

Development of a Nucleocapsid-Based Human Coronavirus Immunoassay and Estimates of Individuals Exposed to Coronavirus in a U.S. Metropolitan Population[∇]

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Coronaviruses cause respiratory infections ranging from common colds to severe acute respiratory syndrome (SARS) in humans. Estimates for exposure to non-SARS coronaviruses are high, particularly for 229E and OC43; however, less information regarding seroprevalence is available for HKU1 and NL63. To measure exposure rates to these four coronavirus strains (229E, HKU1, NL63, and OC43), we devised an immunoassay based on amino- and carboxy-terminally tagged recombinant coronavirus nucleocapsid antigens. Four human and one feline coronavirus antigen were cloned into baculoviruses expressed in insect cells and recovered proteins bound in the solid phase of an enzyme-linked immunosorbent assay-based system. We screened sera from 10 children and 196 adults and established primary cutoff points based on immunoglobulin G (IgG) antibody levels of the predominantly seronegative children. The proportion of seropositive adults for each coronavirus was as follows: 229E, 91.3%; HKU1, 59.2%; NL63, 91.8%; and OC43, 90.8%. No evidence of a significant serological response to the feline coronavirus was observed. Significant associations of coronavirus seropositivity and antibody levels with age, gender, race, socioeconomic status, smoking status, and season of the blood draw were tested with chi-square and regression analyses. The group II coronaviruses (OC43 and HKU1) were significantly associated with race ($P \leq 0.009$ and $P \leq 0.03$, respectively). Elevated OC43 IgG levels were further significantly associated with smoking status ($P \leq 0.03$), as were high NL63 titers with socioeconomic status ($P \leq 0.04$). The high-level immunoreactivity of each coronavirus was significantly associated with the summer season ($P \leq 0.01$ to 0.0001). In summary, high rates of exposure to 229E, NL63, and OC43 and a moderate rate of exposure to HKU1 characterized the seroprevalence among individuals in this population. Demographic factors, such as race, smoking status, and socioeconomic status, may confer an increased risk of susceptibility to these viruses.

Human coronaviruses primarily replicate within the respiratory tract and cause infections ranging from common colds to severe acute respiratory syndrome (SARS) (7, 13). Coronaviruses are single-stranded RNA viruses with outer envelopes that have distinct crown-like morphologies. Non-SARS respiratory infections occur from group I and group II coronaviruses, the prototypes of which (229E and OC43) were first described in the 1960s (4, 9, 18). More recently isolated human coronaviruses described in 2004 and 2005 include NL63, which is a member of group I, and HKU1, which is a member of group II (20, 21). Although current evidence suggests a worldwide distribution of these four coronaviruses (14, 19), seroprevalence studies showing relative exposure rates among the viral strains are lacking.

We developed serological assays specific for each non-SARS human coronavirus (229E, HKU1, NL63, and OC43) and a feline coronavirus, which is not known to cause infections in humans. Antigen targets for these assays were the immuno-

dominant viral nucleocapsid proteins. We prepared recombinant nucleocapsid antigens following baculovirus cloning and expression in insect cell cultures and used these proteins to devise novel enzyme-linked immunosorbent assays (ELISAs) specific for the target coronavirus antigens. With these assays, we quantified antibody levels in a group of 196 generally healthy individuals. Intergroup comparisons of seropositivity, mean antibody levels, and associations with demographic variables were performed to determine the epidemiology of these coronaviruses in a small metropolitan area.

MATERIALS AND METHODS

Coronavirus antigen cloning and expression. Nucleotide sequences encoding nucleocapsid proteins of the human coronaviruses 229E, HKU1, NL63, and OC43 and a feline coronavirus were synthesized with codon optimization for insect cell expression (Genscript Corporation, Piscataway, NJ). The corresponding GenBank coronavirus nucleocapsid gene and protein accession numbers, respectively, are as follows: NL63, NC_005831 and YP_003771.1; OC43, NC_005147 and NP_937954.1; 229E, NC_002645 and NP_073556.1; and HKU1, NC_006577 and YP_173242.1. Insect cell codon-optimized sequences were cloned into a modified pAB-GST baculovirus transfer vector (AB Vector, San Diego, CA) as previously described (22). The pAB-GST vector was mutated to incorporate a 3' 60-nucleotide sequence tag encoding a bovine polyomavirus (BPV) large T antigen peptide. Plasmid constructs were recombined with Pro-Easy linearized baculovirus DNA (AB Vector) and recombinant baculoviruses passaged in sf9 insect cells (Orbigen, San Diego, CA) according to the manufacturer's protocol. Coronavirus nucleocapsid proteins were expressed in

