Rev. Inst. Med. trop. São Paulo
7(2):65-71, março-abril, 1965

UDC 616.981.48

COMPARATIVE STUDY BETWEEN IMMUNOFLUORESCENCE AND COPROCULTURE IN THE DIAGNOSIS OF INTESTINAL INFECTIONS BY ENTEROPATHOGENIC *ESCHERICHIA COLI*

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SUMMARY

The fluorescent antibody and the classical coproculture methods were compared in the research of enteropathogenic *E. coli*, in stool specimens obtained from 147 children suffering from acute diarrhea. Both methods were positive in 44 and negative in 80 cases (84.3% concordance). In 18 cases only immunofluorescence yielded positive results, while coproculture alone was positive in another 5 cases. A larger sensitivity of the technique and 3 instances of false-positive reactions were shown to account for the higher number of positive results obtained with the fluorescent antibody method.

INTRODUCTION

The fluorescent antibody method was first utilized for the identification of enteropathogenic *E. coli* in feces by WHITAKER et al.[15]. They studied 128 stool specimens, obtained from patients with diarrhea during an epidemics caused by *E. coli* 0127:B8, which had been stored for a 3 years period at −20°C. Immunofluorescence yielded 92 positive results in the stored samples, against 53 positive coprocultures carried out at the time of the epidemics. Since then, several comparative studies between both methods have been performed in the diagnosis of intestinal infections caused by enteropathogenic *E. coli* (NELSON & WHITAKER [9], DANIELSON & LAURELL [4], COHEN et al.[3], CHERRY et al.[2], and by LE MINOR et al.[8]). In general, the results have pointed to a higher sensitivity of the immunofluorescence process. Furthermore, its fitness for the rapid detection and identification of enteropathogenic *E. coli* during epidemics of infantile diarrhea has been well established by the experiments of NELSON et al.[10], PAGE & STULBERG [11], and BORIS et al.[1].

The present paper reports the results of a comparative study between fluorescent antibody and coproculture methods in the diagnosis of intestinal infection caused by enteropathogenic *E. coli*. A total of 147 children with acute diarrhea were studied for the presence in the feces of 8 enteropathogenic *E. coli* serotypes.

MATERIAL AND METHODS

A) *Sera*

0B and 0 rabbit antisera against 026:B6, 055:B5, 086:B7, 0111:B4, 0119:B14, 0126:B16, 0127:B8 and 0128:B12 standard enteropathogenic *E. coli* strains were prepared ac-

Research supported by a grant from Johnson & Johnson do Brasil

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cording to the methods recommended by EDWARDS & EWING [5]. The same 0B sera were employed for the preparation of fluorescent conjugates and for slide agglutination.

B) *Fluorescent conjugates*

1. *Preparation:* after salting out with a 50% ammonium sulfate solution, the globulin fraction of 0B sera was coupled to flourescein isothiocyanate in a 100 mg: 5 mg proportion (THOMASON et al.[14]). The excess of isothiocyanate was removed by dialysis against phosphate buffered saline (pH 7.2), the adequate dilution being settled for the use of each conjugate. The highest dilution giving intense staining (3 to 4+) of the homologous *E. coli* was held as optimal to be employed throughout the experiments.

2. *Polivalent conjugates:* taking on account the optimal individual dilution of each conjugate, the following polivalent conjugates were prepared:

   Conjugate I — 026:B6, 055:B5, 086:B7

   Conjugate II — 0111:B4, 0119: B14

   Conjugate III — 0126:B16, 0127: B8, 0128:B12

3. *Specificity of univalent conjugates:* smears of the 8 different *E. coli*, employed in the production of antisera, were submitted to staining with each one of the conjugates at its optimal dilution, in order to test the specificity of the latter (Table I).

4. *Preservation:* stored frozen at −20° C, the conjugates were kept at 4°C after thawing. Dilutions in buffered saline both from uni- and polivalent conjugates were prepared daily just before use.

