Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Examination of seroprevalence of coronavirus HKU1 infection with S protein-based ELISA and neutralization assay against viral spike pseudotyped virus

C.M. Chan\textsuperscript{a,b,c}, Herman Tse\textsuperscript{a,b,c}, S.S.Y. Wong\textsuperscript{a,b,c}, P.C.Y. Woo\textsuperscript{a,b,c}, S.K.P. Lau\textsuperscript{a,b,c}, L. Chen\textsuperscript{a,b,c}, B.J. Zheng\textsuperscript{a,b,c}, J.D. Huang\textsuperscript{a,b,c}, K.Y. Yuen\textsuperscript{a,b,c,}\textsuperscript{∗}

\textsuperscript{a} State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, Hong Kong
\textsuperscript{b} Research Center of Infection and Immunology, The University of Hong Kong, Hong Kong
\textsuperscript{c} Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, People’s Republic of China

\textbf{A B S T R A C T}

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

\textbf{Objectives:} To investigate the seroepidemiology of HKU1 infections in our local population.

\textbf{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.

\textbf{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 Spearmen’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.

\textbf{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.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Coronavirus HKU1 is a newly identified human coronavirus\textsuperscript{7,20} It has a global distribution and was first reported in Hong Kong, USA, Australia and Europe\textsuperscript{4,13,21,38} 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\textsuperscript{3,6,29} 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\textsuperscript{35,40} Ideal antibody test for a viral infection is the presence of neutralizing antibody.

Neutralizing antibodies were shown to be the long lasting protective immune responses to many viral infections including coronavirus\textsuperscript{12,8,28,33,39,42} The utility of assay based on neutralizing antibodies response against pseudotyped human coronaviruses had been successfully reported\textsuperscript{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 |
|----------------------|-----------|---------------|---------------|
| CCGGGATCCCTGATGGCGAATTGATCA | Forward | pGEX-5X3 | Spike, AA 14–367 |
| ATCTTTTCAATGAGATGAGTCTGTCGACATCGCTGGTGCCACAGGCT | Reverse | pGEX-5X3 | Spike, AA 14–367 |
| CCGGGATCCCTGATGGCGAATTGATCA | Forward | pcDNA 3.1(+) | Full length spike, AA 1–1356 |
| AATGCTGCTGATCATCTTCATCCTG | Reverse | pcDNA 3.1(+) | Full length spike, AA 1–1356 |

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

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. Briefly, expressed N protein was bound to nickel his-trap 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 synthesized 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. 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. 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.

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). 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 (ID50), 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, 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 |
| Positive            | 15 (5%)     | 15/15     | 15/15     | 11/15    |
| Negative            | 6 (2%)      | 276       | 0/6       | 0/6      |
SFV viral particles packaging was achieved by cotransfection with other pSFV helper plasmids encoding SFV structural proteins as cited papers.\(^5\),\(^3\)\(^2\)

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. 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–S15) are samples which were S-based-ELISA positive with OD \( \geq 0.6 \) and O–R (S12–S15) with OD between 0.495 and <0.6.

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). 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 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 unculurable 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 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).
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 | 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    |
|                                    | 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    |
| S-based ELISA positive serum samples | C1           | 0.33       | <25   | 21.3    |
|                                    | C2           | 0.12       | <25   | 9.8     |
| 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.

* 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.

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 Kong23,39 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 disease9,37 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. 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.

Acknowledgements

This work was partly supported by a Research Grants Council General Research Fund grant (781008M), St. Paul’s Hospital Professional Development Fund, HKU Special Research Achievement Award, and HKU Internal Award for CAE Membership.

