

Immunofluorescent Demonstration of the Common Enterobacterial Antigen.* (30744)

SHIGEHISA AOKI,[†] M. MERKEL[†] AND W. R. MCCABE (Introduced by E. E. Baker)

Department of Medicine, Boston University Medical Center, Boston, Mass.

Extreme specificity of antigen-antibody reactions is usually desirable for most diagnostic and investigative studies. There are instances, however, in which antisera capable of reacting with several related antigens or common antigens would be useful. One such instance is immunofluorescent localization of bacteria and bacterial antigens in multiple tissue specimens when several bacterial species may be present, but whose differentiation is unimportant. The present investigation was undertaken as a preliminary to studies of bacterial localization in human pyelonephritis. Pyelonephritis is usually caused by a single species of serological type of Enterobacteriaceae in individual instances, but hundreds, or potentially thousands of serotypes are capable of producing the disease. Demonstration of bacteria by fluorescent staining in renal biopsy specimens from patients with pyelonephritis would necessitate preparation of individual antisera for each specimen, but the discovery of an antigen common to most Enterobacteriaceae by Kunin(1-3) suggested that this problem might be circumvented. Kunin's studies demonstrated that rabbit antisera prepared against *E. coli* 0:14, 0:56, 0:124 and 0:144 gave heterogenetic cross reactions by the hemagglutination test with most strains of *E. coli* as well as other enterobacteria. The present investigation was undertaken to evaluate the use of antisera against the common enterobacterial antigen for fluorescent staining of strains of Enterobacteriaceae and to compare hemagglutination tests and fluorescent staining of various bacterial species usually responsible for pyelonephritis.

Material and methods. Hemagglutination

* This study was supported by USPHS Training Grant 5 T1 A1-203-04 and Boston University General Research Support Grant from Nat. Inst. Health 1-SO1-FR-5380-04.

[†] Trainee, Nat. Inst. of Allergy & Infect. Dis., P.H.S., Nat. Inst. Health.

tests. Subcultures of 12 strains of *Escherichia coli* (0:1, 3, 6, 10, 13, 14, 17, 25, 35, 41, 56, and 124) and hyperimmune rabbit sera prepared against *E. coli* 0:14 were obtained from the Communicable Disease Center, U.S.P.H.S. We are indebted to Dr. John Winn for supplying these cultures and the antiserum. Eight strains each of Klebsiella-Aerobacter (Klebsiella 4, Aerobacter 4) and Proteus species (*P. mirabilis* 3, *P. morgani* 2, *P. rettgeri* 2, and *P. vulgaris* 1) and 10 strains each of *Pseudomonas aeruginosa* and *Streptococcus fecalis* isolated from clinical specimens submitted to the University Hospital Bacteriology Laboratory were also used. Peptone (1%) saline suspensions of each organism were inoculated to Heart Infusion Agar (Difco) plates and incubated overnight at 37°C. Cultures were harvested in 0.9% saline (10 ml per plate) and heated at 100°C for 2 hours. The supernatant obtained after centrifugation at 5000 × *g* for 15 minutes at 0°C was removed and 1 ml of 95% alcohol added. A 1:10 dilution of this antigen was incubated with a 2.5% suspension of human type O erythrocytes for one hour in a 37° water bath shaker. Sensitized cells were washed 3 times in balanced Hanks' solution to remove unbound antigen. Hemagglutination tests were performed in plastic trays (Limbro Manufacturing Co.) using 0.25 ml of diluted *E. coli* 0:14 antiserum. Dilutions of antiserum (1:20 to 1:1600) were added to the wells of the plastic trays and 0.25 ml sensitized erythrocytes added. All tests were performed on the same day that the antigen coated erythrocytes were prepared. The mixtures of erythrocytes and antisera were incubated at 37° for one hour. Hemagglutination patterns and clumping were read over an X-ray view box after gentle shaking.

Fluorescent staining of bacteria. The 12 strains of *E. coli*, 8 strains of Klebsiella-Aerobacter, 8 strains of Proteus, 10 strains of *Pseudomonas aeruginosa* and 10 strains of

TABLE I. Comparison of Hemagglutination Results and Fluorescent Staining with *E. Coli* 0:14 Antiserum with Enterobacteriaceae, Pseudomonas and Streptococci.