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Trichoplusia ni (High Five) insect cells (Orbigen) as glutathione *S*-transferase (GST) fusion proteins. To confirm the fusion protein expression, cell lysates were analyzed by immunoblotting using standard methodology. Total protein concentrations were determined using a Bradford-based total protein assay, and 1- μ g amounts were loaded on a precast 2 to 40% gradient Tris-HCl polyacrylamide Criterion gel (Bio-Rad, Hercules, CA). An anti-GST-horseradish peroxidase-conjugated polyclonal antibody (pAb) (GE Healthcare, Piscataway, NJ) was used to verify that expressed antigens contained amino-terminal GST tags and would be properly bound in the solid phase of the ELISA. A pAb against the 20-amino-acid BPV peptide tag was used to detect the presence of carboxy termini and to verify the expression of full-length coronavirus nucleocapsid proteins. This pAb was prepared by immunizing rabbits with the BPV peptide (HNYNLYKSLKN TEEVGKKRY) and isolated using standard methodology. We also probed immunoblots with an anti-OC43 nucleocapsid-specific monoclonal antibody (Millipore, Temecula, CA). Blots were screened with study sera from several representative individuals to determine if coronavirus immunoglobulin G antibodies were present and to what degree antibodies would bind to expressed fusion proteins. The control antigens used in all immunoblots included the feline coronavirus and insect cell lysate with and without empty pAB-GST and pAB-GST-BPV vectors.

Preparation of antigen for the ELISA. For antigen production, *Trichoplusia ni* cells were infected with a recombinant baculovirus at a multiplicity of infection of 1 and grown as adherent cultures in tissue culture plates (245 by 245 mm; Nunc, Naperville, IL) in 100 ml Ex-Cell 400 (Orbigen) medium per plate. After a 72-h incubation at 27°C, the cells were harvested by scraping and then pelleted by low-speed centrifugation. The cells from one plate (~100 million cells) were resuspended in 50 ml phosphate-buffered saline (PBS) containing 1 mM EDTA, 0.5% NP-40, and a protease inhibitor cocktail (Complete Mini; Roche, Indianapolis, IN). After a 30-min incubation on ice, the cell suspensions were sonicated on ice twice for 60 s using a 550 sonic dismembrator with a microtip (Fisher Scientific, Waltham, MA) at a setting of 5. The lysates were centrifuged at 10,000 \times *g* for 15 min. The supernatant was collected and stored at -80°C.

GST fusion protein ELISA. Antibodies to coronavirus nucleocapsid protein were measured by ELISA using the GST capture method of Sehr et al. (16) with some modifications. Briefly, 96-well polystyrene flat-bottom MaxiSorp plates (Nunc) were sensitized overnight at 4°C with 100 ng/well of glutathione-casein in carbonate buffer (pH 9.6) and blocked for 1 h at 37°C with 0.2% (wt/vol) casein and 0.5% (wt/vol) polyvinyl alcohol (PVA) (molecular weight, 30,000 to 70,000) (Sigma, St. Louis, MO) in PBS (casein PVA buffer). The blocked plates were incubated for 1 h at 37°C with cell lysates containing GST fusion protein diluted in casein PVA buffer. Standardized dilutions of each lysate were determined via serial dilutions in ELISAs using the carboxy-terminal BPV peptide tag antibody as the primary antibody. Each antigen preparation was diluted 1:10, 1:40, 1:160, and 1:640, and dilutions that generated an optical density (OD) of 1 following visualization with the BPV pAb tag were used for the ELISAs. The control wells were coated with insect cell lysate expressing GST tag protein alone. Before addition of the serum samples and following each incubation, the plates were washed four times with PBS containing 0.05% (vol/vol) Tween-20 (Sigma) in an automatic plate washer (Skanwasher 300; Skatron, Lier, Norway). Serum samples diluted 1:200 in casein PVA buffer were left for 1 h at 37°C. Antigen-bound immunoglobulin was detected with peroxidase-conjugated goat antibodies against human immunoglobulin G, gamma chain specific (Southern Biotech, Birmingham, AL), diluted 1:4,000 in casein PVA buffer containing 0.8% (wt/vol) polyvinylpyrrolidone (molecular weight, 360,000; Sigma) and 0.05% (vol/vol) Tween 20. After 30 min at 37°C, color development was initiated by adding 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution (KPL Protein Research Products, Gaithersburg, MD). The reactions were stopped after 20 min by 1% dodecyl sulfate and the absorbance measured at 405 nm, with a reference wavelength of 490 nm, in an automated microtiter plate reader (Molecular Devices, Menlo Park, CA).

