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Examination of seroprevalence of coronavirus HKU1 infection with S protein-based ELISA and neutralization assay against viral spike pseudotyped virus

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ABSTRACT

Background: Human coronavirus HKU1 (HCoV-HKU1) is a recently identified coronavirus with a global distribution and known to cause mainly respiratory infections.

Objectives: To investigate the seroepidemiology of HKU1 infections in our local population.

Study design: An ELISA-based IgG antibody detection assay using recombinant HCoV-HKU1 nucleocapsid and spike (S) proteins (genotype A) were developed for the diagnosis of CoV-HKU1 infections. Additionally, a neutralization antibody assay using the HCoV-HKU1 pseudotyped virus was developed to detect the presence of neutralizing antibodies in serum with antibody positivity in an S protein-based ELISA.

Results: Results of the recombinant S protein-based ELISA were validated with Western blot, immunofluorescence analysis and flow cytometry. The coupled results demonstrated good correlation with Spearman's coefficient of 0.94. Seroepidemiological study in a local hospital-based setting using this newly developed ELISA showed steadily increasing HCoV-HKU1 seroprevalence in childhood and early adulthood, from 0% in the age group of <10 years old to a plateau of 21.6% in the age group of 31–40 years old.

Conclusions: Our study demonstrated the usefulness of the S-based ELISA which could be applied to future epidemiological studies of HCoV-HKU1 in other localities.

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1. Introduction

Coronavirus HKU1 is a newly identified human coronavirus.^{7,20} It has a global distribution and was first reported in Hong Kong, USA, Australia and Europe.^{4,13,21,23,38} The reported incidences varied from 0 to 4.4% of patients hospitalized for acute pulmonary and extrapulmonary symptoms. Laboratory detection is mostly achieved by RT-PCR.^{5,6,29} Because the nucleocapsid protein is highly conserved, this has been successfully cloned and used to detect antibody response by enzyme immunoassay (EIA) and Western blot analysis of sera from infected human.^{35,40} Ideal antibody test for a viral infection is the presence of neutralizing antibody.

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Neutralizing antibodies were shown to be the long lasting protective immune responses to many viral infections including coronavirus.^{1,2,8,28,33,39,42} The utility of assay based on neutralizing antibodies response against pseudotyped human coronaviruses had been successfully reported.^{12,16,28,35} As HCoV-HKU1 had not been successfully propagated in culture, it was not possible to determine and measure the neutralizing antibody response to the virus. This is the first report that examined the seroepidemiology of HCoV-HKU1 by making use of HCoV-HKU1 pseudotyped virus to confirm the presence of neutralizing antibodies from serologically positive serum for evaluation of the prevalence of HCoV-HKU1 as a cause of respiratory tract infection in various age groups in our local population.

We analyzed 297 serum samples assayed concurrently with the HKU1 recombinant protein nucleocapsid and spike based ELISA. After establishment of the baseline value by confirmation with Western blot, immunofluorescent microscopy, flow cytometry and the presence of neutralization antibodies to HCoV-HKU1 pseudotyped virus, we screened another 709 serum samples in various age groups and found that >10% of the studied population from age 21

Table 1

Cloning primer sequences cited in the paper.

Primer sequence 5'–3'	Direction	Vector ligated	Encoded insert
<u>CGCGGATCC</u> GTGATTGGCGACTCAACTGC	Forward	pGEX-5X3	Spike, AA 14–367
ATCTTACTCGAGTCA <u>GGAGTCTGTG</u> CACCAGCCT	Reverse	pGEX-5X3	Spike, AA 14–367
<u>CGCGGATCC</u> CACCATGCTGCTGATCATCTTCATCCTG	Forward	pcDNA 3.1(+)	Full length spike, AA 1–1356
<u>CGGAATTC</u> CTAGTCATCATGGGAGGCTTTGAT	Reverse	pcDNA 3.1(+)	Full length spike, AA 1–1356

Note: (1). Underlined sequences are restriction sites. (2). Italic sequences are Kozak sequence.

to 70 has been associated with HCoV-HKU1 infection which implicated that this recently identified virus has already been endemic in our community.