References

1. Bisht H, Roberts A, Vogel L, Bukreyev A, Collins PL, Murphy BR, et al. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc Natl Acad Sci USA 2004; 101(17):6461–6.
2. Bisht H, Roberts A, Vogel L, Subbarao K, Moss B. Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polyepitope containing an N-terminal segment of the spike glycoprotein. Virology 2005; 334(2):160–5.
3. Bosch BJ, de Haan CA, Snuits SL, Rottier PJ. Spike protein assembly into the coronavirion: exploring the limits of its sequence requirements. Virology 2005; 334(2):306–18.
4. Bosis S, Esposito S, Niester HG, Tremolati E, Pas S, Principi N, et al. Coronavirus HKU1 in an Italian pre-term infant with bronchiolitis. J Clin Virol 2007; 39(6):251–3.
5. Chan CM, Woo PCY, Lau SKP, Tse H, Chen HLI, Li F, et al. Spike protein of human coronavirus HKU1: role in viral life cycle and antibody detection. Exp Biol Med (Maywood) 2008; 233(12):1527–36.
6. Chang LJ, Urlacher V, Iwakuma T, Cui Y, Zucali J. Efficacy and safety analyses of the new human coronavirus HKU1: a report of 6 cases. Vaccine 2007; 25(15):2832–8.
7. Chung JY, Han TH, Kim SW, Kim CK, Hwang ES. Detection of viruses identified recently in children with acute wheezing. J Med Virol 2007; 79(8):1238–43.
8. Du L, Zhao G, He Y, Guo Y, Zheng BJ, Jiang S, et al. Receptor-binding domain of SARS-CoV spike protein induces long-term protective immunity in an animal model. Vaccine 2007; 25(15):2884–9.
9. Espier F, Weibel C, Ferguson D, Landry ML, Kahn JS. Coronavirus HKU1 infection in the United States. Emerg Infect Dis 2006; 12(5):775–9.
10. Esposito S, Bosis S, Niester HG, Tremolati E, Beglia E, Rogno A, et al. Impact of human coronavirus infections in otherwise healthy children who attended an emergency department. J Med Virol 2006; 78(12):1609–15.
11. Freymuth F, Vabret A, Dina J, Petitjean J, Gourarin S. Techniques used for the diagnostic of upper and lower respiratory tract viral infections. Rev Prat 2007; 57(17):1876–82.
12. Fukushima S, Mizutani T, Saigo M, Kuranai I, Taguchi F, Tashiro M, et al. Evaluation of a novel vesicular stomatitis virus pseudotype-based assay for detection of neutralizing antibody responses to SARS-CoV. J Virol Methods 2006; 135(1):1509–12.
13. Garbino J, Crespo S, Aubert JD, Rochat T, Ninet B, Defernez C, et al. A prospective hospital-based study of the clinical impact of non-severe acute respiratory syndrome (Non-SARS)-related human coronavirus infection. Clin Infect Dis 2006; 43(8):1009–15.
14. Gerna G, Percivalle E, Sarasin A, Campanini C, Piralla A, Rovida F, et al. Human respiratory coronavirus H1KU1 and other coronavirus infections in Italian hospitalised patients. J Clin Virol 2007; 38(3):244–50.
15. Hofmann H, Pyrc K, van der Hoeck L, Geier M, Berkhout B, Pohlamann S. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proc Natl Acad Sci USA 2005; 102(22):7988–93.
16. Holmes KV. Coronaviruses. In: Fields BN, Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, editors. Fields Virology. 4th ed. Philadelphia: Lippincott, Williams & Wilkins; 2001. p. 1187–203.
17. Kistler A, Aelia PC, Roaskin S, Wang D, Ward T, Vagi S, et al. Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. J Infect Dis 2007; 196(6):817–25.
18. Kupfer B, Simon A, Jonassen CM, Viazos S, Ditt V, Tillmann RL, et al. Two cases of severe obstructive pneumonia associated with an HKU1-like coronavirus. Eur J Med Res 2007; 12(3):134–8.
19. Kuypers J, Martin ET, Heugel J, Wright N, Morrow R, Enghlund JA. Clinical disease in children associated with newly described coronavirus subtypes. Pediatrics 2007; 119(1):e70–76.
20. Kwan LC, Ho YY, Lee SS. The declining HBsAg carriage rate in pregnant women in Hong Kong. Epidemiol Infect 1997; 119(2):281–3.
21. Lau SK, Wuo PC, Yip CC, Tse H, Tsoi HW, Cheng VC, et al. HKU1 and other coronavirus infections in Hong Kong. J Clin Microbiol 2006; 44(6):2063–71.
22. Maache M, Komurian-Pradel F, Rajaharson A, Perret M, Berland JL, Pouzoil S, et al. Corrective effects of TK-90 in a recombinant Ouracise respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid-based western blot assay were rectified by the use of two subunits (S1 and S2) of spike for detection of antibody to SARS-CoV. J Clin Vaccine Immunol 2006; 13(3):409–14.
23. Ndhuma A, Waters AK, Zhou M, Collison DW. Recombinant nucleocapsid protein is potentially an inexpensive, effective serodiagnostic reagent for infectious bronchitis virus. J Virol Methods 2006; 197(1):37–44.
24. Nie Y, Yang W, Shi X, Zhang H, Qiu Y, He Z, et al. Neutralizing antibodies in patients with severe acute respiratory syndrome-associated coronavirus infection. J Infect Dis 2004; 190(6):1119–26.
25. Piacentini A, Gentile M, Di Marco P, Pagnotti P, Scagnolari C, Trombetti S, et al. Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome Italy. J Med Virol 2007; 79(4):463–8.
26. Sirodot MP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 2006; 35(1):99–102.
27. Sivridis D, Liljestrom P. Two-helper RNA system for production of recombinant Semliki forest virus particles. J Virol 1999; 73(2):1092–8.
28. Srivastava RK, Ulmer JB, Barnett SW. Role of neutralizing antibodies in protective immunity against HIV. Hum Vaccine 2005; 1(2):45–60.
29. St-Jean JR, Desforges M, Almazan F, Jacomy H, Enjuanes L, Talbot PJ. Recovery of a neurovirulent human coronavirus OC43 from an infectious cDNA clone. J Virol 2006; 80(7):3670–4.
30. Temperton NJ, Chan PK, Simmons G, Zambon MC, Tedder RS, Takeuchi Y, et al. Longitudinally profiling neutralizing antibody response to SARS coronavirus with pseudotypes. Emerg Infect Dis 2005; 11(2):413–9.
31. Thiel V, Herold J, Schelle B, Siddell SG. Influenza virus in vitro from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. J Gen Virol 2001; 82(1):727–81.
32. Vabret A, Dina J, Gourarin S, Petitjean J, Corbet S, Freymuth F. Detection of the new human coronavirus HKU1: a report of 6 cases. Clin Infect Dis 2006; 42(5):634–9.
33. Wootz PC, Lai SK, Chu CM, Chan KH, Tsoi HW, Huang Y, et al. Characterization and complete genome sequence of a novel coronavirus, human coronavirus HKU1, from patients with pneumonia. J Virol 2005; 79(2):884–95.
34. Wootz PC, Lai SK, Tsoi HW, Huang Y, Poon RW, Chu CM, et al. Clinical and molecular epidemiological features of coronavirus HKU1-associated community-acquired pneumonia. J Infect Dis 2005; 192(11):1856–907.
35. Wootz PC, Lai SK, Wong BH, Chan KH, Hui WT, Kwan GS, et al. False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OCA3 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide. J Clin Microbiol 2004; 42(12):3985–8.
36. Zhou T, Wang H, Luo D, Rowe T, Wang Z, Hogan RJ, et al. An exposed domain in the severe acute respiratory syndrome coronavirus spike protein induces neutralizing antibodies. J Virol 2004; 78(13):7217–26.