THE APPLICATION OF FLUORESCENT ANTIBODY TECHNIQUE IN IDENTIFICATION OF ENTERO-PATHENOGENIC E. COLI IN CHILDREN WITH DIARRHEAL SYMPTOMS

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The fluorescent antibody technique, first applied by Coons[1] in 1942, is now being extensively used in microbiology laboratories as a diagnostic technique.[2] Its special value derives from the fact that the technique enables those dealing with infectious diseases to make a diagnosis in the relatively short time of one to two hours. Studies in this country and abroad indicate that Enteropathic E. Coli (E.E. Coli) plays an important role in epidemic and sporadic diarrhea in infants, especially those under 12 months of age.[3-14] The fluorescent antibody technique (F.A.T.) has been found useful in early diagnosis of this type of diarrhea and the prevention of epidemics among children.[15-22]

We used this technique for the first time in diagnosing cases of enteropathogenic E. Coli in this hospital. During the period from May to November 1961, 602 fecal samples were collected from 454 infants ranging in age from birth to 12 years who were brought to our hospital with complaints of diarrhea. These fecal samples were tested by routine bacteriological methods and by the fluorescent antibody direct technique.

Method and Materials

The F.A.T. involves a conjugation of a fluorescent dye with a specific antibody, which, when united with its matching antigen, becomes visible under a fluorescent microscope. The bacteriae demonstrate typical morphology as well as a bright fluorescent ring around the periphery.

Preparation of antiserum against OB antigen of E.E. Coli : The E.E. Coli types used were: 025:H6; 026:B6; 055:B5; 086:B7; 0111:B4; 0119:B14; 0125:B15; 0126:B16; 0127:B8; and 0128:B12.**

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** E.E. Coli strains from National Salmonella and Shigella Center.

The antiserums were prepared in rabbits by injection of OB antigen of E.E. Coli types in accordance with the directions of Edwards and Ewing. [23]

Conjugation of antiserums with fluoresceine isothiocyanate (FITC) : The prepared antisera of high titers, and serum from one non-immunized rabbit (as a control), were conjugated as follows: Antisera were precipitated with 4 molar ammonium sulphate, and dialized against Ph 7.2 buffered saline. The protein concentration was determined by the biuret method and diluted by adding buffered saline, carbonate-bicarbonate solution to keep the protein level at 15 mg. per milliliter. For each milligram of protein, 0.05 mg. FITC was added. The solution was then dialized, centrifuged, and passed through a millipore filter.

Examination of the Feces by Routine Methods and F.A.T.: In the routine bacteriologic examinations, Endo, triple sugar iron agar, and Endo mediums were used. Serologic typing was first done by the slide agglutination method and the positive ones were then tested by tube agglutination. The fecal material was put on microscope slides cleaned with an alcohol-acetone solution. After the samples had dried, each was fixed with acetone for ten minutes. A drop of conjugated antiserum was added to each sample and the slides allowed to stand at room temperature for 30 minutes in a moist atmosphere. The samples were then washed with buffered saline (Ph 7.2) and examined microscopically, using dark and bright field condensors. The light source for the microscope was an Osram HBO 200 mercury arc lamp. Two filters, A.O. Spencer No. 208 and Kodak Wratten gelatin filter No. 2B, were used. Fluorescence was considered positive if there was typical bacterial morphology and a fluorescent ring or «halo» appeared around the organisms.

Results

In the first part of our study, we determined the titers before and after the conjugation of the OB antiserum by agglutination tests. There are certain parallels between the agglutination titers before and after conjugation, as can be seen in table 1. Sera in higher titers show relatively higher titers after conjugation, but all titers are usually only one-eighth as high as prior to conjugation. Cross-reactions in low titers may disappear following conjugation, and the sera of low titers may, in fact, have titers of zero

