Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1961, **25**, 137-152

Rapid Presumptive Identification of Enteropathogenic *Escherichia coli* in Faecal Smears by Means of Fluorescent Antibody

1. Preparation and Testing of Reagents

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In outbreaks of infantile diarrhoea caused by enteropathogenic Escherichia coli, *rapid detection of the etiological agent is essential for specific and adequate therapy. Fluorescent antibody techniques have proved advantageous for rapid identification of many micro-organisms. The development of fluorescein-labelled antibodies specific for nine serogroups of* E. coli *and the testing of these reagents for specificity are described in this paper. Results of testing pure cultures of various Enterobacteriaceae as well as faecal specimens from cases of diarrhoea are reported. These data show excellent correlation between the fluorescent antibody technique and conventional serological methods for identifying enteropathogenic types of* E. coli. *The immunofluorescent technique is more rapid than the usual bacteriological and serological methods in that smears from faecal specimens can be stained directly with the labelled antibodies. Details of the procedures for carrying out the technique are given in annexes.*

Diarrhoeal diseases constitute one of the leading causes of morbidity and mortality among infants and young children in all parts of the world. Hardy (1959) concluded that " The acute diarrhoeal diseases either have been, or are now, the major disease problem of infants and children in all countries ". The seriousness of the problem is tied to the level of economic, social and sanitary development which has been achieved. Diarrhoeal diseases due to infectious agents are preventable if adequate medical and public health facilities are available.

Although slow in gaining acceptance, the role of the enteropathogenic *Escherichia coli* (EEC) in the causation of infant diarrhoea now is well established. The newborn and infants under one year of age are especially susceptible. In the United States of America the main problem presented by infant diarrhoea caused by EEC is connected with the endemic and epidemic disease situations within hospitals and other institutions. Since accurate diagnosis is a prerequisite for effective control, rapid and simple diagnostic tests are needed. In this and the following papers [3] it will be shown how immunofluorescent techniques may play an important role in this area.

The successful use of the fluorescent antibody (FA) technique in detecting EEC in faecal smears was first reported by Whitaker et al. (1958). In 1957 they examined specimens from a 1954 outbreak of infantile diarrhoea which had been studied extensively (Stulberg et al., 1955) and shown to be caused by *E. coli* O127 : B8. Smears of the specimens which had been maintained in the frozen state for three years were stained with type-specific OB antibody for *E. coli* O127 : B8. Altogether 114 specimens, 46.5% of which had yielded positive cultures when examined at the time of the outbreak, were tested with the fluorescent antibody. Approximately 83% of this group were positive for the *E. coli* O127 : B8 serotype.

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[3] See pages 153 and 159 of this issue.

The increased number of positive specimens obtained by FA staining in comparison with cultural procedures was explained as being due to non-viable organisms and, perhaps, greater sensitivity of the former technique.

Nelson & Whitaker (1960) extended the previous work, using two pools of FA reagents containing antibodies for 10 groups of *E. coli*. These were used to detect EEC in faecal smears from 375 cases of diarrhoea in infants under two years of age. The results obtained by FA examination were compared with those obtained by culture. The two tests agreed in all except seven cases, although the incidence of EEC that belonged to the 10 groups was only 10.4%. They concluded that " the fluorescent antibody test is superior to standard bacteriologic methods in rapidity, economy, simplicity, and in the detection of a greater number of patients with enteropathogenic *E. coli* infection ".

Cohen, Page & Stulberg (1961) prepared two pools containing FA reagents for nine groups of EEC. The groups represented corresponded to those reported in this paper, except that the O124:B17 group was substituted for the O128:B12 group used in our studies. A total of 87 stool specimens were examined by both cultural and FA procedures. A correlation of 87.3% between the two types of examination was obtained, both methods yielding approximately the same percentage of failures. These workers concluded that " The combined use of labeled polyvalent and serotypic antibodies provides a rapid and sensitive method for the early presumptive detection of enteropathogenic *E. coli* in fecal specimens ".

In view of the frequency of false positive reactions encountered in the staining of normal human faecal specimens with labelled antibodies for the salmonellae (Thomason, Cherry & Edwards, 1959), it appeared that additional data were needed to assess the validity of FA staining procedures for *E. coli*. Furthermore, none of the previous workers had reported definitive serotyping of the cultures of EEC isolated from specimens on which comparative cultural and FA tests were performed. Without this information it is impossible to be sure that the cultures being stained and agglutinated are true enteropathogenic serotypes of *E. coli*. This is due to the fact that in most of the OB groups of *E. coli* there are many serotypes, only a few of which have been definitely implicated epidemiologically as etiological agents of infant diarrhoea.

Since members of several OB groups of *E. coli* are associated with diarrhoeal diseases in infants, it is important to have a polyvalent reagent for screening specimens for these organisms. The presence of K antigens in cultures of *E. coli* is known to inhibit their agglutination by O antisera. Therefore, it was assumed that K antigens would inhibit staining of *E. coli* by O antibody labelled with fluorescein. The K antigens of most of the EEC are antigens of the B type, and these are usually abundant in organisms from stool specimens. For this reason, it seemed wise to employ labelled OB antibody, rather than O, for staining tests, since, with a very few exceptions, it is not possible to prepare pure B antisera by methods known at present. Furthermore, on the basis of antigenic analysis of the escherichiae (Ewing et al., 1956), fewer cross-reactions were expected between the EEC and other Enterobacteriaceae when OB, rather than O, antibodies were used for staining faecal specimens.

This paper describes the preparation of pools of labelled antibodies for nine groups of EEC and presents data on the specificity of these reagents when tested with various cultures of the Enterobacteriaceae, as well as with a number of stool specimens. The OB groups for which sera were prepared were those studied by Nelson & Whitaker (1960) with the exception of O124:B17, which was not included in our study. There is no evidence at present to indicate that *E. coli* of this group are an important cause of infant diarrhoea in the USA.

MATERIALS AND METHODS

Detailed procedures used in this study are presented in the annexes to this paper.

Strains used

(*a*) 145 serotypes of *E. coli*, representing O groups 1 through 145.

(*b*) 28 serotypes of salmonellae, representing O groups 1 through 45.

(*c*) 12 serotypes of shigellae, representing subgroups A, B, C, and D.

(*d*) 12 serotypes of the Providence paracolon group, representing O groups 1 through 12.

(*e*) 12 intermediate and coliform strains.

(*f*) 32 serotypes of *Citrobacter freundii*, representing O groups 1 through 32.

