OBSERVATIONS ON THE GROWTH IN NUTRIENT BROTH OF MIXTURES OF *ESCHERICHIA COLI* STRAINS, AND OF FAECAL SPECIMENS HARBOURING PATHOGENIC *ESCHERICHIA COLI* SEROTYPES

By J. M. S. DIXON*

Public Health Laboratory, Cardiff

Topley & Fielden reported in 1922 that a succession of dominant species occurred in broth cultures of faeces on continued incubation. They suggested that advantage might be taken of this change in the predominating species to isolate a required organism by subculture at the appropriate time. The development of fluid media containing selective chemicals favouring the growth of certain intestinal pathogens has hindered the application of this suggestion to routine bacteriology. The success of fluid enrichment media, however, is due not only to the selective agent incorporated but to the fluid character of the medium. Thus Thomson (1955) showed that inoculation of faeces into ordinary nutrient broth resulted in as many isolations of *Salmonella typhimurium* as the use of selenite broth.

Since no selective medium is available for the isolation of pathogenic strains of *Escherichia coli* the behaviour was studied of mixtures of pathogenic and non-pathogenic serotypes of *Esch. coli* in nutrient broth. The first part of this paper concerns the growth in broth of artificial mixtures of *Esch. coli* strains, the second the application of the experimental findings to the isolation of pathogenic serotypes in the faeces of infants.

Part I. Experimental work with laboratory cultures

Studies were made of the growth of certain pathogenic serotypes in association with some non-pathogenic *Esch. coli* strains. To aid identification of the strains, the pathogen and non-pathogen grown in association were chosen with a difference in fermentative activity. Sucrose was found to be a suitable indicator-sugar for this purpose. Differential fermentative activity was found to be readily detectable when suitable dilutions were used for surface viable counts.

MATERIALS AND METHODS

Strains of five 'pathogenic' and five 'non-pathogenic' *Esch. coli* were used; these were classified in accordance with their O-antigen. The O_{26}, O_{55}, O_{119} and O_{128} serotypes were the National Collection of Type Cultures strains 9026, 9055, 9119 and 9128, respectively. The O_{111} strain was isolated from a case of infantile gastro-enteritis in this laboratory. The 'non-pathogenic' coli were isolated from the faeces of healthy infants and behaved as *Esch. coli* type 1. The O-antigens of these

* Present address: Public Health Laboratory, Woodbridge Road East, Ipswich.

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strains were determined after the experimental work was completed and it was found that the strains were serotypes O_{12}, O_{18}, O_{19}, O_{25} and O_{75}. There is some evidence that serotype O_{25} may be associated with diarrhoea in infants (Cefalù & Brancato, 1953), but as no differences in behaviour were noted between the 'pathogenic' and other 'non-pathogenic' serotypes in mixed cultures, this does not significantly affect the results or conclusions. Strains of *Pseudomonas aeruginosa* and *Salmonella typhimurium* were used in a few experiments and were isolated from human faeces. All the organisms were stored on dry Dorset egg slopes at room temperature with infrequent subculture.

Lemco broth was employed for the growth of mixtures and used as 10 ml. tubed media. Inocula were 0·01 or 0·02 ml. volumes of appropriate dilutions of 6 hr. broth cultures grown at 37° C., unless otherwise indicated. All dilutions were made in quarter-strength Ringer solution and the inoculated tubes were incubated in a thermostatically controlled water-bath at 37 or 44° C.

Enumeration of the bacteria in the inocula, and in the cultures after growth, was by a surface viable count method similar to that of Miles & Misra (1938) using a peptone agar incorporating 1 % sucrose and 1/20,000 neutral red. Cultures were thoroughly mixed before removal of an aliquot for counting. Serial tenfold dilutions were made, 1 ml. being transferred by delivery pipette to 9 ml. at each step. Twelve drops of each dilution, each of 0·02 ml. from a dropping pipette, were used and the plates incubated at 30° C. overnight. This temperature of growth facilitated counting by limiting colonial size and hence reducing the risk of confluence of colonies. Control experiments demonstrated that the medium and temperature of incubation used gave results not significantly different from those given by the use of nutrient agar incubated overnight at 37° C. The dilutions chosen for counting were those in which the count from the twelve drops was more than thirty but less than 300 colonies. The results are expressed as the ratio of pathogen to non-pathogen in both inocula and grown cultures, to aid appreciation of the changes in proportion that occurred.

