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Clinical and Diagnostic Virology
2 (1994) 165–179

Clinical and Diagnostic Virology

Antigen detection in human respiratory *Coronavirus* infections by monoclonal time-resolved fluoroimmunoassay

John C. Hierholzer [a,*], Pekka E. Halonen [a,b], Patricia G. Bingham [a], Richard A. Coombs [a], Yvonne O. Stone [a]

[a] *Respiratory and Enteric Viruses Branch, Division of Viral and Rickettsial Diseases, Center for Infectious Diseases, Centers for Disease Control, 1600°Clifton Rd. N.E., Atlanta, GA 30333, USA;* [b] *Department of Virology, University of Turku, Kiinamyllynkatu 13, SF-20520, Turku 52, Finland*

Received 27 May 1993; revised 18 November 1993; accepted 1 December 1993

Abstract

Background: The diagnosis of respiratory infections by detecting viral antigens has received considerable attention using immunofluorescent assays (IFA) and enzyme immunoassays (EIA). Time-resolved fluoroimmunoassay (TR-FIA) has been developed for several viruses.

Objectives: To prepare monoclonal antibodies to coronavirus strains, to incorporate them into a TR-FIA, and test the assay on clinical specimens.

Study design: Monoclonal antibodies were prepared to the N nucleoprotein of the two human respiratory coronaviruses, HCV strains 229E and OC43. Monoclonals to both viruses were completely type-specific; they did not cross-react between themselves or with multiple strains of other respiratory viruses. These antibodies were configured into optimized EIA and TR-FIA tests. The all-monoclonal tests were then compared to polyclonal EIA tests in terms of their ability to detect virus in clinical specimens.

Results: The all-monoclonal TR-FIA was uniformly the most sensitive, detecting virus in all 13 229E-positive specimens compared to 69% for the monoclonal EIA and 54% for the polyclonal EIA test. Similar results were obtained for 10 OC43-positive specimens: 100% in TR-FIA, 90% in monoclonal EIA, and 80% in polyclonal EIA. For 229E in TR-FIA, mean positive/negative (P/N) ratios were 143 for 229E-positive human embryonic lung fibroblast (HLF) cell culture fluids and 10 for positive nasopharyngeal aspirate specimens; for OC43 in TR-FIA, mean P/N values were 964 for OC43-positive rhabdomyosarcoma (RD) cell culture fluids and 174 for positive NPA specimens. The sensitivities of the TR-FIA were determined with purified virions to be 0.308 ng virus per well for HCV-229E and 0.098 ng virus per well for HCV-OC43.

Conclusions: This rapid and sensitive test appears to be much more sensitive than traditional antigen detection assays but will require more extensive field testing on clinical specimens.

*Corresponding author.

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SSDI 0928-0197(94)E0050-P

Key words: Human coronavirus; Monoclonal antibody; Rapid antigen test; Immunoassay

1. Introduction

Rapid testing for the respiratory viruses has become widely appreciated in the clinical laboratory as virus-specific indirect fluorescent antibody (IFA) and spot enzyme immunoassay (EIA) kits have become commercially available and quantitative tests such as EIA and time-resolved fluoroimmunoassay (TR-FIA) have been improved. Both of these tests are of proven greater sensitivity than IFA, and TR-FIA performed with monoclonal antibodies has been shown to be of greater sensitivity even than similarly constructed EIA tests (reviewed in Hierholzer et al., 1990; McIntosh et al., 1993).

We and others have described monoclonal TR-FIA tests for influenza, adenovirus, respiratory syncytial virus, parainfluenza 1–4, mumpsvirus, and the enteroviruses responsible for acute hemorrhagic conjunctivitis (Halonen et al., 1985, 1989; Walls et al., 1986; Hierholzer et al., 1987, 1989, 1990, 1993; Brown et al., 1990; Siitari, 1990; Bucher et al., 1991). Coronaviruses are another group of viruses that cause widespread respiratory infections and thus should be included in the TR-FIA battery for rapid viral diagnosis. Of the four recognized human coronavirus strains, only two have been characterized, and both are associated with upper and occasionally lower respiratory tract disease. IFA and EIA tests for these strains, called human coronavirus (HCV) 229E and HCV-OC43, have been described and applied to epidemiologic surveys with good success (McIntosh et al., 1978; Isaacs et al., 1983; Macnaughton et al., 1983). In the present report, we describe the production of monoclonal antibodies to HCV-229E and HCV-OC43 and describe their use in IFA, EIA, and TR-FIA, with emphasis on the TR-FIA as a highly-sensitive rapid antigen test.

2. Materials and methods

Viruses and cell culture

The prototype strain of HCV-229E was initially obtained from Dorothy Hamre, University of Chicago, at the sHK_2WI38_{11} passage level (Hamre and Procknow, 1966). It has been maintained in a variety of human embryonic lung diploid fibroblast cell lines, e.g., WI38, RU-1, MRC-5, HELF, HLF. The present studies were commenced with strain 229E after a 3× plaque purification in HELF cells, at the $sHK_2WI38_{11}RU_8HELF_5HLF_2$ passage (Hierholzer, 1976; Kemp et al., 1984).