(*g*) 12 serotypes of the Arizona group, representing O groups (1, 2), 5, 6, 7, 8, 11, 12, 17, 18, 22, 25, and 30.

Antisera

OB antisera for strains that belong to O26:B6, O55:B5, O111:B4, O127:B8, O86:B7, O119:B14, O125:B15, O126:B16, and O128:B12 of the EEC were produced in rabbits according to the methods described by Ewing (1956a, 1956b) (see Annex 1). Antisera for comparative study were produced with O antigens prepared with cultures of *E. coli* O55, O125, and O126 using the methods described by the same author.

A portion of each antiserum was reserved for slide agglutination tests. The remainder was used for preparing fluorescein-labelled antibodies.

Preparation and labelling of globulins

See Annex 2 and Annex 3.

Preparation of labelled polyvalent reagents

The conjugated globulin for each OB group was titrated directly against smears of its homologous antigen using twofold dilutions of the conjugate, and a staining titre was determined. The end-points ranged from a dilution of 1:32 to 1:80.

Two pools of labelled reagents were prepared by combining equal quantities of the labelled monospecific reagents. Pool 1 contained labelled antibodies for *E. coli* O26:B6, O55:B5, O111:B4, and O127:B8; Pool 2 contained antibodies for serotypes O86:B7, O119:B14, O125:B15, O126:B16, and O128:B12. A suitable working solution was obtained by diluting each pool so that the final dilution for each serotype was 1:20. Thus, Pool 1 was diluted 1:5, and Pool 2 was diluted 1:4.

Preparation and staining of smears with fluorescent antibody

Smears of saline suspensions of pure cultures of *E. coli* and other Enterobacteriaceae were prepared as described in Annex 4. Frozen rectal swab specimens were thawed at room temperature and soaked in 0.5 ml of sterile 0.85% NaCl solution. These suspensions were used to prepare smears for staining by FA reagents or for inoculation of plates of MacConkey agar. Suspensions of frozen stool specimens were made in similar fashion. All smears were processed as described in Annex 6 and examined for fluorescent organisms (Annex 7).

EXPERIMENTAL RESULTS

Fluorescence staining of various Enterobacteriaceae

The two pools of labelled antibodies were tested against cultures representing all the group of Enterobacteriaceae listed above. Additional smears were made of any culture showing positive fluorescence with the pools and these were tested with the individual components of that pool. Smears of pure cultures of all the designated O groups of *E. coli* were stained with the two pools of labelled globulins followed by the individual OB group reagents as indicated above. Staining of the homologous serotypes was graded as 4+ in all instances. Only two of the nine labelled globulins cross-stained any of the 145 serotypes of *E. coli*. Both homologous and heterologous staining reactions are shown in Table 1. The intensity of fluorescence was recorded as 1+, 2+, 3+, or 4+, the latter being the maximum brightness encountered. All cross-reactions occurred between cultures that have been shown by agglutination tests to be related to each other. One cross-reaction was due to a common O antigen (O127); another, to a common B antigen (B7). In some tests no staining occurred, even though it was expected on the basis of reported O antigen relationships (Ewing et al., 1956).

Further proof of the specificity of fluorescent staining was obtained by the modified FA inhibition test in which unlabelled antibodies were used to inhibit the subsequent staining of homologous antigens by the fluorescein-labelled antibodies (Goldman, 1956). Smears of the test organisms were covered for 30 minutes with a mixture of equal amounts of labelled and unlabelled homologous OB antiglobulin. Smears were washed in the usual manner, mounted, and observed under the fluorescence microscope. When optimal dilutions of each reagent were used, total inhibition of fluorescence was observed. Unlabelled O antiglobulin did not inhibit staining of OB cells by labelled OB antiglobulin. Likewise, inhibition of staining did not occur when the labelled antibody was mixed with unlabelled normal globulin.

Salmonella

Twenty-eight strains of salmonellae representing O groups 1 through 45 were tested with the pooled reagents. Only a few serotypes known to be strongly related to serotypes of *E. coli* were stained, as indicated in Table 2.

Shigella

None of the 12 commonly found *Shigella* serotypes was stained by the pools of *E. coli* conjugates.

Providence

Only one serotype, Providence O6, among 12 O groups tested, was stained with any of the FA reagents for *E. coli*. The heat-stable antigen of this

TABLE 1

HOMOLOGOUS AND HETEROLOGOUS REACTIONS OF 145 SEROTYPES OF *ESCHERICHIA COLI* WITH LABELLED ANTIBODIES FOR NINE OF THE OB GROUPS

Serotypes of *E. Coli*	Labelled antibodies for *E. coli* of OB groups: [a]								
	O26: B6	O55: B5	O86: B7	O111: B4	O119: B14	O125: B15	O126: B16	O127: B8	O128: B12
O26: B6	4+	—	—	—	—	—	—	—	—
O55: B5	—	4+	—	—	—	—	—	—	—
O86: B7	—	—	4+	—	—	—	—	—	—
O111: B4	—	—	—	4+	—	—	—	—	—
O119: B14	—	—	—	—	4+	—	—	—	—
O125: B15	—	—	—	—	—	4+	—	—	—
O126: B16	—	—	—	—	—	—	4+	—	—
O127: B8	—	—	±	—	—	—	—	4+	—
O127ab: B10	—	—	—	—	—	—	—	2+	—
O128: B12	—	—	—	—	—	—	—	—	4+
O19ab	—	—	1+	—	—	—	—	—	—
O20: B7	—	—	4+	—	—	—	—	—	—
O90	—	—	—	—	—	—	—	2+	—

[a] All conjugates used at the diagnostic dilution (1: 20).

TABLE 2

STAINING OF OTHER ENTEROBACTERIACEAE BY REAGENTS FOR *E. COLI*

Number of cultures in each group	Groups and reacting strains tested	Stained by *E. coli* FA reagent				Number of non-reacting cultures
		O55: B5	O111: B4	O127: B8	O128: B12	
28	Salmonella O groups 1-45					25
	Salmonella (35)		4+			
	Salmonella (13, 22)			4+		
	Salmonella (1, 13, 23)			2+		
12	Shigella serotypes					12
12	Providence O groups					11
	Providence O6	4+				
12	Intermediate and coliform strains					12
32	*C. freundii* O groups 1-32					31
	C. freundii O26				2+	
12	Arizona O groups					11
	Arizona O18			1+ to 2+		

strain was known, by agglutination tests, to be related closely to the O55 group of *E. coli*.

