Development and Application of an Enzyme Immunoassay for Coronavirus OC43 Antibody in Acute Respiratory Illness

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Study of coronavirus OC43 infections has been limited because of the lack of sensitive cell culture systems and serologic assays. To improve this circumstance, we developed an indirect enzyme immunoassay (EIA) to detect serum antibody to OC43. Antigen (100 ng) prepared by polyethylene glycol precipitation provided optimal results without a postcoat procedure. Evaluation of intraplate variation indicated that a ≥ 2.5 -fold increase in serum titer was significant. Sixteen of 18 (89%) paired serum samples with previously identified, reproducible increases in the level of hemagglutination inhibition (HAI) antibody to OC43 also showed significant increases as detected by EIA. Specificity for the EIA was established with paired sera obtained from persons given influenza immunizations or experiencing a respiratory infection. No rises in antibody titers occurred among 33 persons with documented coronavirus 229E infection. EIA was then performed on each of 419 paired serum samples from ambulatory chronic obstructive pulmonary disease patients and healthy older adults, from asthmatic adults presenting for emergency room treatment, and from persons hospitalized with acute respiratory symptoms. Twenty-three antibody rises to OC43 were detected; only nine of these were detected by the HAI test, and the HAI test did not detect any increases in antibody titers that were not detected by EIA. Nineteen of 25 coronavirus OC43 infections for which a month of infection could be assigned occurred between November and February. Overall, 4.4% of acute respiratory illnesses in the studied populations were associated with a coronavirus OC43 infection.

Coronaviruses cause a variety of diseases among animals, including gastroenteritis, hepatitis, demyelinating disease, nephritis, and acute respiratory disease. In humans, they are established only as causes of acute respiratory disease, though coronavirus-like particles and antigen have been identified in the feces of both patients with diarrhea (5) and healthy persons in developing countries (18) and in the brains of humans dying from multiple sclerosis (2, 26, 31). The two coronavirus types that cause respiratory illness in humans are represented by the OC43 and 229E strains. Seroprevalence surveys (4, 10, 19, 29) have shown that 86 to 100% of all adults have serum antibody to both types. Initial infections reportedly occur in the first year of life (21, 25), and reinfections occur in all age groups (29). Infections occur predominantly in the winter months, with outbreaks every 2 or 3 years (7, 14). Illnesses primarily involve the upper respiratory tract, although lower respiratory illness among infants and young children has been described. Incidence varies markedly from season to season, with coronaviruses reportedly causing 2 to 35% of colds among adults, frequencies that are second to those of rhinoviruses in this age group (7, 17).

Identification of coronavirus infection has been impaired by difficulty in isolating the virus, so studies have relied primarily on serologic methods to identify infections. Complement fixation (CF), hemagglutination inhibition (HAI), and neutralization antibody tests have been described for OC43 (13, 21, 23), and CF and neutralization antibody tests have been described

for 229E virus (8, 12). In the present report, we describe a sensitive and specific enzyme immunoassay (EIA) for coronavirus OC43 that was used to determine the frequencies of coronavirus OC43 infection in various high-risk populations.

MATERIALS AND METHODS

Virus and tissue culture. Human coronavirus OC43 adapted to growth in cell culture (kindly provided by K. Holmes, Bethesda, Md.) was passaged six times through human rhabdomyosarcoma tissue cultures (Whittaker Bioproducts, Walkersville, Md.). The rhabdomyosarcoma growth medium was Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter (Whittaker) containing 10% fetal calf serum, 0.1 mM glutamine (Whittaker), 100 U of penicillin (Sigma, St. Louis, Mo.) per ml, and 0.05 mg of gentamicin (Sigma) per ml. Cultures inoculated with virus were maintained in Dulbecco's modified Eagle's medium with 2% fetal calf serum, 0.1 mM glutamine, 100 U of penicillin per ml, and 0.05 mg of gentamicin per ml.

HAI. Coronavirus OC43 antigen for HAI assays was prepared by centrifugation of the cell lysate at $10,000 \times g$ for 30 min at 4°C. Supernatants were used for HAI tests, which involved standard methods with incubation of virus-serum mixtures for 45 min at room temperature and the reading of results 30 to 60 min after the addition of chicken erythrocytes (9). Tests were considered positive if consecutive sera demonstrated a repeated fourfold or greater increase in antibody titer.