EXPERIMENTS AND RESULTS

A. *Variation in the pathogen: non-pathogen ratio of the inoculum*

Mixtures of pathogen and non-pathogen were inoculated into tubes of broth. The inocula varied in size but between 50 and 100 organisms of the strain added in the smallest numbers were introduced into 10 ml. broth. The cultures were incubated for 18 hr. at 37° C. Table 1 shows some of the results obtained with various pairs of organisms, which indicated that in broth cultures under these conditions one strain of each pair did not tend to predominate, but that an increase in proportion occurred of the strain inoculated in smaller numbers. Little change was found when the two organisms were inoculated in similar numbers. The change in ratio increased as the disparity in numbers in the inoculum increased. Variation in the degree of the change towards equality in numbers occurred between different pairs of organisms, but there was no indication that either the pathogenic or the non-pathogenic strains used were specially favoured by these cultural conditions. The

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pathogen was favoured when originally numerically inferior and vice versa. In general, there was an approach of the ratio of the two organisms to unity.

Table 1. *Pairs of* Escherichia coli *strains grown in association in broth at* 37° C. *for* 18 *hr. Ratio of pathogen to non-pathogen in inocula and grown cultures*

Organisms		Pathogen: non-pathogen ratio	
Pathogen	Non-pathogen	In inoculum	After incubation
O_{26}	O_{12}	3000:1	50:1
O_{119}	O_{12}	3000:1	15:1
O_{111}	O_{75}	2500:1	20:1
O_{128}	O_{12}	2500:1	2:1
O_{55}	O_{12}	200:1	80:1
O_{119}	O_{25}	100:1	8:1
O_{26}	O_{18}	3:1	2:1
O_{55}	O_{12}	2:1	3:1
O_{111}	O_{75}	2:1	2:1
O_{119}	O_{25}	2:1	3:1
O_{128}	O_{12}	1:1	1:3
O_{26}	O_{12}	1:500	1:10
O_{119}	O_{25}	1:500	4:1
O_{111}	O_{75}	1:500	1:2
O_{128}	O_{12}	1:1000	1:35
O_{55}	O_{18}	1:1000	1:5
O_{55}	O_{18}	1:10,000	1:45
O_{119}	O_{25}	1:10,000	1:80
O_{26}	O_{25}	1:11,000	1:70
O_{55}	O_{12}	1:20,000	1:100
O_{55}	O_{12}	1:200,000	1:135

B. *Growth of a pathogenic strain with multiple non-pathogenic strains*

Experiments were performed in which the inoculum included a pathogenic strain and a mixture of four non-pathogenic strains (serotypes O_{12}, O_{25}, O_{18} and O_{19}) which outnumbered the pathogen. All the non-pathogens in each mixture had the same action on the indicator sugar used in the viable counts. The results of seven such experiments are recorded in Table 2, showing the change in ratio after 18 hr. incubation at 37° C. Type O_{25} outnumbered the other three non-pathogens

Table 2. *Pathogenic strains of* Escherichia coli *grown together with four non-pathogenic strains. Pathogen: non-pathogen ratios before and after incubation at* 37° C. *for* 18 *hr.*

		Pathogen: non-pathogen ratio	
Expt.	Pathogen	In inoculum	After incubation
1	O_{119}	1:45	1:20
2	O_{26}	1:75	1:25
3	O_{119}	1:285	1:16
4	O_{26}	1:500	1:42
5	O_{119}	1:120	1:6
6	O_{26}	1:220	1:25
7	O_{55}	1:600	1:50

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in the inoculum in Expts. 3 and 4 by 70:1. Type O_{12} predominated in Expts. 5 and 6 by 25:1. In the other experiments the non-pathogens were present in equal numbers in the inoculum.

The results indicated that a numerically inferior strain could compete successfully with at least four other coliform strains. The degree of increase of the pathogenic strain was rather less than might be expected when competing with only one non-pathogen.

C. *Addition of other species to two competing* Escherichia coli *strains*

Small inocula of either *Ps. aeruginosa* or *Salm. typhimurium* or both organisms were added to two coli strains (serotypes O_{55} and O_{12}) present in unequal numbers in broth. The number of these organisms added was approximately equal to that of the numerically inferior coli strain (about 50 organisms/10 ml. tube). To reduce the growth of pseudomonas colonies, which tended to overgrow at 18 hr., anaerobic incubation of the plates for the viable coli counts was used. In the dilutions used for enumeration, the salmonella colonies could be readily distinguished. Pseudomonas colonies were counted on duplicate plates incubated aerobically. Table 3 shows the resultant ratios of the two coli strains to one another after incubation. The growth of the other species is also indicated in the table.

