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Cross-reactive antibodies in convalescent SARS patients' sera against the emerging novel human coronavirus EMC (2012) by both immunofluorescent and neutralizing antibody tests

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Summary *Objectives:* A severe acute respiratory syndrome (SARS)-like disease due to a novel betacoronavirus, human coronavirus EMC (HCoV-EMC), has emerged recently. HCoV-EMC is phylogenetically closely related to *Tylonycteris*-bat-coronavirus-HKU4 and *Pipistrellus*-bat-coronavirus-HKU5 in Hong Kong. We conducted a seroprevalence study on archived sera from 94 game-food animal handlers at a wild life market, 28 SARS patients, and 152 healthy blood

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Seroprevalence

donors in Southern China to assess the zoonotic potential and evidence for intrusion of HCoV-EMC and related viruses into humans.
Methods: Anti-HCoV-EMC and anti-SARS-CoV antibodies were detected using screening indirect immunofluorescence (IF) and confirmatory neutralizing antibody tests.
Results: Two (2.1%) animal handlers had IF antibody titer of $\geq$1:20 against both HCoV-EMC and SARS-CoV with neutralizing antibody titer of $<$1:10. No blood donor had antibody against either virus. Surprisingly, 17/28 (60.7%) of SARS patients had significant IF antibody titers with 7/28 (25%) having anti-HCoV-EMC neutralizing antibodies at low titers which significantly correlated with that of HCoV-OC43. Bioinformatics analysis demonstrated a significant B-cell epitope overlapping the heptad repeat-2 region of Spike protein. Virulence of SARS-CoV over other betacoronaviruses may boost cross-reactive neutralizing antibodies against other betacoronaviruses.
Conclusions: Convalescent SARS sera may contain cross-reactive antibodies against other betacoronaviruses and confound seroprevalence study for HCoV-EMC.

Introduction

The emergence of the novel human coronavirus EMC (HCoV-EMC) in the Middle East since April 2012 has so far led to 17 cases of human infection with 11 being fatal as of 26 March 2013.[1–3] The first 2 laboratory-confirmed cases were reported by the World Health Organization (WHO) on 23 September 2012.[1] The index case was a 60-year-old man from Jeddah, the Kingdom of Saudi Arabia, who presented with severe acute community-acquired pneumonia and acute renal failure on 6 June 2012 and later succumbed on 24 June 2012 despite maximal supportive treatment.[1,4] A sputum sample obtained on admission showed cytopathic changes suggestive of virus replication in LLC-MK2 and Vero cells, and was positive for coronavirus by pan-coronavirus RT-PCR. Subsequent phylogenetic analysis of the viral genome sequences showed that the virus was a novel coronavirus with close genetic relatedness to *Tylonycteris*-bat-coronavirus-HKU4 (Ty-BatCoV-HKU4) and *Pipistrellus*-bat-coronavirus-HKU5 (Pi-BatCoV-HKU5) discovered in the lesser bamboo bat (*Tylonycteris pachypus*) and Japanese Pipistrelle bat (*Pipistrellus abramus*) of Hong Kong, China respectively.[4–7] Closely related coronaviruses have also been found in other bat species in Europe and Ghana.[8,9] The second case was a 49-year-old man from Qatar who kept camels and sheep in his farm and had a travel history to the Kingdom of Saudi Arabia before symptom onset.[1,10] He developed severe acute community-acquired pneumonia and acute renal failure requiring extracorporeal membrane oxygenation in an intensive care unit of London. The lower respiratory tract samples were positive for coronavirus using pan-coronavirus RT-PCR. The 250 bp PCR fragments of the viral isolates in the first 2 cases showed 99.5% sequence homology with only 1 nucleotide mismatch over the regions compared.[10] Subsequently, 15 more laboratory-confirmed cases of HCoV-EMC infection were reported in the Middle East and the United Kingdom with a total of 9 in the Kingdom of Saudi Arabia, 2 in Qatar, 2 in Jordan, 1 in United Arab Emirates and 3 in the United Kingdom.[2,3] Most of the cases developed severe pneumonia, at least 6 cases had concomitant acute renal failure, and 11 cases died. This unusually high crude fatality rate of over 50% and the severe clinical manifestations of acute respiratory and renal failure are unique among human coronavirus infections.[11–18]

