4. Pollock, M. E., Kenny, G. E., Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 10.

5. Eagle, H., *Science,* 1959, v130, 432.

6. Merchant, D. J., Kuchler, R. J., Munyon, W. H., *J. Biochem. Microbiol. Tech. Eng.,* 1960, v2, 253.

7. Vogt, M., *Genetics,* 1959, v44, 1257.

8. Darnell, J. E., Jr., Sawyer, T. K., *Virology,* 1959, v8, 223.

9. Murphy, W. H., Landau, B. J., *Nat. Cancer Inst. Monograph,* No. 7, 1962, 249.

Received June 25, 1962. P.S.E.B.M., 1962, v111.

The Importance of Sample Size in Studies Based upon the Serologic Classification of *Escherichia coli.** (27744)

KENNETH LEE VOSTI, ARNOLD S. MONTO AND LOWELL A. RANTZ

Division of Infectious Disease, Department of Medicine, Stanford University School of Medicine, Palo Alto, Calif.

The ability of specific antisera to agglutinate coliform bacteria was recognized prior to 1900. The importance of this observation could not be fully realized until a serologic classification based upon the presence of heat stable O and heat labile K somatic antigens was developed by Kauffmann(1). The original scheme has been extended by many other workers until it is now possible to identify some 138 different O groups of *Escherichia coli.* The importance of this technic has been reflected in contributions to our understanding of the association of *E. coli* of specific O groups with intestinal and extraintestinal diseases(1,2,3,4,5,6). Additionally, it has been shown(3,4,5) that the fecal flora may be composed of a number of different serologic groups of *E. coli* while extraintestinal materials tend to contain only a single serologic group. The relation of sampling technics to total number of groups identifiable in the individual specimens has not been explored. Most investigators have studied only 2 to 4 colonies and rarely as many as 10 per specimen.

During studies of the epidemiology and pathogenesis of infections of the urinary tract, precise information as to the relation of the sample size obtained from specimens from various sources to the total number of groups present was needed. In this paper such information is presented, derived from a systematic study of strains of *E. coli* isolated from stool, urine, vaginal, and blood specimens obtained from diseased and normal patients.

Materials and methods. Clinical materials. Sixty-four stool, 100 urine (containing more than 100,000 organisms per ml), and 50 vaginal specimens were collected from patients with symptomatic infections of the urinary tract. Thirty-six stool samples and 100 urines containing less than 100,000, and usually less than 10,000 *E. coli* per ml, were obtained from students, laboratory personnel, and patients without evidence of active disease of urinary or intestinal tracts. The "clean catch-midstream" voiding technic was used to collect all urine specimens. Urinary tract infection was present in 24 of 35 patients with bacteremia due to *E. coli;* other portals of entry were apparent or could not be determined in the remainder.

Bacteriology. The clinical materials were inoculated on blood, eosin-methylene blue, and MacConkey agar plates. Fifteen to 25 colonies typical of *E. coli* were selected from each stool, 5 from each urine and vaginal, and 2 to 5 from each blood specimen. All strains of *E. coli* studied fermented lactose rapidly, and failed to utilize citrate; most were methyl red and indol positive.

Serologic classification. Unabsorbed sera prepared by immunization of rabbits with standard O groups of *E. coli* were obtained from the Communicable Disease Center, U. S. P. H. S., Atlanta, Ga. Sera for 129 O groups

* This study was supported by a grant from Nat. Inst. of Allergy and Infect. Dis., Nat. Inst. Health, U.S.P.H.S.

TABLE I. Relation of New Groups Identified to Number of Colonies Examined.

	No. of colonies				
	1–5	6–10	11–15	16–20	21–25
New groups added	150	20	13	3	4
Specimens examined	100	100	100	65	65

were available. Recognized O groups that were missing included 30, 37, 52, 66, 68, 87, 88, 92, 95, 101, 107, 108, 129, 130, 131, 134, 135, X3, X5, X6, and X7.

The technic used was that reported by Ewing and Edwards(2,7) and may be briefly summarized. Each of the individual strains of *E. coli* was grown in tryptose broth for 6 to 8 hours at 37°C and heated at 100°C for one hour in an Arnold sterilizer. The final suspensions were used as the test antigens. The individual sera were grouped into pools in which each member had a final dilution of 1:100. The tests were conducted by adding 0.1 ml of pooled serum to 0.9 ml of antigen suspension (final individual serum dilution 1:1000). The mixtures were incubated at 50°C for 18 hours in a water bath and were read for visible agglutination by agitation of the tubes. Each strain was initially tested against all pools. Organisms that agglutinated in any pool or pools were retested against the individual sera in a similar manner. These sera were initially diluted 1:50, therefore the final dilution in the test mixture was 1:500. Those strains reacting equally with more than one individual serum were again tested using serial 2-fold dilutions of each reactive serum. The highest titer determined the final assigned O group in the small number of strains that were thus examined. Absorbed sera were not employed to differentiate between cross reacting strains in this study.