Table 3. *Mixed* Escherichia coli *cultures, some with additional species present. Ratios of inocula and of cultures after* 18 *hr. incubation at* 37° C.

	Ratio—*E. coli* O_{55}:*E. coli* O_{12}:Pseud.:Salm.	
Additional species present	In inoculum	After incubation
—	1:1,655:–:–	1:4:–:–
—	1:16,550:–:–	1:25:–:–
Ps. aeruginosa	1:16,55:1:–	1:4:2:–
Ps. aeruginosa	1:16,550:1:–	1:43:19:–
Salm. typhimurium	1:1,655:–:2	1:15:–:3
Ps. aeruginosa + *Salm. typhimurium*	1:1,655:1:2	1:8:4:2

The results demonstrated that the tendency of these two coli strains to equate numerically was not greatly affected by the presence of either or both of the other species. Subcultures from these tubes at 42 hr. showed that the pseudomonas or salmonella organisms when present tended to replace the *Esch. coli* strains as the predominant organism at this time.

D. *The effect of time on associations of* Escherichia coli *strains*

Six extended experiments were made in which approximately equal numbers (100 organisms of each strain) of pathogen and non-pathogen were inoculated into a number of tubes of broth. Incubation was at 37° C. and viable counts were made at daily intervals for 10 days. There was a gradual fall in the total viable count when incubation was extended beyond 24 hr., but no significant change in the ratio of one strain to the other. In the six associations studied, no strain established significant predominance at any period of the experiment.

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Extended incubation of cultures in which the inoculated organisms were present in vastly different numbers was also made. Some of the results for associations of types O_{55} and O_{12} are presented in Table 4.

Table 4. *Mixed cultures of types* O_{55} *and* O_{12}, *giving ratio of one to the other before and after incubation at* 37° C.

Pathogen:non-pathogen ratio			
In inoculum	After 18 hr.	After 66 hr.	After 168 hr.
200,000:1	> 200:1	> 200:1	60:1
20,000:1	> 200:1	> 200:1	30:1
2,000:1	120:1	50:1	20:1
1:100,000	1:135	1:35	1:10
1:1,000,000	< 1:200	1:30	1:3

Increase of incubation time resulted in a progressive change in the ratio of one to the other until equality was approached within a week, even from extreme inoculum ratios. Subcultures at intermediate times demonstrated the gradual nature of the change.

E. *The effect of incubation at* 44° C. *on mixtures of* Escherichia coli *strains*

The previous experiments were repeated at 44° C. instead of 37° C. Similar results to those at 37° C. were obtained, the trend toward equality being equally noticeable. When the experiments including other species (§C) were repeated at 44° C., the pseudomonas and salmonella strains were much less evident. These experiments are quoted as an example of 44° C. incubation in Table 5. This was a repetition of the experiment recorded in Table 3, the only difference being the temperature of incubation. The changes in the ratio of the two *Esch. coli* strains were of the same order as when incubation was at 37° C. However, the added species grew poorly at 44° C. Thus identification of the outnumbered coliform was easier on plates inoculated from 44° C. cultures than on those from 37° C. cultures.

Table 5. *As Table* 3, *but incubation at* 44° C.

	Ratio—*E. coli* O_{55}:*E. coli* O_{12}:Pseud.:Salm.	
Additional species present	In inoculum	After incubation
—	1:1,655:–:–	1:9:–:–
—	1:16,550:–:–	1:22:–:–
Ps. aeruginosa	1:1,655:1:–	1:15:0:–
Ps. aeruginosa	1:16,500:1:–	1:9:0:–
Salm. typhimurium	1:1,655:–:2	1:4:–:0
Ps. aeruginosa + *Salm. typhimurium*	1:1,655:1:2	1:7:0:0

F. *Variations in the inocula of mixed* Escherichia coli *cultures*

A brief study was made of two other factors that might influence mixed coli cultures, viz. the absolute size of the inoculum and the temperature at which the inoculated organisms had been grown.

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Pairs of organisms were inoculated into broth in the same ratio but with a tenfold increase in absolute numbers from tube to tube. Cultures were counted after 24 hr. at 37 or 44° C. Increasing the inoculum size by as much as a 100,000-fold had no significant effect on the resultant culture at either temperature.

Cultures of *Esch. coli* strains were grown for 6 hr. at 23, 30, 37 and 44° C. and then used as mixed culture inocula in various combinations. Enumeration was made after 18 hr. at 37 or 44° C. No apparent difference was noted in the resultant ratio of the two organisms whatever the temperature of growth of the organisms during the 6 hr. prior to inoculation.

