DIFFERENTIATION OF SERO-FERMENTATIVE TYPES IN *BACTERIUM COLI* O GROUP 55

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Serological studies of the *Bacterium coli* group, based on an analysis of the O, K (A, B and L) and H antigens, have been made by Kauffmann (1947, 1950, 1951), Vahlne (1945), Knipschildt (1945, 1946) and Wramby (1948). Giles, Sangster & Smith (1949) reported the isolation from infants with diarrhoea and vomiting of a specific serological type of *Bact. coli* which they termed the β variety. Smith (1949) described in greater detail the biochemical activities of the type. Kauffmann & Dupont (1950) investigated the antigenic structure and biochemical reactions of six *Bact. coli* β strains from cases of infantile diarrhoea and vomiting and found that they all possessed the antigens O55 B5 H6. Wright (1950) isolated *Bact. coli* β strains of two fermentative types; this paper reports the results of the further work undertaken on them.

SOURCES OF *BACTERIUM COLI* STRAINS

Twelve *Bact. coli* strains giving a positive slide agglutination with *Bact. coli* O 55 B 5 antiserum were isolated during 1949 from the receiving room rectal swabs of 142 cases admitted to one enteritis ward of this hospital. The twelve infants from whom the strains were isolated were all under 8 months of age and were suffering from diarrhoea and vomiting. The laboratory numbers of the strains were 2711, 3771, 3801, 4549, 4798, 5087, 5128, 5297, 5342, 5868, 6105 and 6196. They are referred to throughout this paper as 'the 12 *Bact. coli* strains'.

Bact. coli strains, having O antigens other than O 55, and K antigens other than B 5, were kindly provided by Dr F. Kauffmann, together with information regarding their antigenic components, and are shown in Table 1. With the exception of the O 43 strain, they were included in the Kauffmann Knipschildt-Vahlne diagnostic antigenic schema (see Vahlne, 1945 and Knipschildt, 1945). Strains Bi 7455, F 8188 and F 10018, to which no K antigen had been assigned, were tested by slide and tube agglutination with *Bact. coli* O 55 B 5 antiserum and no reaction was observed.

The strain referred to in this paper as *Bact. coli* β 80 was kindly provided by Dr J. Smith in 1949. This was before Kauffmann & Dupont (1950) had assigned the formula O 55 B 5 H 6 to the β strains. *Bact. coli* β 80 was compared in 1951 with *Bact. coli* Aberdeen 1064, an O 55 B 5 H 6 strain which had been examined by Dr Kauffmann. The biochemical reactions of β 80 and Aberdeen 1064 were found to be the same, and tube agglutination and agglutinin absorption tests showed that β 80 also possessed the antigens O 55 B 5 H 6.

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Table 1

Strain designation	Antigens H	O	K
Bi 7458/41	1	6	2*a*, 2*c*
U 14/41	2	3	2*a*, 2*b*
Bi 7455/41	2	43	.
U 9/41	4	2	1
U 4/41	5	4	3
A 20*a*	6	2	1
U 5/41	7	1	1
F 8188/41	7	19	.
Bi 623/42	10	11	10
Su 4321/41	11	13	11
Bi 316/42	12	9	9
F 10018/41	14	18	.
E 39*a*	15	23	18
K 12*a*	18	17	16

MATERIALS AND METHODS

Media. Nutrient digest of ox-heart with additions, as required, of 0·3 % agar, 2 % agar, 10 % horse blood, 0·1 % chloral hydrate or 0·1 % glucose. MacConkey agar (Mackie & McCartney, 1948). *Containers.* Screw-cap glass ¼ oz. 'bijou' or 1 oz. 'universal containers' for fluid media and for semi-solid agar. *Diluents.* Saline (0·85 % NaCl) or 0·25 % buffered formalin in saline. *Storage.* Stock cultures in 20 ml. plain agar stabs at room temperature (r.t.). Antisera (+ chloroform) and antigens at 4° C. *Incubation.* Aerobic and, unless otherwise stated, at 37° C.