Study participants and serum collection. A total of 196 individuals without a history of psychiatric disorder were recruited by posted announcements to serve as controls for serological studies related to neuropsychiatric disorders (3). As such, this population represents a generally healthy group of individuals in which to measure coronavirus seroprevalence. The participants were between the ages of 18 and 65, inclusive, and had none of the following: current substance abuse over the past 1 month or any history of intravenous substance abuse; mental retardation; a medical disorder that would affect cognitive performance, such as epilepsy, a history of encephalitis or head trauma, or any other reported neurological disorder of the central nervous system; and clinically apparent herpesvirus infection or recent treatment with antiviral medications.

Blood samples were obtained by venipuncture, and sera were separated and assessed for antibodies to coronavirus antigens in the assay described above.

To optimize the assay conditions and to establish the assay seropositivity cutoffs, we used previously obtained sera from 10 healthy children aged 2 to 4 years old. These sera were enrollment sera from healthy children recruited for vaccine studies.

The studies were approved by the institutional review board (IRB) of the Sheppard Pratt Health System and the Johns Hopkins Medical Institution following established guidelines. All participants provided written informed consent after the study procedures were explained. The children's sera were part of prior Johns Hopkins IRB-approved studies, had already been de-identified, and did not require repeat IRB approval for them to be used for the present study.

Statistical analyses. To correct for background reactivity, the absorbance in the wells with the pAB-GST empty vector was subtracted from the absorbance in the wells with the GST-coronavirus nucleocapsid to give an antigen-specific reactivity. Several seropositivity cutoff OD values were assigned. The primary seropositivity cutoff OD values were designated based on the seronegative children (outliers eliminated). The outliers were eliminated if the values fell outside of 2 standard deviations of the mean, and this process was repeated three times. The final cutoff point was assigned as the mean OD unit plus five standard deviations. We also evaluated the seropositivity cutoff OD values based on the mean plus 3, 7, and 10 standard deviations. In order to have a less-sensitive but potentially more-species-specific cutoff, we designated a high cutoff limit based on the upper quartile percentage of data from the adults.

Significant differences in the rates of seropositivity of specific antigens within demographic categories (age, gender, maternal education levels, smoking status, and race) and within the season of the blood draw were identified with chi-square tests (α level, 0.05). The maternal education levels were used as a designation of socioeconomic status. Significant associations of seropositivity with demographic and other factors were further tested with multiple logistical regressions. Significant associations of the antibody levels with age, gender, maternal education, smoking status, race, and season were tested using multiple linear regressions. Potential cross-reactivity between coronavirus strains was assessed using linear regression models. All analyses were performed with STATA version 10 (STATA Corp. LP, College Station, TX). Given the exploratory nature of this study, the data were not corrected for multiple comparisons.

RESULTS

The reactivity of each coronavirus antigen was initially evaluated with immunoblotting (Fig. 1). Using the anti-GST antibody, an immunoreactive band corresponding to approximately 80 kDa for each antigen was observed. Also visualized was a band of approximately 25 kDa, suggesting that small amounts of GST protein are cleaved from the full-length fusion protein by endogenous insect proteolytic processes. The anti-BPV pAb predominantly detected the same 80-kDa protein for each antigen preparation and also recognized a less-abundant protein of approximately 45 to 60 kDa. This lower-yield series corresponds to a fusion protein containing the carboxy BPV tag but lacking the (cleaved) amino GST tag. This GST-cleaved protein would not be bound in the solid-phase portion of the ELISA. The anti-OC43 antibody also recognized these same two proteins, and immunoreactivity was specific for the OC43 preparation only. The study subject sera were specific to the human coronavirus preparations and did not react with the feline coronavirus antigen or the insect cell lysate with or without the empty vector GST and GST/BPV tags.