Hemagglutination		Immunofluorescence			
		Positive	Equivocal	Negative	Total
Positive (titer >100)	<i>E. coli</i>	11			11
	Klebsiella-Aerobacter	8			8
	Proteus	5			5
	<i>Pseudomonas aeruginosa</i>				0
	<i>Streptococcus fecalis</i>				0
Negative (titer <20)	<i>E. coli</i>			1	1
	Klebsiella-Aerobacter				0
	Proteus			3	3
	<i>Pseudomonas aeruginosa</i>		8	2	10
	<i>Streptococcus fecalis</i>			10	10
Total		24	8	16	48

Streptococcus fecalis listed previously were studied. Bacteria were grown in Trypticase Soy Broth for 18 to 24 hours, harvested by centrifugation, washed 3 times in 0.9% saline and diluted to one-tenth the concentration of the original broth culture. Smears of each suspension were made on clean glass slides and air dried. Slides were fixed in acetone for 10 minutes and stained by the indirect technique using a 1:10 dilution of rabbit antisera against *E. coli* 0:14 and undiluted fluorescein isothiocyanate labelled goat anti-rabbit gamma globulin. Commercial goat antiserum to rabbit gamma globulin (Hyland Laboratories) was labelled with fluorescein isothiocyanate by the method of Marshall *et al.*(4). Conjugated antiserum was passed through a DEAE cellulose column(5) for removal of non-specific fluorescent material. Bacterial smears were layered with unlabelled *E. coli* 0:14 antiserum for one hour, then washed with phosphate buffered saline, pH 7.0. These were then stained with the fractionated fluorescein labelled goat anti-rabbit gamma globulin serum at room temperature for one hour and again washed. Stained preparations were examined with a Reichert fluorescent microscope with an Osram HBO 200 watt mercury vapor arc light source with heat filter, Schott and Gen UH1 (1.5 mm) excitor filter, Reichert darkfield condensers and Wratten B2 ultraviolet filter. All photographs were taken using similar conditions of exposure, development and printing.

Results. Comparison of the results of fluo-

rescent staining and hemagglutination of these strains with *E. coli* 0:14 antisera are shown in Table I. The correlation between hemagglutinating titer and fluorescent staining of *E. coli*, Klebsiella-Aerobacter, strains of Proteus and enterococci was excellent. Only rough quantitation of the degree of fluorescent staining was attempted, because of the subjective variation of such observations. Hemagglutinating titers against 11 of the 12 strains of *E. coli* ranged from 100 to 1400 and satisfactory immunofluorescence was obtained with each of these. One strain of *E. coli* 0:25 showed no fluorescence after staining and erythrocytes sensitized with antigen from this organism were not agglutinated by a 1:20 dilution of *E. coli* 0:14 antisera. Hemagglutinating titers from 100 to 1000 were obtained with all 8 strains of Klebsiella-Aerobacter. All of these strains displayed immunofluorescence after staining. Hemagglutination titers from 400 to 800 were present against 5 of the strains of Proteus and satisfactory fluorescent staining was obtained with these strains. No immunofluorescence or hemagglutination (<1:20) was demonstrated with the other 3 strains of Proteus, (*P. morgani*, *P. vulgaris*, and *P. rettgeri*). Hemagglutination tests were negative with the 10 strains of *Streptococcus fecalis* and *Pseudomonas aeruginosa* studied. No fluorescence was observed with any of the strains of enterococci, but equivocal fluorescence was observed with 8 of the 10 strains of *Pseudomonas aeruginosa*.