C) *Fluorescent antibody method*

The technique adopted was in general the same described by THOMASON et al.[14]. A few drops of stool suspension were placed on limited areas of slides and the resulting smears, after heat-fixing, were exposed for 15 minutes at room temperature to the polivalent conjugates. The slides were mounted with slightly alkaline glycerin under a coverslip and sealed with paraffin, after rinsing of smears three times in buffered saline. Microscopical examination in dark field and oil immersion (40 × magnification) was first performed with common light, to confirm the presence of bacteria and then with ultraviolet (HBO-200 bulb) to detect their fluorescence. A BG-12 filter was employed as excitor and a No. 50 Zeiss filter as a barrier. Whenever fluorescent bacteria could be outlined, previously prepared smears of the same stool specimen were stained by the corresponding univalent conjugates and submitted to the same microscopical examination. Only the 3 to 4+ staining were held as specific. The average number of fluorescent bacteria present in each microscopical field was recorded.

D) *Culture*

Stool specimens or common broth culture were seeded in McConkey's medium plates. Ten colonies of each plate were studied, the identification of the *E. coli* being made by the following criteria:

1) agglutination on slides, with polivalent and univalent 0B sera;

2) agglutination in tubes with 0 sera;

3) generic identification by the following tests: indol, VM, VP, citrate (Simmons), H_2S, KCN, phenyl-alanine, lysine, glucose, lactose, sucrose and motility.

E) *Technical procedures*

The 147 stool specimens were collected from infants under 2 years of age, suffering from acute diarrhea, who were brought to the Emergency Service of the "Hospital das Clínicas da Faculdade de Medicina de

TABLE I

Staining of 8 different enteropathogenic *E. coli*, with each of the univalent conjugates at optimal dilution

		Escherichia coli							
Univalent conjugates	Dilution	026:B6	055:B5	086:B7	0111:B4	0119:B14	0126:B16	0127:B8	0128:B12
026:B6	1/20	++++	0	0	0	0	0	0	0
055:B5	1/20	0	++++	0	0	0	0	0	0
086:B7	1/10	0	0	++++	0	0	0	0	0
0111:B4	1/40	0	0	0	++++	0	0	0	0
0119:B14	1/40	0	0	0	0	++++	0	0	0
0126:B16	1/40	0	0	0	0	0	++++	0	0
0127:B8	1/40	0	0	0	0	0	0	++++	0
0128:B12	1/40	0	0	0	0	0	0	0	++++

São Paulo", between September 1963 and February 1964. The feces were obtained by rectal swabing and suspended in approximately 1 ml sterile saline. Within a few minutes, smears were prepared for the fluorescent antibody method, and a sample of the feces was seeded on McConkey's agar. In the first 60 cases, the importance of two enrichment methods was tested as follows: after preparing the smears and seeding the first culture plate, a plain broth culture tube was seeded too; then the original feces-saline suspension and the culture tube were incubated at 37°C for one and three hours, respectively. Fresh smears for immunofluorescence and new plate cultures were made from both preparations. The identification of *E. coli* present in the last two plates was performed only by slide agglutination. In some instances, when immunofluorescence revealed an *E. coli* that was not confirmed in the culture, the growth of one or several tubes of plain agar slants was submitted to immunofluorescence to detect an eventual unespecificity of the staining. On the other hand, when only the cultures yielded positive results, the organisms produced were examined with the fluorescent antibody method as well.

RESULTS

1) In a total of 147 cases studied, the immunofluorescence and coproculture processes were equally efficient in the detection of enteropathogenic *E. coli* in 44 cases (29.9%), being negative in 80 (54.4%). In 18 (12.2%) only immunofluorescence was positive while culture alone was positive in 5 cases (3.5%) (Table II).