2. Materials and methods

2.1. Expression and purification of nucleocapsid (N) antigens and HCoV-HKU1 spike (S)

Recombinant 6xHis tagged N protein was expressed as reported.³⁸ Briefly, expressed N protein was bound to nickel hitrap column (Amersham Biosciences), purified protein was eluted using the AKTA explorer system (Amersham Biosciences). The human codon optimized cDNA coding for HCoV-HKU1-S (genotype A) was synthesized⁵ and served as a template for spike fragment amplifications covering amino acid residues 14–367 and cloned into bacterial expression vector pGEX-5X3 (Amersham Biosciences) with N-terminal fused to glutathione S-transferase (GST) gene. Recombinant protein was expressed in *Escherichia coli* BL21-Gold(DE3) cells. Cloning primer sequences were listed in Table 1.

2.2. Serum samples

Index serum controls were obtained from our previously reported cases of HCoV-HKU1 infection.³⁸ Negative controls were obtained from left-over sera from infants 3–6 months of age. These control sera were used to calibrate our ELISA assays. A total of 1006 random samples from patients hospitalized for acute respiratory illness were used in this evaluation.

2.3. ELISA

An ELISA-based IgG antibody detection assay was designed and standardized as previously reported.³⁸ Briefly, recombinant S and N antigens (0.25 and 0.2 µg/ml, respectively) were coated onto 96-well immunoplate (Maxisorb, Nunc). 100 µl test serum diluted 1:200 was tested in duplicate.

2.4. Confirmation of ELISA result by Western blot analysis

100-ng of purified GST-tagged spike S and His6-tagged nucleocapsid N were loaded into SDS-polyacrylamide gel, separated and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Results were revealed using ECL system (Amersham Biosciences).

2.5. Production of HCoV-HKU1 spike bearing pseudotyped virus

The full length, human codon optimized HCoV-HKU1 spike gene, with which AT-rich codons of the wild-type sequence replaced with the synonymous GC-rich codons that corresponded to the most frequently used human codons, was cloned into pcDNA 3.1(+), cotransfected with lentiviral vector containing reporter gene, GFP was used for pseudotype virus production.⁵

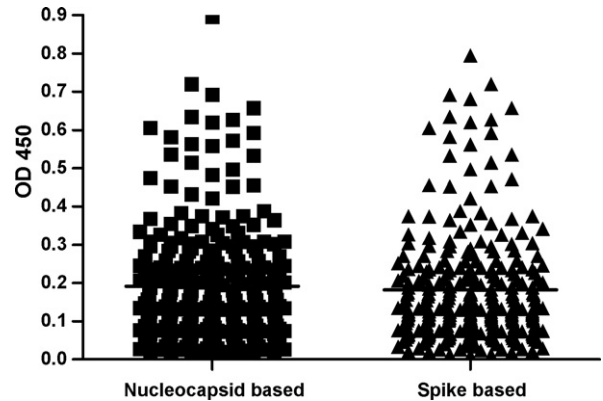


Fig. 1. Determination of cutoff baseline from 297 patients. Two ELISA-based assays against HKU1 recombinant proteins nucleocapsid (N) and spike (S) were compared. Mean OD \pm 3S.D. is 0.534 and 0.495 for N and S-based ELISA. Serum samples with OD above the baseline values were confirmed by Western blot.

2.6. Neutralization assay with pseudotyped virus

The neutralization assay based on the HCoV-HKU1-S pseudotyped virus was performed by measuring the infection of A549 cells (carcinomic human alveolar basal epithelial cells) as indicated by the expression of the green fluorescent protein (GFP).⁵ Pre-heat inactivated serum samples were twofold serially diluted from 1:25 to 1:800, and were mixed with 40 ng pseudotyped virus. Pseudotyped virus was quantitated using p24 ELISA kit (bioMérieux). Virus infectivity was determined 72 h post-infection by measuring the mean fluorescence level expressed in infected cells by flow cytometry (Beckton Dickinson, FACSCalibur). Neutralization antibody titers were determined as the percentage of inhibition of virus infectivity (mean fluorescence) at the final dilution of patient serum inhibiting 50% pseudotyped virus infection (ID_{50}), compared to viral infectivity without treatment with serum.

2.7. Production of Semliki Forest Viral (SFV) particles carrying HCoV-HKU1 S: development of cell-based assay for detection of S-specific antibody

The human codon optimized cDNA coding for HCoV-HKU1-S was subcloned with the C-terminal fused in-frame with FLAG sequence (-DYKDDDDK-), into Bam HI site of pSFV1 Semliki Forest Viral expression vector,³² resulting in the plasmid pSFV1-S-FLAG.