Examples of the fluorescent staining with

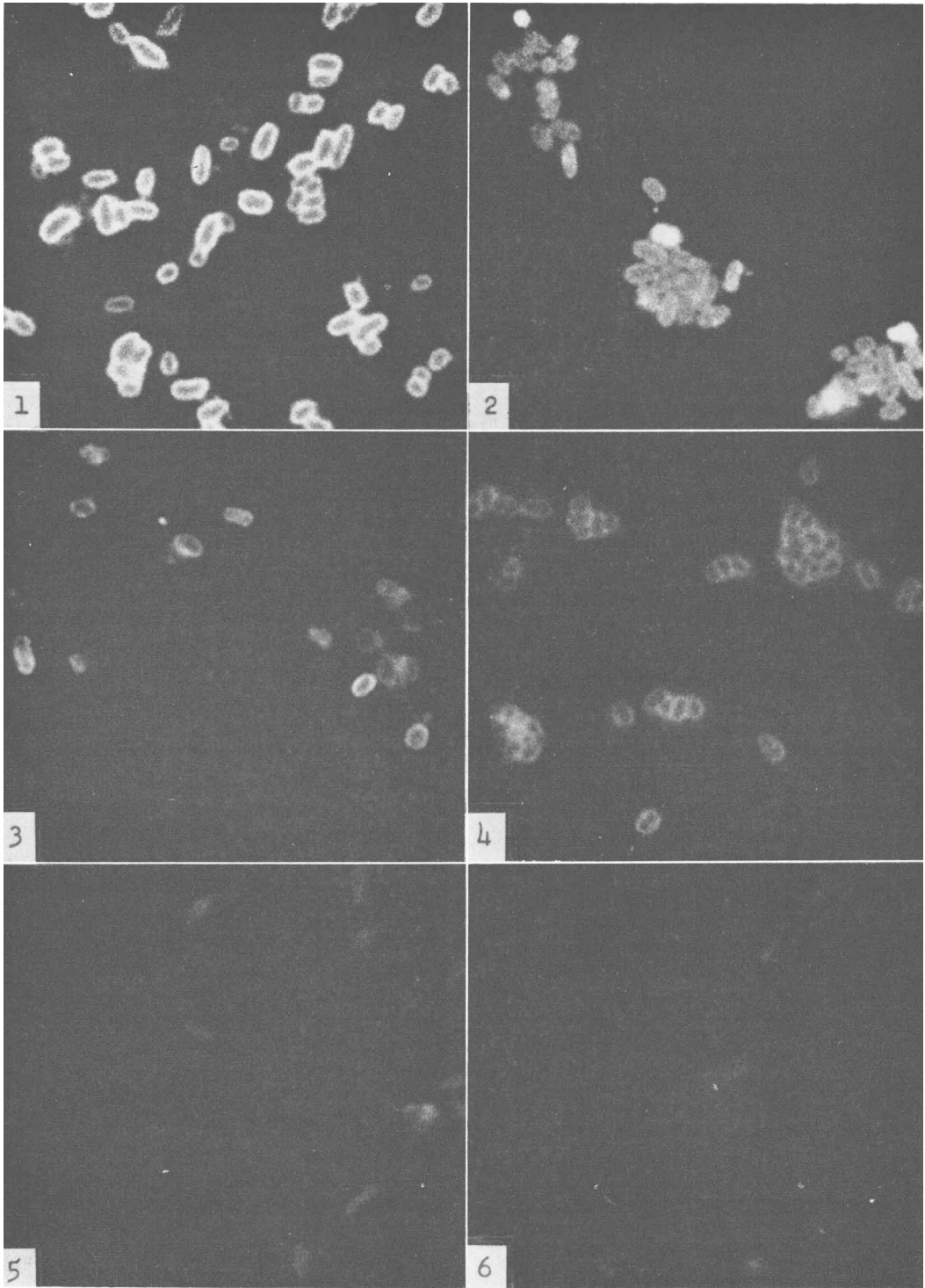


FIG. 1-6. Immunofluorescence of bacteria after staining with unlabelled rabbit anti *E. coli* 0:14 serum followed by fluoresceinated goat anti-rabbit gamma globulin. Magnification ($\times 1800$). Fig. 1. *E. coli* 0:14. 2. *E. coli* 0:13. 3. *A. aerogenes* 5. 4. *P. mirabilis* 3. 5. *P. aeruginosa* 3. 6. *St. fecalis* 3.

TABLE II. Fluorescent Staining and Hemagglutination of *E. coli*, *K. Pneumoniae* and *A. Aerogenes* with *E. coli* 0:14 Antiserum Before and After Adsorption with Homologous and Heterologous Bacteria.

