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Detection of Coronavirus Infection of Man by Immunofluorescence[1] (39761)

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Since primary isolation of the human coronaviruses is difficult to accomplish, much reliance has been placed on serologic methods for identification of infections (1). The techniques that generally have been employed are complement fixation (CF) and hemagglutination inhibition (HI) (2, 3). However, certain problems of sensitivity and specificity are inherent in these methods and difficulty in interpretation of results can sometimes be resolved only by use of the far more complicated neutralization test (2, 4, 5). An additional, more simple test would therefore be of great value. The fluorescent antibody (FA) technique has rarely been employed to detect human coronavirus antibody although it did play a role in the original identification of the organ culture isolates (6). Methods have now been developed for the measurement by immunofluorescence of antibody against human coronavirus OC43 and 229E. The procedure is herein described and results are compared with those obtained with CF, HI, and neutralization.

Materials and Methods. Viruses and sera used. The seed of 229E virus was obtained originally from Dr. Dorothy Hamre and was passed serially in WI-38 cells. Pools of virus grown in WI-38 cells were employed in all procedures. OC43 virus, adapted to growth in mouse brain, was obtained through the courtesy of Mr. H. S. Kaye, Center for Disease Control. It was propagated serially by intracerebral inoculation of suckling mice (7). OC43 was also adapted to growth in BS-C-1 cells, where its presence was detected by the virus-specific hemadsorption technique using rat erythrocytes (8). Titers of pools of both viruses ranged from $10^{4.7}$ to $10^{5.5}$ $TCID_{50}$/ml.

Antisera against 229E and OC43 were prepared in guinea pigs using the same schedule for both viruses. The animals were initially inoculated intramuscularly with 1.0 ml of the virus in an equal volume of incomplete Freund's adjuvant. At 3 weeks, 1.0 ml of the homologous virus was given ip and the final blood specimens were obtained 10 days later. Titers of the antisera were 1:64 for 229E and 1:128 for OC43. Before use in the FA test, these sera were absorbed with the particular noninfected cells to rid them of nonspecific reactivity. In addition, noninfected cell controls were used in all tests, and were uniformly found to be negative. Human specimens were obtained from the collection of the Tecumseh Study of Respiratory Illnesses. These sera had been collected from all those under surveillance at 6 monthly intervals. The particular pairs of sera used had been found by other procedures to exhibit a fourfold rise in coronavirus antibody.

Fluorescent antibody technique. Cultures of WI-38 were commercially obtained (HEM Laboratories) and cells were propagated serially in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum. Coverslip cultures of the WI-38 cells were prepared in Leighton tubes. When a confluent cell sheet had been obtained, the growth medium was removed and the cells were infected with 0.2 ml of a pool of coronavirus 229E. After 2 hr at 37°, maintainance medium (MEM with 2% fetal calf serum) was added. The cultures were then incubated at 34° until CPE began to appear, usually at 2-3 days. At this point, coverslips were rinsed, fixed in acetone, and stored at 4° until used.

OC43 virus pools had been prepared in BS-C-1 cell cultures (Flow Laboratories) maintained on serum-free MEM. For the FA tests themselves, LLC-MK_2 cells were used. Medium employed for growth was 199 with 1% horse serum; when a confluent cell sheet was achieved, the medium was removed from the Leighton tubes and 0.2 ml of undiluted virus was allowed to absorb

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for 2 hr at 37°. Thereafter, fresh medium was added and cultures were incubated at 34° until CPE began to appear, usually at 14 to 16 days; during this time pH was controlled by the addition of small quantities of 1.4% sodium bicarbonate. The coverslips were then washed and fixed as described above.

Coverslips of 229E and OC43 infected cells were cut into small pieces of approximately 4.5 × 5.0 mm. Presence of virus in the cell sheet was first confirmed using guinea pig antisera and then tests involving paired human sera were carried out. In all cases, the antisera were diluted and incubated with the coverslip pieces for 1 hr at 37°. Following two washes with PBS, the fluorescein conjugate was added and incubation was repeated for 1 hr at 37°. For tests involving animal sera, a rabbit anti-guinea pig 7S IgG (Hyland Laboratories) was used; for tests involving human sera, a horse anti-human globulin (Progressive Laboratories, Inc., Washington, D.C.) was employed. The pieces were then washed an additional two times with PBS, counterstained with Eriochrome black, and read on a Leitz fluorescent microscope. On a scale of 1+ to 4+, 3+ or 4+ fluorescence was considered positive.