Table 2

Comparison of results between ELISA using HKU1 spike and nucleocapsid, and confirmation by Western blot. True positive samples against protein spike were tested for presence of antibodies against HKU1-pseudotyped virus with spike envelope.

	ELISA-nucleocapsid		Western blot		Neutralization assay
	Positive	Negative	S-positive	N-positive	Positive
ELISA-spike					
Positive	15 (5%)	0	15/15	15/15	11/15
Negative	6 (2%)	276	0/6	0/6	

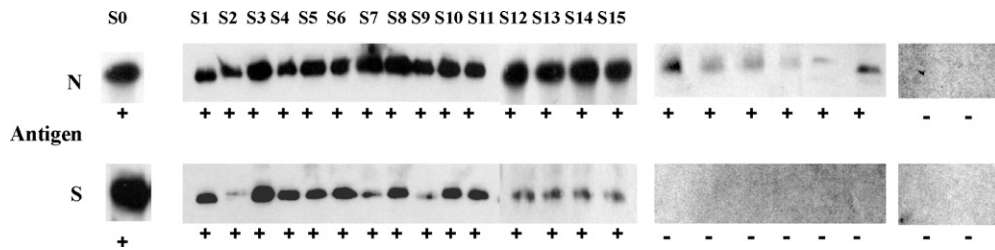


Fig. 2. Western blot analysis can rectify non-specificities of ELISA against recombinant HCoV-HKU1 nucleocapsid (N). Shown are 21 N-based ELISA seropositive samples (lanes 2–22). HKU1-index patient (lane 1, serum sample S0); ELISA positive in both N and S (lanes 2–16, serum samples S1–S15); ELISA positive in N but negative for S (lanes 17–22) and ELISA negative in both N and S (lanes 12–13), chemiluminescent signals were detected using ECL substrate.

SFV viral particles packaging was achieved by cotransfection with other pSFV helper plasmids encoding SFV structural proteins as cited papers.^{5,32}

2.8. Detection of spike-protein specific antibodies by FACS analysis (flow cytometry) and immunofluorescence microscopy

BHK-21 cells were infected with SFV particles.⁵ S-expressed cells were fixed 16–20 h post-infection. Cells were permeabilized and stained with test serum samples, washed and counter-stained with fluorescein isothiocyanate-conjugated goat anti-human IgG antibodies (Invitrogen). S-protein specific antibodies targeted against HCoV-HKU1 S expressed in BHK-21 cells were quantitated by flow cytometry (Beckton Dickinson, FACSCalibur). Corresponding results

were compared to image analysis by fluorescence microscopy (Eclipse 80i Nikon).

3. Results

3.1. Screening for serum antibody against recombinant HCoV-HKU1 nucleocapsid (N) and spike (S)-based ELISA

To establish the baseline for the ELISA tests, the cutoff was determined as mean optical density value plus three standard deviations at 450/620 nm observed. As the result, the mean ELISA OD for S and N-based test was 0.177 and 0.183 with standard deviation 0.106 and 0.117, respectively. Absorbance values of 0.495 and 0.534 were selected as the cutoff values for S and N-based ELISA tests, respec-

