Isolation and Identification of ESCHERICHIA COLI SEROTYPES Associated With Diarrheal Diseases

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CONTENTS

INTRODUCTION ... 1

ISOLATION AND PRELIMINARY IDENTIFICATION ... 2
- General Considerations ... 2
- Isolation ... 4
- Preliminary Identification ... 4
- Fluorescent Antibody (FA) Technics ... 9

FINAL IDENTIFICATION OF ESCHERICHIA COLI SEROTYPES ... 10
- O Antigen Determination ... 10
- K Antigen Determination ... 14
- H Antigen Determination ... 17
- E. coli Serotypes from Animals ... 18

APPENDIX I – Production of Escherichia coli Antisera ... 19
- O Antisera ... 19
- K Antisera ... 20
- H Antisera ... 22

APPENDIX II – Suggested Form for Use in Furnishing Information ... 23

REFERENCES ... 24

LIST OF TABLES AND FIGURES

Table 1 – Escherichia coli Serotypes Reported in Diarrheal Disease ... 3
Table 2 – Preliminary Examination of the K and O Antigens of Escherichia coli Cultures from Cases of Infantile Diarrhea (Slide Tests) ... 6
Table 3 – Biochemical Reactions of Cultures of Escherichia coli ... 8
Table 4 – Example of Tests With O Antigen Suspensions Prepared With an Unknown E. coli Culture ... 11
Table 5 – Relationship of the O Antigens of E. coli O 25 and O 26 Cultures ... 12
Table 6 – The Interrelationship of the O Antigens of Escherichia coli Cultures of O Groups 86, 90, and 127 ... 13
Table 7 – O Antigenic Relationship of Escherichia coli O 86 Cultures ... 13
Table 8 – Differentiation of E. coli 86 and O 127 Cultures (Slide Tests) ... 13
Table 9 – Relationship of the O Antigens of Escherichia coli O 127 Cultures ... 15
Table 10 – Absorption of E. coli Antisera ... 16
Table 11 – Absorption of E. coli H Antisera ... 17
Table 12 – The K Antigens of E. coli ... 20

Figure 1 – Outline of Methods for Examination of Stool Specimens from Cases of Infantile Diarrhea ... 7

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INTRODUCTION

Investigations in the past have made it clear that diarrheal diseases of infants may be caused by a variety of microorganisms. Epidemics and sporadic cases of infantile diarrhea or diarrhea of the newborn may be caused by agents such as *Salmonella* or *Shigella*. Occasionally, a bacterium of another group is thought to be the etiological agent in a particular outbreak and, in certain instances, viral agents have been incriminated. In a large number of otherwise unexplained epidemics of diarrheal disease of infants, however, certain *Escherichia coli* serotypes are recovered. The designation enteropathogenic *E. coli* was suggested for these particular serotypes by the American Public Health Association Committee on Enteropathogenic *Escherichia coli*.

The concept that certain *E. coli* types might be involved in infantile diarrhea is not a new one. For more than 50 years workers have investigated and discussed the role of coliform bacteria in summer diarrhea, infantile enteritis, or diarrhea of the newborn (see Dupont, 1955, for references to earlier literature). Unfortunately, most of the earlier investigators were hampered by the fact that they used only biochemical tests in their attempts to differentiate between the suspected *E. coli* cultures isolated from cases and those recovered from normal infants or adults. By themselves, biochemical methods are inadequate for such differentiation because the *E. coli* group is composed of many different serotypes that often give identical biochemical reactions. However, a few workers used serological methods in addition to biochemical tests. In fact, Goldschmidt (1933) employed a slide agglutination technique to distinguish "Dyspepsiekoli" cultures isolated from cases of infantile diarrhea and to trace the epidemic spread of the bacteria in the investigation of an institutional outbreak. As pointed out by Dupont (1955), it is unfortunate and strange that Goldschmidt's work did not receive the attention it deserved. Also, Dulaney and Michelson (1935) utilized serologic methods to demonstrate the relationship of a number of strains of *Bacterium coli mutabile* which they isolated from cases of infantile diarrhea during an epidemic. Later it was learned that the cultures isolated by Dulaney and Michelson were *E. coli* O 18a, 18c: K77(B21):H7 (Ewing, Tanner, and Tatum, 1956).

The work of Kauffmann (1943, 1944a, 1944b, 1947, 1954) and his collaborators made possible the development of a system for serologic typing of *E. coli* cultures and resulted in an antigenic schema in which the microorganisms may be classified. Interest in members of the *E. coli* group has been stimulated by the association of certain serotypes (Table 1) with diarrheal disease, by studies on hospital acquired infection, and by work on diseases of animals. As a result of this increased interest, the *E. coli* antigenic schema has been extended so that 146 O, 86 K, and 49 H antigens are now known. Further information relative to these antigens may be found in the publications

of Kauffmann (1954), Edwards and Ewing (1962a), and Ewing, Tatum, Davis, and Reavis (1956). The O antigens of the bacteria are the somatic antigens not inactivated by heat at 121° C. K antigens are somatic antigens that occur as envelopes or as capsules. These antigens inhibit the agglutination of living bacteria in O antisera, but this inhibitory effect is inactivated by heat at 100° C., or 121° C. The H, or flagellar, antigens are also inactivated by heat at 100° C. Antisera for O antigens are prepared by injection of smooth cultures that have been heated at 100° C. for two and one-half hours, or at 121° C. for two hours, to inactivate their K and H antigens. In the preparation of K antisera formalinized or living cultures of K forms, selected from platings, are used for injection into animals. Whenever possible, nonmotile strains should be used for K antiserum production. H antisera are produced with cultures of bacteria that have been passed through semisolid agar several times to enhance the development of H antigen. Detailed discussions of methods for the production and use of O, K, and H antisera may be found in Appendix I. Also, this subject is discussed in the publications of Kauffmann (1954), Edwards and Ewing (1962a), Ewing, Tatum, Davis, and Reavis (1956), and Ewing (1956).

The K antigens of *E. coli* cultures are a class composed of at least three varieties, designated L, A, and B. All varieties occur as sheath, envelope, or capsular antigens that inhibit agglutination of living bacteria in O antisera. One of the most important differences between L and B antigens is that the antibody-binding power of L antigen is inactivated by heat at 100° C., whereas the binding power of B antigen is not. A pure L antiserum may be prepared by absorption of an OL antiserum with a heated suspension of the homologous strain. Pure B antiserum cannot be prepared by this procedure because the antibody binding power of B antigen is not inactivated by heat and, if OB antiserum is absorbed by a heated suspension of the homologous culture, both O and B agglutinins are removed from the antiserum (see Table 12, Appendix I).

ISOLATION AND PRELIMINARY IDENTIFICATION

General Considerations. Since complete serologic typing of *E. coli* cultures is a time-consuming and somewhat involved procedure, it may properly be considered a function of a few centers and research institutions that are equipped for the work. The personnel of many laboratories may prefer to limit their activities to isolation and preliminary identification and to forward selected strains to a center equipped for further study. However, it should be emphasized that in addition to providing adequate means for the isolation of *E. coli* types, all laboratories should complete the following steps in the identification of *E. coli* types whether or not the cultures are to be sent to a center: (1) determination of biochemical reactions, (2) slide agglutination tests in which living suspensions are examined in both OB and O antisera prepared with serotypes that have been associated with diarrheal disease (Table 1), (3) slide tests in which heated suspensions are tested in the same OB and O antisera, and (4) titration of heated suspensions in serial dilutions of the indicated O antisera for confirmation of the O antigen group. These steps, as well as methods of isolation, will be discussed in the paragraphs that follow.