EIA antigen preparation. To prepare antigen for EIAs, rhabdomyosarcoma cell monolayers in 150-cm² flasks (Corning Glass Works, Corning, N.Y.), each containing 6 ml of medium, were inoculated with 0.6 ml of OC43 containing 10^6 50% tissue culture infective doses per ml. The virus was allowed to adsorb while rocking for 1 h at 34°C; 10 ml of maintenance medium

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was then added to each flask. The flasks were incubated for 96 h at 34°C and then frozen to -70°C. After thawing, the cell lysate was clarified by centrifugation at 10,000 × g at 4°C for 30 min. The supernatant was stirred with 10% polyethylene glycol (PEG) 8000 and 0.4 M NaCl for 3 h at 4°C. This mixture was pelleted by centrifugation at 10,000 × g at 4°C for 30 min; the pellet was suspended and clarified twice with a total of 20 ml of TMEN buffer (0.05 M Tris hydrochloride, 0.05 M maleic acid, 0.001 M EDTA, 0.1 M NaCl). In some studies, the antigen pool was purified further after PEG precipitation by ultracentrifugation at 82,000 × g through a 20 to 60% sucrose gradient. The virus band was collected, and fractions were then selected by density and peak hemagglutinating titer. The highest-titered fractions, with densities of 1.18 to 1.19 g/cm³, were diluted in TMEN buffer and pelleted by centrifugation at 90,000 × g. Antigen pools were stored at -70°C. The protein concentration of the preparation was determined by a modification of the Bradford procedure (1) (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, Calif.).

EIA. Flat-bottomed, 96-well polystyrene microtiter plates (Immunolon 1; Dynatech Laboratories, Chantilly, Va.) containing 100 ng of OC43 antigen diluted in 100 µl of 0.1 M carbonate-bicarbonate coating buffer (pH 9.6) were incubated overnight at 4°C. After incubation, the mixtures in the plates were decanted and 75 µl of serum diluted in phosphate-buffered saline (PBS) with 0.3% Tween 20 (Tw) and 0.01 M EDTA was added to each well. Sera were tested in serial twofold dilutions beginning initially at 1:100, and the test was repeated at lower or higher dilutions if no end point was reached. The plates were then incubated at room temperature overnight and washed three times in Dulbecco's PBS (without Ca²⁺ or Mg²⁺) with 0.5% Tw (wash solution). Affinity-purified goat antibody to human immunoglobulin G Fc (Caltag, San Francisco, Calif.) at a dilution of 1:3,000 in 0.05% Tw in PBS was added in 75-µl quantities to each well and incubated for 5 h at room temperature. The plates were washed three times, and 75 µl of affinity-purified swine anti-goat immunoglobulin G (heavy and light chain specificity) conjugated to alkaline phosphatase (Caltag) and diluted 1:2,000 in PBS with 0.05% Tw was added to each well. The plates were incubated overnight at room temperature. Following three washes, 75 µl of phosphatase substrate (*p*-nitrophenyl phosphate; Sigma) at a concentration of 1 g/liter in 10% diethanolamine buffer (pH 9.8) with 0.02% sodium azide and 0.01% MgCl₂ · 6H₂O was added to each well. The plates were incubated for 30 min at 34°C, and then the optical density was read at 410 nm with a Dynatech MR600 microplate reader. Serum antibody titers represented a dilution calculated by interpolation for an optical density of 0.2. Sera with EIA antibody titers of 1:40 or less were used as negative controls, and paired sera with antibody rises of greater than fourfold were used as positive controls in each test. Results were discarded if background wells showed optical density readings of >0.08, if negative control sera had calculated titers of ≥1:40, or if paired positive control sera failed to show the expected rise.

Blocking agents tested to decrease background optical density levels were 1% gelatin, 10% gelatin, 1% fetal calf serum, 10% fetal calf serum, 1% bovine serum albumin (BSA), 5% Carnation nonfat dry milk, and Meggablock at 1:250 (Onasco, Houston, Tex.). Diluents for sera evaluated were PBS, PBS with 1% BSA and 0.05% Tw, and PBS with 0.01 M EDTA and 0.3% Tw. Diluents for antisera evaluated were PBS with 1% BSA and 0.05% Tw, PBS with 0.05% Tw, and PBS with 0.01 M EDTA and 0.3% Tw.