Results. Sample size. The relation of the number of colonies examined in each stool specimen to the identification of additional new O groups is shown in Table I. In 100 specimens, at least 15 colonies were classified and an additional 10 colonies were studied in 65 of these same specimens. Nearly 80% of the total number of different O groups was discovered by examination of the first 5 colonies. Thereafter, the number of new groups identified by the study of the next 3 successive collections of 5 colonies grew smaller. The possibility of finding additional groups was never completely exhausted, since examination of the last 5 strains of the 25 colony sequence yielded as many new groups as did the preceding 5. Two specimens that contained *E. coli* of only a single O group after a study of 25 colonies were examined further. In one, the 26th of 43 colonies was of a different O group, and in the other all 65 colonies examined were of the same serologic group.

In Table II the distribution of O groups after an examination of 5 colonies in each of 100 stool specimens is contrasted with that following an examination of an additional 10 to 20 colonies. The outer bold numbers show that after a study of 5 colonies, 59% of the specimens contained *E coli* of only a single O group, and 92% one or 2 groups. In no specimen were 5 different O groups identified. Following the study of a total of 15 to 25 colonies in each specimen, 49% contained a single group, and 75% one or 2 groups. Five different O groups were found in each of 4 specimens.

The horizontal inner squares of Table II illustrate the influence of the study of more than 5 colonies on identification of the coliform flora of the individual specimens. Thus, 49 among 59 specimens that initially contained one group were homogeneous after a study of 15 to 25 colonies. Seven of the remaining 10 ultimately contained 2 groups, 2

TABLE II. Effect of Sample Size on Total Number of Groups per Specimen.

		Groups present						
		1	2	3	4	5	Total	
Groups present	1	49	7	2	1	0	**59**	*5 colonies examined*
	2		19	10	1	3	**33**	
	3			3	4	0	**7**	
	4				0	1	**1**	
	5						**0**	
Total		**49**	**26**	**15**	**6**	**4**	**100**	
15 to 25 colonies examined								

TABLE III. Number of Groups Present in Specimens from Various Sources.

No. of groups present	Stool	Urine*	Urine†	Vagina	Blood
1	59	92	91	38	35
2	33	8	9	12	0
3	7	0	0	0	0
4	1	0	0	0	0
	100	100	100	50	35
	No. of specimens examined				

* Quantitative count 100,000 or greater.
† Quantitative count less than 100,000.

contained 3, and one contained 4. It is apparent that the major shifts in distribution occurred in those specimens that initially contained more than one O group. Thus, the examination of additional colonies uncovered new serologic groups in 10 among 59 stool specimens that contained a single group following the study of 5 colonies. This is in contrast with 19 among 41 specimens that initially contained 2 or more groups.

In specimens containing at least 2 groups, one was usually present in greater numbers (dominant) and the others in lesser numbers (minor). The dominant strain was almost always found among the first 5 colonies examined. In only 3 of 71 stool specimens did an examination of more than 15 colonies reverse the dominant to minor relationship.

Specimens from various sources. The first 5 colonies from each stool, 5 from each urine and vaginal, and 2 to 5 from each blood specimen were examined for purposes of this comparison. The total number of O groups identified in the various specimens is shown in Table III.

Forty-one percent of the stool specimens contained more than a single O group and 8% contained 3 or 4 groups. The heterogeneity of stool specimens is in marked contrast with those from extraintestinal sources. Few specimens from the urine contained more than a single O group. There was no difference in this regard among specimens containing less or more than 100,000 *E. coli* per ml of urine. The vaginal specimens were in an intermediate position when compared with those from the stool and urine. None contained more than 2 groups, but this number was demonstrated more frequently than in materials from other extraintestinal sources. Strains of *E. coli* recovered from blood were invariably of a single O group. At least 5 colonies were studied in 15 of 35 specimens and an additional 5 colonies were studied in 5.

