and had the biochemical characteristics of *E. freundii.* Two days' incubation was required for the production of hydrogen sulphide and the hydrolysis of urea. Acid production in inositol was slight.

(*f*) *Bethesda-ballerup.*

Strains 2, 17, 20, 58, 55, 53, 85, 86, 88, 127, 142, 143 and 145 were considered to belong in the Bethesda-ballerup group. These strains gave variable reactions in dulcitol, lactose, salicin, sucrose, hydrogen sulphide and urease.

Acid production from dulcitol occurred after 24 hours' incubation with strains 86, 142, 143 and 145, and 48 hours in strains 53 and 55.

No strain fermented lactose within 24 hours. Strain 58 did so after two days' incubation, strains 53 and 55 after three days, strains 142 and 143 after five days, strains 2, 17, 28 and 86 after seven days and strain 145 after 12 days.

Strains 85, 86, 88 and 127 were not tested for salicin fermentation. Positive results for salicin were obtained within 24 hours in strains 2, 17, 20, 53 and 58, and 48 hours in strain 55. Acid production in sucrose was delayed in strains 85, 88, 142, and 145. The longest period noted was 16 days for strain 142.

Hydrogen sulphide production was slight with strains 85, 88, and 127, and delayed for two and five days with two strains, 142 and 145.

(*g*) *Klebsiella ozaenae*

Strains 1, 33 and 105 had the biochemical characteristics of *K. ozaenae.* Strains 1 and 33 were isolated from chickens and strain 105 from a bovine milk sample. Strain 33 was isolated from liver and lung from a week-old chicken. Strain 1 was isolated from the eye of a 6-week-old Australorp chicken. Eight birds out of 200 seen had conjunctivitis with swollen eyelids. On autopsy, the bird submitted had a sero-tracheitis and *Klebsiella ozaenae* and *Lactobacillus* sp. were isolated from the eye discharge and alpha haemolytic streptococci from the trachea.

(*h*) *Klebsiella pneumoniae*

Strains 13, 91, 92, 94, 95, 98, 99, 104, 116, 135, 147, 149, 150, 151, 157, 158, 159, 162 and 163 had biochemical characteristics similar to those dsecribed by Cowan (1956) for *Klebsiella pneumoniae.*

Ludford and Stevens (1958) have described the isolation of Strain 13.

Strains 91, 92, 94, 95, 98, 99, 104, 116 and 117 were isolated from bovine milk samples obtained from clinical cases of mastitis. The ovine strain (No. 135) was isolated from a mesenteric lymph node from which *Salmonella*

typhimurium was also isolated. The clinical history of the affected animal included enteritis with profuse diarrhoea.

Urea hydrolysis was sometimes delayed. Strains 13, 91, 92, 94, 95, 98, 99, 104, 116, 135, 157 and 162 were positive after 24 hours' incubation but the remaining seven strains gave only a weak positive reaction after 24 hours, requiring 48 hours' incubation to give the strongly positive result.

Table 3.

BIOCHEMICAL REACTIONS OF KLEBSIELLA STRAINS WHICH COULD NOT BE GIVEN SPECIES CLASSIFICATION.

Test.	Strain No.										
	107.	108.	109.	110.	111.	112.	113.	114.	115.	125.	161.
Motility	—	—	—	—	—	—	—	—	—	—	—
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+
Acid from glucose ..	+	+	+	+	+	+	+	+	+	+	+
Acid from mannitol ..	+	+	+	+	+	+	+	+	+	+	+
Acid from adonitol ..	—	—	—	—	—	—	—	—	—	+	+
Acid from dulcitol ..	+	+	+	+	+	+	+	+	—	—	+
Acid from inositol ..	+	—	—	—	+	+	—	—	+	+	+
Acid from lactose	+	+	+	+	+	+	+	+	+	+	+
Acid from salicin	+	+	+	+	+	+	+	+	+	+	+
Acid from sucrose ..	+	+	+	+	+	+	+	+	+	+	+
Indole	+	—	—	—	—	—	—	—	—	+	+
Gelatin liquefaction ..	—	—	—	—	—	—	—	—	—	+	—
H_2S production	—	—	—	—	—	—	—	—	—	—	—
Voges–Proskauer test ..	+	+	+	+	+	+	+	+	+	+	+
Methyl red test	—	—	—	—	—	—	—	—	—	+	+
Urea hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Growth in KCN	+	+	+	+	+	+	+	+	+	+	+
Growth in Koser's citrate	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine test ..	—	—	—	—	—	—	—	—	—	—	—

(*i*) *Klebsiella* spp.