Intermediate and coliform bacteria

Twelve miscellaneous cultures in this category were not stained by any of the labelled antibodies for the pathogenic *E. coli*.

Citrobacter freundii

Cultures representing 32 different O antigen groups of the Bethesda-Ballerup group of paracolon bacilli were tested with the nine OB conjugates of *E. coli*. Table 2 indicates a single minor cross-reaction of *C. freundii* O26 and *E. coli* O128:B12. Although O128 was not included, earlier studies of *C. freundii* (West & Edwards, 1954) gave no evidence of agglutinative relationships between their O antigens and those of the other pathogenic types of *E. coli* used in the present study. Likewise, there was little evidence of similarity of the K antigens of *E. coli* and those which occur occasionally in *Citrobacter freundii*.

Arizona group

Twelve serological O groups of *Arizona* paracolon bacilli were tested with the nine OB conjugates. Only one, *Arizona* O18, was stained by any of the EEC conjugates (Table 2).

Comparison of O and OB conjugates

When the staining titres of O and OB conjugates were determined against their homologous antigens using boiled and living cultures, respectively, it was found that the OB conjugates gave titres twofold to fourfold higher than the corresponding O conjugates. There was no appreciable difference in the type of staining. No significant inhibition of fluorescence was observed when the O conjugates were tested against strains containing large amounts of B antigen.

Fluorescent antibody and cultural examination of faecal specimens

A total of 80 frozen cotton swabs of faecal material were examined by FA and cultural techniques. These swabs were frozen soon after they were obtained from the patients and were shipped to the laboratory under dry-ice refrigeration. Four of the swabs were positive for EEC by FA staining techniques. A culture from one of these was subsequently isolated and definitively identified as an EEC. Seventy-nine of the swabs failed to yield any growth on MacConkey agar. The results are presented in Table 3.

Frozen stool specimens

During this phase of the study, 227 frozen stool specimens from children were examined (Table 3).

TABLE 3

RESULTS OF FLUORESCENT ANTIBODY AND CULTURAL STUDIES ON VARIOUS TYPES OF FAECAL SPECIMENS

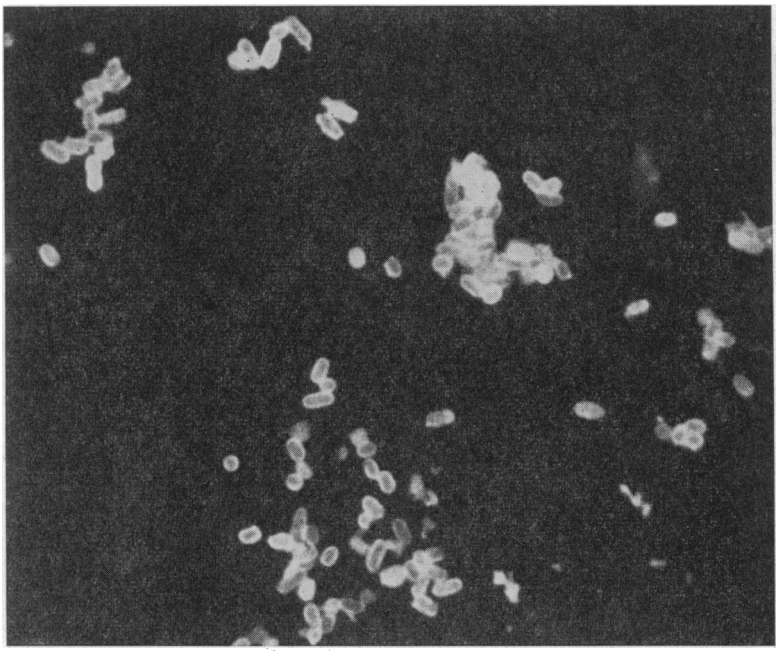
Type of specimen		Number of specimens	Specimens yielding viable organisms [a]				Non-viable specimens	
			Total number viable	Number positive by FA	Number positive by agglutination	Number confirmed as EEC	Total number non-viable	Number positive by FA
Frozen cotton faecal swabs	A	80	1	1	1	1	79	4
Frozen stool specimens	B	136	129	46	33	31 [b]	7	2
	C	9	9	9	8	8	0	0
	D	40	24	15	15	13 [c]	16	5
	E	42	20	17	10	10	22	12
Total		307	183	88	67	63	124	23

[a] Specimens yielding growth of any type on MacConkey agar plates.

[b] 31 of the 33 isolates were definitively typed as EEC. One was a related serotype not known to be pathogenic; the other belonged to a type not known to be antigenically related to EEC.

[c] Two of the 15 isolates were serotypes antigenically related to EEC. All the other isolates were definitively typed as EEC.

ENTEROPATHOGENIC *ESCHERICHIA COLI* OF THE O127: B8 GROUP IN A FAECAL SMEAR FROM A CASE OF INFANTILE DIARRHOEA



Stained with homologous antibody labelled with fluorescein isothiocyanate. Photographed at a magnification of 1200×.

These were collected in various parts of the USA and Canada and were shipped to us preserved with dry-ice. Some specimens had been collected just prior to shipment, while others had been kept frozen for several years in other laboratories. When these were cultured, only 183 gave growth of any kind on MacConkey medium. Of these, 88 were positive for EEC when examined with fluorescent globulins. Sixty-seven of the FA positive specimens subsequently yielded cultures agglutinated by sera for the EEC and all except four of this group proved to be EEC when definitively typed. Three of the latter cultures were serologically related to EEC types; one had no known antigenic relationship to any of the EEC types but gave positive FA and slide agglutination tests. It should be noted that in the total of 307 specimens, 124 (or 40%) yielded no growth on the isolation plates. Twenty-three of the non-viable group were positive by FA tests, giving a total of 111 FA positive specimens, as compared with 67 positive by culture and agglutination. The typical appearance of EEC in a faecal smear stained with FA is shown in the accompanying photomicrograph.

Controls

A control group consisting of 32 specimens from hospitalized children with no symptoms of diarrhoeal disease, 25 specimens from healthy, non-hospitalized adults, and 25 specimens from adult patients with diarrhoea were examined by FA for the presence of EEC. None of the adults was found positive. However, three specimens, one from a healthy adult and two from adult patients, were found to contain small organisms that fluoresced weakly with all labelled reagents. Since their morphology was atypical, they were recorded as FA negative. Organisms of this type were not isolated from the corresponding faecal specimens. Specimens from four of the hospitalized children were FA positive for EEC, and cultures isolated from three of these were subsequently typed as EEC. The fourth specimen was culturally negative for EEC. These results are summarized in Table 4.