TABLE 1

AGGLUTINATION TITERS BEFORE AND AFTER CONJUGATION PREPARED AGAINST HOMOLOGUE AND HETEROLOGUE TYPES OF E.E. COLI BACILLI

Antigens	ANTISERUMS								
	025:H6	026:B6	055:B5	086:B7	0111:B4	0119:B14	0125:B15	0127:B8	0128:B12
025:H6	1:2560+ (1:320)		1:80						
026:B6		1:1280 (1:160)							
055:B5			1:5120 (1:160)						
086:B7				1:5120 (1:320)		1:80			
0111:B4					1:2560 (1:320)				
0119:B14					1:640 (1:80)	1:2560 (1:320)			
0125:B15							1:640 (1:40)		
0127:B8								1:640 (1:80)	1:80
0128:B12									1:640 (1:160)

The numbers in parentheses are titers after conjugation, those without parentheses are titers before conjugation.
Note: Type 0126:B16 antiserum had a titer of 1:160 before conjugation and fell to zero after conjugation.

TABLE 2

FLUORESCENT REACTIONS OF HOMOLOGUE AND HETEROLOGUE TYPES WITH CONJUGATED ANTISERUMS

Antigens	Conjugated Antiserums								
	025:H6	026:B6	055:B5	086:B7	0111:B4	0119:B14	0125:B15	0127:B8	0128:B12
025:H 6	++++								
026:B 6		+++							
055:B 5			++++		++				
086:B 7				+++				++	++
0111:B 4					++++	++			++
0119:B14				++	+++	++++			
0125:B15							++		
0126:B16									
0127:B 8								+++	++
0128:B12					++				++++

Note: No fluorescence was noted between 0126:B16 antiserum and the homologue type organism.

following conjugation. For example, the titer of 0126:B16 type antiserum fell to zero after conjugation, whereas it was 1:160 before (table 1).

Table 2 shows homologous and heterologous types of E.E. Coli conjugated antiserums and the results of the staining. Three plus or four plus positive fluorescence was seen in the homologous types. Conjugated antisera used for staining were not diluted at all, as dilution makes it difficult to produce a strong fluorescence.

Cross-staining occurred sometimes to a weak (one or two plus) degree with the following organisms: S. typhii, S. typhimurium, S. gärtner, S. dispar (type II), A. aerogenes, B. alcaligenes, P. vulgaris, E. coli var. communior, E. coli 36, Strep. fecalis, Staph. aureus, Staph. albus, L. acidophilus, L. bifidus, Sh. flexneri (type 2B), Sh. flexneri (type 3). This cross-staining of other organisms was never strong enough to cause any confusion with homologous E.E. Coli, since the latter uniformly produced a three or four plus reaction. Staining of E.E. Coli types with normal conjugated rabbit serum also gave negative results.

In the second phase of the study, after testing the specificity of the conjugated antiserums, the specimens of feces were examined by both routine methods and the F.A.T. for identification of the E.E. Coli types. The results, according to type of test and age group, are given in table 3.

TABLE 3

COMPARATIVE RESULTS OF EXAMINATIONS BY F.A.T. AND CULTURE METHOD ACCORDING TO AGE

	Age Group				
	0 to 6 months	6 to 12 months	13 to 24 months	2 years	Total
F.A.T. + Culture +	30(8.8%)*	9(7.4%)	0	3(4.2%)	42(7.0%)
F.A.T. + Culture —	49(14.3%)	23(19.0%)	5(7.9%)	9(12.7%)	86(14.3%)
F.A.T. — Culture +	8(2.5%)	3(2.5%)	3(4.4%)	1(1.4%)	15(2.5%)
F.A.T. — Culture —	255(74.5%)	86(71.4%)	60(00.8%)	58(81.7%)	459(76.2%)
Total.	342(56.8%)	121(20.0%)	68(11.3%)	71(11.8%)	602

* Numbers in parentheses represent per cent of total age group.

These results indicate that the fluorescent antibody technique alone was positive in 14.3 per cent of children in the O to 6 months age group, and in 19 per cent of the 6 to 12 months age group. Culture and F.A.T. were both positive in 8.8 per cent and 7.4 per cent respectively in these age groups. If all the specimens are considered, the correlation between the two methods is 83.2 per cent.