The prototype of HCV-OC43 was obtained from Ken McIntosh, National Institutes of Health, after adaptation from human embryonic tracheal organ culture to suckling mouse brain (SMB), at the HET_5SMB_7 passage level (McIntosh et al., 1967). For this study, OC43 was further adapted to human embryonal rhabdomyosarcoma (RD) cells (ATCC No. CCL-136) as suggested by Schmidt et al. (1979;

Schmidt and Kenny, 1982). OC43 was then used at the $HET_5SMB_{18}RD_6$ passage level throughout this study.

The $xHLF_2$ and xRD_6 working stock passages of 229E and OC43, respectively, were made under a fortified maintenance medium consisting of RPMI-1640 with 5% fetal calf serum, and incubated on a roller or rocker platform at 35°C for 28–36 h post-adsorption as previously described (Hierholzer, 1976). The flasks were harvested by 2 cycles of freeze/thawing and stored in aliquots as crude working stocks for the preparation of test antigens and for antibody production. Due to the extreme lability of coronaviruses and the association of biological activities with intact virions, an aliquot was used only once, and tests were run in a manner to minimize the exposure of virus to ambient temperatures (Kaye et al., 1970; Hierholzer, 1976; Hierholzer and Tannock, 1988).

Prototype and wild strains of other respiratory viruses were obtained from our reference virus collection, maintained at −100°C. They were used in this study without further passage.

Specimens

Nasopharyngeal aspirates positive for 229E or OC43 were obtained from the Department of Virology, University of Turku, and had been identified there by EIA tests with hyperimmune rabbit and guinea pig antisera made with purified virions (Halonen et al., 1985; Mertsola et al., 1991). Specimens positive for other respiratory viruses were obtained from the CDC diagnostic service and from prior studies (Halonen et al., 1983, 1985; Hierholzer et al., 1987, 1989). All specimens had been stored at −70°C as a 1:5 dilution in PBS containing 20% FCS, 2% Tween-20, and 0.004% merthiolate (Halonen et al., 1985).

Antisera. Polyclonal (hyperimmune) antiserums used in some EIA formats were prepared in adult rabbits, guinea pigs, mice, and chickens by immunization with crude virus supernatants or purified virions as previously described (Kaye et al., 1970; Hierholzer et al., 1972; Hierholzer, 1976; Halonen et al., 1985).

Production of monoclonals. The production of monoclonal antibodies to human coronaviruses was more problematic than usual because of the prevalence of latent murine coronaviruses (the mouse hepatitis viruses) in mouse colonies and the strong antigenic cross-reactions between OC43 and many strains of MHV (reviewed in Robb and Bond, 1979; Macnaughton, 1981; Siddell et al., 1983; Hierholzer and Tannock, 1988). Thus, our mouse colonies were carefully monitored by EIA for serologic evidence of MHV infection (No. 36218/8191-20, Organon Teknika Corp., Durham, NC), as well as by EIA for infection by other murine viruses, to ensure that adventitious viruses would not compromise the OC43 monoclonals.

Monoclones to 229E and OC43 viruses were prepared as previously described (Hierholzer et al., 1993). For 229E, Balb/C mice were immunized with crude virus supernatants from HLF cultures that were frozen/thawed 3× and clarified at $1000 \times g$ for 10 min at 4°C; the immunizing antigen had an infectivity titer of $10^{8.3}$ $TCID_{50}$/ml in 5 days in the HLF microneutralization system (see below). For OC43, mice were immunized with an SMB seed stock after 4× freeze/thawing and clarification at $6\ 600 \times g$ for 20 min at 4°C to remove the maximum amount of cellular

debris while retaining full infectivity (Kaye et al., 1970); this preparation had an infectivity titer of $10^{7.9}$ LD_{50}/ml in 9 days in suckling mice inoculated i.c. with 0.02 ml of virus dilution. The spleen cells from immunized mice were fused with Sp2/0-Ag14 myeloma cells (ATCC No. CRL-1581) in the presence of 50% PEG [2 ml basic Opti-MEM medium at 37°C plus 2 ml of melted polyethylene glycol-1500 (No. 29575, BDH Ltd., Poole, UK), filter-sterilized], and the hybridoma cells were selectively grown out by standard media.