Table 3 gives the biochemical reactions of the 12 strains of Klebsiella-type strains which could not be placed in any of the three groups of Klebsiella. Strain 161 was isolated from the heart blood of a cockatoo, and all the others were isolated from bovine milk samples. Strains 107-114 resembled *K. pneumoniae* in all reactions except that they failed to ferment adonitol. Strains 107 and 111 produced acid from inositol after 10 days' incubation and strain 113 after 14 days incubation. Strain 115 did not ferment dulcitol but otherwise it also resembled *K. pneumoniae.*

(*j*) *Cloaca*

Strains 10, 11, 12, 21, 22, 23, 24, 25, 26, 31, 35, 36, 38, 39, 51, 67, 81, 93, 96, 97, 117, 131, 139, 140, 141, 148 and 156 could be placed in the group Cloaca.

Table 4.

BIOCHEMICAL REACTION OF 19 STRAINS WHICH WOULD NOT FIT INTO THE GENERAL SCHEME OF CLASSIFICATION.

Test.	Strain No.																		
	15.	18.	19.	34.	52.	69.	75.	78.	79.	82.	83.	84.	126.	160.	144.	47.	122.	64.	136.
Motility	−	+	−	+	+	+	+	+	−	+	+	−	+	−	+	+	−	−	+
Gas from glucose	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from mannitol ..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
Acid from adonitol	−	+	+	+	+	−	+	−	+	−	+	−	+	−	−	−	−	−	+
Acid from dulcitol	−	+	+	+	+	−	+	−	+	+	−	+	$+^{5}$	+	−	−	−	−	+
Acid from inositol	+	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	$-^{2}$	+
Acid from lactose	−	*2	$+^{3}$	−	$+^{5}$	−	$+^{8}$	+	+	$+^{7}$	+	+	$+^{9}$	$+^{6}$	−	−	−	−	$+^{12}$
Acid from salicin	+	+	+	$+^{3}$	+	−	$+^{2}$	$+^{3}$	+	−	$+^{2}$	$+^{3}$	+	$+^{3}$	−	−	−	+	
Acid from sucrose	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−
Indole	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−
Gelatin liquefaction ..	−	$+^{15}$	$+^{20}$	−	−	−	−	−	−	−	−	−	$+^{20}$	−	−	−	$+^{2}$	+	−
H_2S production	−	−	−	+	+	+	+	−	−	−	+	+	−	−	−	−	+	−	+
Voges-Proskauer test ..	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Methyl red test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Urease hydrolysis	$+^{5}$	+	$+^{4}$	−	$+^{18}$	+	−	$+^{2}$	+	−	$+^{3}$	−	−	−	−	−	$+^{6}$	−	$+^{4}$
Growth in KCN	+	+	−	−	−	+	+	+	+	−	+	−	−	+	−	−	+	−	−
Growth in Koser's citrate ..	+	−	−	+	−	+	−	+	+	−	−	−	$+^{5}$	−	−	+	−	−	+
Phenylalanine test	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* Number of days is specified when positive result was recorded after first day's incubation in those tests observed daily.

Sixteen of the 27 strains were isolated from cattle.

The strains fermenting adonitol, dulcitol and sucrose all did so within 24 hours. Strains 21, 31, 117, 24, 93, 139, 81, 141 required two to five days to ferment lactose and strains 96, 97, 24 and 141 fermented inositol weakly only after two to seven days. Gelatin liquefaction occurred only after periods of six to 30 days. Strain 131 hydrolysed urea in 24 hours and the remaining 26 strains which gave a weak reaction in 24 hours were positive after 48 hours' incubation.

(*k*) *Providencia.*

Strains 65 and 66 isolated were classified as Providencia, although motility could not be demonstrated in either case. They were isolated from the lung and pleural fluid of a cow. Both gave a positive result for the deamination of phenylalanine but did not hydrolyse urea.