The source, transmissibility and seroprevalence of HCoV-EMC are not well established at present. As with other highly pathogenic viruses which are capable of causing epidemics such as SARS coronavirus (SARS-CoV) and avian H5N1 influenza A virus, an animal source of the virus leading to interspecies jumping to humans is possible.[7,11,19–22] This hypothesis is supported by the epidemiological link to animal exposure in some of these patients with laboratory-confirmed HCoV-EMC infection,[1,3] the close phylogenetic relatedness between HCoV-EMC and Ty-BatCoV HKU4 and Pi-BatCoV HKU5,[5,6] and the broad species tropism of HCoV-EMC in different animal cells including bat, primate, swine, civet, and rabbit.[23,24] Human-to-human transmission appears to be limited at this stage with only 4 epidemiologically-linked clusters being identified so far. The Jordanian cluster was retrospectively traced back to April 2012 with no further evidence of spread. Moreover, none of 2400 residents in the Kingdom of Saudi Arabia had serum antibody against HCoV-EMC.[4] Thus, HCoV-EMC is likely different from other human coronaviruses associated with mild respiratory tract infections, namely HCoV-OC43, HCoV-229E, HCoV-NL63 and HCoV-HKU1 which account for 5–30% of all respiratory infections with up to 21.6% of the general population having serum antibodies.[25,26] Rather, it may be similar to SARS-CoV which crossed species barriers from its natural bat reservoir to intermediate amplification animal hosts and humans and caused severe infection or subclinical non-pneumonic infection in about 0.5% of the general population.[12]

In order to further substantiate the hypothesis of HCoV-EMC being a zoonotic agent and elicit evidence for intrusion of HCoV-EMC and its related viruses into humans, we studied the antibody titers using immunofluorescence (IF) as screening and neutralization as confirmatory tests in at-risk groups working in a wild life market in Guangzhou of Southern China who were constantly exposed to a wide range of game food animals, SARS patients who might have acquired their infection directly from wild animals, and healthy blood donors.

Materials and methods

The study was approved by the Institutional Review Board of the Hospital Authority in Hong Kong.

Subjects and sera

Archived sera obtained from 94 subjects belonging to at-risk groups working in a wild life market in Guangzhou, 28

patients with laboratory-confirmed SARS by RT-PCR, and 152 healthy blood donors in Hong Kong Special Administrative Region, Southern China were retrieved from −70 °C refrigerator. The at-risk groups consisted of game food animal market retailers, animal slaughterers and animal transporting personnel. All subjects were aged 18 years or above. The 94 animal handlers had a mean age of 35.4 years (range, 19—76 years), and the male-to-female ratio was 60:34. All of them had exposure to live and/or dead chickens, ducks, geese, pigeons, sparrows, seagulls, turtledoves, cranes, foxes, wild boars, sika deers, rabbits, and/or cats. Their average exposure time was 3.91 years (range, 1 month to 16 years).

Viral isolate

A clinical isolate of HCoV-EMC was kindly provided by Fouchier and Zaki et al.[4] The isolate was amplified by one additional passage in Vero cell lines to make working stocks of the virus. All experimental protocol involving live HCoV-EMC coronavirus isolate followed the standard operating procedures of the approved biosafety level-3 facility as we previously described.[27]

Preparation of antigens of human betacoronaviruses as infected cell smears

HCoV-EMC and SARS-CoV-infected Vero, HCoV-OC43-infected BSC-1, HCoV-229E-infected MRC-5 and HCoV-NL63-infected LLC-MK2 cell smears were used for the study. Smears were prepared as we previously described.[28] Briefly, when 60%—70% of cells had early evidence of cytopathic effect (CPE) as shown by rounding up of cells under inverted microscopy, the cells were harvested by trypsinization and air dried on Tefllon slides (Immuno-cell Int, Mechelen, Belgium), and fixed with chilled acetone for 10 min at −20 °C and were stored at −80 °C until use.

Indirect immunofluorescent antibody test

(Fig. 1) Anti-HCoV-EMC and anti-SARS-CoV IF antibody detection was performed using indirect IF as we previously described with slight modifications.[28] Sera were screened at a dilution of 1 in 20 on infected and non-infected control cells at 37 °C for 45 min. The cells were washed twice in PBS for 5 min each time. Anti-human IgG (INOVA Diagnostic, San Diego) were then added and the cell smears further incubated for 45 min at 37 °C. Sera positive at a screening dilution of 1 in 20 were further titrated with serial 2-fold dilutions. A positive result was scored when fluorescent intensity equaled or was higher than that of a positive control used in our previous studies.[28—32] For HCoV-EMC antibody testing, Vero cells were infected with 0.01 MOI for 36—40 h before harvesting. The infected cells were then coated on Teflon slides 8-well, air dried and fixed with chilled acetone at 20 °C for 10 min, and kept at −80 °C until use. Guinea pig anti-N hyper-immune sera were prepared as positive controls for testing with each new batch of infected and non-infected cells together with non-immune guinea pig sera as a negative control.[23] Positive and negative guinea control sera were included in each run of antibody testing. The IF antibody titer was taken to be the highest serum dilution giving a positive result. Anti-HCoV-OC43 IF antibody titers were further determined for sera with positive anti-HCoV-EMC IF antibody titers.