Normal antibodies in the sera of various animals are being recognized with increasing frequency (Thomason, Cherry & Edwards, 1959; Cohen,

TABLE 4
FLUORESCENT ANTIBODY AND CULTURAL STUDY OF CONTROL GROUPS OF FAECAL SPECIMENS FOR ENTEROPATHOGENIC *E. COLI*

Type and status of patients	Number of specimens examined	Number of specimens positive by FA	Number of specimens positive by culture	Serotype of EEC isolated
Children: Hospitalized, no diarrhoea	32	4 [a]	3	O126: B16: NM; O55: B5: H7; O128ac: B12: H12
Adults: Hospitalized, diarrhoea	25	—	—	
Adults: Normal	25	—	—	

[a] One specimen was positive for O126 by fluorescent staining, but the organisms could not be isolated.

Cowart & Cherry, 1961). These are especially important in work with fluorescent antibody reagents since they may result in unexpected and unexplained cross-reactions. To determine whether or not EEC antibodies were present in non-immunized rabbit serum, 16 undiluted normal rabbit serum conjugates were tested against the nine serotypes of EEC used in the study. No fluorescence was exhibited by any strain with any conjugate. It is emphasized that rabbits serving as the source of the conjugates were an inbred strain considered to be " specific-pathogen-free ". It cannot be assumed that animals from commercial sources would be equally free of normal antibody.

DISCUSSION

Specificity and sensitivity

It has been demonstrated that the specificity of staining of enteropathogenic *E. coli* by fluorescent antibodies for nine OB groups is roughly equivalent to that shown by agglutination tests. Data are not available to illustrate directly a comparison between the agglutination of cultures of EEC by OB sera and staining them by the corresponding fluorescent globulins. However, the desired information may be obtained indirectly from the work of Ewing et al. (1956), and it has been assembled in Table 5. If this table is examined in conjunction with Table 1, it is

TABLE 5
HOMOLOGOUS AND HETEROLOGOUS AGGLUTINATION REACTIONS OF 145 SEROTYPES OF *ESCHERICHIA COLI* WITH O SERA FOR NINE OF THE SEROTYPES

O Antigens	Agglutination titre with O sera of *E. coli*											
	O26	O55	O86	O111	O119	O125	O126	O127	O128	O19ab	O20	O90
O26	20 480	—	—	—	—	—	—	—	—	—	—	—
O55	—	20 480	—	—	—	—	—	—	—	—	—	—
O86	—	—	20 480	—	—	—	—	2 560	—	320	—	640
O111	—	—	—	20 480	—	—	—	—	—	—	—	—
O119	—	—	—	—	20 480	—	—	—	—	—	—	—
O125	—	—	—	—	—	20 480	—	—	—	—	—	—
O126	—	—	—	—	—	—	5 120	—	—	—	—	—
O127	—	—	2 560	—	—	—	—	20 480	160	—	—	1 280
O128	—	—	—	—	—	—	—	640	5 120	—	—	—
O19ab	640	—	20 480	—	—	—	—	—	—	20 480	—	320
O20	—	—	—	—	—	—	—	—	—	—	10 240	—
O90	—	—	1 280	—	—	—	—	2 560	—	—	—	5 120

seen that cross-agglutination reactions given by O sera correspond closely to the cross-staining reactions given by the conjugates. Most of the differences are attributed to lower sensitivity of the fluorescent antibody tests under these conditions. It should be remembered that the conjugates were tested at or near to the highest dilutions (1 : 20) giving the maximum staining reaction (4+) with the homologous antigen. This procedure minimized cross-staining reactions. The staining of antigen O20:B7 by the O86:B7 conjugate is entirely due to the identity of their B antigens. Failure of the O20 antigen to be agglutinated by O86 serum was the expected result. Conversely, cross-staining of O127ab:B10 antigen by O127:B8 conjugate (Table 1) may be attributed to their O antigenic relationship. The staining of the O90 group culture of *E. coli* by the O127:B8 conjugate was expected from the strong cross-agglutination reactions previously reported (Ewing et al., 1956; Kampelmacher, 1959). Staining of the O group 19ab culture by the O86:B7 conjugate reflects antigenic similarities noted by Ewing et al. (1956). It is emphasized that the staining reactions shown in Table 1 represent the total of those observed when the nine OB conjugates were tested at the working dilution with 145 serotypes of *E. coli*. These results contrast sharply with the several hundred O antigen cross-reactions demonstrable by agglutination tests (Ewing et al., 1956; Kampelmacher, 1959). It appears that false positive tests for enteropathogenic *E. coli* due to the staining of related O antigens in other *E. coli* types are not a serious problem.

Since OB conjugates were used to detect the EEC, cross-reactions with other *E. coli* having related B antigens could give erroneous results. It seems unlikely that this occurred except in the case of the O86:B7 and O20:B7 serotypes, as mentioned above. Examination of the K antigen relationships (K1-K79) of *E. coli* revealed that by agglutination tests there were no other relationships between the B antigens of the EEC included in this study and those of the remaining serotypes (Ewing et al., 1956). Thus, there appeared little reason to anticipate errors from this source.

No unexpected major cross-reactions were encountered in the examination of pure cultures of the various Enterobacteriaceae. In some cases staining did not occur even though known O-antigen relationships existed. Among the salmonellae, a serotype belonging to O group 35 was stained as well by the O111:B4 coli conjugate as was the homologous culture. This relationship is due to identical OB antigens and was reported first by Varela et al. (1946). The serological relationship of salmonellae O groups (13, 22) and (1, 13, 23) to *E. coli* of serotype O127:B8 has been reported by Kampelmacher (1959) on the basis of agglutination test results.

On the basis of the studies of Ewing et al. (1956), no serological relationships of the heat-stable antigens of these particular *E. coli* to those of the shigellae were expected, and none was found among the 12 serotypes of shigellae tested (Table 2). Certain strong antigen relationships do exist between *E. coli* and shigellae, but these involve coli types other than those for which labelled antibodies were prepared.

All specimens from which enteropathogenic *E. coli* were isolated were also stained by the corresponding fluorescent antibody. This fact suggests that the FA technique can be applied successfully to the detection of these organisms. For purposes of evaluation, all specimens giving a positive FA test should be cultured and the isolates typed. Since the FA technique is more sensitive than culture methods in that non-viable organisms may stain specifically, it is felt that a negative FA specimen may be safely reported as negative for the nine EEC, thus eliminating many specimens that would normally be cultured.