There are certain principles that may be applied to the investigation of any epidemic of infantile diarrhea. Materials for culture should be collected before antibiotic therapy is begun. Specimens may be taken from freshly soiled diapers or collected by means of rectal swabs. If stools or other materials must be held several hours before inoculation of media or are to be sent through the mails, specimens should be emulsified in a preservation medium such as buffered glycerol saline preservative. Samples of stools to be examined for viruses

TABLE 1

ESCHERICHIA COLI SEROTYPES[1] REPORTED IN DIARRHEAL DISEASE

O Antigen	K Antigen	H Antigens (and synonyms)
26	60 (B6)	NM (nonmotile) (E893), 11, 32
55a	59 (B5)	NM (β), 6, 7
86a	61 (B7)	NM (E990), 11, 34
111a, 111b	58 (B4)	NM (α), 2 (D433), 4, 12, 21
112a, 112c	66 (B11)	NM (Guanabara)
119	69 (B14)	NM, 6 (Aberdeen 537-52)
124	72 (B17)	NM, 30
125a, 125b	70 (B15)	19 (Canioni)
125a, 125c	70	15, 21
126	71 (B16)	NM, 2 (E611), 27
127a	63 (B8)	NM (Holcomb)
128a, 128b	67 (B12)	2 (Cigleris), 7, 8, 9, 12
128a, 128c	67	NM, 12

The simple numerical designations applied above to the K antigens are in accordance with the nomenclature recommended by Kauffmann *et al.*, 1956, International Bulletin of Bacteriological Nomenclature and Taxonomy, 6, 63-64.

1 For references to descriptive literature regarding these serotypes, the reader is referred to Ewing, Davis, and Montague (1963).

N.B. Only the more prevalent serotypes are listed (see text).

should be frozen. Such specimens may be placed in ointment boxes, resistant glass or plastic tubes, or other small containers, then quickly frozen in dry ice or in a deep freeze box and maintained at low temperature (- 40° C. to - 70° C.). It should be noted that Neter and coworkers (1953) were able to recover *E. coli* 0111:B4 from specimens after 5 years in the frozen state. Specimens taken at autopsy from cases and materials collected for control purposes may be treated in the manner outlined above. If specimens are collected at autopsy, samples of the intestinal contents and tissue specimens should be taken at various levels, such as ileum, ileocecal junction, transverse colon, and descending colon.

Salmonellae or shigellae are incriminated in certain epidemics of infantile diarrhea, hence a thorough search for members of these groups always should be made. Methods for the isolation and identification of these microorganisms may be found in many publications (*e.g.*, Edwards and Ewing, (1962a, 1962b).

Isolation

Since *E. coli* strains usually do not grow well on the highly selective media in general use for the isolation of salmonellae and shigellae, it is imperative that less inhibitory differential media such as MacConkey agar, eosin methylene blue (EMB) agar, brom cresol purple lactose agar, or other similar media be employed. **In addition to the aforementioned plating media, a blood agar plate or a plain infusion agar plate should be** streaked with a very small inoculum of stool specimen because it has been noted that, in a few instances, *E. coli* serotypes associated with infantile diarrhea appeared on blood agar plates and did not grow on MacConkey agar and similar media. Blood agar is recommended also because it is easier to avoid contaminants when picking colonies from this medium. In addition, *E. coli* serotypes that belong to 055:B5 and 0111:B4 generally are not hemolytic, a fact which may aid in the recognition of colonies. Undue reliance should not be placed upon this observation since many other *E. coli* serotypes also are nonhemolytic. However, the observation may be utilized provided that its limitations are recognized.

Rappaport and Henig (1952) described a modified MacConkey agar plating medium to be used for the differentiation of *E. coli* serotypes 055:B5 and 0111:B4. The medium was prepared by substituting sorbitol for lactose in a medium similar to MacConkey agar. These investigators reported that freshly isolated *E. coli* 055:B5 and 0111:B4 cultures did not produce acid from sorbitol and therefore colonies of these serotypes were colorless on the sorbitol agar plates. Many other *E. coli* types fermented sorbitol and produced red colonies. In the examination of a large number of freshly isolated strains of *E. coli* serotypes that belonged to 055:B5 and 0111:B4, the writers found that although the majority of cultures did not produce acid rapidly from sorbitol, there were a significant number of exceptions in which cultures of both groups fermented this carbohydrate within 24 hours. With the exception of 0127:B8 strains, the majority of which failed to ferment sorbitol or fermented it slowly, cultures that belonged to the other groups listed in Table 1 usually produced acid rapidly from this substrate. Again, it should be remembered that other *E. coli* serotypes may fail to ferment sorbitol. Therefore, it is believed that the sorbitol plating medium has value only after the characteristics of the epidemic strain involved in a particular outbreak are established. If it is found that the epidemic strain produces colorless colonies on sorbitol agar, this medium may be of value in finding further cases or carriers and in the estimation of the number of colonies of the serotype in a specimen. Similar plating media could be prepared with any of several carbohydrates, such as sucrose or salicin, if the epidemic strain did not ferment the sugar and if common *E. coli* types did produce acid from it. The sole use of sorbitol agar plates, or other plating medium based on the same principle, is potentially dangerous in that a search for a sorbitol negative epidemic strain may cause the bacteriologist to miss not only other significant *E. coli* serotypes but also *Salmonella* or *Shigella* strains that may be introduced into a nursery during the course of an epidemic. However, the sorbitol medium may be used in the manner mentioned above provided that it is employed in conjunction with other media such as MacConkey agar and blood or infusion agar.

Preliminary Identification

After incubation for 16 to 20 hours, the primary plating media may be examined for colonies of coliform bacteria. No specific directions can be given as to the selection of colonies for transfer because colonies of the various *E. coli* serotypes all appear quite similar. Portions of ten or more individual colonies that appear on the blood agar or plain infusion agar plates should be tested directly in antiserums. If strongly positive slide agglutination tests are obtained by this procedure, it may be considered presumptive evidence that one of the particular *E. coli* serotypes is present in the specimen. This procedure not only affords a rough estimate of the presence or absence of serotypes for which antisera are available, but it also indicates the prevalence of a given sero-

type in the specimen. Examinations of this sort should be made with colonies on blood agar or infusion agar plates rather than colonies from MacConkey agar plates since it has been shown that the presence of bile salts may cause confusing agglutination reactions, which are not confirmed in subsequent work (McNaught and Stevenson, quoted by Stevenson, 1956). Three or more entire, smooth, opaque, escherichia-like colonies from each plating medium should be transferred to infusion agar slants (long slants prepared from plain infusion agar, without added sugar). Additional transfers should be made if possible. The slants should be inoculated over the entire surface in order to obtain maximum growth. After incubation these slants are used for biochemical and serological studies.

A generous portion of the growth from each of the agar slant cultures should be emulsified in about 0.5 ml. of 0.5 percent sodium chloride solution to make a very dense suspension. Droplets of the heavy suspensions may be tested for agglutination on slides with droplets of *E. coli* O and OB antisera, first as living suspensions and again after being heated. Agglutination of a living antigen in an OB antiserum and lack of a reaction in the corresponding O antiserum are indicative of the presence of B antigen in the strain. If such a reaction occurs in one of the OB antisera, the suspension is heated at 100° C. for one hour, cooled, and retested in the indicated OB and O antisera. If the culture belongs to the O antigen group, the heated antigen may be expected to react in both OB and O antisera. An example of results that may be anticipated with freshly isolated cultures is given in Table 2. It is to be emphasized that in slide agglutination tests with the unabsorbed antisera, **cognizance should be taken only of strong reactions,** and undue significance should not be attached to weak and delayed agglutination. Also, it must be pointed out that if a living suspension reacts strongly in O, as well as in the OB antiserum, the culture should be plated, and colonies that are inagglutinable in O antiserum should be selected for re-examination.