TABLE II

Number of positive and negative cases detected by immunofluorescence and coproculture techniques, in 147 stool specimens studied

	Coproculture		Total
	Positive	Negative	
Immunofluorescence-positive	44	18	62
Immunofluorescence-negative	5	80	85
	49	98	147

2) Regarding the 44 cases pointed as positive by both methods, the fluorescent antibody method, besides conforming the results of the cultures, revealed the association of 2 or 3 enteropathogenic *E. coli* (Table III).

TABLE III

Fluorescent antibody and coproculture methods in 7 cases in which an association of two or three enteropathogenic *E. coli* was revealed by the first method only

Cases	Identification by	
	Immunofluorescence	Coproculture
1	0111:B4 0127:B8	0111:B4
2	0111:B4 0119:B14	0111:B4
3	0111:B4 055:B5	0111:B4
4	0128:B12 086:B7	0128:B12
5	0128:B12 0126:B16	0128:B12
6	0127:B8 055:B5	055:B5
7	0111:B4 055:B5 026:B6	0111:B4

3) In 3 of the 18 cases where only immunofluorescence was positive, antigenically related enterobacteria were isolated by culture. Treated by the fluorescent conjugates, these organisms presented a 3^+ to 4^+ fluorescence degree (Table IV).

4) The data concerning the number of positive cultures in relation to the amount of stained bacteria present per microscopical field are shown in Table V. Cultures were negative in 3 cases where less than 1 organism per field was recorded, and positive in 20%, 73.3% and 88.2% of cases with 1 to 5, 5 to 10 and over 10 bacteria per field, respectively.

TABLE V

Positive coproculture in relation to the number of fluorescent bacteria present per microscopical field (40×)

Number of fluorescent bacteria per microscopical field	Number of cases	Positive cultures	
		No.	%
< 1	3	—	—
1 to 5	15	3	20.0
5 to 10	15	11	73.3
> 10	34	30	88.2

5) The fluorescent antibody method applied to freshly prepared stool suspensions, to stool suspensions incubated for one hour at 37°C, and to broth culture incubated for 3 hours, was positive 35, 34 and 35 times, respectively. Cultures of the same material were positive 31, 25 and 24 times, respectively (Table VI). However, a marked tendency to an increase in number of stained bacteria was noticed on the slides prepared from broth cultures.

TABLE IV

Immunofluorescence of stool specimens, cultural identification and immunofluorescence of the isolated bacteria, in 3 of the 18 cases where only immunofluorescence yielded positive results

Cases	Immunofluorescence identification	Isolated bacteria	
		Biochemical and serological identification	Intensity of staining
1	0111:B4	*Aerobacter* related to 0111:B4 *E. coli* (1/2,560)	+++
2	086:B7	*E. coli* related to 086:B7 *E. coli* (1/320)*	++++
3	086:B7	*E. coli* related to 086:B7 E. coli (1/180)*	+++

* The 086 serum had homolog titer of 1/5,120

TABLE VI

Results of immunofluorescence and coproculture in 60 cases, according to different treatment of the material

Process of treatment	Number of positives	
	Immuno-fluorescence	Coproculture
Freshly prepared saline suspension of stool specimens	35	31
Same, incubated for 1 hour at 37°C	34	25
Broth culturing for 3 hours at 37°C	35	24

DISCUSSION

The comparison of the data obtained by immunofluorescence and the classical coproculture methods offers coincident results in 124 cases (84.3%); in 18 (12%) cases *E. coli* was only demonstrated by immunofluorescence and in 5 (3%) by the coproculture alone. Such results resemble closely those reported in literature, since a high agreement (87-91%) between the two methods has so far been the rule, the slight discordance depending upon a larger number of positive results by the immunofluorescence (COHEN et al.[3], THOMASON et al.[14], CHERRY et al.[2], LE MINOR et al.[8], BORIS et al.[1]).

A number of factors, taken individually or as a whole, has been pointed out as accounting for the higher frequency of positive results in immunofluorescence tests: higher sensitivity of the method, inespecific staining and specific staining of non-viable organisms (DANIELSON et al.[4]; THOMASON et al.[14]; NELSON & WHITAKER [10]).