Biochemical methods

Methods described by Mackie & McCartney (1948) were used for fermentation reactions (read up to 14 days), gelatin liquefaction (r.t., read up to 28 days) and indole production (tested at 3 days); by Ministry of Health Report no. 71 (1939) for methyl-red and Voges-Proskauer reactions (tested at 3 days), citrate utilization (read up to 14 days) and MacConkey broth at 44° C.; by Topley, Wilson & Miles (1946) for nitrate reduction (tested at 5 days). Urease production was tested (and read up to 14 days) in a fluid modification (Maslen, in preparation) of Christensen's (1946) urea medium.

Serological methods

Reference may be made to the publications of Kauffmann (1947, 1950), Vahlne (1945), Knipschildt (1945) and Wramby (1948) for particulars of the methods used by them in their studies of the coliform group. The methods used in this investigation in connexion with O and K reactions were based on those described by these workers or were modified from them; certain other methods were used in the preparation of H antigens (see Table 2).

O antigens. Broth cultures for tube agglutination tests were incubated for 6–8 hr. only, stored overnight at 4° C. and then steamed. The period of 18–24 hr. incubation previously used was found to result in spontaneous clumping in control

tubes (without antiserum), but this difficulty was not encountered with cultures incubated for the shorter period.

Table 2. *Preparation of antigens*

Antigen	Purpose of antigen	Culture medium	Diluent	Treatment
O	Immunization	Plain broth	—	Steamed 2½ hr.
O	Tube agglutination	Plain broth	—	Steamed 1½ hr.
O	Suspension for agglutinin absorption	Plain agar	Saline	Steamed 2½ hr. washed once
K	Slide agglutination	Plain, MacConkey or chloral hydrate-blood agar	Saline	—
K	Immunization	Glucose agar	Saline	—
K	Tube agglutination	Glucose or MacConkey agar	Saline or formol saline	—
K	Suspension for agglutinin absorption	MacConkey agar	Formol saline	Washed once
H	Immunization	Plain broth	—	0·25 % formalin added
H	Tube agglutination	Semi-solid agar	Formol saline	—
H	Suspension for agglutinin absorption	Semi-solid agar	Formol saline	Washed once

K antigens. K antigens for tube agglutination tests were at first prepared from 18 hr. glucose agar slope cultures in bijou containers. Later, because it was found that considerable H antigen might be present in living suspensions from even slightly moist culture medium, whether plain, glucose or MacConkey agar, K antigens for test were prepared on glucose or MacConkey agar plates which had been left, before inoculation, in the incubator for several days to ensure a dry surface. The presence of H 6 agglutinin in the K antisera (see section on K antigen results) indicated that similar precautions should be taken in preparing K antigens for immunization and the absence of H antigens from them confirmed before use.

K antigens for tube agglutination tests and for immunization were diluted to about 500×10^6 organisms/ml.

H antigens. All cultures used for the preparation of H antigens were incubated at room temperature.

H antigen for tube agglutination tests was prepared by harvesting, at the time of optimal motility, the outer surface growth from a Craigie tube culture in semi-solid agar. The suspension was diluted to about $1,000 \times 10^6$ organisms/ml.

For preparation of H suspensions for absorption tests a modification of the Craigie tube method was used. Two inner tubes, in a ½ lb. screw-cap glass jar containing semi-solid agar, were inoculated with the strain. The antigen was harvested when the outer surface of the medium was well covered with growth, at which stage active motility was found in all instances.

Preparation of antisera

Antisera were prepared in rabbits by intravenous injection, usually at intervals of 3 days, but in some instances of 5–6 days. For O or K antiserum, the immunizing course was 0·25, 0·5, 0·5, 1·0, 1·0 ml. of antigen, and for H antiserum, 0·1, 0·2,

0·3, 0·5, 0·75, 1·0 ml. of antigen, followed in four of the six rabbits used by a further 1·0 ml.

A blood sample from each rabbit, taken immediately before immunization, was tested by the appropriate agglutination method with the homologous immunizing antigen. Only two of these sera reacted, and these to insignificant titre.