The hybrid cells were screened for antibody production by the tissue culture EIA procedure (Anderson et al., 1985). For 229E, HLF cells at 20 000 cells per well in 100 μl of Eagles MEM/10% FCS growth medium were added to all wells of 96-well tissue culture plates (No. 3596, Costar, Cambridge, MA). Entire rows were alternately supplemented with 50 μl of RPMI-1640/5% FCS maintenance medium for the cell control wells or infected with a standard dose of 229E working stock virus in 50 μl of maintenance medium to give minimal CPE after 3 days of incubation at 35°C under CO_2. The plates were then washed 3 × (see EIA methods), fixed with 75 μl of 80% acetone/20% PBS for 20 min at 4°C, air-dried, and stored in protective plastic at −20°C until needed. For testing, 50 μl of PBS/GT diluent (see EIA methods) was added to all wells, 25 μl of supernatant from the hybridoma wells was added into a virus well and a cell control well, and the test incubated for 1 h at 37°C in a moist chamber. The plates were then washed 3 × and 75 μl of goat anti-mouse peroxidase (No. 14-18-06, Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a 1:3000 dilution in PBS-GT was added; the test was incubated for 1 h at 37°C as above. After a 6 × wash step, 125 μl of TMB (see EIA methods) was added, reacted 15 min at ambient temperature, stopped with 25 μl of 2 M sulfuric acid, and read spectrophotometrically at 450 nm. Monoclones for HCV-OC43 were screened in similar manner, with OC43 working stock virus infecting RD cells seeded at 15 000 cells/well.

Positive cultures were cloned by limiting dilutions and again screened for specific antibody. Mouse ascitic fluids were prepared with cells from a subsequent second cloning, clarified by centrifugation at 2850 × *g* for 30 min at 4°C, pooled, and stored at −80°C. These ascitic fluid-derived monoclonal antibodies were tested by the same EIA procedure as the cell culture supernatants, except that they were titrated to higher dilutions.

Monoclonal antibody characterization

The antibodies as mouse ascitic fluids were characterized as previously described (Hierholzer et al., 1993). Antibody subclass was determined by commercial EIA kit. IFA assays utilized HLF and RD cell cultures, as appropriate, and incubation following inoculation was for 3 days at 35°C. Hemagglutination (HA) and hemagglutination-inhibition (HI) tests for OC43 were done by standard procedures, using human and chicken erythrocyte suspensions at ambient temperature (Hierholzer et al., 1969, 1972). For biotinylation, antibody IgG was purified and labeled at 1 mg/ml concentration with the Enzo biotin reagent (No. EBP-406, Enzo Biochemical Inc., New York, NY).

For Western blots, the coronaviruses were grown in HLF or RD cells, as appro-

priate, under Medium 199 with 5% FCS for 24 h (before any cytopathology was evident), then the monolayers were washed and incubated briefly under PBS to remove non-viral proteins. The cultures were harvested by 2× freeze-thaws and light centrifugation. The viral proteins in the supernatant fluids were then solubilized, electrophoresed under both reducing and non-reducing conditions, and identified by the blotting technique of Tsang et al. (1983).

Microneutralization test. Serum neutralization (SN) tests for 229E and OC43 were carried out in HLF and RD cells, respectively, in 96-well, flat-bottom styrene microculture plates (Costar No. 3596, Cambridge, MA). Working stock passages at 1:1000 starting dilutions were first titrated in replicas of 6 in growth medium (GM, RPMI-1640 with 10% FCS, L-glutamine, penicillin, and streptomycin) in 2-fold dilution series using an autodiluter (Cetus Pro/Pette Model 1077, Perkin-Elmer/Cetus Corp., Norwalk, CT); cell control passages were titrated in the other 2 rows in the plate. Then, 0.10 ml of cell suspension (1×10^5 cells/ml) was added per well to the entire plate, and the plate was gently agitated for mixing, wrapped in plastic film, and incubated for 5 days at 35°C under 5% CO_2/80% humidity. For reading, the cells were stained by immersion for 20 min in a fixative/stain solution consisting of 1.46 g of crystal violet in 50 ml 95% ethanol, 300 ml of 37% (wt/wt) formaldehyde, 650 ml distilled water, and 1000 ml 0.01 M phosphate-buffered saline, pH 7.2; final concentrations were 5.55% formaldehyde and 0.07% crystal violet, and the final pH was 6.5. Endpoints were read in the wells which showed $\leqslant$25% of the stained area (i.e., uninfected cells) present in the uninoculated cell control wells (rows 7,8). The virus titer was thus defined as the reciprocal of the highest dilution of virus showing a 0 to 1+ staining in 5 days; this dilution was called 1 U of virus/0.05 ml.

SN tests were carried out in replicas of 6, with rows 7 and 8 as serum control wells. The untreated antibody samples at 1:4 starting dilution were diluted in GM through 2-fold series; 0.05 ml of virus dilution containing 2 U of virus/0.05 ml was added to each well in the first 6 rows; 0.05 ml of GM was added to each well in the last 2 rows; and the plates were incubated at room temperature for 1 h. Then, cells were added and the test was mixed, incubated, and stained as above. A virus back-titration was done to determine the actual dosage of virus in the test. The serum titer was defined as the reciprocal of the highest dilution of serum inhibiting 2 U of virus for 5 days.