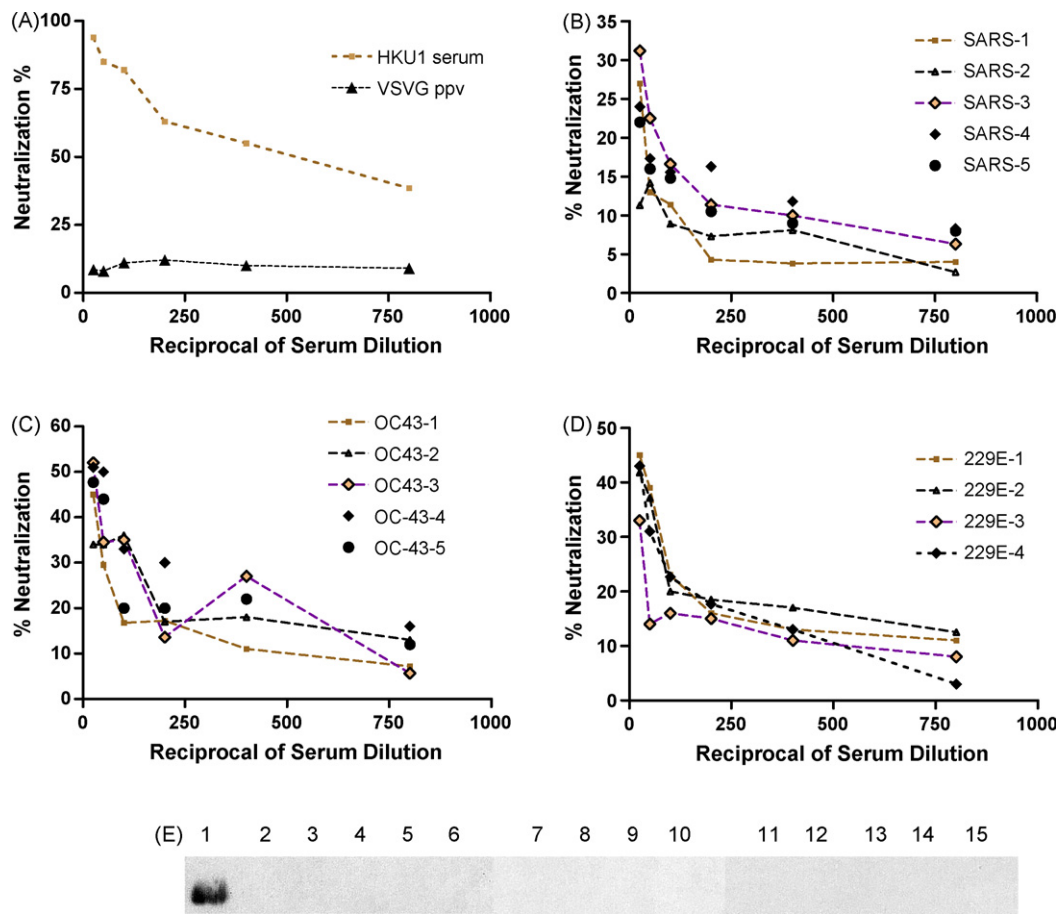


Fig. 3. (A) Detection of neutralizing antibodies in serum samples with HCoV-HKU1 infection which inhibit the infection of A549 cells by blocking entry of pHIV-GFP/HKU1 pseudotyped virus. Neutralizing antibodies targeted against pseudovirus bearing HKU1-spike do not neutralize the infection by pseudotyped virus bearing VSV-G protein. (B–D) Specificities of HKU1 pseudotyped viruses were shown against other convalescent patient serum from other SARS and other non-SARS human coronaviruses, OC-43 and 229E. (E). Western blot showed no cross-reactivity between HKU1-S antigen with other coronavirus infected patient serum tested in (B). Lane 1: HKU1 index patient serum, lanes 2–6 HCoV-OC43 patient sera, lanes 7–10 HCoV 229E patient serum and lanes 11–15 SARS patient sera.