The use of two pools of FA reagents facilitates the screening of large numbers of faecal specimens. This technique should be of interest to paediatric hospitals for routine examination of admissions and for rapid diagnosis of infantile diarrhoea caused by the *E. coli*.

Value of OB as against O conjugates

The relative value of OB as against O conjugates for detection of EEC requires comment. Whitaker et al. (1958) used conjugates derived from an OB serum (O127:B8). Nelson & Whitaker (1960) prepared FA staining reagents from O sera and used these successfully in the study of 375 cases of diarrhoea in infants. Cohen, Page & Stulberg (1961) employed conjugates derived from OB sera for detection of EEC in faecal smears. We prefer OB sera for the following reasons. First, data given in this paper indicate that higher-titred reagents result from conjugation of OB globulins than from O globulins, even though the B antigen agglutination titre of the former is small as compared with the corresponding O antigen titre. The most potent reagents possible are desirable from the standpoint both of economy and of ability to dilute away from small

amounts of normal antibody which may be present. Secondly, labelled OB globulins should be used for detection of EEC in faecal smears, since freshly excreted organisms may contain B antigens that inhibit staining by O conjugates. Thirdly, the B antigens of the EEC are far more specific than are the O antigens (see Tables 5 and 12 of Ewing et al., 1956).

An additional comment concerning the second point mentioned above is in order. In our laboratory, inhibition by B antigen of the staining of OB cells of *E. coli* with labelled O conjugates could not be demonstrated unequivocally even though such cells were inagglutinable with the corresponding O serum. Unless careful quantitative work is done, it may be quite difficult to demonstrate inhibition of staining. For example, the fluorescent staining of the organisms in a smear containing both O and OB forms in the ratio of 4 : 1 may not appear to be inhibited when treated with a labelled O conjugate. In reality, staining of 20% of the bacteria may be completely inhibited. Thus, FA experiments involving the presence of two antigen forms, one of which inhibits the serological reaction of the other, may be misleading unless the relative density of each type is known. Furthermore, the report of Kauffmann (1947, 1954) that the antigenicity of B antigens of some strains of *E. coli* was not completely destroyed by heating at 100° C for 2½ hours and the statement of Ewing et al. (1956) that the binding power of these antigen also resisted 121°C for 2 hours suggest that the O sera used in the inhibition experiments may have contained small amounts of B antibody not detectable otherwise. In view of these considerations the problem cannot be resolved without further study.

ACKNOWLEDGEMENTS

We wish to express our gratitude to several investigators who supplied specimens for the evaluation of the fluorescent antibody technique for detection of enteropathogenic *E. coli*. Dr Merlin L. Cooper, of the University of Cincinnati, furnished frozen rectal swab specimens from patients admitted to the Children's Hospital there. Dr James Connor, of Variety Children's Hospital in Miami, and Mr Don C. Mackel, of the Phoenix, Arizona, Field Station of the Communicable Disease Center, each supplied a number of frozen faecal specimens from children with diarrhoea. Miss Margaret Finlayson, of the Ontario, Canada, Department of Health Laboratories, kindly sent us a number of frozen faecal specimens which had been collected in that area.

We are indebted to Mr Wallis Jones, of the Communicable Disease Center, Atlanta, Ga., for the fluorescein-labelled conjugates of normal rabbit globulin. We wish to thank the Diagnostic Reagents Section of the Communicable Disease Center for supplying some of the sera used in the series of studies reported in this and the following papers.

RÉSUMÉ

La mise en évidence de certains antigènes au moyen d'anticorps marqués par des substances fluorescentes facilite depuis plusieurs années déjà le diagnostic de diverses maladies, en permettant de dépister rapidement les antigènes cherchés.

Les auteurs décrivent l'application qu'ils ont faite de cette méthode, proposée dès 1958 pour la recherche d'*Escherichia coli*, l'un des agents de la diarrhée des nourrissons et des jeunes enfants, dont le rôle dans cette affection a été longtemps discuté. Ils ont préparé des globulines marquées par la fluorescéine, correspondant à neuf groupes sérologiques d'*E. coli* entéropathogènes, dont la spécificité avait été éprouvée par rapport à tous les groupes sérologiques d'*E. coli* et à divers autres groupes d'entérobactériacées.

La recherche était faite sur des frottis de fèces provenant de diverses parties du monde. Les prélèvements par écouvillonnage rectal, congelés, ne se prêtèrent ni à la culture ni à l'examen par fluorescence, au contraire des échantillons de selles, conservés de la même manière (pendant un temps limité cependant) qui fournirent un matériel d'étude satisfaisant.

Les résultats ont confirmé et étendu les conclusions d'autres auteurs, quant à la spécificité et à la rapidité du diagnostic d'une infection à *E. coli* par cette méthode. Ils encouragent son application à une étude sur le terrain, dans une région où la diarrhée des nourrissons est fortement endémique, et son étiologie discutée.

Annexes

PROCEDURES FOR THE IDENTIFICATION OF *ESCHERICHIA COLI* IN FAECAL SMEARS BY FLUORESCENT ANTIBODY TESTS

General methods for the preparation and fractionation of sera and for the labelling of the globulin are given in the following annexes. Specific methods for the detection of enteropathogenic *E. coli* in faecal smears are included. This information is not to be considered a complete exposition of the technique but rather is designed to serve as a practical guide for public health laboratory workers. For detailed information on the many facets of immunofluorescence the reader is referred to the reviews of Coons (1956, 1958, 1959), Cherry et al. (1960), Goldman (1960) and Liu (1960).

In the description of the procedures given in the following annexes, the use of trade names is for identification purposes only and does not necessarily imply endorsement by the Public Health Service of the US Department of Health, Education, and Welfare or by the World Health Organization.

Annex 1

PREPARATION OF KO ANTISERA

(Ewing, 1956a, 1956b)

1. Plate cultures on infusion agar medium. After 18-24 hours' incubation at 37° C, test colonies of the smooth, opaque, rather mucoid K forms with O and KO antisera.

2. Select for transfer colonies that are agglutinable with KO antisera and inagglutinable, or only slightly agglutinated, by O sera.