Bacterial strain (used for fluorescent staining and hemagglutin- ating antigen)	<i>E. coli</i> 0:14 antiserum adsorbed with	Hemagglutination	Fluorescent staining
<i>E. coli</i> 0:14	—	1:1400	++++
" " 0:14	<i>E. coli</i> 0:14	<1:20	—
" " 0:14	<i>E. coli</i> 0:41	1:1300	+++
" " 0:14	<i>A. aerogenes</i> 7	1:1000	+++
<i>E. coli</i> 0:41	—	1:700	++
" " 0:41	<i>E. coli</i> 0:14	<1:20	—
" " 0:41	<i>E. coli</i> 0:41	<1:20	—
" " 0:41	<i>A. aerogenes</i> 7	<1:20	—
<i>E. coli</i> 0:6	—	1:600	++
" " 0:6	<i>E. coli</i> 0:14	<1:20	—
" " 0:6	<i>E. coli</i> 0:41	<1:20	—
" " 0:6	<i>A. aerogenes</i> 7	<1:20	—
<i>A. aerogenes</i> 7	—	1:1000	+++
" " 7	<i>E. coli</i> 0:14	<1:20	—
" " 7	<i>A. aerogenes</i> 7	<1:20	—
" " 7	<i>E. coli</i> 0:41	<1:20	—
<i>K. Pneumoniae</i> 5	—	1:800	++
" " 5	<i>E. coli</i> 0:14	<1:20	—
" " 5	<i>A. aerogenes</i> 7	<1:20	—
" " 5	<i>E. coli</i> 0:41	<1:20	—

E. coli 0:14 antiserum against the homologous strain *E. coli* 0:14 (Fig. 1) and heterologous bacteria *E. coli* 0:13 (Fig. 2), *A. aerogenes* (Fig. 3) and *M. mirabilis* (Fig. 4), *Pseudomonas aeruginosa* (Fig. 5) and *Streptococcus fecalis* (Fig. 6) are demonstrated in Figs. 1-6. The most brilliant fluorescence was observed with the homologous organism, *E. coli* 0:14 and the stain was homogeneously distributed throughout a smooth relatively thick cell wall. Heterologous bacteria (Fig. 2-4) stained less intensively and the walls of these organisms appeared thinner, with irregular distribution of the fluorescence. Similar results were observed with other strains studied. Faint fluorescence can be seen with *Pseudomonas aeruginosa*, but no cell wall fluorescence could be observed with *Streptococcus fecalis*. In view of the faint, indistinct staining of *Pseudomonas aeruginosa*, additional studies with these strains were undertaken. Smears of strains of *Pseudomonas* untreated with either *E. coli* 0:14 antiserum or fluoresceinated anti-rabbit gamma globulin demonstrated autofluorescence when examined under conditions similar to those used for stained bacteria.

Adsorption experiments were carried out

to insure that the cross reactions with *E. coli* 0:14 antiserum were directed against a common antigen rather than being due to the presence of antibodies to specific O antigens of multiple species of Enterobacteriaceae. The results of these studies are illustrated in Table II. Fluorescent staining and determination of hemagglutination titers to *E. coli* 0:14, *E. coli* 0:41, *E. coli* 0:6, *A. aerogenes* 7, and *K. pneumoniae* 5 before and after adsorption of *E. coli* 0:14 antiserum with *E. coli* 0:14, *E. coli* 0:41 and *A. aerogenes* 7 are shown. One hundred milligrams of acetone dried bacteria were added to one ml aliquots of antiserum, shaken for 1 hour at 4°C and the mixture centrifuged at 13,000 r.p.m. for 30 minutes. Adsorption of 0:14 antiserum with *E. coli* 0:14 completely removed hemagglutinating antibody and rendered the sera incapable of staining not only *E. coli* 0:14, but also *E. coli* 0:41, *E. coli* 0:6, *A. aerogenes* 7 and *K. pneumoniae* 5. In contrast, adsorption with either *E. coli* 0:41 or *A. aerogenes* 7 reduced the hemagglutinating titer of 0:14 antiserum from 1:1400 to 1:1300 and 1:1000 respectively and reduced the ability of the serum to stain *E. coli* 0:14 only slightly. Adsorption with *E. coli* 0:41