Next, the heated suspensions prepared from agar slant cultures are diluted and titrated in serial dilutions (1:200 to 1:6,400) of the indicated O antiserum. If desired, broth cultures that have been incubated for 4 to 6 hours and then heated at 100° C. for one hour may be used in the titrations and, in the absence of O antisera, OB antiserum may be employed. Such tests are read after 16 to 18 hours' incubation in a water bath at 48° to 50° C. If the results of slide agglutination tests performed in the first steps were clear-cut and the culture was inagglutinable in O antisera in the living state, confirmation by agglutination to or near the titer of the antiserum may be expected in the titrations. Other *E. coli* cultures, however, are related to certain of the serotypes associated with infantile diarrhea through possession of common O antigenic fractions. A strain that belongs to one of these related O groups may be expected to cross-react in the titrations. Also, it should be remembered that living suspensions of *E. coli* cultures that belong to related O antigen groups may react in OB and O antisera in slide tests if they are not O-inagglutinable, and heated suspensions may be expected to cross-react. As an example of this, the O antigens of O group 25 strains are related to those of 026, and an O group 25 suspension may react in 026:B6 and 026 antisera in slide tests, but when tested by titration in 026 antiserum, only a relatively low-titered reaction is obtained.

The serotypes listed in Table 1 occurred more frequently among cultures submitted to the Enteric Bacteriology Laboratories from laboratories within the United States (Ewing, Tatum, and Davis, 1957; Ewing, Davis, and Montague, 1963). However, serotypes of OB groups 86:B7, 112a, 112c:B11, and 124:B17 occurred less frequently than the others listed. If the data reported by the above-mentioned workers may be taken as an index for the selection of antisera, it may be suggested that O and OB antisera *E. coli* 055:59(B5), 111:58(B4), and 127:63(B8) should be available in all laboratories and that O and OB antisera for all of the *E. coli* groups listed in Table 1 should be added if time and facilities permit their proper use.

After preliminary serological tests are completed, the biochemical reactions of the cultures should be determined to make certain that they belong to the *E. coli* group. If all of the bacterial

TABLE 2

PRELIMINARY EXAMINATION OF THE K AND O ANTIGENS OF *ESCHERICHIA COLI* CULTURES FROM CASES OF INFANTILE DIARRHEA (SLIDE TESTS)

Antigen Suspensions	*E. coli* Antisera							
	026:B6	026	055:B5	055	0111:B4	0111	0127:B8	0127
K antigen (living)	–	–	–	–	4	–	–	–
O antigen (100° C., 1 hr.)	–	–	–	–	4	4	–	–

Symbols: –, no reaction; 4, complete agglutination.

suspensions prepared with strains from a particular specimen are agglutinated by one of the antiserums, one of these strains may be selected for biochemical studies. If the coliform types present in the specimens are not agglutinated by any of the antisera available, it is suggested that the biochemical reactions of three or more strains from each specimen should be determined. All strains that are examined biochemically also should be placed in stock culture medium.*

Although any desired number of biochemical tests may be done (Table 3), the tests listed in Figure 1 are essential to determine whether a culture belongs to the *E. coli* group. In many instances it may be advantageous to determine whether the epidemic strain involved in a particular outbreak ferments sorbitol, dulcitol, salicin, adonitol, or other substrates. Several biotypes of *E. coli* 0111:B4, 055:B5, and 026:B6 were described by Kauffmann and Dupont (1950) and by Ørskov (1951).

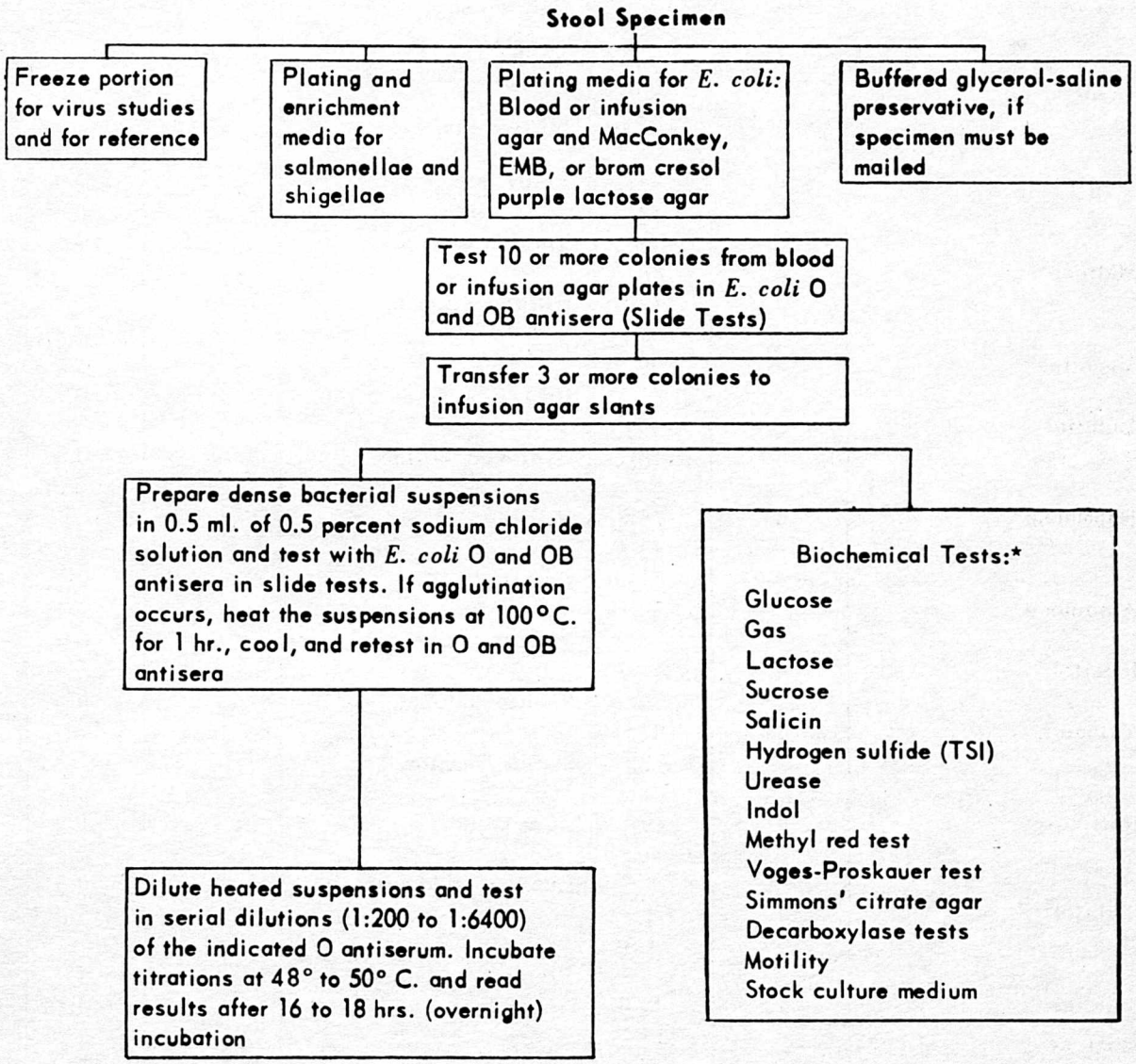
*Stock cultures should be inoculated by stab inoculation into a plain infusion agar medium such as blood agar base. Alternatively, a medium prepared by mixing equal parts of extract agar and nutrient broth may be used. After inoculation the tubes should be stoppered with corks that have been soaked in hot paraffin. Stock cultures should be kept at room temperature in the dark.

If one of the *E. coli* OB groups known to be associated with infantile diarrhea is isolated, it is recommended that one or more cultures from each stool specimen be sent to the State health department laboratory with a request for confirmatory studies. Pertinent information regarding each case should be submitted with the cultures (see Appendix II). In addition to confirmation of the O group, the B antigens, and biochemical characteristics, further studies should be made on the O antigen components of the cultures and their H antigens should be determined. In attempts to trace the source of infection in an outbreak or in studies of intrahospital and interhospital spread of infection, determination of O antigenic components and of H antigens of the strains involved would seem to be essential, just as it is in the investigation of salmonellosis. These determinations also are necessary for the detection of other serotypes of the same O antigen group that may be introduced into a ward by newly admitted cases during the course of an epidemic and for the delineation of serotypes isolated from diverse sources. For a more complete discussion of the values of complete serotyping of selected *E. coli* strains, the reader is referred to Ewing (1956).