3. Inoculate these colonies into infusion broth and on to infusion agar slants.

4. After overnight incubation, heat the broth cultures to 100° C for 1 hour to check the stability of the suspensions. Discard any culture that is auto-agglutinable after heating.

5. Inoculate a tube of infusion broth from the agar slant culture. After incubating at 37° C for 4-5 hours, add formalin to give a final concentration of 0.5%. Store the formalinized broth culture in the refrigerator overnight. This antigen is to be used for the first injection of animals.

6. On the next day start immunizing rabbits according to the following schedule:

1st day, inject 0.3 ml
5th day, inject 0.5 ml
9th day, inject 1.0 ml
13th day, inject 2.0 ml
18th day, inject 2.0 ml
22nd day, inject 2.0 ml.

For the second and third injections prepare new antigens. These may be used within 1-2 hours after the addition of formalin. For subsequent injections, use 4-5-hour living broth cultures. Bleed the rabbits 6-8 days following the last injection.

7. Select colonies for titration of K antibody and prepare antigens in the manner outlined above. Formalinize 4-5-hour broth cultures and add equal amounts of twofold serial dilutions of KO and O antisera. The titration in O antiserum is a necessary control on the inagglutinability of the formalinized K antigen suspension. Incubate the tests and controls at 37° C for 2 hours and refrigerate overnight. In KO agglutination the cells settle out in the form of a disc or pellicle at the bottom of the tubes. Record titres as the reciprocal of the highest dilution that gave good disc formation. Antisera having a K titre of 1:160 or higher are satisfactory.

For fluorescent antibody work, the sera should be frozen or a preservative other than glycerol should be added. If glycerol is used, the sera must be dialysed before the globulin can be precipitated.

Annex 2

SERUM FRACTIONATION

(Cherry et al., 1960)

1. Prepare a saturated solution of chemically pure ammonium sulfate in water at room temperature. The last portion of salt will take several days to dissolve at room temperature so the solution should be prepared well in advance.

2. To a measured amount of undiluted serum, add an equal amount of this solution (Note 1). The serum should be kept cold (ice-bath or refrigerator) and should be in a centrifuge tube or bottle large enough to allow thorough mixing. The ammonium sulfate solution, still at room temperature, should be added slowly from a pipette while the serum is rotated constantly.

3. Allow the precipitated globulin to stand overnight at 0°-5° C and then separate the sediment by centrifugation. Usually, 30 minutes at 1000 *g* in a horizontal-head machine will pack the precipitate present in 100 ml of solution. The sediment contains the crude globulin fraction that is to be labelled. Decant the supernatant fluid.

4. Dissolve the precipitated globulin by slowly adding measured amounts of distilled water. Gently

triturate the precipitate in order to get a homogeneous suspension. Water may be added in larger amounts than the volume of the original serum in order to wash the globulin more thoroughly.

5. Add to the dissolved globulin an amount of the saturated ammonium sulfate solution equal to the volume of water used to dissolve the precipitate. This is done as described in paragraph 2 above. The precipitated globulin may be centrifuged immediately, the supernatant fluid discarded, and the precipitate dissolved, reprecipitated and redissolved a third time. Three precipitations usually eliminate most of the haemoglobulin that may be present. The third precipitation may be omitted if the original globulin solution appears free of haemoglobin.

6. Dissolve the final precipitate in as small an amount of water as possible. Usually this will be slightly less than that of the original volume of serum. Occasionally, when considerably greater volumes are required, slightly alkaline water (about pH 7.5) may dissolve the globulin more readily.

7. Dialyse the dissolved globulin at 5° C against 0.85% sodium chloride solution, using frequent changes during the process. Dialysis is continued until sulfate is no longer detectable in the liquid outside the sac following overnight dialysis. This is determined by adding a few millilitres of saturated barium chloride to an equal amount of dialysate. If the mixture does not become cloudy or opalescent, the globulin is considered to be free of sulfate.

8. When working with sera containing lipids to the extent that they are milky or opalescent, they may be clarified by treatment with chloroform (Updyke & Conroy, 1956). Either whole serum or the globulin fraction may be used. Add one part of cold chloroform to five parts of cold protein solution. Shake vigorously. Centrifuge the mixture at 4000 *g* for 10-15 minutes. Discard the thick sediment. If the solution is not clear enough, the process may be repeated.

NOTES

1. If serum preserved with glycerol is used, dialyse it to remove the glycerol prior to fractionation. Since the volume will increase approximately 100% during dialysis, allow for this factor in selection of the sac. Dialyse for a period of 18-24 hours. Fractionate as described above. After the last precipitation, dissolve the globulin in enough distilled water to bring the total volume up to one-half the original volume. This is necessary because the original serum contained 50% glycerol.

2. It has been shown that essentially complete removal of ammonium sulfate may be effected within a 4-hour period under the following conditions (Kaufman & Cherry, 1961). Place up to 20-ml amounts of 1% to 2% globulin in 20/32 inch (15.88 mm) or 27/32 inch (21.43 mm) diameter tubing and dialyse with agitation for 2 hours in a 2-litre volume of 0.85% saline solution. Change to a fresh volume of saline and continue dialysis for an additional 2-hour period. The wall thicknesses of the two sizes of dialysis tubing are 0.0008 inch (0.020 mm) and 0.0010 inch (0.025 mm), respectively. They may by obtained as " Visking " dialysis tubing from the Visking Co., Food Casing Division, 6733 West Sixty-Fifth St., Chicago 38, Ill., USA.

Annex 3

LABELLING OF SERUM GLOBULINS

(Marshall et al., 1960; Cherry et al., 1960)

Fluorescein is still the most satisfactory label available, and conjugation methods employing fluorescein isothiocyanate are the simplest to perform. Therefore the use of this reagent only will be described here.

1. Determine by a standard procedure the protein content of the globulin to be labelled. This information is necessary in order to compute the amount of isothiocyanate needed. The Biuret method (Gornall et al., 1949) has been used routinely in our laboratories. However, protein determination by means of the refractometer is faster and simpler than by spectrophotometry. The accuracy of the refractometer should be sufficient for routine FA work.

2. Adjust the protein solution to 1% (10 mg/ml) by diluting it with physiological saline or by concentration if necessary. Globulins may be concentrated by evaporation from a dialysis sac that is placed before a fan.

3. Add carbonate-bicarbonate buffer (0.5 M, pH 9.0) to the chilled globulin in an amount equal to 10% by volume of the 1% globulin solution.