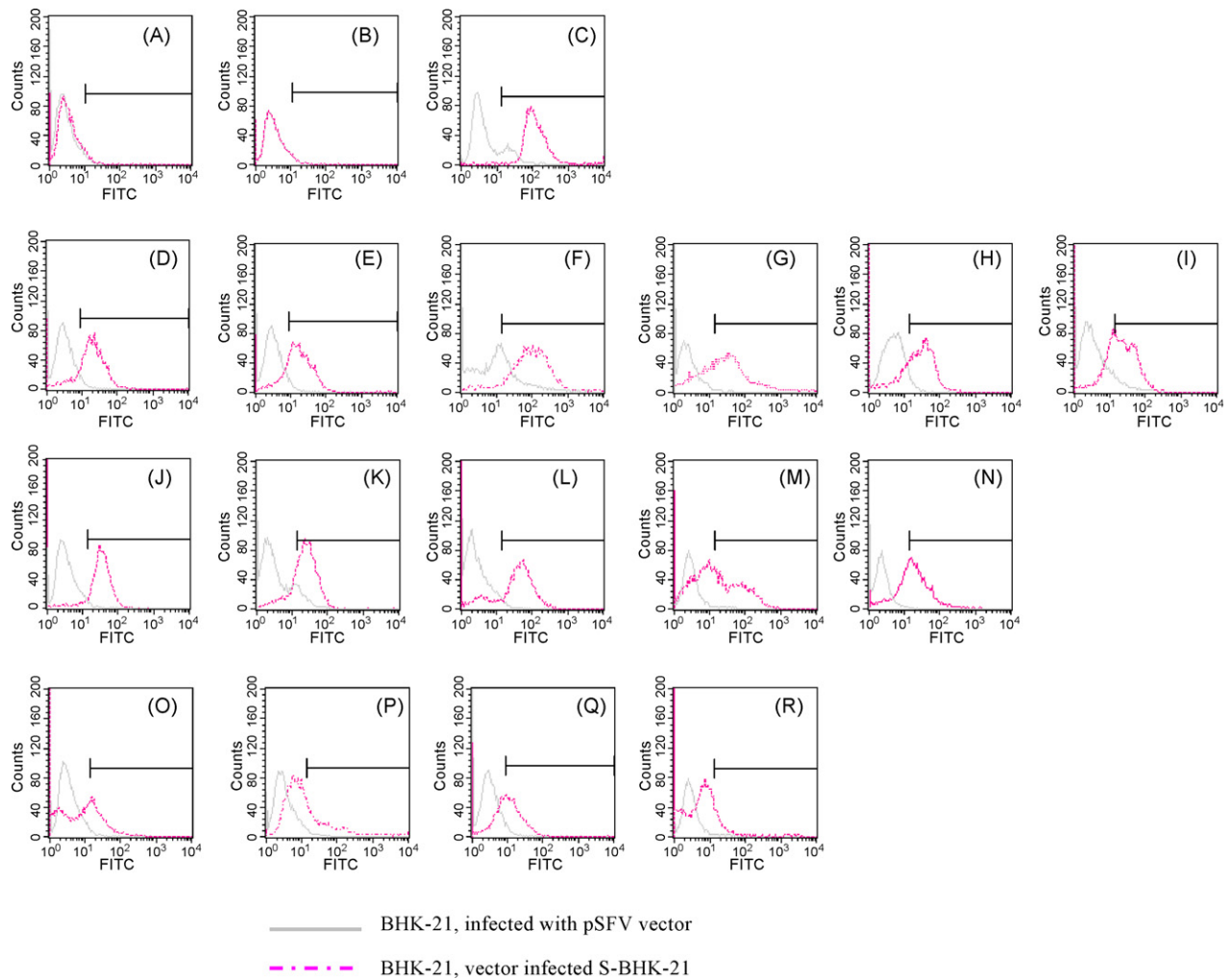


Fig. 4. Detection of antibodies against native HCoV-HKU1 S expressed in BHK-21 cells by flow cytometry. Sera A and B (C1–2) are S-based-ELISA negative samples. Serum C (S0) was taken from a patient who had recovered from HKU1 infection. Sera D–N (S1–S11) are samples which were S based-ELISA positive with OD ≥ 0.6 and O–R (S12–S15) with OD between 0.495 and <0.6 .

tively (Fig. 1). With reference to this standard, we found that 5% (15/297) samples were positive for both S and N, and 2% (6/297) were positive only against N but negative in S-based ELISA (Table 2). No samples were found to be positive only against the S but negative to N antigen.

3.2. Confirmation of ELISA test with Western blot

A confirmatory Western blot was done against 21 ELISA seropositive samples. All 15 samples (S1–S15) tested positive by both S and N-based ELISA were also positive by Western blot of their respective antigens (Table 2 and Fig. 2). The other 6 (2%) positive N-based ELISA samples were found to produce weakly positive protein band to N (50 kDa) but none to S (66 kDa) by Western blot. Seronegative samples all remained negative in Western blot. There is no discrepancy in of results between our ELISA system and the Western blot assay.

3.3. Index patient serum specifically neutralized HCoV-HKU1 S-pseudotyped virus infection

To achieve an assay for detection neutralizing antibodies (Nab) to unculturable HCoV-HKU1. It was shown that the infection could be blocked by convalescent patients serum recovering from

HCoV-HKU1 infection. The inhibition appeared to be specific to the HCoV-HKU1 as the same serum did not neutralize VSV-G enveloped pseudotyped retroviral particles (Fig. 3A). Serum from other patients recovering from other coronavirus infections, such as SARS and non-SARS human coronavirus, 229E and OC-43, did not block the HCoV-HKU1-pseudotyped virus infection (Fig. 3B–D) and no cross-reactivity with HKU1-S antigen shown by Western blot (Fig. 3E). This demonstrated that our HCoV-HKU1-pseudotyped virus can serve as a surrogate tool to detect neutralizing antibodies to HCoV-HKU1.