Antisera obtained after immunization were tested by the appropriate tube agglutination methods for the presence of agglutinins to α-antigen of Stamp & Stone (1944), β-antigen of Mushin (1949), a non-specific O antigen (strain 'Straughan'), and Vi antigen. Col. H. J. Bensted kindly gave his advice regarding these tests and provided the strains and Vi suspension. Strain 'Straughan' was agglutinated by one O antiserum at 1/100 and by one K antiserum at 1/1600; this K antiserum was rejected.

Agglutination tests

Slide agglutination tests were used only in the isolation of *Bact. coli* strains from primary plates and in checking their purity in subculture. Undiluted O55 B5 antiserum was used. Reactions were read with a ×8 dissecting microscope. Prompt and complete agglutination with large aggregates was regarded as a positive result; fine, slow or no agglutination as a negative result.

Tube-agglutination tests were performed in Dreyer's tubes, using an equal volume (0·3 ml.) of antigen and of twofold serial dilutions of antiserum. Controls of antigen and diluent only, and of antiserum dilutions with homologous antigen, were included in each test. Reactions were read, using a ×3 hand-lens, by oblique light against a dark background (Table 3).

Table 3. *Tube-agglutination tests*

Nature of test	Antigen	Antiserum	Serial dilutions of antiserum	Conditions of test: Temp. (° C.)	Conditions of test: Time (hr.)
Agglutination titre	O	O	1/400–1/3200	50	20
Agglutination titre	K	K	1/100–1/1600	37 } r.t. }	2½ } 18 }
Agglutination titre	H	H	1/1600–1/51,200	50	2½
O-inagglutinability	K	O	1/10–1/1280	37 } r.t. }	2½ } 18 }

O-inagglutinability tests were performed by adding K and O antigens of the strain under test to the 1st and 2nd respectively of four sets of serial dilutions of O antiserum, and K and O antigens of the homologous strain to the 3rd and 4th sets. Absence of agglutination of K antigen by final dilutions of O antiserum 1/160–1/2560, in the presence of satisfactory O reactions in the control tubes, was regarded as evidence of O-inagglutinability (see Kauffman, 1944).

Agglutinin absorption tests

Diluted antiserum (O, 1/25; K, 1/12·5; H, 1/100) was added to absorbing bacterial deposit and the mixture left at 37° C. for 2½ hr. and then at 4° C. for 18 hr. Twofold serial dilutions of absorbed and of control, unabsorbed, antisera (O, 1/25–

1/400; K, 1/12·5–1/1600; H, 1/100–1/51,200) were tested by methods similar to the direct agglutination titre tests. K antiserum (1/12·5) was absorbed with O deposit for B agglutinin absorption and was then tested with K antigen.

RESULTS

The results of the biochemical and serological tests made on the 12 *Bact. coli* strains are given in the following sections.

BIOCHEMICAL ACTIVITIES

The 12 *Bact. coli* strains showed the following biochemical reactions in common: produced acid and gas on the 1st day in arabinose, galactose, glucose, lactose, laevulose, mannitol and xylose; on the 1st–2nd day in raffinose; on the 1st day in MacConkey broth at 44° C.; and acid and clot in litmus milk; failed to ferment inositol or inulin or to liquefy gelatin; were methyl-red+, indole+, nitrate reduction+, Voges-Proskauer−, citrate utilization−, and urease−.

The strains fell into three fermentative types on the basis of their reactions with six further fermentable substances:

I. Nine strains (2711, 3771, 4549, 5128, 5297, 5342, 5868, 6105 and 6196) produced acid and gas on the 1st day in sucrose and rhamnose, on the 2nd day in dulcitol, fermented maltose slowly with the production of acid and a small amount of gas on the 2nd–5th day, and failed to ferment salicin or adonitol.

II. Two strains (4798 and 5087) produced acid and gas on the 1st day in maltose and rhamnose, acid and gas on the 2nd day in salicin, fermented sucrose slowly with production of acid and a small amount of gas on the 2nd–5th day, fermented dulcitol slowly with production of acid but no gas on the 10th–14th day, and failed to ferment adonitol.