Sera from populations with documented coronavirus OC43 infections (including 17 pairs of acute- and convalescent-phase

serum samples kindly provided by J. Gwaltney) were used to develop the test. Results for serum pairs with reproducible fourfold or greater increases in HAI titers were compared with results from the EIA. Serum pairs from volunteer challenge studies with coronavirus 229E (kindly provided by R. Turner), rhinovirus, and *Mycoplasma pneumoniae* and from recipients of influenza virus vaccine were tested by the EIA to assess the specificity of the test.

Populations. The populations used for evaluation of the coronavirus EIA were those for which a longitudinal evaluation of respiratory viral infections was being conducted (Table 1). Sequential sera collected over a period of 2 years from a group of 55 patients older than 55 years with chronic obstructive pulmonary disease (COPD) and 48 healthy, age-matched controls were available. Acute- and convalescent-phase sera from 178 persons hospitalized with an acute respiratory illness and from 46 adults with acute asthma who were seen in an emergency room were available.

RESULTS

EIA development. (i) Reproducible detection of OC43 EIA antibody rises among sera. Within-plate reproducibility of the original antigen pool, which included lots prepared by PEG precipitation followed by isopycnic centrifugation and lots prepared by PEG precipitation alone, was assessed with 44 serum samples tested in duplicate. Minimal differences in antibody titers of duplicate sera were found, with calculations indicating that the difference in titers would be ≤2.5-fold 99% (mean ± 2.58 standard deviations) of the time. A second antigen pool, prepared by PEG precipitation alone, was similarly assayed for reproducibility with 60 serum samples tested in duplicate. For this pool, the differences in antibody titers between duplicate sera were less than 2.5-fold 95% (mean ± 1.96 standard deviations) of the time and less than 3.3-fold 99% (mean ± 2.576 standard deviations) of the time. Therefore, a reproducible 2.5-fold increase or a 4-fold or greater increase between 2 consecutive serum samples was accepted as evidence of an antibody rise. HAI and EIA titers of serum antibody were compared, and there was no correlation between values obtained by the two test methods, though all pairs which showed an increase by the HAI test also showed increases by EIA. Of serum samples obtained from earlier studies in which reproducible 4-fold or greater HAI rises against OC43 were demonstrated, 16 of 18 (89%) also demonstrated an EIA rise of 2.5-fold or greater.

(ii) EIA specificity. The EIA was evaluated for specificity with acute- and convalescent-phase sera bracketing challenge infections with other microorganisms that caused respiratory illness or before and after vaccination of older adult outpatients in the COPD population with influenza virus vaccine. Eighty-eight paired serum samples from these various sources were tested by both the HAI test and EIA for coronavirus OC43. None of 33 subjects with coronavirus 229E infection or 34 subjects with influenza virus vaccination with a serum antibody response showed a rise in EIA titer. Two of 15 subjects infected with rhinovirus in a volunteer challenge study and 1 of 6 infected in a *Mycoplasma pneumoniae* challenge study showed an increase in antibody titer by EIA; all 3 of these also showed an increase in HAI titer against OC43, suggesting a dual infection in these persons. These three infections all occurred within a 3-month period (April 1971 to July 1971) in a closed population with which studies were being conducted.

OC43 infections detected by EIA rises in high-risk populations. Among the 327 persons whose sera were tested, only 2

TABLE 1. Coronavirus infections among persons with respiratory illnesses

Population	<i>n</i>	Mean age (years)	No. of illnesses	No. of HAI rises	No. of EIA rises	Total with rises ^a (%)
Outpatients						
Older adults ^b						
COPD	55	65.2	87	4	7	7 (8.0)
Control	48	66.9	87	0	1	1 (1.1)
Asthma patients ^c	46	38.4	57	1	5	5 (8.8)
Inpatients						
Public hospital ^d						
≤10 years old	60	2.2	62	1	4	4 (6.5)
>10 years old	85	46.8	91	1	3	3 (3.3)
Private hospital ^d						
≤10 years old	3	2.3	3	0	0	0 (0.0)
>10 years old	30	49.7	32	2	3	3 (9.4)
Total						
≤10 years old	63	2.2	65	1	4	4 (6.2)
>10 years old	264	53.6	354	8	19	19 (5.4)
All ages	327	43.7	419	9 ^e	23 ^e	23 (5.5)

^a HAI and/or EIA antibody rise; all HAI antibody rises were accompanied by EIA antibody rises.