Enzyme immunoassays (EIA)

The EIA procedures were modifications of those previously described (Hierholzer et al., 1993). EIA Format 1 was an all-monoclonal test designed to parallel that used for the TR-FIA as closely as possible, and required the use of biotinylated IgG. The EIA was carried out in 96-well polystyrene microtiter plates with purified monoclonal IgG diluted in carbonate buffer, pH 9.6 as capture antibody. After overnight incubation at 4°C, the plates were washed with 0.01 M PBS, pH 7.2/0.05% Tween-20. The specimen (NPA or virus culture) was added at 1:5 dilution in PBS-GT diluent (0.01 M PBS, pH 7.2, with 0.5% gelatin and 0.15% Tween-20) or in the TR-FIA antigen diluent, and the plates incubated and washed. Next, the biotinylated detector antibody diluted in PBS-GT was added, and the test again incubated and

washed. The test was finally developed with streptavidin/peroxidase and TMB substrate, and absorbance read at 450 nm in an EIA reader.

EIA Format 2 for each virus was our optimal polyclonal test derived from hyperimmune antisera. The steps were the same as for Format 1 except that the capture antibody was a polyclonal IgG and the detector antibody was the same IgG after biotinylation or a different species antiserum. When a different species IgG was tried as detector, followed by the appropriate anti-species IgG-peroxidase conjugate, the diluent for the detector was tried with 2% normal goat serum or 1.5–2% normal species serum, as needed to reduce background signal; the same diluent was then used for the conjugate.

Time-resolved fluoroimmunoassay

The purification and europium labeling of monoclonal antibodies from mouse ascitic fluids was done as described (Hierholzer et al., 1993). The one-incubation TR-FIA procedure also was done as described therein. Briefly, purified monoclonal IgG, diluted to optimal concentration in pH 9.6 carbonate buffer, was added to wells of 12-well polystyrene strips (Flow Titertek No. 78-591-99, ICN Biomedicals, Inc., Horsham, PA) in 250-μl volumes and adsorbed overnight at ambient temperature in a moist chamber. The wells were washed 3 × with aqueous 0.9% NaCl/0.05% Tween-20. Wells were then saturated with 250 μl of 0.1% gelatin (Difco, Detroit, MI) in 0.05 M Tris/0.9% NaCl/0.05% NaN_3 buffer, pH 7.75, again with overnight incubation at ambient temperature. After removing the excess volume (leaving ~25 μl/well), the strips could be stored in sealed plastic up to 1.5 years at 4°C at this point with no loss of activity.

For the test, the wells were washed 3 ×, and 100 μl each of antigen and Eu-detector antibody were added to appropriate wells. The antigen (NPA or culture suspensions) was diluted 1:5 in specimen diluent, consisting of 50 mM Tris, pH 7.75, 0.9% NaCl, 0.01% NaN_3, 0.5% gelatin, 0.01% Tween-40, 20 μM DTPA, and 2% BSA; the Eu-labeled detector antibody was diluted to the appropriate concentration in the same diluent. The strips were then incubated for 1 h at 37°C in a moist chamber, washed 6 ×, and 200 μl per well of enhancement solution was added. The plates were gently agitated on a shaker for 10 min at ambient temperature and then placed in the fluorometer for counting. The fluorometer was programmed to take the mean and coefficient of variation (CV) of 12 reagent blanks and to take the mean and CV of the duplicates or triplicates of each specimen minus the reagent blanks. We then further analyzed the printed data by computing the mean and standard deviation (SD) of the negative specimens run in the same test, and used this mean + 3 SDs as the cut-off value for positive specimens (Hierholzer et al., 1990, 1993).

3. Results

Evaluation of monoclonals

Mouse ascitic fluids to all monoclonals which possessed useable homologous titers by EIA were considered for use in TR-FIA. The protein specificity of these clones

was determined by Western blots. The first blots reacted the electrophoresed viral proteins with hyperimmune rabbit antisera to 229E and mouse, guinea pig, and rabbit antisera to OC43, followed by the appropriate anti-species peroxidase, to confirm the presence of coronavirus proteins in the test lanes (Schmidt and Kenny, 1982; Hierholzer and Tannock, 1988; Arpin & Talbot, 1990); replicate lanes were then reacted with the individual monoclonals, followed by anti-mouse peroxidase. All eleven 229E and OC43 monoclonals with suitable EIA titers were directed to the nucleoprotein (N), which had a mean molecular weight of 51.3 kDa in the reducing gels (Table 1).