III. One strain (3801) produced acid and gas on the 1st day in maltose, acid and gas on the 2nd day in dulcitol, fermented sucrose slowly with the production of acid and a small amount of gas by the 3rd day, produced acid in adonitol on the 4th day and gas later, fermented rhamnose slowly with the production of acid by the 7th day but no gas, and failed to ferment salicin.

SEROLOGICAL RESULTS

O antigens

O agglutination and agglutinin absorption tests

β 80 O antiserum, using O antigens: (*a*) agglutinated the homologous strain and each of the 12 *Bact. coli* strains to a titre of 1/3200; (*b*) after absorption with the homologous strain, was tested with the homologous strain and with each of the 12 *Bact. coli* strains and, after absorption with each of the 12 *Bact. coli* strains, was tested with the homologous strain and with the strain used for the absorption; in all instances, absorption of O 55 agglutinin was complete. It was concluded that each of the 12 *Bact. coli* strains possessed O 55 antigen.

K antigens

O-inagglutinability tests

β 80 and each of the 12 *Bact. coli* strains were found to be O-inagglutinable, indicating that each possessed a thermolabile surface antigen.

K agglutination tests

β 80 K antiserum, using K antigens, gave a positive slide agglutination reaction, and a tube agglutination titre of 1/800, with the homologous strain and with each of the 12 *Bact. coli* strains.

K agglutinin absorption tests

β 80 K antiserum, using K antigens: after absorption with the homologous strain, was tested with the homologous strain and with each of the 12 *Bact. coli* strains, and, after absorption with each of the 12 *Bact. coli* strains, was tested with the homologous strain and with the strain used for the absorption; in all instances, the absorption of the K agglutinin was complete.

B antigen

β 80 K antiserum, absorbed with O suspension of each of the 12 *Bact. coli* strains, was tested with K suspension of Aberdeen 1064 and with the strain used for the absorption. The results showed that the 12 *Bact. coli* strains each possessed B antigen since, in all instances, the K agglutinin was completely absorbed by the O suspension.

In view of the results of the above tests with K antiserum, each of the 12 *Bact. coli* strains was presumed to possess B 5 antigen. No attempt was made to prepare a pure B antiserum.

β 80 K antiserum was examined for the presence of O 55 and of H 6 agglutinins. In order to obtain results comparable with those of the K agglutination tests, the tubes were read after 2½ hr. at 37° C. and again after 18 hr. at room temperature. The K antiserum agglutinated O antigen of the homologous strain to a titre of 1/1600. Since, however, the 12 *Bact. coli* strains were O-inagglutinable, it was concluded that agglutination of their K antigens by the K antiserum was not due to its O agglutinin content. The K antiserum agglutinated an H antigen of A 20*a* (O 2 K 1 H 6) to a titre of 1/6400 with production of floccular agglutination at 2½ hr. To ensure that reactions between K antiserum and K antigens were not due to H antigen, each K suspension used was checked for absence of H 6 antigen by testing it with A 20*a* H antiserum (1/400).

H antigens

Preliminary H antigen determination

H 6 antigen. β 80 H antiserum, using H suspensions: (*a*) agglutinated the homologous strain to a titre of 1/12,800; (*b*) when tested with *Bact. coli* strains not having O 55 or B 5 antigens, (i) agglutinated the strain with H 6 antigen (A 20*a*) to a titre of 1/12,800, and (ii) failed to agglutinate the strains having H 1, 2, 4, 5, 7, 10, 11, 12,

14, 15, or 18 antigen; (*c*) when tested with the 12 *Bact. coli* strains, agglutinated nine of them to a titre of 1/12,800 and failed to agglutinate the other three. The nine positive strains (2711, 3771, 4549, 5128, 5297, 5342, 5868, 6105 and 6196) were presumed to possess H 6 antigen and the three negative strains (3801, 4798, 5087) to possess H antigen other than H 6.