^b Prospectively evaluated adult outpatients with COPD and healthy, age-matched controls. Subjects were monitored longitudinally from September 1991 to May 1993, with blood drawn three times yearly and at the time of any respiratory illness.

^c Adults presenting for emergency room treatment of asthma from October 1992 to August 1993.

^d Persons hospitalized at Ben Taub General Hospital (public), St. Luke's Episcopal Hospital (private), or Texas Children's Hospital (private) for respiratory symptoms from June 1991 to January 1993.

^e For the HAI test versus EIA, $P = 0.0005$ (McNemar's test).

had EIA titers of <1:40 (overall seroprevalence, 99.3%). One of the subjects with no detectable serum antibody at the lowest dilution tested (1:20) was an 8-month-old child; the other was a 62-year-old woman. For all serum pairs bracketing episodes of illness in the study population, the HAI test detected nine coronavirus OC43 seroconversions. The EIA confirmed these 9 and detected an additional 14 antibody rises (Table 1). The antibody response frequency for the EIA in the present study was significantly greater than that for the HAI test ($P = 0.0005$, McNemar's test). The seasonal distribution of OC43 infections for which a month of infection could be assigned (including infections in populations in both the present study and an earlier survey for OC43 in COPD patients) is shown in Fig. 1. Nineteen of 25 infections occurred between the months of November and February.

The 103 outpatients (Table 1) older than 55 years were recruited and monitored longitudinally for acute respiratory illness. The mean age of the COPD patients was 65.2 years, and the mean age of healthy controls was 66.9 years. A total of 268 pairs of serum samples were obtained from COPD patients and 323 were obtained from healthy controls, but 181 of the serum pairs from COPD patients and 236 from the controls bracketed time periods when the subjects were not ill. Eighty-seven pairs from each group bracketed periods in which the subject experienced a respiratory illness that was reported to the study coordinator (Table 1). Among COPD patients, seven infections (8%) were detected by EIA in relation to an illness while only one was detected during a period without illness (7 of 87 versus 1 of 181; $P = 0.04$, Fisher's exact test). One infection occurred among age-matched controls during 87 illness episodes (1.1%), while two infections were detected during 236 periods without illness (0.8%).

In 46 asthma patients (mean age, 38.4 years) with 57 exacerbations requiring emergency room treatment, five OC43

infections were detected by EIA; only one of these was detected by the HAI test. Among 63 children 10 years old or younger (mean age, 2.2 years) hospitalized for 65 illnesses, EIA detected four infections. Among 115 persons older than 10 years of age (mean age, 47.6 years) hospitalized with 123 illnesses at public or private hospitals, EIA detected six infections. The HAI test detected only 4 of these 10 infections in hospitalized patients ($P = 0.04$, McNemar's test).

DISCUSSION

To evaluate the role of coronaviruses in acute respiratory infections, we developed an indirect EIA for detection of coronavirus infections with OC43-like viruses. Of the initial sera used for test development, 16 of 18 (89%) pairs of serum samples having reproducible HAI titer rises of fourfold or greater also had EIA titer rises. In our high-risk study populations, 100% (9 of 9) of infections detected by the HAI test were also detected by EIA. Fourteen additional antibody rises that were not detected by the HAI test were detected by EIA in our study, indicating that the true sensitivity of the EIA for detecting OC43 serum antibody rises is greater than that of the HAI test.

On the basis of the criteria adopted for detecting antibody responses, the specificity of our test appeared to be high; in 52 subjects who had experienced infections with other respiratory pathogens and in 34 who had received influenza virus vaccinations, no EIA antibody rises occurred. Three persons from those same populations exhibited EIA antibody rises, but those increases were accompanied by HAI antibody rises, a finding more consistent with a dual infection than with a false-positive increase. The closed population with which those studies were done and the brief time period during which all antibody rises occurred support such a conclusion.

FIG. 1. Distribution of coronavirus OC43 infections by month. For 25 of 31 infections, a month could be assigned. Information on month of illness was not available for six additional infections.