Neutralizing antibody test

All sera were inactivated at 56 °C for 30 min before neutralizing antibody test. Starting with a serum dilution

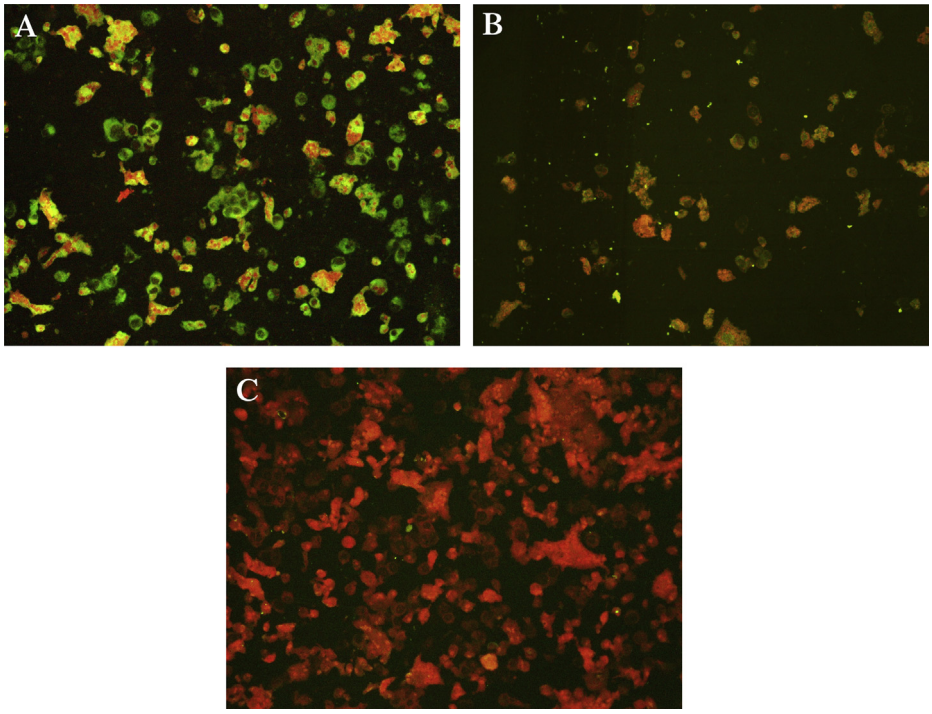

Figure 1 Indirect immunofluorescent antibody test for anti-HCoV-EMC IgG. (1A): positive; (1B): borderline; (1C): negative.

of 1 in 10, serial 2-fold dilutions of sera were prepared in 96-well microtiter plates as we have previously described.[28] Each serum dilution of 0.05 ml was mixed with 0.05 ml of 200 50% tissue culture infectious doses ($TCID_{50}$) of HCoV-EMC or SARS-CoV (HK39849), and incubated at 37 °C for 1.5 h in a CO_2 incubator. Then 0.1 ml of the virus-serum mixture was inoculated in duplicate wells of 96-well microtiter plates with preformed monolayers of Vero cells and further incubated at 37 °C for 3—4 days. A virus back-titration was performed to assess the actual virus titer used in each experiment. CPE was observed using an inverted microscope on day 3 and 4 post-inoculation. The neutralizing antibody titer was determined as the highest dilution of serum which completely suppresses the CPE in at least half of the infected wells. The experiment was read when the virus back-titration showed the virus dose to be 100 $TCID_{50}$ as expected. Mouse anti-whole HCoV-EMC hyper-immune sera were used as positive controls. All sera with positive neutralizing antibody titers were repeated for confirmation. Anti-HCoV-OC43 neutralizing antibody titers were further determined for sera with positive HCoV-EMC IF antibody titers.