3.4. Correlation of neutralization assay with different serological tests

To assess the correlation between the presence of neutralization antibodies and ELISA baseline, we analyzed 15 S-based ELISA positive sera (S1–S15). Two randomly selected negative samples along with the index patient serum (S0) as positive control for the neutralization assay. Neutralizing antibodies were detected in 11 serum samples (S1–11) with S-based ELISA absorbance values score >0.6 gave results corresponding to neutralizing antibodies titers (ID_{50}) between 1:55 and 1:292 while no detectable neutralization activities (corresponding to titers of $<1:25$) were found in samples scored <0.6 (S12–S15).

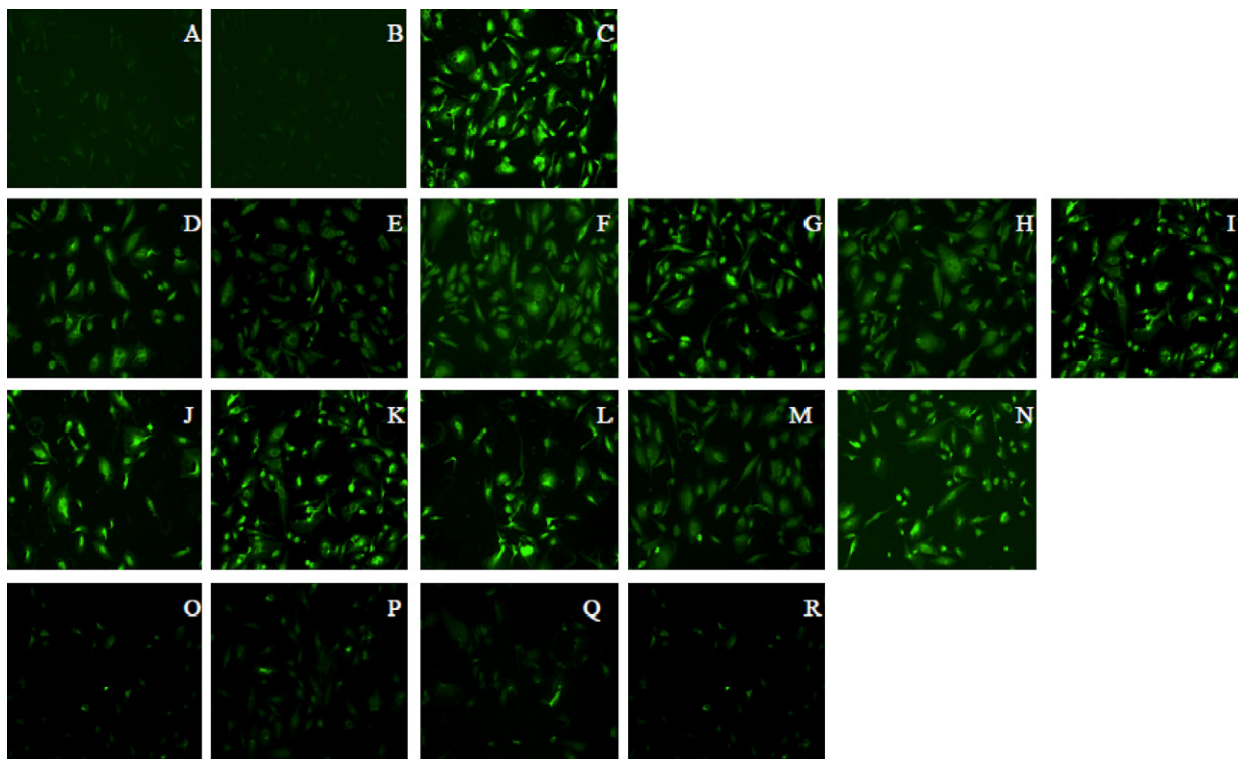


Fig. 5. Detection of antibodies against native HCoV-HKU1 S protein expressed in BHK-21 cells by indirect immunofluorescent microscopy. Sera A and B (C1–2) are S-based ELISA negative samples. Serum C (S0) was taken from a patient who had recovered from HKU1 infection. Sera D–N are samples (S1–S11) which were S-based ELISA positive with OD ≥ 0.6 and O–R (S12–S15) with OD between 0.495 and <0.6 .