Summary of experimental findings

These experiments demonstrated that, with the strains of *Esch. coli* used, incubation together in nutrient broth of widely different numbers of two or more strains resulted in a gradual decrease in the disparity in numbers between the strains. This tendency to equate numerically increased with increasing length of incubation. Behaviour was similar at 37 and 44° C. The presence of certain other bacterial species did not greatly affect the tendency. The absolute size of the inoculum had little bearing on the composition of the culture after growth. Variation of the temperature at which the organisms were grown in the 6 hr. prior to inoculation did not affect the result.

Part II. The use of nutrient broth to facilitate isolation of pathogenic *Escherichia coli* serotypes from the faeces of infants

The experimental results suggested that it might be possible to increase the proportion of a pathogenic *Esch. coli* serotype, present in a scantily positive faecal specimen, by growth overnight in nutrient broth. The tendency to numerical equation in broth might increase the relative number of a serotype present in such small numbers in the faeces as to be unlikely to be detected by the usual routine of picking a certain number of colonies for slide agglutination. Thus positive results additional to those of direct plating might be expected with such specimens. It is well known, however, that in the acute stages of infantile gastro-enteritis, the pathogen is frequently present in huge numbers in the faeces and is the predominant organism isolated on MacConkey agar. In such cases, broth incubation might be expected, from the experimental results reported above, to favour the non-pathogens; some specimens, positive by direct plating, might even become negative after broth subculture.

It is not easy to evaluate a potential enrichment method that might be of value in demonstrating scanty positives but which might lose some of the more strongly positive specimens. Specimens harbouring few pathogens would be needed for this purpose, and the occurrence of an outbreak of gastro-enteritis in a children's residential nursery provided some such specimens. Material from convalescent children in whom the pathogens were becoming more scanty was included, and an assessment of broth enrichment for the isolation of the causative strain (serotype $O_{55}:B_5:H_7$) made. These specimens are considered as series A. Later the methods were applied to another series of faecal specimens. These were from sporadic cases

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collected over a period of a year which were sent in the acute stage of the disease for diagnosis and are considered under series B. Incubation of broth cultures was at both 37 and 44° C.

A suspension of faeces was made in 2 ml. nutrient broth. One drop (0·02 ml.) of this was spread on MacConkey agar and incubated at 37° C., and comprised the direct examination. Tubes of 10 ml. Lemco nutrient broth were inoculated with two drops (0·04 ml.) of the suspension and incubated at 37 or 44° C. for 18 hr. in a thermostatically controlled water-bath. Subculture was made on to MacConkey agar and the methods of inoculation, spreading, incubation and examination were identical with those used for the direct plate. Identification of pathogenic serotypes was by slide agglutination with O:B sera, which was later confirmed by tube agglutination. Eight colonies from each plate were tested, taking colonies with different morphology where apparent.

SERIES A

Specimens from an outbreak of gastro-enteritis associated with Escherichia coli O_{55}:B_5:H_7

Esch. coli type O_{55}:B_5:H_7 was isolated from seventy faecal specimens from infants in a residential nursery. They were from patients in all stages of the disease and included clearance specimens from convalescent cases. The results obtained by direct plating and the two fluid methods were: direct plating—54 positive (77 %), 37° C. nutrient broth—57 positive (81 %), 44° C. nutrient broth—65 positive (93 %).

Thus nutrient broth incubated at 44° C. was the most successful method and resulted in 11 (16 %) more positive isolations of this serotype than the routine direct method.

SERIES B

Specimens from acute cases of gastro-enteritis associated with various serotypes

A series of fifty specimens of faeces harbouring other pathogenic serotypes (viz. O_{26}, O_{111}, O_{119} and O_{128}) was then used. Nearly all were from specimens in the acute stage of the disease. The results obtained were: direct plating—43 positive (86 %), 37° C. nutrient broth—36 positive (72 %), 44° C. nutrient broth—42 positive (84 %).

In this trial the direct plating was the most successful method, but culture in broth did result in seven additional positive specimens being reported. It was to be expected from the experimental work that some of the strongly positive specimens might be lost during broth incubation. The pathogen was replaced by previously scanty non-pathogens.

The combined results for all specimens in series A and B during the 12-month trial period are given in Table 6.

44° C. nutrient broth proved to be the most efficient method of isolation of the pathogenic serotypes; when used as a complement to direct plating it resulted in

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an additional nineteen specimens being found positive—an increase of 19·6 %. Analysis of the results of direct plating alone and direct plating plus 44° C. broth culture by the χ^2-test, showed the latter technique to be a significant improvement ($\chi^2 = 13{\cdot}52$; $P < 0{\cdot}01$).