Human serum pairs were diluted in two-fold steps from 1:8. A rise in antibody titer was considered to have occurred if there was a fourfold increase or if antibody appeared in the second specimen at 1:16 and none had been present at 1:8 in the first specimen of the pair. Included in all of these tests were positive cell controls using human sera of known antibody titer, in addition to the uninfected cell controls.

Other serologic procedures. The complement fixation (CF) test was used with both 229E and OC43. It was performed by the microplate technique with sheep erythrocytes, 1.8 units of complement, and 4 units of antigen (4, 5). The 229E antigen was prepared by inoculation of WI-38 bottle cultures with undiluted virus. After incubation for 34–38 hr at 33°, cultures were frozen and thawed three times, fluids were clarified by centrifugation, and supernatant was used as antigen (10). OC43 antigen was a 20% suspension of infected mouse brain in veronal-buffered saline (11). The HI test was also used for OC43 virus. The method employed was that described by Kaye and Dowdle and involved the use of chick red cells and mouse-brain antigen suspension (3). The tests were performed in microplates.

Neutralization tests were carried out for 229E virus in WI-38 cells. Maintenance medium consisted of Eagle's MEM with 2% fetal bovine serum. Virus was used at a dose of 32–100 $TCID_{50}$/0.1 ml. After inactivation at 56° for 30 min the sera were serially diluted. To 0.3 ml of the diluted sera, 0.3 ml of the appropriate dilution of virus was added and the mixture was incubated at 24° for 1 hr. Thereafter 0.2 ml of each mixture of serum and virus was inoculated into each of two cell culture tubes. The tubes were placed in a roller drum and incubated at 33°. After 4 days, endpoints were read on the basis of presence of CPE. For OC43, BS-C-1 cells were used. The growth medium was removed; the cells were washed three times with balanced salt solution and serum-free MEM was added as maintenance medium. The test itself was carried out as described for 229E except that a dose of 100–320 $TCID_{50}$ was employed. After the tubes had been incubated for 4 days at 33°, endpoints were read by virus-specific hemadsorption. Rat erythrocytes were washed three times and 0.2 ml of 0.4% suspension was added to each cell-culture tube. Following 45 min at 24° tubes were examined microscopically for the presence of hemadsorption (8).

Results. Characteristics of FA tests for 229E and OC43. Coverslip cultures were stained first with the homologous guinea pig antisera to confirm the presence of virus; in subsequent tests, paired human antisera from known infections were used. Granular fluorescence was present in the cytoplasm of cells infected with both viruses. An example of fluorescence exhibited by WI-38 cells infected with 229E virus is shown in Fig. 1 and fluorescence exhibited by LLC-MK_2 cells infected with OC43 is shown in Fig. 2. The major difference seen between the two is referable not to the location of the fluorescence, which in both cases was clearly cytoplasmic, but rather to the characteristics of the cell line used, with the WI-38 cells