Bioinformatic analysis of spike proteins

Amino acid sequences of the S proteins of HCoV-EMC, SARS-CoV, HCoV-OC43 and HCoV-HKU1 were downloaded from NCBI GenBank. Structure-based sequence alignment of the S1 and S2 domains of HCoV-EMC, SARS-CoV, HCoV-OC43 and HCoV-HKU1 were performed by PROMALS3D server.[33] Immunogenic regions containing potential human B-cell epitopes were predicted using Epitopia.[34] The transmembrane domain preceding the cytoplasmic tail was predicted using TMHMM version 2.0.[35] Heptad repeat regions within the S2 domains were predicted using MARCOIL.[36]

Statistical analysis

Fisher exact test was used to determine the differences in proportion of the 3 groups with positive antibody titers by IF and NT between animal handlers and healthy blood donors, SARS patients and healthy blood donors, and animal handlers and SARS patients. Computation was performed using the Predictive Analytics Soft Ware (PASW) Version 18.0 for Windows. Correlation between the IF and neutralizing antibody titers against HCoV-EMC, SARS-CoV and HCoV-OC43 was performed using IBM SPSS Statistics 19, with titers of <1:20 and <1:10 regarded as 1:10 and 1:5 respectively. A p-value of <0.05 was considered as statistically significant.

Results

Indirect IF and neutralizing antibody titers

Two of 94 (2.1%) animal handlers working at a wild game food animal market in South China had positive anti-HCoV-EMC IgG detected by indirect IF with titer of 1:20 and 1:40 (Table 1). Case 1 was a 38-year-old man with exposure to pigeons for more than 2 years. Case 2 was a 39-year-old man with exposure to chickens, ducks, and geese for more than 3 years. Both of them also had positive anti-SARS-CoV IgG by indirect IF with a titer of 1:40 and anti-HCoV-OC43 IgG with titers >=1:320 (Table 2). Case 2 who had adequate archived serum for testing of anti-HCoV-OC43 neutralizing antibody had a titer of 1:80. Another 11 animal handlers had positive anti-SARS-CoV IgG by indirect IF and 4 of them had anti-SARS-CoV neutralizing antibodies (Table 1). None of the animal handlers had anti-HCoV-EMC neutralizing antibody.

Among the 28 SARS patients, 17 (60.7%) had positive anti-HCoV-EMC IgG detected by indirect IF with titers ranging from 1:20 to 1:320 (Table 1). Most had a titer between 1:80 to 1:160 (6/28 or 21.4% each). All 17 patients had anti-HCoV-OC43 IgG detected by indirect IF (Table 2). Surprisingly, 7 (25%) of the SARS patients also had low titers of anti-HCoV-EMC neutralizing antibody of 1:20 or less, and all 17 of them had anti-HCoV-OC43 neutralizing antibodies. Anti-SARS-CoV IF and neutralizing antibodies were found in the majority (96.4%) of the SARS patients as expected. Most of them had high titers of 1:80 or above. Four of the 28 SARS patients had paired acute and convalescent sera available for comparison (Table 3). The anti-HCoV-EMC IF IgG titer rose from <1:20 in the acute sera to 1:40 and 1:320 in the convalescent sera in 2 of these patients, while there was no significant rise in the other two. These patients also had 4-fold rise in IF antibody titer against another human betacoronavirus HCoV-OC43.

None of 152 (0%) healthy blood donors had anti-HCoV-EMC or anti-SARS-CoV antibodies by indirect IF and neutralization (Table 1). There was an overall significant correlation between the indirect IF IgG titers against HCoV-EMC and SARS-CoV (Pearson correlation 0.587, $p < 0.01$), and between the neutralizing antibody titers against HCoV-EMC and SARS-CoV (Pearson correlation 0.422, $p < 0.01$). For subgroup analysis of SARS patients with positive anti-HCoV-EMC IF and/or neutralizing antibodies, the correlation was strongest between antibodies against SARS-CoV and HCoV-OC43 (Pearson correlation 0.593 and 0.605 for IF and neutralizing antibodies respectively; $p < 0.01$ in both cases).