If *Salmonella*, *Shigella*, or *E. coli* strains for which antiserums are available are not iso-

FIGURE 1

OUTLINE OF METHODS FOR EXAMINATION OF STOOL SPECIMENS FROM CASES OF INFANTILE DIARRHEA



* See Table 3

TABLE 3
BIOCHEMICAL REACTIONS OF CULTURES OF *ESCHERICHIA COLI*

Substrate or Test		% + (% +)	Substrate or Test		% + (%+)
Glucose	+	100	Cellobiose	–	8.5
gas	+	93			
			Glycerol	+	97
Lactose	+	90.5 (5.5)	Indol	+	99
Sucrose	d	54 (7)	Methyl red	+	99.9
			Voges-Proskauer	–	0
Maltose	d	88 (4)	Citrate, Simmons'	–	.3 (.8)
Mannitol	+	>99			
			Urease	–	0
Dulcitol	d	49 (18)	Hydrogen sulfide	–	0
Rhamnose	d	78 (3.5)	Nitrite	+	100
			Gelatin (22 C)	–	0
Arabinose	+	>99			
			Phenylalanine deaminase	–	0
Inositol	–	.6			
			Sodium malonate	–	0
Xylose	d	87 (2)	Sodium mucate	+	93
Raffinose	d	49 (1.5)	KCN broth	–	1
			Lysine decarboxylase	d	69.5* (14) **
Sorbitol	d	75 (1.3)			
			Arginine dihydrolase	d	4* (40)**
Salicin	d	44.5 (17)			
			Ornithine decarboxylase	d	52* (9)**
Adonitol	–	6			
Trehalose	+	>99	Motility	+ or –	70

+, 90 per cent or more positive in 1 or 2 days.
–, 90 per cent or more negative in 1 or 2 days.
d, different reactions [+, (+), –]
(% +), per cent positive delayed 3 or more days.
*, per cent positive in 1 day.
**, per cent positive on second or third day.

lated from cases in an outbreak, three or more cultures of the *E. coli* types that are recovered from each case should be forwarded to the State health department with a request for serologic typing. Such cultures may belong to an *E. coli* serotype previously found in association with infantile diarrhea, or they may represent a serotype not hitherto recognized as being associated with the disease. In any event, additional information about the association of *E. coli* serotypes with cases of infantile diarrhea will become available as a result of the studies.

It should be emphasized that anyone may enlarge the scope of their work on *E. coli* as time and facilities permit. In fact this is to be commended and encouraged, for additional centers are needed. It is for this reason that methods for more complete serologic characterization of *E. coli* cultures are included in this manual (*v. inf.*).

Fluorescent Antibody (FA) Technics. In recent years the use of antibody globulins labeled with fluorescin has been advocated for identification of the etiological agents in a wide variety of infectious disease processes (*v.* Cherry, Goldman, Carski, and Moody, 1960, for an excellent review of methods and applications). Whitaker *et al.* (1958) reported upon the identification of *E. coli* 0127:B8 by FA technics and Thomason *et al.* (1959) gave the results of preliminary studies on the use of labeled globulins from OB antisera in rapid presumptive identification of *E. coli* associated with infantile diarrheal disease. Cherry *et al.* (1961) and Thomason *et al.* (1961, 1961b) outlined the results of a field evaluation of the use of FA technics for rapid presumptive identification of *E. coli* OB groups in fecal smears. These investigators also gave detailed procedures for the preparation and testing of the reagents used.

A review of the literature on *E. coli* cited above indicated that within certain limitations, immunofluorescent tests should prove to be valuable in the management and control of diarrheal disease in institutions. As pointed out by Cherry *et al.* (1961), FA technics are not a substitute for isolation and definitive serotyping of the microorganisms, but once the etiological agent in an outbreak has been established through complete characterization by conventional methods, then the FA technic becomes a valuable tool for rapid examination of fecal smears. However, it should be pointed out that all new cases must be examined critically and not through the use of a single labeled OB antiserum, since other types of *E. coli* (or of *Salmonella* or *Shigella*) may be introduced during the course of an outbreak and these would be missed.

As far as the Enterobacteriaceae are concerned, no particular type can be identified by means of FA technics alone any more than it could be by means of slide agglutination tests alone. One has only to consider the known intra- and intergroup antigenic relationships of members of the family to realize that this is true (also, see Davis and Ewing, 1963). Apparently, the reason that FA technics give good results with certain *E. coli* OB groups lies in the specificity of the B antigens and the fact that the OB antisera may be diluted to a point at which O antigen-antibody reactions are minimized.

FINAL IDENTIFICATION OF E. COLI SEROTYPES

Although emphasis is placed upon the identification of serotypes from cases of diarrheal disease the methods outlined for the identification of *E. coli* serotypes may be employed in the examination of all strains. The procedures are based upon those of Kauffmann (1947, 1954) and collaborators but have been modified in certain respects by the author and coworkers.

O Antigen Determination

Heated O antigen suspensions are prepared as outlined in the discussion of methods for testing O antisera (Appendix I). Such suspensions prepared from unknown *E. coli* cultures are tested first in O antiserum pools in single tube tests in which the final dilution of each component O antiserum is 1:1000. Eight pools, organized according to close antigenic relationships, were used by Ewing, Tatum, Davis, and Reavis (1956) for this preliminary procedure, but these were modified (Ewing and Davis, 1961b) as a result of studies on the incidence of members of the various *E. coli* O antigen groups among materials examined over an 11-year period (Ewing and Davis, 1961a). For example, it was found that about 26 percent of the total number of cultures examined reacted in O antiserum pool No. 1, which contained antisera for 22 O antigen groups. However, 82.5 percent of the strains that reacted in pool No. 1 belonged to eight O antigen groups, while 14.5 percent belonged to seven other O groups, and 3 percent belonged to the remaining seven O antigen groups, for which antisera were present in the pool. Hence, pool No. 1 was divided into 1A (eight antisera), 1B (seven antisera), and 1C (seven antisera). Pools 2 to 7 inclusive were divided into 2A, 2B, 2C, etc. on the same basis, since in every instance from 78 to 92 percent of cultures examined belonged to six to eight O antigen groups represented in the A pools. Since the majority of cultures reacted in only one of the subdivided pools (e.g., 1A), further examination of a strain usually required the use of six to eight individual O antisera instead of 13 to 22 or more as was the case with the original pools. Some cross reactions occurred in the above-mentioned subdivided pools, but these generally formed patterns that are easily recognized after some experience with the use of the pools was acquired. For further information concerning the preparation and use of O antiserum pools for preliminary examination of *E. coli* cultures, the reader is referred to Ewing and Davis (1961b). It is possible to prepare pools of *E. coli* O antisera for special purposes such as the pool 4 mentioned in the first edition of this book. This pool contained O antisera for O antigen groups 26, 55, 111, 127, etc., and may be used for the examination of strains from cases of infantile diarrheal disease. Similarly, pools may be prepared for preliminary examination of cultures from extraintestinal sources or for use in veterinary bacteriology, as mentioned by Ewing and Davis (1961a). The pools are diluted with phenolized isotonic saline solution so that the final concentration is 1:100 for each component O serum. Nine-tenths ml of heated O antigen suspension is added to 0.1 ml of each O serum pool and the tests are placed in a water bath at 48 to 50 C for overnight incubation. Parenthetically, antigens heated at 100 C. are tested first in the pooled serums but if these tests are negative they are repeated with autoclaved antigen suspensions. If agglutination occurs in one, or possibly more, of the pools, O antigen suspensions are then tested in each of the individual O antisera that comprise that pool or pools. The latter tests are also single tube tests and the dilution employed is 1:500 (Table 4). If agglutination occurs in one of the O antisera the O antigen then is titrated in that antiserum for confirmatory purposes. In the case of many *E. coli* cultures the above-mentioned procedures for O grouping may suffice and further examination of the O antigens may not be warranted. However, several of the serotypes associated with diarrheal disease contain O antigenic factors which

Table 4
EXAMPLE OF TESTS WITH O ANTIGEN SUSPENSIONS PREPARED WITH AN UNKNOWN *E. COLI* CULTURE

O Antiserum Pools (1:1000)																		
1A	2A	3A	4A	5A	6A	7A	1B	2B	3B	4B	5B	6B	7B	1C	2C	3C	4C	5C
–	–	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Individual O Antisera (Components, Pool 5A)																		
					062	075	078	086	0126	0127	0128							
					–	–	–	4	–	–	–							
Titration in O antiserum for 086 and slide tests in absorbed O antisera																		

are present in other O antigen groups.* In such instances further examination of the O antigen components is necessary if they are to be exactly identified. Also, many of the O groups may be subdivided into two or more subgroups which may be identified by analysis of the component O antigen factors. For analysis of the factors involved in such relationships, absorbed O antisera may be employed in slide agglutination tests.