The most frequently isolated types of E.E. Coli were 055:B5 (49 times) and 0111:B4 (46 times). The others, in order of frequency, were: 0128:B12, 0125:B15, 0119:B14, 0127:B8, 025:H6, 086:B7, 026:B6, and 0126:B16. There had been no previous reports of isolations in Turkey of the following types: 0128:B12, 0125:B15, 0119:B14, 0126:B16, and 025:H6. Double infections were noted in ten specimens. Nine infections were discovered only after application of the F.A.T. In only one case was a double infection found by two methods. In double infections, E.E. Coli types 055:B5 and 0111:B4 were most commonly found together.

In four of our patients with positive results by both the culture and F.A.T. the cultures were negative within 24 hours following antibiotic treatment, whereas findings by F.A.T. remained positive 48 to 72 hours after antibiotics were given. Two specimens gave positive results to the F.A.T. 24 hours before similar results could be obtained from the culture method. Six specimens could not be proven positive by the culture method but did yield positive results to the F.A.T.

Discussion

The decrease in agglutination titers after conjugation of the serum can be minimized by careful control of every step of the procedure. In practice, this means that sera to be conjugated should have high initial titers which can be diluted later, if need be.

Most of those who have used the F.A.T. to identify E.E. Coli, contrary to La Brec and his colleagues, [21] have not encountered cross-reactions with other enterobacteriaceae. [25] In our studies, the rate of positive findings with the F.A.T. correlates well with the reports of others. [18, 19, 20] This technique yielded 14.3 per cent more positive findings than the culture methods. It certainly seems to be advantageous in differentiating E.E. Coli in fecal specimens containing a small number of these organisms. Also, dead bacteria bearing antigenic characteristics but having lost the ability to grow, seem to be differentiated by this technique. In some cases

reported here, there were positive rsults with F.A.T. in specimens with bacterial counts too low to give a positive culture result. In four cases, the culture was negative after antibiotic treatment but positive results continued to appear in testing with F.A.T. Two and a half per cent of the specimens were positive in the culture but negative according to the F.A.T. This may have been due to a lack of fecal material in the swab to be tested by the F.A.T. This problem can be avoided by collecting double fecal samples in the swab. Other investigators [18, 19, 20] report as much as 5.8 per cent of cases with positive culture and negative F.A.T. results.

The E.E. Coli types most commonly isolated both in this country and elsewhere are 055:B5 and 0111:B4. 0128:B12 and 0125:B15 types were not isolated in Turkey previously because no one had worked with sera of these types before. Our studies, however, revealed that these were among the most common types and second in frequency to 055:B5 and 0111:B4. We stress the need for further studies on the sera of these two types for identification of E.E. Coli in bacteriology laboratories attached to children's clinics.

Application of the F.A.T. in the identification of E.E. Coli has an advantage over the culture method because it enables diagnosis within one or two hours and gives more positive results. On the other hand, the culture method is more reliable from the point of view of antigenic typing and precise identification of the bacteria. By the application of the new method it is possible to test fecal specimens of infants, and particularly premature infants, within a short time and thus prevent dangerous epidemics.

Summary

Out of 602 fecal smears examined for E.E. Coli, seven per cent proved to be positive by both the culture method and the F.A.T. In 14.3 per cent of the tests, the results were positive using only the F.A.T. Using the culture method, 2.5 per cent were positive.

The most common types among the isolated E.E. Coli were 055:B5, 0111:B4. Other types isolated, according to the rate of frequency, were: 0128:B12, 0125:B15, 0119:B14, 0127:B8, 025:B6, 086:B7, 026:B6 and 126:B16.

Of these, types 0128:B12, 0125:B15, 0119:B14 and 0126:B16 were isolated for the first time in Turkey.

The need of an antiserum of high titer and specificity after conjugation is discussed, as well as the advantages and disadvantages of the F.A.T. compared to routine culture methods.

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