Characterization and labeling data for these antibodies are listed in Table 1 also. Most had type-specific EIA titers of $>10^{-4}$ after purification; their Eu:IgG molar ratios varied from 5.6 to 10.1, indicating acceptable labeling of the antibody. IFA titers of the 229E monoclonals ranged from 1:10 to 1:160 and of the OC43 antibodies from 1:10 to 1:40, with no cross-reaction between serotypes and no reactions with a large panel of heterologous viruses (prototype plus 1–4 strains each of parainfluenza 1, 2, 3, and 4, measles, mumps, respiratory syncytial virus groups A and B, influenza A and B, herpes simplex 1 and 2, adenovirus 1, 2, 4, 7, 8, 11, and 37, polio 2, coxsackie A9 and B2, and echo 11). None of the coronavirus monoclonals had neutralizing activity (i.e., $\geq$1:4 antibody titer with 50 $TCID_{50}$'s of virus per 0.05 ml), and none of the OC43 monoclonals possessed HI activity (i.e., $\geq$1:2 antibody titer with 4 HA units of virus/0.05 ml). [HCV-229E does not exhibit HA activity, and therefore the 229E MAbs were not tested by HI.] The lack of SN and

Table 1
Characteristics of coronavirus monoclonals and labeled antibodies

Virus	Monoclone number	MAb properties			Purified ab		Eu^{3+}-labeled ab	
		Immun. strain	Protein specif.	IgG subcl.	Protein (mg/ml)	EIA titer*	Eu/IgG ratio	Protein (mg/ml)
229E:	400-10H	229E	N	1k	13.88	3.0	7.4	0.310
	401-3C	229E	N	2Ak	8.15	5.2	7.3	0.305
	401-4A	229E	N	1k	18.27	7.2	6.8	0.297
	402-6F	229E	N	1k	13.43	6.4	7.9	0.286
	402-8H	229E	N	2Ak	16.90	5.2	5.6	0.365
OC43:	540-4D	OC43	N	2Ak	4.60	5.7	7.5	0.290
	541-8F	OC43	N	1k	3.26	5.5	10.0	0.332
	541-11H	OC43	N	2Ak	3.41	4.0	8.2	0.166
	542-7D	OC43	N	1k	10.18	6.2	10.1	0.354
	543-10E	OC43	N	2Ak	3.04	3.3	8.4	0.184
	543-11F	OC43	N	2Ak	3.55	5.2	8.6	0.260

*EIA titers are expressed as the $\log_{10}$ of the highest dilution of antibody whose absorbance values exhibit a P/N ratio of $\geq$3.0 in 229E- or OC43-infected cells, respectively, where the absorbance value in uninfected cell controls constitutes N.

HI activities and the low IFA titers were all consistent with the characterization of the antibodies as anti-N.

Formatting the EIAs

The monoclonal EIA (Format 1) for each virus using the same antibodies as TR-FIA was optimized by checkerboard titrations. We also biotinylated the other purified antibodies and tested all possible combinations in EIA. The optimal monoclonal EIA formats for antigen detection are given in Table 2. For 229E, several capture/detector combinations were reasonably sensitive in detecting virus, but the most sensitive combination was the same as the one chosen as the best TR-FIA format. Antibody 401-3C as capture with 401-4A as detector gave mean OD values of 1.683 with 229E virus in HLF cultures, 0.016 with OC43 in SMB and RD cultures, and 0.011 with negative HLF and RD cell controls, for a mean P/N value of 153. The second best format (401-4A capture/401-4A detector) gave a mean negative OD value of 0.026 and a mean P/N with 229E of 47.

For OC43 virus, the optimal Format 1 was also the same as that found for TR-FIA: monoclonal 541-8F as capture with 542-7D as detector. This combination gave mean OD values of 1.165 with OC43 virus in RD cultures, 0.043 with 229E virus in HLF cultures, and 0.045 with negative RD cell controls, for a mean P/N value of 26. Another format (542-7D capture/542-7D detector) gave very similar values: 1.013/0.040, for a mean P/N with OC43 cultures of 25. The same panel of heterologous viruses tested by IFA was also tested by direct EIA, with the antigen coating the wells followed by a monoclonal antibody and anti-mouse peroxidase,

Table 2
Parameters for optimal coronavirus EIA tests for antigen detection

Capture ab (IgG)		Antigen dilution	Detector ab (IgG)		Developing system	
Antiserum	Dilution		Antiserum	Dilution	Conjugate	Dilution
Format 1: Monoclonal sera						
229E 401-3C	1:3,000	1:10	401-4A/biotin	1:10,000	streptav.-peroxidase	1:3,000
OC43 541-8F	1:3,000	1:10	542-7D/biotin	1:3,000	ibid	
Format 2: Polyclonal sera						
229E g.pig	1:10,000	1:10	rabbit	1:3,000	anti-rab.-peroxidase	1:3,000
OC43 mouse	1:3,000	1:10	rabbit	1:10,000	anti-rab.-peroxidase	1:3,000

Diluent for capture antibodies was pH 9.6 carbonate buffer, incubated overnight at 4°C. Antigen was diluted in TR-FIA antigen diluent; incubation was 1.5 h, 37°C. Diluent for the detector antibodies and developing system was PBS-GT (0.01 M phosphate-buffered saline, pH 7.2, with 0.5% gelatin and 0.15% Tween-20) throughout, except that the 229E polyclonal test required 2% normal goat serum added to the PBS-GT for these steps; incubation was 1 h, 37°C. The developing system for Format 1 was incubated 10 min at ambient temperature, and for Format 2 was incubated 1 h at 37°C.

and again the coronavirus monoclonals were uniformly negative and were thus type-specific.