O Groups 25 and 26. The O antigens of cultures that belong to O groups 25 and 26 are related to each other (Table 5) and to those of a number of other O antigen groups as well. The relationship shown in Table 5 is known as an a, b - a, c relationship. The factor that is common to the two cultures and which accounts for the cross agglutination in unabsorbed antisera is labeled a, while the factors that remain after reciprocal absorption are labeled b and c, respectively. However, these two absorbed antisera are not entirely specific because cultures of O groups 4, 18, 62, and 102 may react in O 25 serum absorbed with O 26 and cultures of O groups 4, 18, and 102 may react in O 26 antiserum absorbed by O 25. Hence, *E. coli* O 26 antiserum should be absorbed by suspensions of O groups 4, 18, and 25, as shown in Table 10 *E. coli* O group 25 antiserum may be absorbed by O groups 4, 18, 26, and 102 to render it more specific.

O Group 55. The O antigens of cultures that belong to *E. coli* O group 55 are not related significantly to those of other *E. coli* O antigens but the O group has been subdivided. Ørskov and Fey (1954) described cultures of O group 55 that differed from the type culture in that they contained an O antigenic factor that was not present in the type culture (i.e., O 55a and O 55a, 55b).* Although no absorption is listed in Table 10 for the preparation of antiserum for differentiation of cultures that belong to these subgroups, such antiserum could

*It should be emphasized that the reactions and relationships mentioned herein are those obtained with lots of antisera prepared by the writers and that some of the relationships may not be apparent in antisera prepared in other laboratories. It is a recognized fact that antisera prepared in different laboratories with the same cultures may vary in their agglutinin content for minor factors. It is believed that this may be caused by differences in the titers of such antisera, to variations in the manner in which individual animals respond to certain agglutinogens, and to unknown variations in the minor factors.

*See Table 7 for an example of the a-a,b variety of relationship.

Table 5
RELATIONSHIP OF THE O ANTIGENS OF *E. COLI* O 25 AND O 26 CULTURES

O antigen Suspensions (100 C, 1 hr.)	*E. coli* O Antisera			
	25		26	
	Unabsorbed	Absorbed by *E. coli* O 26	Unabsorbed	Absorbed by *E. coli* O 25
E. coli O 25	20,480	10,240	1,280	0
E. coli O 26	1,280	0	20,480	20,480

be prepared by absorption of O 55a, 55b antiserum with a suspension of O 55a. It should be noted that some *E. coli* O 55 antisera, prepared in this and other laboratories, react with *E. coli* O 22 cultures. The reason for this phenomenon is unknown but O 55 antiserum should be tested with a suspension of *E. coli* O 22 and absorbed with it if necessary.

O Group 86. The important O antigenic relationships of *E. coli* O 86 cultures are listed in Table 6 and the relationship between the O antigens of O 86a and O 86a, 86b cultures are given in Table 7 (Ewing, Tanner and Tatum, 1955). It will be noted that in the type of relationship (a - a,b) shown in Table 7, only one of the cultures contains a special factor (b). The absorbed O antisera advised for the differentiation of *E. coli* O 86 cultures are given in Table 8 together with the reactions that may be expected.

O Group 111. The O antigens of *E. coli* O 111 cultures are not related significantly to those of other *E. coli* O antigen groups but the O group may be subdivided into at least three factors. Ewing, Galton, and Tanner (1955) described the relationships extant between cultures of *E. coli* O group 111 from cases of infantile diarrhea (O 111a, 111b: B4) and strains from monkeys (O 111a, 111c: B4). Reciprocally absorbed O antisera may be used to differentiate these subgroups. Also, Ørskov (1954b) described a culture of *E. coli* O 111 which contained a factor not present in strains from cases of infantile diarrhea.

O Group 112. As mentioned by Ewing and Kauffmann (1950), this O antigen group is divisible into O 112a, 112b and O 112a, 112c and these subgroups may be differentiated by the use of cross absorbed O antisera. *E. coli* O 112a, 112c cultures are not related to other O antigen groups in any important way but there is a relationship between the O antigens of O 112a, 112b and O 113 strains (Ewing, Hucks, and Taylor, 1952; Ewing, Tatum, Davis, and Reavis, 1956).

O Group 119. Cultures of O group 119 are not known to react significantly in O antisera for other *E. coli* O antigen groups but O 48 suspensions react in O 119 antiserum prepared by the writers. This unilateral relationship necessitates the absorption of O 119 antiserum by *E. coli* O 48.

O Group 124. The O antigens of *E. coli* O 124 cultures are not significantly related to those of other *E. coli* O antigen groups.

O Group 125. There is a reciprocal (a,b - a,c) relationship between the O antigens of *E. coli* O 125 and those of *E. coli* O 73 which necessitates cross absorption of the O antisera. Also there are unilateral relationships which cause agglutination of *E. coli* O 7 and O 11 suspensions in O antiserum prepared with *E. coli* O 125. The latter relationships generally are slight but may require absorption of O 125 antiserum with O group 7 and 11 cultures. Further, O antigen group 125 is divisible into two subgroups, O 125a, 125b and O 125a, 125c (Ewing and Tanner, unpublished data; Ewing, Tatum, and Davis, 1957).

Table 6
THE INTERRELATIONSHIP OF THE O ANTIGENS OF *ESCHERICHIA COLI* CULTURES OF O GROUPS 86, 90, AND 127.

O Antigen Suspensions (100 C, 1 hr.)	*E. coli* O Antisera (unabsorbed)				
	86a	86a,86b	90	127a	127a,127b
86a	5,120	5,120	640	0	5,120
86a,86b	20,480	20,480	640	0	10,240
90	2,560	160	5,120	1,280	5,120
127a	1,280	0	640	20,480	5,120
127a,127b	10,240	640	1,280	10,240	20,480

Table 7
O ANTIGENIC RELATIONSHIP OF *ESCHERICHIA COLI* O 86 CULTURES

O Antigen Suspensions (100 C, 1 hr.)	O Antisera			
	86a		86a 86b	
	Unabsorbed	Absorbed by 86a, 86b	Unabsorbed	Absorbed by 86a
86a	5,120	0	5,120	0
86a, 86b	20,480	0	20,480	5,120

Table 8
DIFFERENTIATION OF *E. COLI* O 86 AND O 127 CULTURES (SLIDE TESTS)

O Antisera	Absorbed by	*E. coli* O Antigen Suspensions (100 C, 1 hr.)				
		O 86a	O 86a,86b	O 90	O 127a	O 127a,127b
O 86a, 86b	O 86a + O 90	0	4	0	0	0
O 86a	O 90	4	4	0	0	0
O 90	O 127a	0	0	4	0	0
O 127a,127b	O 127a + O 90	0	0	0	0	4
O 127a	O 90	0	0	0	4	2

O Group 126. There is a unilateral relationship between *E. coli* O groups 126 and 75 which produces agglutination of O 75 in O 126 antiserum. The writers employ reciprocally absorbed antisera for the differentiation of these O groups. The O group 126 is not significantly related to other known O groups of *E. coli.*

O Group 127. The relationships between *E. coli* O groups 86, 90, and 127 were mentioned. Furthermore, O group 127 may be divided into subgroups O 127a and O 127a, 127b (Tables 6 and 9). The absorbed O antisera advised for differentiation of cultures that belong to these subgroups are given in Table 8. A relatively minor relationship is known which causes agglutination of *E. coli* O 128 cultures in O 127a and O 127a, 127b O antisera but this usually does not cause difficulty in diagnosis.