Two different binding assays were applied to detect S-specific antibodies from the seropositive samples (S1–S15), using flow cytometry and immunofluorescent microscope analysis (IF), against S-expressed BHK-21 cells⁵ (Figs. 4 and 5) in order to correlate the results between ELISA and neutralization assays (Table 3). Distinctive antibody signals by both IF and flow cytometry were detected in those samples, scored absorbance values >0.6 (Figs. 4 and 5D–N, sera S1–S11) with net geometric mean fluorescence intensity (MFI) of 53.8 ± 5.2 although weaker signal was observed in samples with lower absorbance values <0.6 with net MFI of 37.35 ± 3.0 (Figs. 4 and 5O–R; samples S12–S15). Very good

positive correlations with the neutralization assay were shown with ELISA and flow cytometry which suggested our S protein-targeted serological assay is a reliable indicator to predict the presence of neutralization antibodies in S-based seropositive samples with ELISA absorbance scored above 0.6.

3.5. Determination of seroprevalence from different age groups in local community

709 blood samples were collected from patients who had attended Queen Mary Hospital and were found to be clinically free

Table 3
Correlation between ELISA, neutralization and flow cytometry assays.

Group	Serum sample	Absorbance	Titer ^a	Net MFI
HCoV-HKU1 index patient convalescent serum	S0	0.83	494	81
	S1	0.68	120	48.6
	S2	0.62	59	45.2
	S3	0.71	227	55.7
	S4	0.65	151	52.3
	S5	0.67	55	47.1
	S6	0.73	288	57.3
S-based ELISA positive serum samples	S7	0.7	178	56.4
	S8	0.67	118	55.1
	S9	0.69	266	52.8
	S10	0.76	262	62
	S11	0.79	292	59.3
	S12	0.53	<25	37.6
	S13	0.58	<25	33.2
	S14	0.52	<25	38.2
	S15	0.57	<25	40.4
Negative control serum samples	C1	0.33	<25	21.3
	C2	0.12	<25	9.8

ELISA absorbance was measured at OD450/620. Fluorescence level of S-protein antibodies binding measured by flow cytometry was expressed in term of Net geometric mean fluorescence intensity (MFI) calculated as MFI of test serum samples against S-expressed BHK-21 cells minus background made against uninfected BHK-21.

^a Titer: Dilution of serum at the HCoV-HKU1 pseudotyped virus ID₅₀.

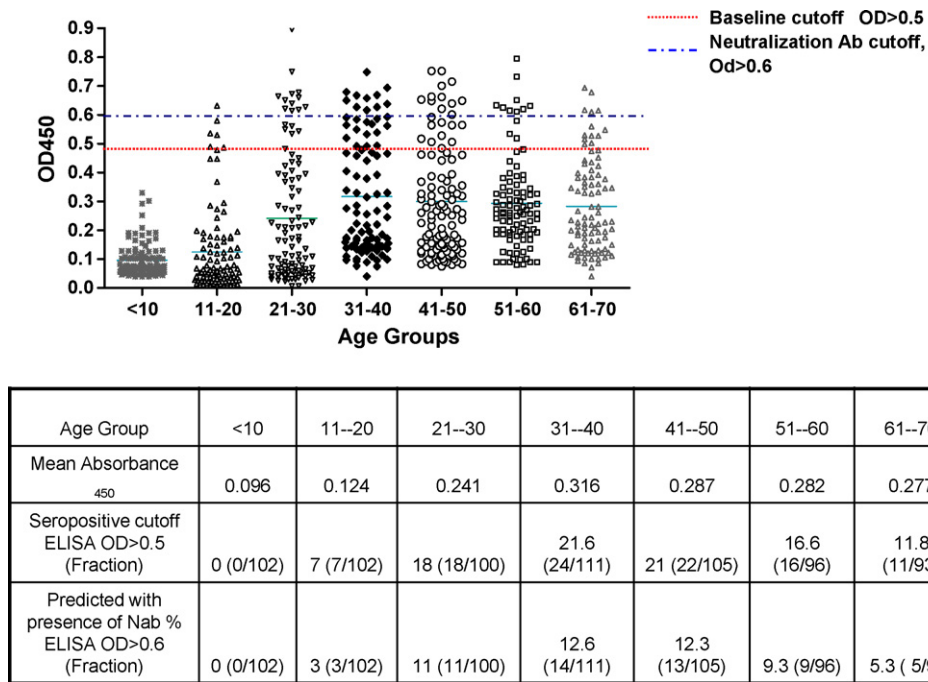


Fig. 6. Seroprevalence of different age groups in HK-SAR were determined by presence of antibodies specific to recombinant HCoV-HKU1 S-based ELISA. Percentage positive above ELISA and neutralization antibodies cutoffs determined previously at OD₄₅₀ > 0.495 and 0.6 among various age groups were shown in the table.

of active respiratory infections. These were categorized into different age groups and analyzed for IgG level against HCoV-HKU1 spike protein by ELISA method as described above (Fig. 6). With reference to cutoff standard determined in previous tests, the mean absorbances and percentage of population predicted with neutralizing antibodies against S protein in each group are shown in Fig. 6. ANOVA analysis showed that there are no significant differences in sample means among age groups of 31–40, 41–50, 51–60 and 61–70.