Table 6. *Results of examination of* 120 *positive faecal specimens for pathogenic* Escherichia coli *serotypes by various methods*

Method	Positive results
Direct plating	97
37° C. nutrient broth	93
44° C. nutrient broth	107
Direct plating + 44° C. nutrient broth	116

DISCUSSION

The experiments demonstrate that when two or more coliform organisms are inoculated into nutrient broth in unequal numbers, incubation at either 37 or 44° C. results in an increase in the proportion of the more scanty strain. When certain other organisms are included in the inoculum the same change occurs at both temperatures, but at 37° C. growth of the other species tends to obscure both the coli strains and so render isolation of either of the strains more difficult. When the mixture is incubated at 44° C. there is less interference by other species, as many organisms grow poorly or not at all at this temperature. When faeces in broth are incubated at 44° C., the faecal flora probably become greatly simplified, and *Esch. coli* strains are left to dominate the resultant culture.

The results of the longer-lasting experiments are of interest in that no succession of dominant organisms was found with mixtures of *Esch. coli* strains, such as other workers have noted with mixtures of different species. Topley & Fielden (1922) working with mixed broth cultures of faecal organisms, and Fulton (1937), using *Esch. coli* and *Salm. paratyphi* B in a synthetic medium, noted a tendency for species to succeed one another as the dominant organism of their cultures. The studies of Dean & Hinshelwood (1954) on the competitive growth of strains of coliforms in a synthetic medium were, however, more analogous to the present work. Using lactose-positive and lactose-negative strains of *Bact. coli mutabile*, they found that one type or another might predominate according to circumstances. The determining factor was not the initial relative proportion of the strains, but the degree of general adaptation of each strain to the medium in which the competitive growth occurred. Very little adaptation to growth in the nutritive broth used in the present work must have been necessary and thus the strains might have been expected to compete on almost equal terms. The brief study of differing growth temperatures of the inocula suggests that the short period of cooling undergone by many faecal specimens before examination will not affect significantly subsequent competitive growth of the coliform strains in broth.

The field trial with faecal specimens demonstrated that the use of 44° C. nutrient broth has some value for the isolation of pathogenic coli strains from scantily

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positive specimens. The use of 37° C. was of little value generally, because other species obscured the *Esch. coli* strains present. 44° C. broth would seem to be of most value as a complement to direct plating; it presents no advantage when used alone, since a number of specimens positive by direct plating are lost in the broth cultures. The continued enrichment of a very scanty pathogen in mixed coli cultures after 24 hr. is not applicable to practical isolation procedures. Competition of species other than *Esch. coli* can be controlled to a sufficient degree by temperature (44° C.) for 24 hr., but not for longer than this. Subcultures of scantily positive faeces grown in broth at 37 or 44° C. for 42 hr. were often found to be negative.

The experimental findings and the results of the field trial both point to the value of nutrient broth incubated at 44° C. for the isolation of pathogenic *Esch. coli* serotypes from specimens in which they are present in small numbers. Little or no gain can be expected with specimens from acute cases. Probably at least 1 % of children under 1 year of age and living at home are harbouring one of these serotypes at any one time (Thomson, Watkins & Gray, 1956). The discovery of this carrier condition in these infants, in whom the pathogens are often scanty, is difficult, and the use of the 44° C. broth culture method might be valuable for its detection. Employing this method, effective screening of entrants to hospitals and institutions could probably cause a useful reduction in the incidence of infantile gastro-enteritis, and an increase in the value of the examination of specimens from convalescent patients might be expected.

SUMMARY

Experiments were made with five pathogenic serotypes and five non-pathogenic *Esch. coli* strains to study the competitive growth of mixed coli cultures in nutrient broth. In associations incubated at either 37 or 44° C. the organism inoculated in smallest numbers increased proportionately after 18 or more hours' incubation. The greater the initial disparity, the greater was the increase. Broth culture favoured the pathogenic strains only when these were numerically inferior in the inoculum. The tendency to numerical equation increased with increasing length of incubation.

A study was made of the use of nutrient broth as an aid to the isolation of pathogenic *Esch. coli* strains from the faeces of infants. 120 positive specimens were examined. Nutrient broth incubated at 44° C. was the most successful method, and when used as a complement to direct plating resulted in an additional 19·6 % of positive specimens. The main value of nutrient broth as an enrichment method is in the examination of the faeces of suspected carriers and convalescent patients, in which the pathogen is likely to be outnumbered by non-pathogenic *Esch. coli* strains.

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