We tested our assay and established a primary seropositivity cutoff value by measuring the reactivity to these antigens in 10 children. We found that the majority (60%) of the children did not have detectable reactivity to the coronavirus antigens tested. Among the children who showed reactivity, one child responded to all four human coronaviruses; a second was reactive to 229E, HKU1, and OC43; a third was seropositive for 229E, NL63, and OC43; and a fourth was reactive for NL63 only. Using the primary seropositivity cutoff value established

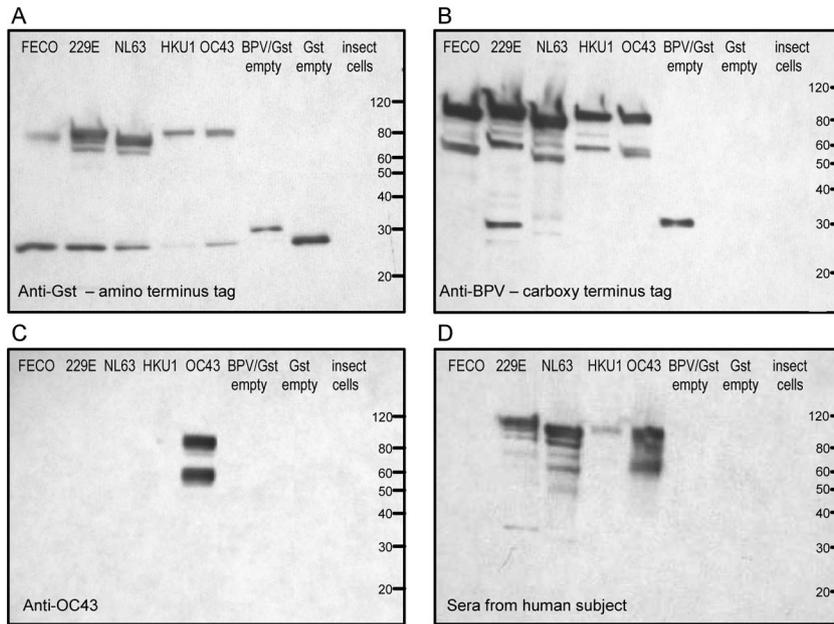


FIG. 1. Immunoblot series of human coronavirus recombinant antigen fusion proteins. FECO refers to the feline coronavirus nucleocapsid antigen. BPV refers to the bovine polyomavirus. Insect cells refer to a negative control containing baculovirus-free and cloning vector-free insect cell protein extracts. Empty refers to a negative control containing baculovirus and cloning vector, but the cloning vector lacks a nucleocapsid insert.

from the children’s data, we found that 91.3% of the 196 adults were seropositive for 229E, 59.2% for HKU1, 91.8% for NL63, and 90.8% for OC43. The range of antibody levels of the children compared to the adults for each antigen is shown in Fig. 2. A significant seroresponse was not observed for the feline control coronavirus.

For each demographic and other factor (age, gender, race, socioeconomic status, smoking status, and season of the blood draw), we tested the number of negative versus positive individuals in each category using chi-square contingency tables. For analyses based on cutoff values from the children’s data, HKU1 was significantly associated with race ($\chi^2 = 7.1047$; $df = 2$; $P \leq 0.029$) (Table 1). Using this primary cutoff limit, none of

the other coronaviruses showed any significant associations with any of the listed variables. We then tested the high seropositivity cutoff designations and found that OC43 was significantly associated with race ($\chi^2 = 7.4335$; $df = 2$; $P \leq 0.024$) and smoking status ($\chi^2 = 4.6912$; $df = 1$; $P \leq 0.030$) and NL63 was significantly associated with maternal education ($\chi^2 = 10.2358$; $df = 4$; $P \leq 0.037$) (Table 2). High-cutoff-point seropositivities for 229E ($\chi^2 = 17.5643$; $df = 3$; $P \leq 0.001$), NL63 ($\chi^2 = 22.4689$; $df = 3$; $P \leq 0.0001$), and OC43 ($\chi^2 = 11.1342$; $df = 3$; $P \leq 0.011$) were also significantly associated with the season of the blood draw, with the highest levels of antibodies observed during the summer months.

Significant chi-square associations of coronavirus seroresponse with demographic variables were further verified in multivariate models that included gender, age, maternal education, smoking status, and race as covariates. We used antibody level as the dependent variable in multiple linear regressions and seropositivity as the dependent variable in multinomial logistic regressions. In multiple linear regressions, OC43 antibody levels were again significantly associated with race ($t = 2.65$; $df = 5$; $P \leq 0.009$). Similarly, with multiple linear regressions, all coronavirus antibody levels were significantly associated with the summer season (for 229E, $t = 3.97$, $df = 6$, and $P \leq 0.0001$; for HKU1, $t = 2.62$, $df = 6$, and $P \leq 0.009$; for NL63, $t = 4.49$, $df = 6$, and $P \leq 0.0001$; and for OC43, $t = 2.62$, $df = 6$, and $P \leq 0.01$). Using the primary cutoff point of seropositivity in multinomial logistic regressions, none of the coronaviruses were significantly associated with demographics. Using the high cutoff value for seropositivity, however, OC43 was again significantly associated with race ($z = 2.47$; $df = 5$; $P \leq 0.014$) and with smoking status ($z = 2.03$; $df = 5$; $P \leq 0.043$), and NL63 was again significantly associated with