O Group 128. The O antigens of cultures of this *E. coli* O group are related reciprocally to those of strains that belong to O groups 48 and 87 but the relationships are minor and generally are not detected in O antiserum pools used in a dilution of 1:1000. However, if tests indicate that absorption of O 128 antiserum is necessary, the serum may be absorbed by O 48 or O 87 because either of these O group cultures removes agglutinin for the other. O antisera for O groups 48 and 87 should be absorbed in the manner given in Table 10. Further, Taylor and Charter (1955) indicated that the O antigens of all *E. coli* O group 128 cultures are not identical and that the group may be subdivided. This observation was confirmed by Ewing and Tanner (unpublished data), Ewing, Tatum, and Davis (1957) who demonstrated subgroups O 128a, 128b and O 128a, 128c within O group 128. Reciprocally absorbed O antisera (Table 10) may be used to differentiate cultures that belong to these subgroups.

The methods used for the preparation of the absorbed O antisera listed in Table 10 are similar to those employed with other Enterobacteriaceae. Growth from the required number of infusion agar plates is suspended in physiological saline solution. The suspensions are heated at 100 C for 1 hour and then centrifuged. One-half of the absorbing dose of sedimented bacteria is suspended in O antiserum diluted 1:10 with phenolized physiological saline solution and placed in a water bath at 48-50 C for 4 hours. Following this, the mixtures are centrifuged and the diluted O antiserum is added to the second half of the absorbing dose of bacteria. The mixtures are again placed in the water bath for an additional 2 hours' incubation, after which they may be placed in a refrigerator overnight. After centrifugation, the antisera are tested with O antigen suspensions prepared from the homologous and absorbing strains. If absorption is incomplete, the antisera may be reabsorbed with the growth from a few additional plates.

K Antigen Determination

Results obtained by slide agglutination should be confirmed by titration of living or formalinized antigen suspensions in KO antisera. Infusion broth cultures prepared from selected KO colonies, and incubated for 4 or 5 hours at 37 C are suitable as antigen suspensions. These may be preserved by addition of 0.5 percent formalin. **Such tests should be controlled by titration of the same K antigen suspension in the appropriate O antiserum in order to demonstrate inhibition of O agglutination.** Control tests are incubated in the same manner as K agglutination tests at 37 C for 2 hours, followed by overnight incubation in a refrigerator. Confirmation of the results of slide tests are obtained when the K antigen suspension is agglutinated in characteristic manner to, or near, the K titer of the KO antiserum and no agglutination or minimal reactions are seen in the O antiserum control. If a number of similar strains are recovered from different patients in connection with an outbreak of diarrheal disease, it is advisable to absorb the KO antiserum in which the cultures reacted with a heated suspension prepared from at least one of the cultures. This procedure aids in the identification of both the K and O antigens of the epidemic strain. For example, if the B and O antigens of the epidemic strain are identical with those of the culture with which the antiserum was prepared, a heated suspension prepared from the former may be

Table 9
RELATIONSHIP OF THE O ANTIGENS OF *ESCHERICHIA COLI* O 127 CULTURES

O Antigen Suspensions (100 C, 1 hr.)	O Antisera			
	127a		127a, 127b	
	Unabsorbed	Absorbed by 127a, 127b	Unabsorbed	Absorbed by 127a
127a	20,480	0	5,120	0
127a, 127b	20,480	0	20,480	10,240

expected to remove both O and K agglutinins from the serum. It is felt that all cultures isolated from sources other than epidemic diarrhea which are agglutinated by antisera for diarrheal strains should be examined by absorption tests as outlined above. It is known that many cultures from diverse sources are not identical with serotypes isolated from infantile diarrhea cases although they may exhibit varying degrees of relationship.

Cultures from cases of diarrhea of the newborn that do not react in KO antisera prepared with those serotypes known to be associated with diarrheal disease, as well as strains from other conditions, must be examined in antisera prepared with the other K antigens of the *E. coli* group. Slide agglutination tests with pooled K antisera may be employed for preliminary examinations (*v.* Ewing, Tatum, Davis, and Reavis, 1956). A number of these pools may be prepared, each containing equal quantities of five K antisera and if tests indicate its practicability, an equal volume of phenolized saline solution may be added to each. If a K antigen suspension is agglutinated in one of the several pooled K antisera, it is then tested on a slide in the constituent sera that compose that pool. Results obtained in slide agglutination tests should be confirmed by titration of K antigen suspensions in KO antisera in the manner outlined above for confirmation of K antigens. When possible it is advisable also to examine K antigen suspensions on slides in pure K antisera prepared by absorption. An outline of absorption methods for use with K antisera was given by Ewing, Tatum, Davis, and Reavis (1956). As pointed out by Kauffmann (1954), K antisera should not be diluted more than 1:5 for absorption because of their comparatively low titers.

In the determination of A antigen (and frequently the L and B antigens, as well) in *E. coli* cultures it is possible to use the quellung reaction since these antigens occur as capsules. Kauffmann (1954) recommends this method if cultures are not entirely inagglutinable in O antisera.

The presence of H agglutinins in *E. coli* K antisera prepared with motile cultures is a potential source of error in slide agglutination tests and must be considered as such. Studies made in this laboratory indicate that H agglutinins can be demonstrated in about 75 percent of K antisera prepared from motile strains when tube agglutination tests are made with passaged H antigen preparations. However, the results of slide agglutination tests were negative in all but a few instances when the same K antisera were tested in 1:5 dilutions with antigens prepared from infusion agar slant cultures of freshly isolated strains that had not been passaged in semisolid medium or treated in any other way to enhance H antigen development. In all of the above-mentioned tests cultures were employed which contained common H antigens but different O and K antigens. The results of the writer's studies tend to confirm those of Kauffmann (1947). The important points here are that nonmotile cultures should be employed for K antiserum production whenever possible and that if motile strains must be used for this purpose, the K antisera should be tested for their H agglutinin content. If the H agglutinin content of any of the antisera is sufficient to cause confusion, it should be removed by appropriate absorption.

Table 10

ABSORPTION OF *E. COLI* O ANTISERA

E. coli O Antiserum 1.0 ml	Absorbed by *E. coli* O group	No. of Plates
26	4+	5
	18+	5
	25+	5
	102	5
86a,86b	86a+	5
	90	5
86a	90	15
90	127a	15
127a	90	15
127a,127b	127a+	5
	90	5
111a,111b	111a,111c	15
111a,111c	111a,111b	15
112a,112b	112a,112c	15
112a,112c	112a,112b	15
119	48	10
125a,125b	73	15
125a,125b	125a,125c	15
125a,125c	125a,125b	15
126	75	15
75	126	15
128a,128b	87	15
87	128a,128b	15
48	128a,128b+	10
	87	10
128a,128b	128a,128c	15
128a,128c	128a,128b	15

H Antigen Determination

The H antigens of freshly isolated *E. coli* cultures generally are poorly developed and do not flocculate in characteristic manner when tested in H antisera. Therefore, it usually is necessary to transfer cultures through several passages in semisolid medium in order to promote H antigen development. Methods for the preparation of satisfactory H antigen suspensions are outlined in Appendix I under *E. coli* H antiserum production and testing. Also, the reader is referred to Kauffmann (1947, 1954) and to Ewing, Tatum, Davis, and Reavis (1956) for further discussions of H antigen preparation.