The polyclonal EIAs (Format 2) also were optimized as shown in Table 2 but were of much less utility because of higher background signals or lower serum antibody levels. All of the polyclonal 229E tests had relatively high backgrounds which were lowered only slightly by the addition of normal goat serum to the detector side of the formats. Thus, the optimal 229E test with guinea pig capture antibody gave mean OD values of 1.466 with 229E cultures, 0.214 with OC43 cultures, and 0.212 with negative cell controls in the presence of 2% normal goat serum; the mean P/N was 7. None of the OC43 tests were improved by addition of any normal serum to the detector antibodies; thus, the OC43 test with mouse capture antibody gave similar positive OD values (1.402/0.034); the mean P/N ratio was 41, and the test was as type-specific as the 229E test. These results show that for both viruses as cell culture supernatants the all-monoclonal format was more sensitive than the polyclonal test in antigen detection. Limited testing with NPA specimens confirmed both the optimally-sensitive tests described above and the monoclonal tests as superior to polyclonal tests.

Formatting the TR-FIA

The one-incubation TR-FIA was formatted for the coronaviruses by testing all combinations of the monoclonals at serial dilutions with tissue culture isolates and then with NPA specimens. The optimization utilized checkerboard titrations of capture antibodies at 0.25, 0.5, 0.75, and 1.0 μg/well and detector antibodies at 6.25, 12.5, 25, 50, 75, and 100 ng/well. For each concentration of capture antibody, the optimal concentration of detector antibody was determined by the P/N ratio for representative positive and negative samples to obtain the greatest sensitivity. In the TR-FIA, system background was the mean of 12 reagent (system) controls and was automatically subtracted out when samples were run. Test background was measured as the fluorescence values in known negative specimens that were tested in parallel with positive specimens; thus, a P/N value was the mean of the replicate tests for a positive specimen divided by the mean of all the negative specimens tested (in replicates) in the run.

The P/N values were used to ascertain the best format in TR-FIA (Table 3). Of 11 MAbs evaluated in this study, only 4 were found to be useable in either capture or detector position in the TR-FIA. For 229E virus, clone 401-3C in capture position @0.50 μg/well, with clone 401-4A in detector position @25 ng/well, gave a mean P/N value of 143 with 229E cultures and 1 with OC43 cultures, and a mean P/N of 10 with 229E clinical specimens and 0.9 with OC43 specimens. In 1:10-diluted test samples, signals for 229E cultures ranged from 6 260 to 138 780 (mean = 61 329) cps (mean of negatives = 429 cps), and for 229E clinical specimens were 640–4 411 (mean = 2 520) cps. For OC43 virus, clone 541-8F in capture position @0.50 μg/well, with clone 542-7D in detector position @50 ng/well, gave a mean P/N value of 964 with OC43 cultures and 1 with 229E cultures, and a mean P/N of 174 with OC43 clinical specimens and 1 with 229E specimens. In 1:10-diluted test samples, signals for OC43 cultures ranged from 2 105 to 1 498 080 (mean = 756 595) cps (mean of

Table 3
Comparison of the most sensitive combinations of monoclonals for coronavirus TR-FIA tests

Virus	Capture antibody	Conc. (µg/well)	Detector antibody	Conc. (ng/100 µl)	Mean P/N of specimens	
					Cultures	Clinical
229E:	*401-3C*	*0.50*	*401-4A*	*25*	143	10
	401-4A	0.50	401-4A	50	111	6
	401-4A	0.50	401-4A	25	89	3
OC43:	*541-8F*	*0.50*	*542-7D*	*50*	964	174
	541-8F	0.50	542-7D	25	899	142
	541-8F	0.50	541-8F	50	548	131

Italics denotes optimal test system for direct detection TR-FIA.

negatives = 785 cps), and for OC43 clinical specimens were 3 976–734 406 (mean = 347 937) cps.

The 401-3C/401-4A format was thus the most sensitive test for 229E in both cell cultures and clinical specimens, and the 541-8F/542-7D format was the most sensitive for OC43 in both cultures and specimens. Both tests were more sensitive than the next best combinations (clones 401-4A/401-4A for 229E; 541-8F/542-7D at lower detector concentration for OC43). As in the EIA tests, the optimized TR-FIA formats for coronaviruses 229E and OC43 were type-specific when tested against the same panel of heterologous viruses used in the IFA and EIA tests.

To determine the dose–response curves for the optimized TR-FIA formats, working stock cultures of 229E in HLF cells and of OC43 in RD cells were clarified, concentrated 10-fold by ultrafiltration, and purified through glycerol/tartrate gradients at 107 000 × *g* (Hierholzer, 1976; Hierholzer et al., 1981). The visible band for whole virus at the 1.18 density position was harvested, dialyzed, confirmed to be coronavirus by electron microscopy, measured for total protein by the Lowry method, and titrated in a 10-fold dilution series in TR-FIA. Sham-inoculated cell control cultures for each virus were treated, the 1.18 density position harvested from gradients, and the harvests titrated in TR-FIA in identical fashion; the cps values for these samples constituted the negative values averaged for the cut-off calculations. The cut-off point was defined as the mean + 3 standard deviations of the negative samples, and was 384 and 892 cps for 229E and OC43, respectively.