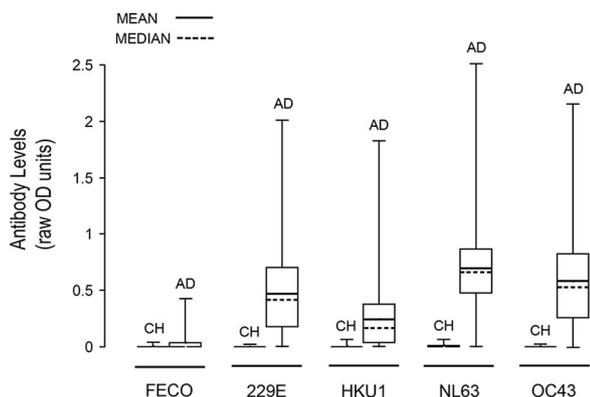


FIG. 2. Box plots of coronavirus antibody distribution in sera from children (CH) and adults (AD). Boxed portions indicate the middle 50% of the data. Small horizontal bars indicate the range of data, with the lower bar indicating the minimum value and the upper bar representing the maximum value.

TABLE 1. Demographics of coronavirus seropositivity using primary seropositivity cutoff values^a

Demographic	Total no. of subjects (n = 196)	229E		HKU1		NL63		OC43	
		No. of positive subjects (%) (n = 179 [91.3%])	P value	No. of positive subjects (%) (n = 116 [59.2%])	P value	No. of positive subjects (%) (n = 180 [91.8%])	P value	No. of positive subjects (%) (n = 178 [90.8%])	P value
Age (yr)			0.357		0.211		0.523		0.944
20–29	93	88 (94.6)		62 (66.7)		88 (94.6)		85 (91.4)	
30–39	43	37 (86.0)		22 (51.2)		38 (88.4)		39 (90.7)	
40–49	34	30 (88.2)		17 (50.0)		30 (88.2)		30 (88.2)	
≥50	26	24 (92.3)		15 (57.7)		24 (92.3)		24 (92.3)	
Race			0.804		0.029 ^b		0.690		0.493
African American	56	50 (89.3)		39 (69.6)		50 (89.3)		53 (94.6)	
Caucasian	129	119 (92.2)		68 (52.7)		120 (93.0)		115 (89.1)	
Other	11	10 (90.9)		9 (81.8)		10 (90.9)		10 (90.9)	
Gender			0.657		0.069		0.232		0.789
Male	71	64 (90.1)		36 (50.7)		63 (88.7)		65 (91.5)	
Female	125	115 (92.0)		80 (64.0)		117 (93.6)		113 (90.4)	
Maternal education			0.498		0.846		0.858		0.912
<12 yr	30	27 (90.0)		18 (60.0)		27 (90.0)		27 (90.0)	
12 yr (high-school graduate)	74	69 (93.2)		40 (54.1)		69 (93.2)		66 (89.2)	
12–16 yr (1–3 yr of college)	30	25 (83.3)		19 (63.3)		28 (93.3)		27 (90.0)	
16 yr (college graduate)	40	37 (92.5)		25 (62.5)		37 (92.5)		37 (92.5)	
>16 yr (postcollegiate)	22	21 (95.5)		14 (63.6)		19 (86.4)		21 (95.5)	
Smoking status			0.911		0.738		0.379		0.981
Smoker	44	40 (90.9)		27 (61.4)		39 (88.6)		40 (90.9)	
Nonsmoker	152	139 (91.5)		89 (58.6)		141 (92.8)		138 (90.8)	
Season			0.886		0.113		0.484		0.946
Winter (Dec-Feb)	39	35 (89.7)		29 (74.4)		35 (89.7)		36 (92.3)	
Spring (Mar-May)	7	6 (85.7)		3 (42.9)		6 (85.7)		6 (85.7)	
Summer (June-Aug)	79	72 (91.1)		47 (59.5)		71 (89.9)		72 (91.1)	
Fall (Sept-Nov)	71	66 (93.0)		37 (52.1)		68 (95.8)		64 (90.1)	

^a Cutoff values established using children's data means plus 5 standard deviations.