H 2 antigen. 4798 H antiserum, using H suspensions: (*a*) agglutinated the homologous strain to a titre of 1/25,600; (*b*) when tested with *Bact. coli* strains not having O 55 or B 5 antigens, (i) agglutinated the strains with H 2 antigen (Bi 7455 and U 14) to a titre of 1/25,600, and (ii) failed to agglutinate the strains having H 1, 4, 5, 6, 7, 10, 11, 12, 14, 15, or 18 antigen; (*c*) when tested with the 12 *Bact. coli* strains, agglutinated, to a titre of 1/25,600, one strain only (5087) in addition to the homologous strain 4798; both these strains therefore were presumed to possess H 2 antigen. The antiserum failed to agglutinate the nine strains already presumed to possess H 6 antigen. It also failed to agglutinate one other strain (3801), which therefore was presumed to possess H antigen other than H 6 or H 2.

H 7 antigen. 3801 H antiserum, using H suspensions: (*a*) agglutinated the homologous strain to a titre of 1/102,400; (*b*) when tested with *Bact. coli* strains not having O 55 or B 5 antigens, (i) agglutinated the strains with H 7 antigen (U 5 and F 8188) to a titre of 1/102,400, and (ii) failed to agglutinate the strains having H 1, 2, 4, 5, 6, 10, 11, 12, 14, 15, or 18 antigen; (*c*) when tested with the 12 *Bact. coli* strains, agglutinated the homologous strain (3801) to a titre of 1/102,400 and failed to agglutinate the other eleven strains. 3801 was presumed to possess H 7 antigen.

Confirmation of H antigen determination

H antisera were prepared with three *Bact. coli* strains having O antigens other than O 55 and K antigens other than B 5, and, respectively, H 6, H 2 and H 7 antigens. The three antisera were tested with H antigen of each of the 12 *Bact. coli* strains and the results are shown in Table 4.

Table 4. *H antigen determinations of the twelve* Bact. coli *strains*

H antigen of		H antiserum prepared with		
Homologous strain	The 12 *Bact. coli* strains	A 20*a* H 6 O 2 K 1	Bi 7455 H 2 O 43 K.	U 5 H 7 O 1 K 1
A 20*a*	2711, 3771, 4549, 5128, 5297, 5342, 5868, 6105, 6196	51,200	0	0
Bi 7455	4798, 5087	0	25,600	0
U 5	3801	0	0	51,200

Figures denote reciprocal of titre. 0 = no agglutination.

Representative H 6, H 2 and H 7 agglutinin absorption tests were performed, using six H antisera and six H antigens. The results are shown in Table 5.

Table 5. *Results of cross-agglutinin absorption tests with H antisera and antigens*

H antiserum prepared with strain	Absorbed with H suspension of strain	Tested with H antigen of strain					
		2711	A 20*a*	4798	Bi 7455	3801	U 5
β 80	Unabsorbed	12,800	12,800	.	.	.	.
	Abs. A 20*a*	0	0	.	.	.	.
A 20*a*	Unabsorbed	51,200	102,400	.	.	.	.
	Abs. 2711	0	1,600 (0)	.	.	.	.
4798	Unabsorbed	.	.	51,200	51,200	.	.
	Abs. Bi 7455	.	.	0	0	.	.
Bi 7455	Unabsorbed	.	.	12,800	25,600	.	.
	Abs. 4798	.	.	0	0	.	.
3801	Unabsorbed	.	.	.	.	102,400	102,400
	Abs. U 5	.	.	.	.	400 (0)	800 (0)
U 5	Unabsorbed	.	.	.	.	12,800	51,200
	Abs. 3801	.	.	.	.	0	1,600 (0)

H antisera were diluted 1/100 before absorption. Figures denote reciprocal of titre. (0) = no agglutination after a second absorption. A 20*a* possesses antigens O 2 K 1 H 6; Bi 7455, O 43 K. H 2; and U 5, O 1 K 1 H 7.