EIA has been used previously to detect coronavirus antibody. EIA systems have several advantages over other serologic methods; the foremost is the increased sensitivity over more traditional serologic procedures. EIA titers were higher than HAI titers by at least 100-fold, and a greater number of antibody rises were detected by EIA than by the HAI test in our study. Indirect EIAs, in which an unlabeled second antibody is used and quantitated by an enzyme-labeled anti-immunoglobulin, generally have a greater sensitivity than direct EIAs; moreover, a single labeled anti-immunoglobulin may be used in a number of test systems that use antibody from the same animal species for the second antibody (33). In the past, antigen preparation methods for coronavirus EIAs have varied from simple clarification (16) to procedures that include rate zonal and isopycnic centrifugation (28). The simpler method, however, resulted in cross-reactivity between 229E and MHV3 (an OC43 serogroup virus) (15), and when the method was used to detect 38 coronavirus antibody increases in 37 adults, 4 simultaneous OC43 and 229E antibody rises were noted (19). In the simpler procedure, an antibody rise is expressed as an EIA ratio, i.e., the highest ratio of postinfection to preinfection absorption values obtained at any of the serum dilutions tested. With an EIA ratio of 1.5, this method was more sensitive than the neutralizing antibody test in detecting 229E infection among experimentally infected volunteers who shed 229E virus. No sensitivity data with coronavirus OC43 were described for this method. With rabbit antisera, the more complex method yielded EIA antibody titers 100- to 1,000-fold greater than CF test titers, depending on the antigen preparation and antisera used, and at least 100-fold greater than neutralizing antibody test titers with all antigen preparations. With human convalescent-phase sera, 100-fold-higher antibody titers were obtained by EIA than by the neutralizing antibody test, and 16 of 20 serum samples with neutralizing antibody test titers of $<1:4$ were positive by EIA.

To assess the utility of the EIA for detection of OC43 coronavirus infections, we used sera from a variety of studies of acute respiratory illnesses. The EIA indicated that about 5% of all respiratory illnesses studied during this period were associated with an OC43 coronavirus infection. Among COPD patients with respiratory illnesses, 7 (8%) of 87 respiratory illnesses were associated with an OC43 infection. In contrast, only one OC43 antibody rise occurred during 181 periods without illness (0.6%). In healthy, age-matched controls, only one coronavirus infection was detected during 87 time periods in which a respiratory illness was identified and two infections were detected during 236 periods (0.8%) in which patients were not ill. Coronavirus infections also occurred among other populations during the surveillance period. Among adult asthmatics, coronavirus OC43 infection was detected in 8.8% of patients presenting for emergency treatment, and in children and adults hospitalized for respiratory illnesses, 5.3% had a coronavirus OC43 infection.

Coronavirus infections have been previously examined with a variety of populations. In children under 6 years of age, coronaviruses 229E and OC43 were detected (by an antigen detection EIA) in nasal secretions of 30% of 159 persons with acute respiratory tract infections over a 2-year period (11, 20); 27% were due to 229E alone. Using CF and HAI tests, McIntosh and associates (22, 24) found coronavirus OC43 and 229E antibody rises in 8 to 19% of infants and children, but dual infections with other respiratory viruses were detected in 15 to 43%. Kaye and associates (14) surveyed 1,328 respiratory illnesses and found 44 (3%) OC43 antibody responses by the HAI test in a children's home over a 7-year period. Other than the study using EIA for antigen detection in nasal secretions, all these studies used the HAI or CF test to detect coronavirus antibody.

Coronavirus infections in military recruits who were hospitalized for acute respiratory disease were studied over a 3-year period (32); the CF test was used for detection of antibody to

229E, and the HAI test was used for detection of antibody to OC43. Though infections with both coronaviruses were detected in all three years, 52% of the 75 men hospitalized one winter season had rises of antibody to coronavirus OC43. This severe presentation in an otherwise healthy group of young adults was possibly attributable to the conditions of unusual crowding and physical exertion.

In elderly nursing home residents, 7% of upper respiratory illnesses in 11 homes over one winter were attributed to coronavirus OC43 by Kraaijeveld's EIA method (27). Among adults with COPD, coronavirus infections have been detected by both the CF test and the HAI test in 1.7 to 20% of exacerbations (3, 6, 30). Thus, even when the insensitive HAI and CF tests are used, coronavirus appears to be a significant contributor to illness in patients with pulmonary disease or advanced age.

The published literature and our experience with an indirect EIA for coronavirus OC43 suggest that coronavirus OC43 is of importance as a cause of illness in several high-risk populations, including patients with COPD and asthma.

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