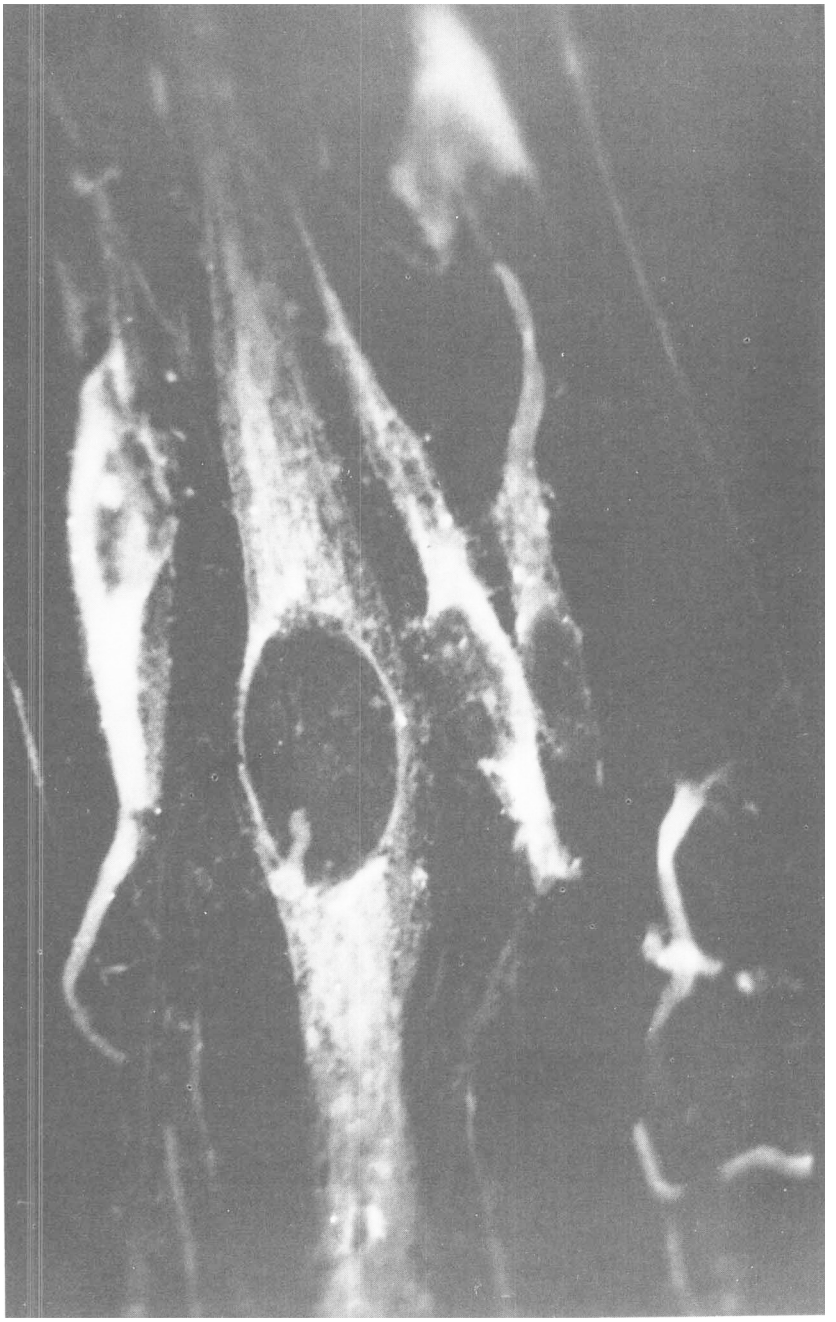

FIG. 1. Cytoplasmic granular fluorescence exhibited by WI-38 cells infected with 229E virus.