^b Significant difference at $P \leq 0.05$ among categories within the demographic variable.

maternal education ($z = 1.93$; $df = 5$; $P \leq 0.054$). Multinomial logistic regressions using the high cutoff seropositivity also showed significant associations of the summer season with 229E ($z = 4.04$; $df = 6$; $P \leq 0.0001$), NL63 ($z = 4.31$; $df = 6$; $P \leq 0.0001$), and OC43 ($z = 2.75$; $df = 6$; $P \leq 0.006$).

Multiple linear regressions showed a high degree of correlation between antibody levels of the different strains (for all comparisons, $P \leq 0.0001$). The highest R^2 value was observed between the group II coronaviruses HKU1 and OC43 ($R^2 = 0.69$; $df = 195$), followed by the group I coronaviruses 229E and NL63 ($R^2 = 0.54$; $df = 195$). The lowest R^2 values were observed between the group I and group II coronaviruses: for HKU1 and 229E, $R^2 = 0.30$ and $df = 195$; for HKU1 and NL63, $R^2 = 0.31$ and $df = 195$; for 229E and OC43, $R^2 = 0.39$ and $df = 195$; and for OC43 and NL63, $R^2 = 0.48$ and $df = 195$.

DISCUSSION

In this study, we show a generally high level of seroprevalence (90.8 to 91.8%) in 196 adults for three of the four human coronaviruses (229E, NL63, and OC43) and a moderate expo-

sure rate for the fourth (59.2% for HKU1), an indication that these viruses have an established presence in the study population. Our estimates support previously reported seroprevalence rates for 229E (53 to 100%) and OC43 (88 to 100%) (6, 10, 15). Until now, seroprevalence rates of the more newly discovered viruses NL63 and HKU1, particularly in asymptomatic adult controls, generally have been lacking. Our results suggest that exposures to HKU1 are less common for unknown reasons, whereas NL63 exposures are as frequent as 229E and OC43 exposures. The lack of a detectable increase in antibodies to the feline coronavirus, which is not thought to infect humans, indicates that we are not measuring a nonspecific antibody response.

In our demographic analyses, race, socioeconomic status, and smoking status were all identified as risk factors for coronavirus exposure. Higher rates of seropositivity were observed in African Americans, smokers, and individuals of low socioeconomic status. All three of these risk factors have been previously shown to render individuals more prone to a variety of respiratory illnesses (2, 5, 11). The demographic factor as-

TABLE 2. Demographics of coronavirus seropositivity using secondary, higher-limit seropositivity cutoff values^a

Demographic	Total no. of subjects (n = 196)	229E		HKU1		NL63		OC43	
		No. of positive subjects (%) (n = 49 [25%])	P value	No. of positive subjects (%) (n = 49 [25%])	P value	No. of positive subjects (%) (n = 52 [26.5%])	P value	No. of positive subjects (%) (n = 51 [26.0%])	P value
Age (yr)			0.257		0.078		0.105		0.130
20–29	93	29 (31.2)		30 (32.3)		32 (34.4)		29 (31.2)	
30–39	43	7 (16.3)		5 (11.6)		7 (16.3)		7 (16.3)	
40–49	34	7 (20.6)		8 (23.5)		8 (23.5)		6 (17.6)	
≥50	26	6 (23.1)		6 (23.1)		5 (19.2)		9 (34.6)	
Race			0.559		0.516		0.077		0.024 ^b
African American	56	12 (21.4)		17 (30.4)		19 (33.9)		22 (39.3)	
Caucasian	129	33 (25.6)		29 (22.5)		28 (21.7)		26 (20.2)	
Other	11	4 (36.4)		3 (27.3)		5 (45.5)		3 (27.3)	
Gender			0.345		0.345		0.778		0.617
Male	71	15 (21.1)		15 (21.1)		18 (25.4)		17 (23.9)	
Female	125	34 (27.2)		34 (27.2)		34 (27.2)		34 (27.2)	
Maternal education			0.182		0.531		0.037 ^b		0.199
<12 yr	30	3 (10.0)		8 (26.7)		4 (13.3)		7 (23.3)	
12 yr (high-school graduate)	74	18 (24.3)		14 (18.9)		14 (18.9)		19 (25.7)	
12–16 yr (1–3 yr of college)	30	10 (33.3)		10 (33.3)		12 (40.0)		10 (33.3)	
16 yr (college graduate)	40	10 (25.0)		10 (25.0)		14 (35.0)		6 (15.0)	
>16 yr (postcollegiate)	22	8 (36.4)		7 (31.8)		8 (36.4)		9 (40.9)	
Smoking status			0.693		0.236		0.794		0.030 ^b
Smoker	44	12 (27.3)		14 (31.8)		11 (25.0)		17 (38.6)	
Nonsmoker	152	37 (24.3)		35 (23.0)		41 (27.0)		34 (22.4)	
Season			0.001 ^b		0.135		0.0001 ^b		0.011 ^b
Winter (Dec-Feb)	39	4 (10.3)		10 (25.6)		5 (12.8)		8 (20.5)	
Spring (Mar-May)	7	1 (14.3)		1 (14.3)		0 (0.0)		0 (0.0)	
Summer (June-Aug)	79	32 (40.5)		26 (32.9)		35 (44.0)		30 (38.0)	
Fall (Sept-Nov)	71	12 (16.9)		12 (16.9)		12 (16.9)		13 (18.3)	