In spite of the fact that some of our cases were receiving antibiotics at the moment of feces collection, no significant differences between medicated and non-medicated subjects were found in the present research. Thus, the larger number of positive results outlined by the fluorescent antibody method is, partially at least, a consequence of the higher sensitivity of the process and of eventual inespecific staining.

As a matter of fact, Table VI shows that the percentage of positive culture increased progressively (0 to 88%) and in accordance with a numerical increase of fluorescent bacteria per microscopical field, thus evidentiating the higher sensitivity of the immunofluorescence. On the other hand, in 3 of the 18 cases positive only by immunofluorescence, the culture revealed enterobacteria antigenically related to those identified by the former method; once submitted to the fluorescent conjugates, these organisms presented a fluorescence as intense as the specific one (Table IV). This finding strongly supports the presence of "false-positive" influencing the results afforded by immunofluorescence and, in consequence, the number of cases shown as positive by this method only, was reduced from 18 to 15 (10.2%). We can afford no explanation for the positive cases demonstrated by the culture alone. Any failure in the conjugates, besides being improbable, was excluded by proving that *E. coli* isolated in the 5 cultures could be deeply stained by them.

As seen from Table VI, neither the incubation for one hour at 37°C of stool saline suspensions nor the seeding of feaces samples in broth with subsequent culturing for 3 hours at 37°C, contributed to a numerical increase in the positive results of immunofluorescence, reducing, much on the contrary, the number of positive cases by the culture. The smaller number of positive cultures probably resulted from an intensive proliferation of some bacteria, like *Aerobacter*, whose colonies overthrived *E. coli* in the isolation plates.

The rather small incubation period observed for the broth culturing seems to have influenced the number of positives by immunofluorescence in our experiments. As already pointed out, a sharp tendency to an increase in number of the stained bacteria was recorded, which would support the statement that broth culturing over longer periods (8 to 24 hours) improves the results of subsequent immunofluorescence tests, as previously demonstrated by COHEN et al.[3] and DANIELSON et al.[4].

We conclude that the fluorescent antibody method is an extremely useful tool not only as a quick diagnostic test for identification

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of enteropathogenic *E. coli* strains, but also as a more sensitive one. In addition to the identification of a larger number of positive cases, it allowed the diagnosis of frequent mixed infections (Table III) not revealed by the standard bacteriological methods of coproculture. By all these attributes, it is particularly fit for epidemiologic and clinical control of infantile diarrhea and can be recommended as an excellent diagnostic aid. Nevertheless, the thorough identification of an *E. coli* strain cannot be based only on quantitative or semi-quantitative serological tests, as in immunofluorescence. Close antigenic relations are known to prevail among different antigenic groups of *E. coli* and between these and other enterobacteria groups; such relations have been demonstrated both by immunofluorescence (THOMASON et al.[14]) and agglutination (EWING et al.[6]; KAMPELMACHER[7]). On this ground, the positive results obtained by immunofluorescence must be confirmed, whenever possible, by the isolation, and complete study of the involved *E. coli*.

RESUMO

Estudo comparativo entre a imunofluorescência e a coprocultura no diagnóstico das infecções intestinais por colibacilos enteropatogênicos

Os AA. compararam os métodos de imunofluorescência e de coprocultura na pesquisa de colibacilos enteropatogênicos, nas fezes de 147 crianças portadoras de diarréia aguda. Ambos os métodos foram positivos em 44 casos e negativos em 80 (concordância de 84,3%). Em 18 casos, sòmente a imunofluorescência revelou colibacilos enteropatogênicos e, em 5, apenas a cultura.

O maior número de resultados positivos pela imunofluorescência decorreu de resultados falso-positivos (3 casos) e de maior sensibilidade da técnica.

ACKNOWLEDGMENT

We wish to express our indebtedness to Dr. W. Ewing, from the CDC in Atlanta (Georgia), who kindly provided us with *E. coli* standard strains.

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Recebido para publicação em 3/3/1965.