and *A. aerogenes* 7 did prevent hemagglutination, and fluorescent staining of heterologous strains of *E. coli* 0:6 and *K. pneumoniae* 5, however. The failure to demonstrate hemagglutination or fluorescent staining of *E. coli* 0:6 and *K. pneumoniae* 5 after adsorption with *E. coli* 0:41 or *A. aerogenes* 7 indicated that 0:14 antiserum did contain antibody against an antigen common to these strains rather than multiple enterobacterial antibodies.

Discussion. Earlier studies by Kunin indicated that two types of antigenic determinants exclusive of flagellar, fimbrial and envelope antigens were possessed by *E. coli* and other Enterobacteriaceae. One is the classical specific O antigen; the other is a hapten common to most members of this family(1-3). The present studies lend additional confirmation to Kunin's observations and demonstrate the presence of the common antigen on the surface or within the cell wall of such bacteria. Kunin was unable to demonstrate bacterial agglutination with the Enterobacteriaceae utilizing antisera against the common antigen(1). These studies indicate that the failure of agglutination is not due to the absence of the common antigen from the surface of the bacterial cell. Fluorescent staining of *E. coli* 0:14 and hemagglutination results after adsorption of antibody against the common antigen demonstrate that antibody to the specific 0:14 somatic antigen comprises a much greater proportion of the antibody of 0:14 antiserum than antibody against the common antigen. Since both hemagglutination(6) and fluorescent staining appear to be more sensitive than bacterial agglutination, Kunin's failure to demonstrate agglutination with 0:14 antiserum may reflect only the relatively low content of antibody against the common antigen in 0:14 antisera.

The correlation between immunofluorescence and the results of hemagglutination were excellent in general. Only *Pseudomonas* proved to be an exception and demonstrated equivocal fluorescence in the absence of hemagglutination. *Pseudomonas aeruginosa* produces a fluorescent pigment which shows

ultraviolet excitation at 400 to 410 m μ with a smaller maxima at 525 m μ (7). The latter is similar to that produced by the fluoresceinated antibody. Since autofluorescence was also observed with unstained smears of *P. aeruginosa*, the faint fluorescence of these strains may be the result of "fluorescein" or "pyoverdine" production by these strains.

The present studies do demonstrate the feasibility of utilizing antiserum against the common enterobacterial antigen for the localization of bacteria and bacterial antigen in renal tissue in pyelonephritis in man. Since the objective of such studies is antigenic localization rather than species differentiation this technique obviates the necessity of using multiple type specific antisera and should prove useful in such investigations.

Summary. The fluorescent antibody technique was used to study the presence of an antigen common to many species of Enterobacteriaceae and demonstrated localization of this antigen in the cell wall of these species. Although the studies indicate that specific O antigen comprised the largest portion of the cell wall of these organisms, satisfactory cell wall fluorescence was obtained utilizing antiserum against the common antigen. In addition, correlation between immunofluorescence and hemagglutination results were excellent. The use of antiserum against the common antigen allows studies of immunofluorescence of multiple strains of Enterobacteriaceae without the necessity of multiple type specific antisera.

1. Kunin, C. M., Beard, M. V., Halmagyi, N. E., Proc. Soc. Exp. Biol. and Med., 1962, v111, 160.
2. Kunin, C. M., Beard, M. V., J. Bact., 1963, v85, 541.
3. Kunin, C. M., J. Exp. Med., 1963, v118, 565.
4. Marshall, J. D., Eveland, W. C., Smith, C. W., Proc. Soc. Exp. Biol. and Med., 1958, v98, 898.
5. Peters, J. H., McDevitt, H. O., Pollard, L. W., Harter, J. G., Coons, A. H., Fed. Proc., 1961, v21, 17.
6. Marrack, J. R., Brit. Med. Bull., 1963, v19, 178.
7. Wasserman, A. E., Applied Microbiol., 1965, v13, 175.

Received September 9, 1965. P.S.E.B.M., 1966, v121.