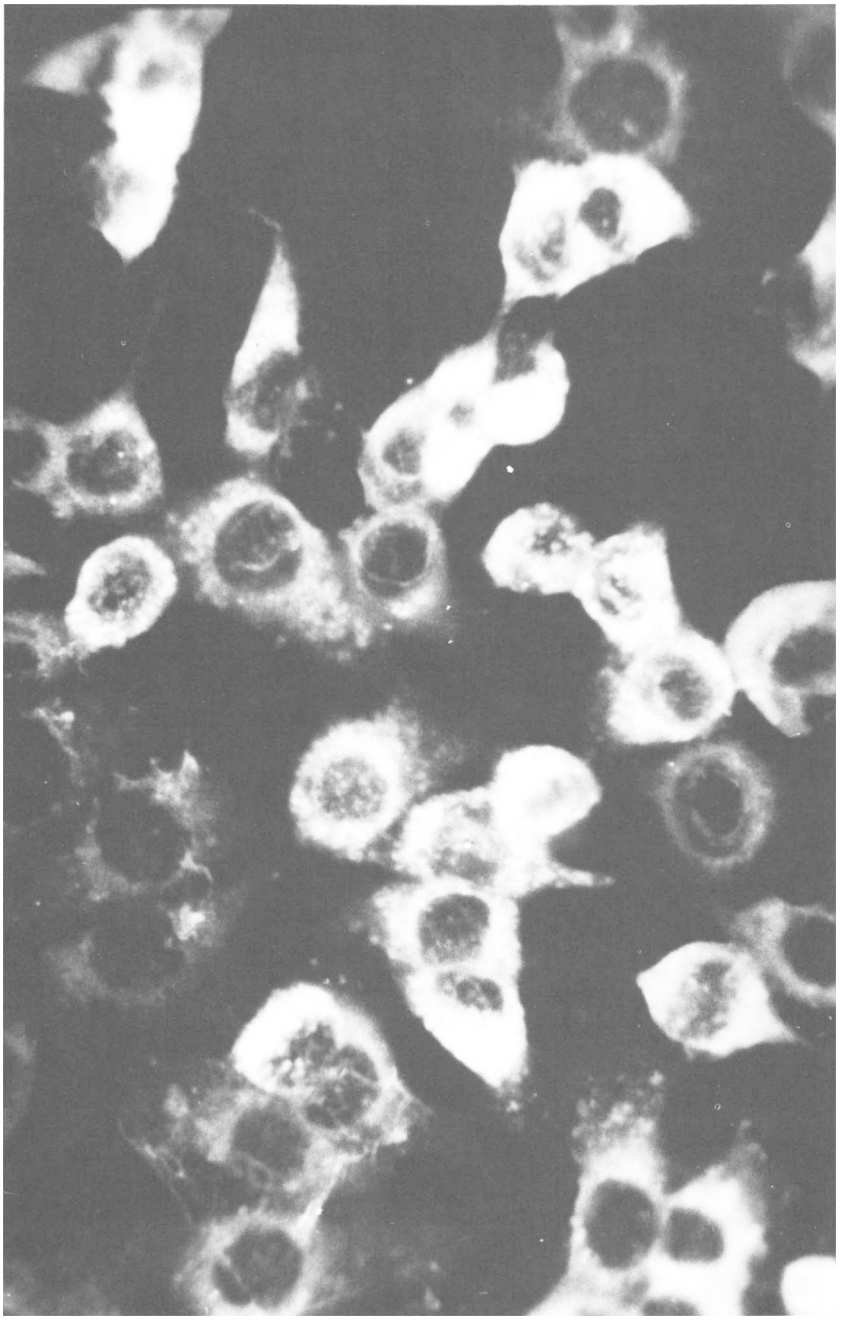

FIG. 2. Cytoplasmic granular fluorescence exhibited by LLC-MK_2 cells infected with OC43 virus.

exhibiting their typical elongated fibroblastic appearance. No cross reactions were seen when OC43 animal antisera were used against 229E-infected cells or when the reverse test was carried out. Fluorescence identical with that shown in the figures was seen when homologous postinfectious human sera were employed.

Comparison of FA with other serologic techniques. A total of 55 serum pairs known to exhibit a rise in antibody titer for 229E by CF or neutralization tests were available from among the blood specimens collected in the community of Tecumseh, Mich. When tested by FA, 69.1% of these sera also showed a rise in titer by this method. However, as shown in Table I, the correlation was far better when a serum had exhibited rise in titer by both neutralization and CF than when it had shown a rise in titer in one test alone. In fact, 94.7% of the 19 serum pairs with rises in titer by both CF and neutralization also had a rise by FA. For those pairs with only one rise in titer, the FA correlated slightly better with the CF at 62.5% than with the neutralization at 50.0%. Since the sera were selected for inclusion on the basis of showing evidence of infection, there were no pairs tested which had neither CF nor neutralization rises.

A similar comparison of serologic results for OC43 is shown in Table II. The pairs included were all those available from the Tecumseh collection which were known to exhibit a rise in antibody titer by HI and/or CF. These sera were now further tested by FA as well as by neutralization. The results again demonstrate that the FA test is most likely to be positive when there is evidence of infection by more than one other test. This was especially true when neutralization was one of the tests with an increase in titer; within this group, whether the pair had a rise in titer by CF, HI, or both, the percentage also positive by FA ranged from 58.3 to 66.7%. Within the group of paired sera without a rise in neutralization titer, sera were most frequently positive by FA in the CF–HI combined group and least frequently positive in those with a rise by HI alone. Overall, pairs in this group were much less likely to show a rise in FA titer (31.4%) than pairs in the group of sera with a rise in neutralization titer (60.7%).