^a Cutoff values established using the 75th percentile cutoff from the adult data.
^b Significant difference at the listed P value among categories within the demographic variable.

sociations were particularly evident in individuals with higher antibody titers, suggesting that these risk factors are linked to recent infections. Similarly, higher antibody levels were significantly associated with the season of the blood draw, and the highest coronavirus immunoreactivity occurred during the summer months. It is possible that in this population of adults and especially in those individuals deemed at risk, these summer bouts could represent either a persistent infection or a reinfection with the same coronavirus strain (1).

Amino acid identity among the four coronavirus nucleocapsids is overall generally low (35.4 to 68.2%), thus making the nucleocapsid protein a useful strain-specific diagnostic antigen (8). Nevertheless, defining the extent of the cross-reactivity among the coronavirus nucleocapsids remains an ongoing issue. Recent results regarding the degree of cross-reactivity between the SARS nucleocapsids and each of the non-SARS nucleocapsids (8, 12) are mixed, and in these studies, it was not possible to assess the degree of cross-reactivity among the non-SARS strains. Our study was not designed specifically to test cross-reactivity; however, the correlation between the antibody levels of different strains was evident, with the expected

highest association observed between viruses within the same phylogenetic group (i.e., between NL63 and 229E [group I] and between OC43 and HKU1 [group II]). The cross-reactivity between group I strains has previously been suggested in a serological study of NL63 and 229E in children and young adults (17). In this study, portions of the nucleocapsid genes were expressed and certain amino acid regions of the NL63 recombinant proteins were detected by 229E antisera in Western blots. Although our use of the whole nucleocapsid sequence may have contributed to a certain degree of cross-reactivity, the absence of reactivity to the feline coronavirus nucleocapsid indicates that genus- and group-specific cross-reactivity cannot completely explain the correlation between antibody levels to the different human coronavirus strains. Because the feline virus is more closely related to NL63 and 229E than to OC43 and HKU1, the correlation observed between the group I and group II coronaviruses is likely to reflect a shared exposure rather than cross-reactivity. In our study, we also have evidence for intragroup monospecificity of individual strains. For example, 32% of the 196 individuals who were seropositive for OC43 were seronegative for HKU1. With re-

spect to the group I viruses, 5.6% of individuals were seropositive for either NL63 or 229E but not for both. The extent of the cross-reactivity to the nucleocapsid proteins of human coronaviruses will need to be addressed in future studies by competitive inhibition experiments with purified nucleocapsid proteins.

Documenting relative levels of seroprevalence of coronavirus strains is important, particularly given the recent emergence of the SARS virus as a serious pathogen. The assay that we have developed is a straightforward and sensitive means of tracking the four known human non-SARS coronaviruses, and its application will likely benefit epidemiological studies of these viruses.