SERO-FERMENTATIVE TYPES

The serological work undertaken on the 12 *Bact. coli* strains showed that nine of them possessed O 55 B 5 H 6 antigens, two of them O 55 B 5 H 2 antigens and one of them O 55 B 5 H 7 antigens. The same 12 *Bact. coli* strains, on the basis of their reactions in six fermentable substances, could be assigned to three fermentative types, I, II and III. Correlation of the serological and the biochemical results (Table 6) revealed that the nine strains with H 6 antigen were the nine strains of fermentative type I, the two strains with H 2 antigen were the two strains of fermentative type II and the one strain with H 7 antigen was the one strain of fermentative type III.

Table 6. *Division of the twelve* Bact. coli *strains into three sero-fermentative types*

Strains nos.	Antigens	Fermentative type	Sucrose	Maltose	Dulcitol	Salicin	Rhamnose	Adonitol
2711, 3771, 4549, 5128, 5297, 5342, 5868, 6105, 6196	O 55 B 5 H 6	I	+	− +	+	−	+	−
4798, 5087	O 55 B 5 H 2	II	− +	+	− +	+	+	−
3801	O 55 B 5 H 7	III	− +	+	+	−	− +	− +

+ = prompt production of acid and gas (1st–2nd day); − + = slower production of acid and gas (usually in small amount), or of acid only; − = no fermentation during 14 days' incubation.

The possibility was then considered that the association between antigenic structure and biochemical reaction might not be confined to the *Bact. coli* strains which had been isolated during 1949. Four *Bact. coli* O 55 B 5 strains had been obtained during 1951 from infants admitted to the gastro-enteritis unit; investigation of the flagellar antigens and biochemical activities of these strains showed that two of them possessed H 6 antigen and fell into fermentative type I and two pos-

sessed H 2 antigen and fell into fermentative type II. Thus the same association was found to be present after an interval of 2 years. No H 7 strains were isolated during 1951. Reference to the work of Kauffmann & Dupont (1950) showed that the biochemical activities of the six *Bact. coli* O 55 B 5 H 6 strains which they examined were the same as those of our *Bact. coli* O 55 B 5 H 6 strains. Their cultures had come from workers in Denmark, Scotland and England. Association of antigenic structure with biochemical activity was therefore shown to hold good for *Bact. coli* O 55 B 5 H 6 strains isolated in different countries. Recently, Laurell (1952) has reported, but without particulars of the fermentative reactions, the isolation of *Bact. coli* O 55 B 5 H 2 strains in Sweden.

The 12 *Bact. coli* O 55 B 5 strains already described in this paper came from rectal swabs taken at the time of admission of the infants to the hospital. Further *Bact. coli* O 55 B 5 strains, which had been isolated from infants in the gastro-enteritis ward and which apparently had been acquired by them as a result of cross-infection, were investigated with the object of ascertaining whether the sero-fermentative types were of a stable character. The flagellar antigen types of the strains were first determined. Eighteen of these *Bact. coli* O 55 B 5 strains, six with H 6, six with H 2 and six with H 7 antigens, were then selected for investigation of their biochemical activities; each strain had been isolated from a different infant. In all instances, *Bact. coli* O 55 B 5 H 6, H 2 and H 7 corresponded with fermentative types I, II and III respectively. The H antigen type and the biochemical activity, and the association between these two attributes, appeared therefore to be strain-characteristics of sufficient stability to be maintained during passage through infants in the ward.

It seemed justifiable to assume as a working hypothesis that *Bact. coli* O 55 B 5 strains are divisible into sero-fermentative types on the combined basis of their flagellar antigen determination and their biochemical activities and that these sero-fermentative types have a stability adequate for their practical application in problems relating to the spread of *Bact. coli* infections. They were employed therefore in a cross-infection study in a gastro-enteritis ward. The results of this investigation will be the subject of another paper (Roden & Wright, in preparation).

SUMMARY

Biochemical and serological investigations were made upon *Bact. coli* O 55 B 5 strains isolated from infants with diarrhoea and vomiting. On the basis of their reactions with six fermentable substances and of their H antigen types, the strains could be differentiated into three sero-fermentative types, I, II and III, which comprised, respectively, those having H 6, H 2 and H 7 antigens.

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