Although OC43 and 229E are antigenically among the most distinct human coronaviruses, cross-reactions have been reported by CF (1). Therefore, it was of importance to define the specificity of the FA test. Two groups of ten serum pairs, each showing

TABLE I. RISES IN FA TITERS IN PAIRED SERA WITH SIGNIFICANT CHANGE IN CF AND/OR NEUTRALIZATION TITER (229E VIRUS).

	Fourfold rises in titer by FA in sera with indicated neutralization titer changes					
	Neutralization rise present		Neutralization rise absent		Total	
Change in CF titer	Number	%	Number	%	Number	%
CF rise present	18/19[a]	94.7	10/16	62.5	28/35	80.0
CF rise absent	10/20	50.0	–	–	10/20	50.0
Total	28/39	71.8	10/16	62.5	38/55	69.1

[a] Number of rises in titer by FA for 229E per number of paired sera in group.

TABLE II. COMPARISON OF SEROLOGIC TECHNIQUES IN SERA WITH SIGNIFICANT RISE IN CF AND/OR HI TITER (OC43 VIRUS).

	Four-fold rises in titer by FA in sera with indicated neutralization titer change					
	Neutralization rise present		Neutralization rise absent		Total	
Type of rise in antibody titer	Number	%	Number	%	Number	%
CF rise, no HI rise	4/6[a]	66.7	5/15	33.3	9/21	42.9
HI rise, no CF rise	6/10	60.0	4/15	26.7	10/25	40.0
CF and HI rise	7/12	58.3	2/5	40.0	9/17	52.9
Total	17/28	60.7	11/35	31.4	28/63	44.4

[a] Number of rises in titer by FA for OC43 per number of paired sera in group.

fourfold rises in titer by FA for OC43 or for 229E were selected at random from among the specimens described above, which had been collected in periods of time in which the other coronavirus was not known to be circulating (4, 5). Each group of sera was run by FA against the heterologous virus. Among the 10 sera which had rises in titer for OC43, there were no rises found using infected cover slips. However, among the sera with 229E rises, 1 of the 10 pairs did show a rise in titer for OC43. This specimen was collected at the height of the community-wide outbreak of 229E, when no evidence of OC43 activity was found. Whether this represents a true cross-reaction or is the result of a dual infection is impossible to determine, considering the difficulty of isolation of the viruses in question. In any event, cross-reactions between these viruses by FA cannot be a frequent event and should not be a major problem in most studies.

Discussion. Identification by serology of infections caused by the two coronaviruses adapted to cell culture, 229E and OC43, is complicated by a number of factors. In experiments with volunteers, it has been shown that coronavirus infections are sometimes not accompanied by rises in antibody titer, a reflection either of the poor antigenicity of the viruses or, more likely, of the relative insensitivity of the procedures available (12). For 229E, the CF test, using the antigen described by Hamre and Procknow, is positive for only a short time after the infection has taken place (10). The neutralization test is rather difficult to perform because of the nonspecific nature of CPE produced by 229E. For OC43, the HI test is also available, but results obtained by this test and by CF often disagree; the disagreement can only partially be resolved by the neutralization test. The problem here is partially related to the circulation of other coronaviruses related antigenically to OC43 which further confuses the serodiagnosis of infection (11).

The FA test for these two viruses can be viewed as an aid in a difficult area. It would be of special value to laboratories already using FA for other viruses since it offers another means of resolving diagnostic problems. For 229E, it was positive in nearly 95% of those pairs in which a recent infection had produced a rise in both neutralization and CF titer. For OC43, the FA test correlated reasonably well with neutralization. However, this correlation was never as good as that seen with 229E, which again probably relates to the antigenic diversity of the OC43 virus group. Thus, the FA test, while clearly not a complete solution to the problem of identifying coronavirus infection serologically, does offer a reasonable and relatively simple addition to techniques now being used.

Summary. An indirect fluorescent antibody technique has been developed for use with coronaviruses 229E and OC43. For the former virus, WI-38 cells were used as the host system, and for the latter LLC-MK_2 cells were employed. Human serum pairs which were known to show a rise in antibody titer by other serologic techniques were tested by the FA method. Comparison of results obtained by different procedures showed that FA was specific and would be a useful addition to available methods for identifying infection serologically.

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