4. Chill the buffered globulin in an ice-bath and add slowly, with constant stirring, 0.05 mg of isothiocyanate powder per mg of protein in the solution.

5. Stir overnight in the cold.

6. Dialyse the product in the cold against 0.85% saline for a day or two, then against buffered (0.01 M phosphate, pH 7.5) saline until a 100-ml beaker of overnight dialysate shows no fluorescence with a portable Wood's lamp. An alternative procedure is to shake the conjugate intermittently for one hour with an equal volume of Dowex 2-X4 (chloride form), 20-50 mesh, anion-exchange resin (Dow Chemical Co.). Centrifuge the mixture and remove the conjugate by pipette or separate from the resin by centrifugation through a few layers of cheesecloth fastened in place in the mouth of a centrifuge tube. Dialyse against buffered saline overnight to restore the pH to the proper range.

7. Clear the conjugate by centrifugation if necessary and add 1 : 10 000 thiomersal. It is convenient to freeze the bulk of the conjugate and to keep small aliquots available at 5° C.

NOTES

1. Preparation of carbonate buffer:

Solution A: Na_2CO_3, 5.3 g;
H_2O to make 100.0 ml.

Solution B: $NaHCO_3$, 4.2 g;
H_2O to make 100.0 ml.

Theoretically, a pH of 9.0 should result from mixing 4.4 ml of solution A with 100 ml of solution B. In practice, it is sometimes necessary to add as much as 17 ml of solution A to 100 ml of solution B. The pH should be checked on a meter.

2. Preparation of phosphate buffer:

Solution A: Na_2HPO_4, 1.4 g;
H_2O to make 100.0 ml.

Solution B: $NaH_2PO_4.H_2O$, 1.4 g;
H_2O to make 100 ml.

Add 84.1 ml of solution A to 15.9 ml of solution B; add H_2O to make 1 litre. For buffered saline, add 8.5 g of NaCl before adding water.

3. Variation has been found in the staining quality of different batches of dye. This is presumably due to variations among lots of aminofluorescein, resulting in different labelling efficiencies. If results are disappointing with one lot of dye, it may be worth while labelling another aliquot of the same antiserum with a different lot of dye.

Annex 4

PREPARATION OF SMEARS FOR STAINING WITH FLUORESCENT ANTIBODY

1. Prepare suspensions of pure cultures or faecal specimens by mixing either a small amount of growth scraped from an agar slant or a loopful of faeces in 0.5-1.0 ml of sterile 0.85% saline. Enteropathogenic *E. coli* often multiply in saline faecal suspensions held at room temperature or at 37° C. Advantage may be taken of this fact to enhance detection of *E. coli* by incubation of the suspension for an hour or so before making smears. This procedure accentuates the difference between positive and negative specimens by the FA test. Incubation of the specimen may not be desirable if cultural procedures are to be carried out.

2. Prepare smears on clear glass slides which are 1.0-1.1 mm thick and which are free from aberrations (Note 1). Thicker or thinner slides may prevent bringing the objective into focus (Note 2). On a single slide, make two smears from each culture or faecal suspension. One of these will be stained with Pool 1 and the other with Pool 2 conjugates for *E. coli* as described in Annex 6. Etch circles approximately 1.5 cm in diameter on slides prior to use, or use a diamond-point pencil to circumscribe the smears after preparation.

3. Air-dry the smears and fix by passing the slides very quickly through a gas flame. Take care not to overheat the smears. Some investigators have found acetone fixation to be satisfactory (Nelson & Whitaker, 1960).

NOTES

1. Etched slides for FA work are available commercially as follows: Cat. STO-SAF-T-GRIP, double ring, fluorescent antibody slides, packed 10 gross per shipping container, from Propper Manufacturing Co., Inc., 10-34 44th Drive, Long Island City 1, N.Y. These may also be obtained through Aloe Scientific, A.S. Aloe Co., St. Louis, Mo.

The cost of these slides varies with allowable thickness tolerance. We have purchased slides under specifications allowing a range of thickness from 1.0 mm to 1.3 mm

with a request that they be cut as close as possible to 1.0 mm. The slides obtained were approximately 1.0 mm and were satisfactory. At least one laboratory has not been so fortunate since their slides were too thick to use. The purchaser must decide whether or not the risk is justified in view of the reduced cost or whether he should specify a thickness within known usable limits. Information concerning thickness of the stocks of slides may be obtained from the supplier prior to purchase.

2. The American Optical Co. recommends slides of thickness 1.15 to 1.25 mm for use with their cardioid dark-field condenser. Condensers obtained from other optical companies may require slides of thicknesses different from those mentioned here.

Annex 5

TITRATION OF LABELLED ANTIBODIES

1. Prepare twofold dilutions of the labelled antibody in sterile 0.85% saline (Note).

2. Stain smears of the appropriate strains with each of the dilutions of conjugate, as directed in Annex 6.

3. Examine under the fluorescence microscope. Record fluorescence intensity as follows:

4+: Brilliant yellow-green, maximal fluorescence.
3+: Bright yellow-green fluorescence.
2+: Visible fluorescence, but much less bright.
1+: Barely visible fluorescence.
−: No fluorescence.

4. Record staining titre as the reciprocal of the highest dilution that gave 4+ staining. For diagnostic purposes use the conjugate at one-half this dilution.

NOTE

If it is desired to preserve the conjugates, dilute them in sterile 0.85% saline containing a final concentration of 1 : 10 000 thiomersal. They will retain activity for one to two weeks if kept refrigerated and uncontaminated. Undiluted conjugates have been stored for periods of three years or more with no appreciable loss of titre. However, they should be retitrated prior to use if stored for long periods.

Annex 6

STAINING SMEARS WITH FLUORESCENT ANTIBODY

1. Cover one of the fixed smears with one drop of the fluorescein-labelled conjugate designated Pool 1; cover the second smear with Pool 2. Pool 1 contains antibodies for the following types: O26:B6, O55:B5, O111:B4, and O127:B8. Pool 2 contains antibodies for O86:B7, O119:B14, O125:B15, O126:B16, and O128:B12.

2. Cover the slides with a large Petri dish top lined with moistened filter paper. This prevents evaporation of the staining reagents.

3. After 15-30 minutes, wash away the excess FA reagent by dipping the slides briefly into a vessel of 0.85% saline. Then gently place them in a bath of buffered saline (pH 7.5) for 5-10 minutes. Change the saline after each group of slides has been washed. This will reduce the chance of stained organisms from positive smears becoming attached to negative smears.