Discussion. The composition and dynamics of change in the *E. coli* populations of feces have been extensively studied by several authors(8,9,10,11,12). Their work emphasizes that several different serologic groups may be present in a single specimen and may change from week to week. Two investigators have studied individuals sequentially over several months and have identified as many as 10 serologic groups of *E. coli* in their feces. Sears *et al.*(11) have noted that some strains persist for longer periods, "Residents," and others remain for only a short time, "Transients." The total number of different serologic groups that can be identified in single specimens had not been fully explored by any of these authors as their sample sizes have varied from only 4 to 10 colonies.

During a study of the pathogenesis of *Escherichia coli* infections of the urinary tract, precise information as to the relation of sample size to number of different serologic groups present in an individual specimen was needed. These data represent an extensive, systematic study of materials from several people. The heterogeneity of the coliform flora of stool has been confirmed. Approximately 50% of the specimens from feces contained more than one group, and 10% contained 4 or 5 groups. The importance of sample size in regard to total number of different O groups identified has been demonstrated. Nearly 80% of the serologic groups identified after an examination of 15 to 25 colonies were present in the first 5 colonies studied. Although the new groups identified by a study of increments of 5 colonies grew smaller, the possibility of discovering additional new groups was never completely exhausted. It was also clear that the chances of uncovering new serologic groups by a study of additional colonies were increased in specimens that contained *E. coli* of several different O groups. The impor-

tance of these relationships to a study of the pathogenesis of infections of the urinary tract is obvious when one considers that in approximately 25% of such patients the serologic group identified in the urine could not be found in feces obtained at the same time (13).

Several authors(3,4,5) have suggested that organisms from extraintestinal sites tend to be serologically homogenous as contrasted with those from stool. The present studies of *E. coli* isolated from the urine and blood are in keeping with these observations; however those from the vagina are at variance. Of particular interest, in view of the importance of quantitative urine culture in recent years, was the fact that urine specimens with counts of greater or less than 100,000 per ml did not differ in this respect. Therefore, it seems that the suggestions that fecal contamination causes the low counts, or that the presence of a rapidly multiplying organism crowds out other serological groups in specimens with high counts, are not applicable.

Summary. The total number of different serological O groups in individual specimens was determined by a study of *Escherichia coli* isolated from feces, urines, the vagina, and blood. The serologic heterogeneity of the fecal coliform flora was confirmed. Ninety percent of urines contained only a single *E. coli* group regardless of the number of organisms present. The vaginal flora was composed of a single group less frequently than the organisms isolated from urine, but was not so heterogenous as that of the feces. *E. coli* recovered from blood was invariably of a single group. The importance of sample size was demonstrated in the study of stool specimens. Most O groups present were identified by an examination of only 5 colonies but the possibility of identifying additional strains was not exhausted by a study of as many as 25 colonies. The chances of finding new O groups by a study of more than 5 colonies were greater in those specimens that initially contained more than one O group.

The authors gratefully acknowledge the assistance of Patricia S. Austill, Mary G. Kocher, and Elizabeth Randall.

1. Kauffmann, F., *J. Immunol.*, 1947, v57, 71.
2. Ewing, W. H., Tatum, R. W., Davis, B. R., Reavis, R. W., *Studies on the serology of the* Escherichia coli *group,* CDC Publication, Communicable Disease Center, Atlanta, Ga., 1956.
3. Vahlne, G., *Acta Path. Microbiol. Scand.*, 1945, Suppl. 62, 1.
4. Ujvary, G., *Zbl. f. Bakt. Orig.*, 1958, v170, 394.
5. Leppanen, M. K., *Am. Med. Exp. et Biol. Fenn.*, 1958, v36, Suppl. 2, 1.
6. Rantz, L. A., *Arch. Int. Med.*, 1962, v109, 37.
7. Edwards, P. R., Ewing, W. H., *Identification of Enterobacteriaceae,* Burgess Publishing Co., Minneapolis, 1955.
8. Wallick, H., Stuart, C. A., *J. Bact.*, 1943, v45, 121.
9. Kauffmann, F., Perch, B., *Acta Pathol. Microbiol. Scand.*, 1943, v20, 201.
10. Perch, B., *ibid.*, 1944, v21, 239.
11. Sears, H. J., Brownlee, I., Uchiyama, J. K., *J. Bact.*, 1950, v59, 293.
12. Sears, H. J., Brownlee, I., *ibid.*, 1952, v63, 47.
13. Vosti, K. L., Monto, A. S., Rantz, L. A., *Clin. Res.*, 1962, v10, 113.

Received June 27, 1962. P.S.E.B.M., 1962, v111.