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REFERENCES

1. Callow, K. A., H. F. Parry, M. Sergeant, and D. A. Tyrrell. 1990. The time course of the immune response to experimental coronavirus infection of man. *Epidemiol. Infect.* **105**:435–446.
2. Chatila, W. M., E. A. Hoffman, J. Gaughan, G. B. Robinswood, G. J. Criner, and the National Emphysema Treatment Trial Research Group. 2006. Advanced emphysema in African-American and white patients: do differences exist? *Chest* **130**:108–118.
3. Dickerson, F. B., J. J. Boronow, C. Stallings, A. E. Origoni, S. Cole, B. Krivogorsky, and R. H. Yolken. 2004. Infection with herpes simplex virus type 1 is associated with cognitive deficits in bipolar disorder. *Biol. Psychiatry* **55**:588–593.
4. Hamre, D., and J. J. Procknow. 1966. A new virus isolated from the human respiratory tract. *Proc. Soc. Exp. Biol. Med.* **121**:190–193.
5. Hegewald, M. J., and R. O. Crapo. 2007. Socioeconomic status and lung function. *Chest* **132**:1608–1614.
6. Hrusková, J., F. Heinz, E. Svandová, and S. Pennigerová. 1990. Antibodies to human coronaviruses 229E and OC43 in the population of C.R. *Acta Virol.* **34**:346–352.
7. Kahn, J. S. 2006. The widening scope of coronaviruses. *Curr. Opin. Pediatr.* **18**:42–47.
8. Lehmann, C., H. Wolf, J. Xu, Q. Zhao, Y. Shao, M. Motz, and P. Lindner. 2008. A line immunoassay utilizing recombinant nucleocapsid proteins for detection of antibodies to human coronaviruses. *Diagn. Microbiol. Infect. Dis.* **61**:40–48.
9. McIntosh, K., J. H. Dees, W. B. Becker, A. Z. Kapikian, and R. M. Chanock. 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc. Natl. Acad. Sci. USA* **57**:933–940.
10. Mourez, T., A. Vabret, Y. Han, J. Dina, L. Legrand, S. Corbet, and F. Freymuth. 2007. Baculovirus expression of HCoV-OC43 nucleocapsid protein and development of a Western blot assay for detection of human antibodies against HCoV-OC43. *J. Virol. Methods* **139**:175–180.
11. Nuorti, J. P., J. C. Butler, M. M. Farley, L. H. Harrison, A. McGeer, M. S. Kolczak, and R. F. Breiman for the Active Bacterial Core Surveillance Team. 2000. Cigarette smoking and invasive pneumococcal disease. *N. Engl. J. Med.* **342**:681–689.
12. Patrick, D. M., M. Petric, D. M. Skowronski, R. Guasparini, T. F. Booth, M. Kraiden, P. McGeer, N. Bastien, L. Gustafson, J. Dubord, D. Macdonald, S. T. David, L. F. Srouf, R. Parker, A. Andonov, J. Isaac-Renton, N. Loewen, G. McNabb, A. McNabb, S. H. Goh, S. Henwick, C. Astell, J. P. Guo, M. Drebot, R. Tellier, F. Plummer, and R. C. Brunham. 2006. An outbreak of human coronavirus OC43 infection and serological cross-reactivity with SARS coronavirus. *Can. J. Infect. Dis. Med. Microbiol.* **17**:330–336.
13. Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, T. K. Ng, K. Y. Yuen, and SARS study group. 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* **361**:1319–1325.
14. Pyrc, K., B. Berkhout, and L. van der Hoek. 2007. The novel human coronaviruses NL63 and HKU1. *J. Virol.* **81**:3051–3057.
15. Schmidt, O. W., I. D. Allan, M. K. Cooney, H. M. Foy, and J. P. Fox. 1986. Rises in titers of antibody to human coronaviruses OC43 and 229E in Seattle families during 1975–1979. *Am. J. Epidemiol.* **123**:862–868.
16. Sehr, P., K. Zumbach, and M. Pawlita. 2001. A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. *J. Immunol. Methods* **253**:153–162.
17. Shao, X., X. Guo, F. Esper, C. Weibel, and J. S. Kahn. 2007. Seroepidemiology of group I human coronaviruses in children. *J. Clin. Virol.* **40**:207–213.
18. Tyrrell, D. A. J., and M. L. Bynoe. 1965. Cultivation of novel type of common-cold virus in organ cultures. *Br. Med. J.* **1**:1467–1470.
19. van der Hoek, L. 2007. Human coronaviruses: what do they cause? *Antivir. Ther.* **12**:651–658.
20. van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. Berkhout, K. C. Wolthers, P. M. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout. 2004. Identification of a new human coronavirus. *Nat. Med.* **10**:368–373.
21. Woo, P. C., S. K. Lau, C. M. Chu, K. H. Chan, H. W. Tsoi, Y. Huang, B. H. Wong, R. W. Poon, J. J. Cai, W. K. Luk, L. L. Poon, S. S. Wong, Y. Guan, J. S. Peiris, and K. Y. Yuen. 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J. Virol.* **79**:884–895.
22. Yolken, R. H., H. Karlsson, I. Bossis, L. Aspl, F. Dickerson, C. Nellaker, M. Elashoff, E. Rubalcaba, and R. P. Viscidi. Endogenous retroviruses and human neuropsychiatric disorders, p. 66–86. *In* F. Gage and Y. Christen (ed.), *Retrotransposition, diversity and the brain*, in press. Springer-Verlag, Berlin, Germany.