4. Remove the slides, allow to drain and gently blot off excess saline with absorbent paper. (Caution! Never use the paper more than once, since transfer of organisms may give an erroneous result.)

5. Place a small drop of mounting fluid (Note) on the smear and cover with a No. 1 cover-slip. Cover glasses should be selected with care. For use with high-power objectives they should usually be between 0.16 mm and 0.18 mm in thickness. If an excess of mounting fluid is used, it may mix with the immersion oil on the top surface of the cover-slip impairing observation of the smear. Seal the cover-slip to the slide with fingernail polish if the preparation is to be transported or preserved. Stained smears will remain fluorescent for several weeks if kept refrigerated.

NOTE

Mounting fluid may be prepared by adding one part of phosphate buffered saline to 9 parts of reagent grade glycerol. The refractive index of this mixture is approximately 1.46. Since glycerol tends to become acid, only a small quantity of mounting fluid should be prepared at one time and the pH of this should be adjusted to 7.5. The authors prefer to add one part of the carbonate buffer as given in Annex 3 to 9 parts of glycerol. This furnishes a margin of safety and may enhance fluorescence.

Annex 7

EXAMINATION OF SMEARS

1. Examine smears under a microscope fitted with a cardioid-type darkfield condenser and illuminated by an intense light source such as the Osram HBO-200 mercury vapour lamp. This light source is available in the equipment supplied by several manufacturers. An auxiliary incandescent lamp aids in focussing the smear and supplies useful information concerning the unstained bacteria and debris in the preparation.

2. Oil the condenser to the slide employing fluorescence-free immersion oil. Cargille's Type A, Very Low Fluorescence, Non-drying Immersion Oil for Microscopy may be obtained from Fisher Scientific Co., USA. Its refractive index is 1.515 at 25° C.

3. Bring the area to be examined in focus under the low power objective.

4. Switch to the oil immersion lens.

NOTE

Maximal fluorescence is observed only when all components of the optical system are properly aligned. The filter combination employed influences the intensity and colour of the fluorescent organisms. The optical and lighting system employed by the authors consisted of the Leitz Ortholux microscope with a darkfield condenser illuminated by the Leitz fluorescent lamp assembly utilizing either the Philips CS-150 high-pressure mercury vapour lamp or the Osram HBO-200 lamp. The authors have used the Schott UG-1 (Ultraviolet 2 mm) primary filter, which is comparable in transmission with the Corning 5840 (4 mm). The ocular filter was the Leitz UV-2 (yellow-green, 1 mm), which is approximately the same as the Schott GG-9 (1 mm). A combination of the Schott BG-12 (3 mm) and the Schott OG-1 (1 mm) should also give satisfactory results. Photographs were taken on Super Anscochrome Daylight Film (135) employing the Leitz Micro Ibso attachment. Exposure times ranged from two to four minutes. The reader should refer to publications dealing with the theory and use of lighting and optical equipment for fluorescence microscopy. The review of Price & Schwartz (1956) is excellent.

Annex 8

INTERPRETATION OF RESULTS

Fluorescent antibody procedures applied to the detection of *E. coli* in faecal smears are usually definitive in terms of the numbers and intensity of staining of the bacteria. Differentiation of positive and negative specimens is enhanced by incubation of the faecal suspension for an hour or two at room temperature or at 37°C. Under these conditions each microscopic field of a positive smear often contains numerous organisms. Usually, no organisms are seen in smears read as negative. However, since practical considerations dictate the simultaneous rinsing of smears on several slides, the possibility exists that an occasional stained organism may be transferred from a positive to a negative smear. For this reason, the authors usually re-examine, after incubation of the faecal suspension, any specimen in which less than 10 stained organisms resembling *E. coli* were found. Smears are scanned for one to two minutes before being recorded as negative. A positive result usually can be determined in a matter of seconds.

Faecal smears occasionally contain unidentified bacteria which stain brightly but which are morphologically unlike the enteropathogenic *E. coli*. Such organisms do not present a problem except to the novice. Occasionally, bacteria are encountered which are stained typically by the *E. coli* conjugates but which prove to be serologically related to heterologous types of *E. coli* or to other enteric organisms. In one study, involving 291 infants with diarrhoea, such an organism was encountered only once. In another study of 130 patients there were two occurrences of this type. In a few specimens, bacteria have been encountered which were stained by all of the conjugates for *E. coli* with which they were tested. These organisms were not naturally fluorescent, but were stained by conjugates for other enteric bacteria. The data suggest that staining was due to the presence of naturally occurring antibody in the serum of the rabbits.

Occasionally, when only small numbers of stained organisms were observed in faecal smears treated with a conjugate pool, they could not be located in

smears stained with the components of that pool. This finding may be due to chance alone, but it also may result from the existence of slight serological relationships between the antigens of an unknown enteric bacterium and antibodies to two or more of the EEC. Thus, a cumulative staining effect produced by the conjugate pool is not demonstrable with individual components of that pool. However, if failure to observe stained cells is due to insufficient organisms, FA examination of additional smears from the incubated faecal suspensions may yield positive results. Cohen, Page & Stulberg (1961) made observations similar to the above but did not attempt an explanation.

The possibility of encountering multiple infections with pathogenic *E. coli* or, at least, observing two or more OB groups in a faecal smear must be recognized. The authors have often seen specimens in which organisms were stained by two and occasionally three OB conjugates. In many of these cases, multiple serotypes of EEC have been isolated. Complete examination of each specimen is the only method which will give the essential information without distortion.

Abbreviation of the procedure must be avoided. Such a temptation may arise in the study of epidemics in which it has been established that a given serotype of *E. coli* is the principal cause of illness. Screening of the population for that type only usually cannot be justified, owing to the possibility that two or more serotypes of *E. coli* are etiologically implicated in the outbreak. Even when it is established that only a single type is present, there can be no assurance that other types will not be introduced immediately thereafter. If an epidemic requires immediate control, a decision to screen for a single OB group of *E. coli* should be made only after careful consideration of the risks involved. Responsibility for obtaining misleading results must be assumed by those who alter the recommended procedure.

It is imperative that representative specimens from any group of individuals studied be cultured and the *E. coli* serotyped according to conventional procedures (Ewing et al., 1956). The FA technique permits rapid selection of positive specimens for further study. The K, O, and H antigens of cultures from outbreaks of infantile diarrhoea should be identified accurately. It can then be determined if they are serotypes of *E. coli* known to be associated with diarrhoea.

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