(*l*) *Ungrouped Strains*

Strains 15, 18, 19, 34, 52, 69, 75, 78, 79, 82 83, 84, 126, 160, 144, 47, 122, 64 and 136 could not be placed with confidence into any of the groups. Their biochemical reactions are given in Table 4.

IV. DISCUSSION.

Most specimens submitted for bacteriological examination to this veterinary diagnostic laboratory suffer a considerable degree of contamination either from post-mortem spread of bacteria within the carcase or by contamination when exposed to the environment at post-mortem. A large number of such contaminants are Enterobacteriaceae. The problem is to decide which isolates other than Salmonellae should be studied in more detail to permit recognition of possible pathogens.

Among the strains described above, there are some other than Salmonellae which could be pathogens.

Most Salmonellae isolated in this laboratory conform to the biochemical pattern given by Cowan (1956), but the scheme suggested by that author does not satisfy requirements for *Salmonella pullorum*. The potassium cyanide test is of considerable value in distinguishing Salmonellae from organisms of the Bethesda-ballerup group.

Arizona strains which may be potential pathogens may also be confused with Salmonellae, but in contrast with most Salmonellae they do not ferment dulcitol. Kauffman and Moller (1956) have described a method for separating Salmonella, Arizona and Bethesda strains based on growth in KCN and the production of amino acid carboxylases.

Escherichia coli and the Alkalescens-Dispar group have similar IMVIC reactions, but the latter are differentiated from *E. coli* by lack of motility, not fermenting lactose and failure to form gas from glucose. Three strains

isolated from a duck resembled *K. pneumoniae* in colonial appearance, but biochemically had characteristics of *E. coli.* Several strains (18, 52, 75, 82, 83, 84, 122) had IMVIC reactions of *E. coli* but could not be classified as such because of the number of atypical results given in the other tests.

The Bethesda-ballerup group may be confused with Salmonellae. Pathogenicity has been ascribed to them by several workers. Their colony types are identical with those of the Salmonellae, and as mentioned above, except for the KCN test the biochemical tests of the two groups may be similar. Some strains differ from Salmonellae in their ability to ferment lactose, dulcitol, salicin and sucrose and to hydrolyse urea. *Escherichia freundii* is clearly related to the Bethesda group but ferments lactose within 24 hours.

Pathogenicity has not been ascribed to *Klebsiella ozaenae.* The strains isolated were VP negative and MR positive whereas *K. pneumoniae* is VP positive and MR negative.

Klebsiella pneumoniae is a potential pathogen and it has been isolated from a number of bovine milk samples submitted to this laboratory.

Eleven of the Klebsiella strains could not be placed in any species. From the aspect of pathogenicity it is important that this group be studied so that potential pathogens may be recognised.

Cloaca sp. have colonial morphology similar to Salmonellae on blood agar, and on MacConkey's agar and Salmonella-Shigella agar if they are non- or late-lactose fermenters. Cloacae most closely resemble the *K. pneumoniae* group in their reactions, distinguishing features being that the former are motile, non-capsulated and liquefy gelatin, whereas *K. pneumoniae* is non-motile, frequently capsulated and does not liquify gelatin. All strains of Cloaca examined hydrolysed urea, but except in one case, hydrolysis was slower than that of *K. pneumoniae* strains. The majority of Cloaca strains did not ferment inositol or adonitol whereas acid was produced from these sugars by all *K. pneumoniae* strains. The MR-VP tests separate Cloaca and *K. pneumoniae* from the other Enterobacteriaceae. No pathogenicity has been ascribed to Cloaca strains.

Providencia strains have been associated with urinary tract infections and diarrhoeic diseases in man (Edwards and Ewing 1955). Their colonial morphology is similar to Salmonellae but biochemically they are unique in that they do not produce urease but do deaminate phenylalanine.

Nineteen strains could not be placed in any of the groups and require further detailed investigation using both biochemical and serological methods to determine their positions in the Enterobacteriaceae.

Even with the limited biochemical tests done in this study, it is apparent that there would be a considerable delay in reporting results if they were to be routinely done. In most cases, the examination requested is for Salmonellae and these can be usually isolated and identified within a few days.

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