The purified 229E sample had a protein content of 230 µg/ml and a fluorescence value at 1:10 dilution of 183 410 cps. The OC43 sample had a higher protein content (736 µg/ml), higher fluorescence value at 1:10 dilution (1 242 360 cps), and a higher background. The log-log relationship of signal-to-antigen concentration in the optimized tests is shown in Fig. 1. The all-monoclonal TR-FIA had a threshold of detection of 0.31 ng/well (3.082 ng/ml) of 229E virus and 0.098 ng/well (0.986 ng/ml) of OC43 virus; these correspond to 16.57×10^5 and 5.30×10^5 virions per well, respectively.

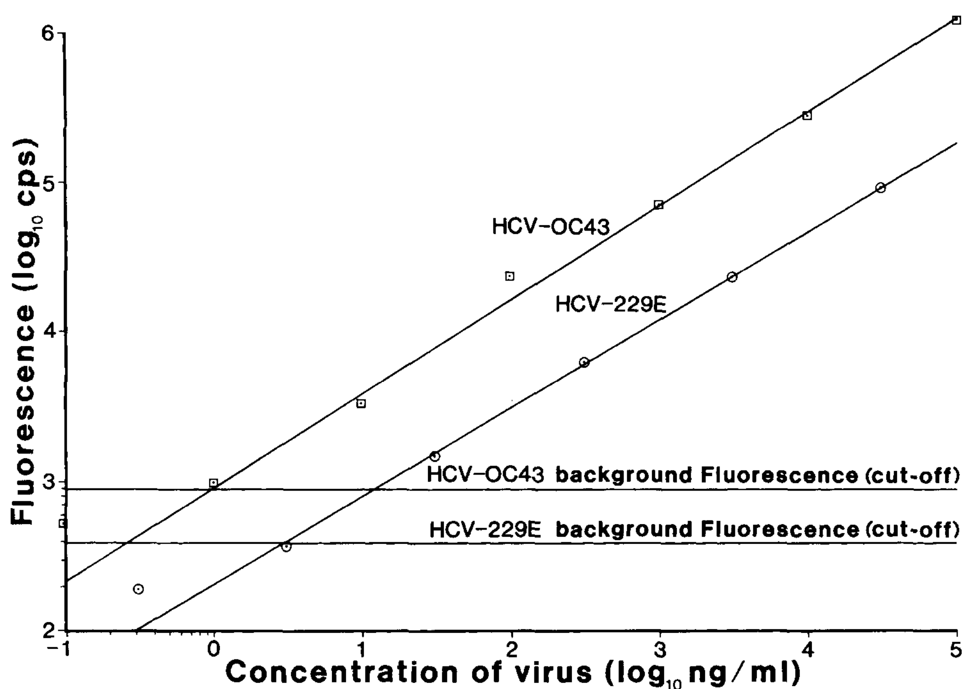

Fig. 1. Dose-response curve for the optimized coronavirus 229E and OC43 TR-FIA tests. Purified virus was titrated to endpoint dilutions in the TR-FIA antigen buffer and quantitated by the optimized tests. The HCV-229E test can detect 3.082 ng virus per ml and the HCV-OC43 test, 0.986 ng virus per ml.

Comparison of TR-FIA with EIAs using clinical specimens

Using the optimum reagents and dilutions just described, the TR-FIA was compared with EIA Formats 1 and 2 in testing original clinical specimens (Table 4). For 229E, the comparative evaluation included 13 229E-positive specimens, 10 OC43-positive specimens, and 10–20 negative specimens. Seven negative specimens were positive for adenovirus, RSV, or parainfluenza types 1 and 3 and thus were important controls in the study; the remaining specimens were negative for any recognized virus by culture and TR-FIA. All specimens were tested at 1:10 dilution in the specimen diluent. None of the negative specimens were positive by any of the three tests, but several positive specimens failed to be detected by the EIA tests. In all of these cases, the absorbance values were slightly below the EIA cutoff value. The 5 specimens (4 229E, 1 OC43) which were not detected in the monoclonal EIAs were also not detected in the respective polyclonal EIAs, and furthermore were minimally positive in TR-FIA, suggesting that these specimens had deteriorated to the point where the EIA tests were not sufficiently sensitive to detect the viruses.