After two or three passages in semisolid agar medium, formalinized infusion broth cultures may be tested in pooled H antisera. If these preliminary tests are negative, further passages in semisolid agar are indicated. The writer employs ten pools, each of which contains five or six H antisera with the exception of pool 10 which contains two antisera. Because of important reciprocal relationships, antisera for several H antigens are paired in the pools. One ml of the unknown H antigen suspension is added to an amount of each pooled H antiserum calculated to give a final dilution of 1:1000 of each constituent serum contained in a pool. Tests for H agglutination are incubated in a water bath at 48 to 50 C. and are read after periods of 15 minutes, 30 minutes, and 1 hour. If characteristic H agglutination occurs in one of the H antiserum pools, the suspension then is tested in each of the H antisera contained in that particular pool. These also may be single tube tests in which the final antiserum dilution is 1:1000. Results obtained in the latter tests are confirmed by titration and, if necessary, by tests in absorbed antisera (*v. inf.*).

Although there are several minor relationships among the 49 recognized *E. coli* H antigens, most of these do not appear in tests made at the 1:1000 level of antiserum dilution. However, important relationships exist between the pairs of *E. coli* H antigens listed in Table 11 and absorbed H antisera usually are required for their exact identifica-

Table 11
ABSORPTION OF *E. COLI* H ANTISERA

H Antiserum (1.0 ml)	Absorbing Cultures	No. of Plates
1	12	Growth from 10 plates
12	1	do
8	40	Growth from 10 plates
40	8 +	do
	11	do
11	21 +	Growth from 10 plates
	40	do
21	11	Growth from 20 plates
37	41	Growth from 10 plates
41	37 +	do
	39*	do
49	39*	Growth from 10 plates

*These absorptions may not be necessary.

tion. For these absorption tests one-half of the absorbing suspension may be added to H antiserum diluted 1:50 with phenolized isotonic saline solution. The mixture is placed in a water bath at 48-50 C. for 1 hour and then centrifuged. The diluted antiserum is then added to the second half of the absorbing suspension, reincubated for 1 hour and then centrifuged. Absorbed H antisera may be used in single tube tests in a dilution of 1:1000 or more.

E. coli Serotypes from Animals

Cultures of *E. coli* isolated from various pathologic processes in animals may be examined by the same methods outlined in the foregoing sections. However, O and KO antisera for the serotypes frequently found in swine edema disease, in calf scours, or in infections in poultry or other animals will be required, depending upon the interests of the investigator. Slide agglutination tests in O and KO antisera and other preliminary steps may be carried out in the manner described for presumptive identification and final identification may be made by means of complete biochemical and serologic characterization as outlined. Further information on complete antigenic analysis of *E. coli* strains may be found in the publications of Kauffmann (1954), Ørskov (1951 *et seq.*), and Ewing, Tatum, Davis, and Reavis (1956) and some data on the occurrence of members of the various *E. coli* O antigen groups among materials from animal sources were recorded by Ewing and Davis (1961a) and Ewing (1962).

The following references are cited for the purpose of providing sources for further studies by readers who may be concerned with veterinary bacteriology and the importance of *E. coli* serotypes to it.

Diarrheal Disease: Wramby (1948), Lovell (1955), Bokari and Ørskov (1952), Ulbrich (1954a, 1954b), Wood (1955), Fey (1956, 1957a, 1957b), Charles (1957), Rees (1958a, 1958b), Roberts (1958), Glantz *et al* (1959) and Smith (1962). **Mastitis:** Fey (1958), Murphy and Ryan (1958), and Dunne (1959). **Swine edema disease:** Timoney (1949), 1950, 1956), Gregory (1958, 1960), Ewing *et al.* (1958), Kelen *et al.* (1959), and Campbell (1959). **Avian disease:** Hjarre and Wramby (1945), Hjarre (1949), Ulbrich (1951), Edwards and Ewing (1954), Gross (1956, 1957), and Bankowski (1961).

APPENDIX I.

Production of E. coli Antisera

Three classes of antigens are important in the serology of *Escherichia coli*, the O, the K, and the H antigens. The O antigens are somatic antigens that are not inactivated by heat at 121 C., as in an autoclave. K antigens also may be considered somatic antigens but these occur as envelopes or as capsules. K antigens inhibit agglutination of living bacterial suspensions in O antisera, this inhibitory effect upon O agglutination being annulled by heat either at 100 C., or 121 C., depending upon the variety of K antigen present in a given culture. The more important differences between the three varieties of *E. coli* K antigens are given in Table 12. The H or flagellar antigens of *E. coli* cultures also are inactivated by heat at 100 C., 2½ hours. Methods for the preparation of O, K, and H antiserums differ considerably and will be dealt with separately in the following paragraphs. It should be mentioned that alternative methods for the preparation of each type of antiserum are known,* but the procedures outlined here may be expected to give good results.

Cultures to be used for antigen preparation and antiserum production should always be plated on an infusion agar medium and smooth (S) colonies selected for transfer to infusion agar slants, or, as is the case of H antigens, to semisolid agar.

O antisera. The treatment of bacterial suspensions to be used for O antiserum production is dependent upon the variety of K antigen present in a particular *E. coli* O group culture. If the culture is known to possess an L or a B antigen, S colonies are inoculated into infusion broth and incubated at 37 C. for about 6 hours, after which the broth cultures should be heated at 100 C. for 2½ hours. This treatment inactivates the antigenicity of the L or B antigens as well as the H antigen of the culture. Only suspensions that remain homogenous after the heat treatment should be employed as vaccines, and cultures that are autoagglutinable should be discarded. After they are cool, such O antigen suspensions may be preserved by the addition of 0.5 percent formalin.

When an O antiserum must be prepared with a culture that is known to contain a K antigen of the A variety, K minus or O colony forms should be selected from platings. These colonies are inoculated into broth, incubated for about 6 hours, and then heated at 100 C. for 2½ hours. When it is impossible to obtain K minus colony forms, the more opaque K plus or KO forms may be used, but if such is the case, the broth cultures should be heated at 121 C. for 2½ hours.

If it is necessary to prepare O antiserum from a culture for which the K antigen is undetermined, it is advisable to prepare antigen suspensions for injection in the manner outlined in the preceding paragraph, since the culture may possess an A antigen and should be treated accordingly.

It is advisable to inject at least two rabbits with each O antigen preparation, all inoculations being made in the marginal ear vein. The first dose is 0.5 ml, the second 1.0 ml, the third 2.0 ml, and the fourth and fifth 4.0 ml each. The interval between each injection should be 4 or 5 days and the animals should be exsanguinated 7 or 8 days following the last injection. After separation, the antisera may be preserved with an equal volume of glycerol or with another suitable preservative.

O antigen suspensions employed in the testing of O antisera may be prepared in the manner outlined above except that cultures known to contain L or B antigens need to be heated for 1 hour at 100 C. Agglutination tests used in the study of *E. coli* O antigens are incubated in a water bath at 48 to 50 C. for about 16 hours.

Alternatively, *E. coli* O antisera may be produced with antigens prepared according to the method of Roschka (1950). Selected colonies are inoculated over the entire surface of infusion agar plates or slants and after incubation for 16 to 18 hours, the growth is removed by washing with 0.5 percent sodium chloride solution. The suspensions

*For discussions of other methods, see Kauffmann (1954) or Edwards and Ewing (1962a).