4. Discussion

HCoV-HKU1, a newly identified human coronavirus, had been consistently detected in the respiratory specimens of patients suffering from respiratory tract infections, in a multitude of studies around the world.^{4,9,13,31,37,39} Its prevalence was found to be generally comparable to the other non-SARS human coronaviruses, such as 229E, OC43 and NL63 in our local population particularly in winter season.^{17,23} Any individual may probably experience coronavirus infections and carry antibodies. This is the first report that examined seroepidemiology and seroprevalence of HCoV-HKU1 including neutralization test as one of the determination parameters.

In the first publication on HCoV-HKU1 an ELISA based antibody test was made against nucleocapsid (N) proteins and seroconversion was observed in index patient.^{25,27,38,40} Its high percentage in sequence conservation results false-positivity renders it not an ideal single marker for serodiagnosis despite its antigenicity.^{25,27,40} In regard to minimize cross-reactivity, we incorporated spike (S) protein as an additional marker which exhibits least exhibition of sequence conservation among coronavirus proteins.^{3,39}

The results of WB analysis support the specificities of both the N and S-based ELISA. No seropositive serum, above the cutoff values, were failed by WB tested by its target antigen. As expected, 28.5% (6/21) of N-based seropositive samples were tested negative by S-based assay which further indicates the inclusion of

double markers is critical in curtailing the false-positive rates and non-specificities.

Our neutralizing results are in general, consistent (73.3%, samples S1–S15) with those obtained by ELISA with scores above the cutoff (0.495). For the 4 neutralization-negative, S-based positive samples (S12–S15), were detected containing low level of S-protein-specific antibodies by binding assays immunofluorescent microscopy and flow cytometry using S-expressed BHK-21 cells (Figs. 4 and 5, Table 3). 100% consistency of the results in the binding assays and our pseudovirus neutralization tests can be achieved if ELISA cutoff was raised to 0.6, as in samples S1–S11. It is justifiable to set the cutoff to a high level to insure as a reliable index in determination the seroprevalence and excludes the false-positivities posed by the presence of other human coronavirus antibodies.^{23,40}

Based on the standard we determined, our results show a rising trend of seroprevalence from age group 11–20, peaks in group from 31–50 (~12%) and declines to 5.3% in age group 61–70, while with no seropositive cases identified in the age group <10 (Fig. 6). This pattern is within our expectations, as the incidence of HCoV-HKU1 infections were found to be relatively low (0.3%) in Hong Kong^{23,39} while other reports were mostly targeted to patients with respiratory symptoms and used RT-PCR for viral detection, which would only identify cases with active disease.^{9,10,11,14,19,21,22,29,31,34,37} In contrast, our study excluded sera from patients with respiratory symptoms, and the detection of specific IgG antibodies would allow a better estimation of the incidence in the wider population. Our study is not truly population-based, especially not including extra-respiratory disease^{9,37} the present results still demonstrates that a substantial adult population has no demonstrable immunity to HCoV-HKU1.

One limitation of the newly developed assay is that we have utilized the S protein from HCoV-HKU1 genotype A only. There are currently three known genotypes (A, B and C) of HCoV-HKU1 in circulation, with the S proteins of genotypes A and B sharing about 84% amino acid similarity. The S protein of genotype C, arising from the recombination of genotypes A and B, is identical to that of genotype B. It is likely that there will be an appreciable degree of cross-

reactivity between the two closely related S proteins and hence the test may pick up some of the patients infected with HCoV-HKU1 genotype B. The incorporation of the S protein of genotype B will be an important area of improvement in the future development of the assay.

The development of a vaccine is possibly the best strategy to protect against HCoV-HKU1 infections in the predominantly non-immune population and reduce the risk of a major outbreak. Recent success in producing infectious full length cDNA clones would pave way for the development of genetically engineered live attenuated protective vaccines.^{34,36} The assays developed in the present work would be valuable for studying the humoral immune response to HCoV-HKU1 and in guiding further drug and vaccine design.

Conflict of interest

The authors do not have a commercial or other association that might pose a conflict of interest.

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