Thus, the TR-FIA was the most sensitive of these tests, detecting 100% of both 229E and OC43 in clinical specimens considered positive by the initial polyclonal EIAs carried out with fresh specimens, compared with 69% and 90% detected by the monoclonal EIAs, and 54% and 80%, respectively, detected by the polyclonal

Table 4
Comparison of TR-FIA with EIA for detection of coronaviruses in clinical specimens

Specimen/dilution	TR-FIA	EIA-1 (mono.)	EIA-2 (poly.)
HCV-229E (1:10):			
Negative ($n=20$) range	84 to 380	0.007 to 0.021	0.089 to 0.146
Mean & SD	242 ± 81	0.011 ± 0.003	0.132 ± 0.012
Mean + 3 SDs	485	0.020	0.168
Positive ($n=13$) range	596 to 3623	0.011 to 0.157	0.093 to 0.281
Number (%) positive	13 (100)	9 (69)	7 (54)
HCV-OC43 (1:10):			
Negative ($n=10$) range	739 to 2885	0.011 to 0.022	0.018 to 0.079
Mean & SD	1568 ± 542	0.017 ± 0.002	0.035 ± 0.012
Mean + 3 SDs	3194	0.025	0.071
Positive ($n=10$) range	3784 to 692850	0.024 to 0.750	0.048 to 0.330
Number (%) positive	10 (100)	9 (90)	8 (80)

The original specimens tested here included all the NPA specimens tested for Tables 2 and 3; TR-FIA data are mean counts/second; EIA data are mean absorbance at 450 nm.

EIAs. Due to the small number of coronavirus specimens available, however, these numbers must be considered provisional.

4. Discussion

Fluorescent antibody tests – the first rapid antigen tests developed for respiratory viruses – are still widely used in clinical laboratories and have been successfully applied to the human respiratory coronaviruses as well (McIntosh et al., 1978). Enzyme immunoassays have become even more important for rapid viral diagnosis, because EIA is more sensitive than IFA and is therefore the critical test with which to compare any new test (Halonen et al., 1983, 1985; Grandien et al., 1985; Hornsleth et al., 1986; Hughes et al., 1988; Hierholzer et al., 1990; Takimoto et al., 1991; McIntosh et al., 1993). Thus, this study included optimized EIA tests with monoclonal and polyclonal antibodies in a direct comparison with optimized TR-FIA tests with monoclonal antibodies.

We have shown previously that the TR-FIA with polyclonal antibodies was more sensitive for detecting respiratory virus antigens than other rapid antigen tests (Halonen et al., 1983, 1985). Subsequent studies with monoclonal antibodies revealed even greater sensitivities for detecting influenzavirus (Walls et al., 1986), adenovirus (Hierholzer et al., 1987), enterovirus 70 and coxsackie A24 viruses in eye swabs (Hierholzer et al., 1990), respiratory syncytial virus and parainfluenza type 1–3 viruses (Hierholzer et al., 1989), and parainfluenza type 4(A and B) and mumps viruses (Hierholzer et al., 1993) directly in clinical specimens. In the present study, we describe monoclonal antibodies for the human respiratory coronaviruses which are useable in similarly-constructed, all-monoclonal TR-FIA formats. Like the other

direct-antigen TR-FIA tests, these tests are also highly useful for identifying viruses in cell culture supernatants.

For coronavirus 229E, the most sensitive EIA was a monoclonal assay using clone 401-3C as capture antibody and clone 401-4A as detector, and for OC43 virus was a monoclonal assay with clone 541-8F as capture and clone 542-7D as detector. These tests detected virus in 69% of 229E- and 90% of OC43-positive clinical specimens, respectively. The TR-FIA, on the other hand, detected virus in 100% of 229E- and 100% of OC43-containing specimens. These detection rates may change with additional experience with the formatted tests, because the numbers of original specimens that were known EIA-positive and were available for testing were too low for an accurate determination; we have no culture-positive coronavirus specimens owing to the extreme lability of these viruses.

The most reactive monoclonal antibodies made in this study were directed against the N nucleoprotein. They were type-specific and did not possess neutralizing activity, consistent with the known biological functions of coronavirus proteins (reviewed in Schmidt et al., 1982; Schmidt, 1984; Hierholzer and Tannock, 1988). The anti-N antibodies of HCV-OC43 also did not exhibit HI activity, which resides in the peplomers; HCV-229E does not possess HA activity, and thus the 229E antibodies could not be tested for HI titers (Hierholzer et al., 1981). Anti-N monoclonals, in addition to anti-E1 (envelope) and anti-E2 (peplomer) antibodies, were type-specific in other studies as well (Arlene Collins and Ernesto DeNardin, SUNY, Buffalo NY, personal communication, 1987; Fleming et al., 1988). Furthermore, probes and PCR primers based on the N gene sequences of 229E and OC43 have also been successfully used in type-specific diagnostic tests (Kamahora et al., 1989; Myint et al., 1989, 1990; Stewart et al., 1992). Thus, our findings of the utility of the N protein in TR-FIA tests is consistent with other tests based on this highly conserved protein or its gene.

The optimized TR-FIA formats described herein should be well-suited to the detection of coronavirus types 229E and OC43 directly in clinical specimens, to the identification of virus isolates recovered in cell culture should this be successful, and to much-needed epidemiologic studies of the role of these viruses in upper and lower respiratory disease.

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