TABLE 12

THE K ANTIGENS OF *E. COLI*

Variety of K Antigen	Characteristics
L	1. Agglutinability of L antigen in L antiserum inactivated by heat at 100 C, 1 hr. 2. Suspensions rendered agglutinable in O antiserum by heat at 100 C, 1 hr. 3. Antibody binding power inactivated by heat at 100 C, 1 hr. 4. Antigenicity inactivated by heat at 100 C, 2 hrs. 5. Occur as envelopes or sheaths, occasionally as capsules.
A	1. Agglutinability of A antigen in A antiserum inactivated by heat at 121 C, 2-1 2 hrs. 2. Suspensions rendered agglutinable in O antiserum by heat at 121 C, 2-1/2 hrs. 3. Antibody binding power not inactivated by heat at 100 C, 2-1/2 hrs. or at 121 C, 2 hrs. 4. Antigenicity inactivated by heat at 121 C, 2-1/2 hrs. 5. Known forms occur as capsules.
B	1. Agglutinability of B antigen in B antiserum inactivated by heat at 100 C, 1 hr. 2. Suspensions rendered agglutinable in O antiserum by heat at 100 C, 1 hr. 3. Antibody binding power not inactivated by heat at 100 C, 1 or 2 hrs. or by 121 C, 2 hrs. 4. Antigenicity inactivated by heat at 100 C, 2 hrs. 5. Known forms occur as envelopes or sheaths.

then are heated at 100 C. for 2 and 1/2 hours or at 121 C. for 2 hours, as required. After centrifugation, the sediment is resuspended in 95 percent or in absolute ethyl alcohol and placed in an incubator at 37 C. for 2 hours. The suspensions are recentrifuged, resuspended in alcohol, and placed in an incubator for an additional 2 hours. Following this the suspensions are centrifuged, washed twice with acetone, resuspended in a small amount of acetone, and placed in an incubator overnight or until the acetone is evaporated. The dried bacteria are ground in a mortar until finely powdered and placed in an airtight container. For use, a small amount of the dried material is suspended in isotonic saline solution and injected according to the above-mentioned schedule of inoculations. The density of the suspensions may approximate that of a 24-hour broth culture.

K antisera. Whenever possible, nonmotile strains should be selected for K antiserum production since this eliminates concern over the presence of H agglutinin in K antisera. If nonmotile cultures are unavailable, freshly isolated strains that have not been passaged in semisolid agar should be used. Cultures should be plated on infusion agar plates and colonies of the smooth, more opaque, somewhat mucoid K forms should be selected and transferred to an agar slant and a tube of infusion broth. Often K forms are not clearly discernible

on agar plates that have been incubated at 37 C. for 20 to 24 hours; but if the plates are allowed to remain at room temperature for an additional 24 hours, K antigen production usually is enhanced and selection of colonies is made easier. Parenthetically, it may be mentioned that if K and O antisera are available, colonies that agglutinate rapidly and completely in K antiserum and do not react, or react very slightly, in O antiserum, should be selected for K antiserum production. After overnight incubation, the above-mentioned broth cultures are heated at 100 C. for 1 hour. This procedure is merely a control of the stability of the culture, and if the microorganisms in any broth culture are rendered autoagglutinable by the heat treatment, the corresponding agar slant should be discarded and not used for K antiserum production. The heated broth cultures may be discarded since their only purpose is that of a control.

Tubes that contain about 10 ml each of infusion broth should be inoculated from the agar slant cultures selected in the manner outlined in the preceding paragraph. These broth cultures are incubated at 37 C. for about 4 to 5 hours, after which 0.05 ml of formalin is added to each tube to give a final concentration of 0.5 percent formalin. Formalinized broth antigens are refrigerated overnight and then used for the first inoculation of the animals. Fresh 4- to 5-hour broth cultures are employed for the second and third injections, but the animals may be injected an hour or two after the addition of formalin. Similar broth cultures are used for subsequent injections, but formalin is omitted and living bacteria are inoculated. Agar slant cultures used to inoculate broth are transferred two or three times a week in order to assure rapid growth of the broth cultures. If the K and O antisera are available, it is advisable also to retest the agar slant cultures several times during the course of immunization.

A modification of the Roschka (1950) method described above in connection with O antiserum production has been found useful for the production of K antisera of the B variety. The heat treatment of the suspensions is omitted and the bacteria are removed from the agar plates and placed directly into the alcohol, in which they are emulsified and suspended as well as possible. This modification is particularly useful when motile cultures must be used for OB antiserum production. Alcohol treatment may not completely inactivate the flagellar antigens of the bacteria, but production of H agglutinins is minimized by its use.

As is the case in O antiserum production, all injections are made in the marginal ear vein, an interval of 4 or 5 days is allowed between injections, and the animals are exsanguinated 7 or 8 days after the last dose of vaccine is given. However, in K antiserum production it is advisable to increase the number of inoculations to six; thus, the first dose should be 0.2 to 0.3 ml, the second 0.5 ml, the third 1.0 ml, and the fourth, fifth, and sixth 2.0 ml each. The last two injections may be increased to 4.0 ml each if desired.

Antigen suspensions employed in evaluation of newly prepared K antiserums generally are prepared by the inoculation of broth from isolated colonies selected for inagglutinability in O antisera. Broth cultures are inoculated from selected KO colonies, incubated for 4 or 5 hours, and then formalinized. Such K antigen suspensions are then tested in serial dilutions of antiserum ranging from 1:20 to 1:2,560. The O inagglutinability of the antigen should be controlled by titration of the same formalinized antigen suspension in serial dilutions of O antiserum. Both the test in K antiserum and the control titration are placed in an incubator at 37 C. for 2 hours and then removed to a refrigerator for overnight incubation. Characteristic K agglutination results in the formation of compact membranes at the bottom of the tubes, and the K agglutinin titer of an antiserum is generally considered to be the highest dilution in which such a compact membrane or pellicle is seen. The K agglutinin titer of K antiserums should be at least 1:160 but titers of 1:320 or 1:640 are preferable. Such antisera may be used in dilution of 1:5 or 1:10 in slide tests. There should be no reaction in the control titration, but slight incomplete agglutination in low dilutions (<1:160) is acceptable. However, if complete agglutination occurs, even in low dilutions, the results are considered equivocal and the titrations must be repeated with antigens prepared from newly selected colonies. The O

agglutinin titer of K antisera should be determined by titration of a heated broth antigen in serial dilutions of antiserum.

If any K antiserum is prepared with a motile culture, the H agglutinin content of the antiserum should be determined and if appreciable H agglutinin is present, it should be removed by absorption before the antiserum is used for K antigen determinations. For removal of H agglutinin from K antiserum, a strain that possesses the same H antigen as the homologous antiserum strain but a different K antigen should be employed.

H antisera. To prepare H antigen suspensions for antiserum production and evaluation it is necessary to promote motility and enhance H antigen development by passage of the cultures through semisolid agar medium. Poorly motile strains do not react in a characteristic manner when tested in H antisera nor do they elicit adequate H antibody production when injected into animals. Usually five or six passages through semisolid agar are required for H antigen development but more may be necessary. A medium that contains 0.2 percent agar is recommended for the first few passages. Subsequent passages may be made in a semisolid medium that contains 0.4 percent agar. After enhancement of H antigen development, flasks of infusion broth are inoculated and incubated at 37 C. for 15 to 18 hours. The broth cultures then are preserved by the addition of 0.5 percent formalin. Such H antigen preparations may be injected into rabbits using the same route and schedule of inoculations as that given above for O antiserum production. After immunization of animals is complete, H antigen suspensions may be kept at room temperature, under which conditions they remain usable for several years as test antigens. The titer of H antisera should be determined by titration of the antigen in serial dilutions of 1:100 to 1:25,600. Tests for H antigen determination are incubated in a water bath at 48 to 50 C., and are read after intervals of 15, 30, and 60 minutes.

APPENDIX II.

Suggested Form for Use in Furnishing Information

Patient's name: _____
(or other significant designation)

Age: _____ Sex: _____

Clinical diagnosis: _____

_____

Degree of severity: Mild ☐ Moderate ☐ Severe ☐

Date of onset of symptoms: _____

Sporadic case? Yes ☐ No ☐

Other cases in family? Yes ☐ Number ☐
No ☐

Is case one of an outbreak? Yes ☐ No ☐
Number of cases in outbreak: _____

Presence of parenteral infection: Present ☐ Absent ☐
Nature and location, if present: _____

Primary gastroenteritis? Yes ☐ No ☐
Duration of symptoms: _____
Final outcome and disposition of case, with date: _____

_____

_____

Remarks: (Include therapy)

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