Bioinformatic analysis of spike proteins

While there was little amino acid sequence identity (16.6%) between the receptor-binding domain in the S1 proteins of HCoV-EMC and SARS-CoV, their S2 proteins showed an amino acid sequence identity of 40.3%. Epitopia was used to predict immunogenic regions that might be B-cell epitopes in the S1 and S2 domains.[34] While epitopes were predicted in aligned regions of S1 from HCoV-EMC and SARS-CoV, it is unlikely that cross-neutralization by antibodies would occur in these regions as the sequence identity of the predicted epitopes between the two viruses is low (Fig. 2). Three and two immunogenic regions were predicted in the S2 domains of HCoV-EMC and SARS-CoV respectively (Fig. 3). The immunogenic regions identified in S2 of HCoV-EMC overlapped the predicted regions in S2 of SARS-CoV. Notably, the identified immunogenic regions sars-I and emc-II overlapped the heptad repeat 2 region of the S2 domain of both HCoV-EMC and SARS-CoV, which is known to harbor an epitope for broadly neutralizing antibody in the case of SARS-CoV.[37]

Table 1 Titers of anti-HCoV-EMC and anti-SARS-CoV antibodies by immunofluorescence and neutralization among animal handlers, SARS patients and healthy blood donors.

	HCoV-EMC IF		HCoV-EMC NT		SARS-CoV IF		SARS-CoV NT	
Animal handlers	<1:20	92 (97.9%)	<1:10	94 (100%)	<1:20	81 (86.2%)	<1:10	90 (95.7%)
($n = 94$)	1:20	1 (1.1%)	1:10	0 (0%)	1:20	6 (6.4%)	1:10	1 (1.1%)
	1:40	1 (1.1%)	1:20	0 (0%)	1:40	7 (7.4%)	1:20	3 (3.2%)
	1:80	0 (0%)	1:40	0 (0%)	1:80	0 (0%)	1:40	0 (0%)
	1:160	0 (0%)	1:80	0 (0%)	1:160	0 (0%)	1:80	0 (0%)
	≥1:320	0 (0%)	≥1:160	0 (0%)	≥1:320	0 (0%)	≥1:160	0 (0%)
SARS patients	<1:20	11 (39.3%)	<1:10	21 (75.0%)	<1:20	1 (3.6%)	<1:10	1 (3.6%)
($n = 28$)	1:20	1 (3.6%)	1:10	5 (17.9%)	1:20	0 (0%)	1:10	0 (0%)
	1:40	3 (10.7%)	1:20	2 (7.1%)	1:40	0 (0%)	1:20	1 (3.6%)
	1:80	6 (21.4%)	1:40	0 (0%)	1:80	0 (0%)	1:40	0 (0%)
	1:160	6 (21.4%)	1:80	0 (0%)	1:160	5 (17.9%)	1:80	13 (46.4%)
	≥1:320	1 (3.6%)	≥1:160	0 (0%)	≥1:320	22 (78.6%)	≥1:160	13 (46.4%)
Healthy blood donors	<1:20	152 (100%)	<1:10	152 (100%)	<1:20	152 (100%)	<1:10	152 (100%)
($n = 152$)	1:20	0 (0%)	1:10	0 (0%)	1:20	0 (0%)	1:10	0 (0%)
	1:40	0 (0%)	1:20	0 (0%)	1:40	0 (0%)	1:20	0 (0%)
	1:80	0 (0%)	1:40	0 (0%)	1:80	0 (0%)	1:40	0 (0%)
	1:160	0 (0%)	1:80	0 (0%)	1:160	0 (0%)	1:80	0 (0%)
	≥1:320	0 (0%)	≥1:160	0 (0%)	≥1:320	0 (0%)	≥1:160	0 (0%)
No. of patients with significant antibody titer[a]								
Animal handlers vs SARS patients	2/94 vs 17/28	$p < 0.01$	0/94 vs 7/28	$p < 0.01$	13/94 vs 27/28	$p < 0.01$	4/94 vs 27/28	$p < 0.01$
Animal handlers vs blood donors	2/94 vs 0/152	$p = 0.15$	0/94 vs 0/152	$p = 1.0$	13/94 vs 0/152	$p < 0.01$	4/94 vs 0/152	$p = 0.02$
SARS patients vs blood donors	17/28 vs 0/152	$p < 0.01$	7/28 vs 0/152	$p < 0.01$	27/28 vs 0/152	$p < 0.01$	27/28 vs 0/152	$p < 0.01$

IF, immunofluorescence; NT, neutralization.
[a] Antibody titer ≥20 for immunoflourescence assay and ≥10 for neutralization assay.

Discussion

While looking for evidence of intrusion by the novel betacoronavirus HCoV-EMC into at-risk groups and the general population, convalescent SARS patients' sera were found to contain significant titers of antibodies against other betacoronaviruses. There was a positive correlation between the antibody titers against the SARS-CoV and HCoV-EMC using both the indirect IF and neutralization antibody tests. The finding of cross-reactive IF antibodies was not that unexpected because these could be induced by cross-reactive epitopes against structural proteins such as the nucleoprotein which is the most abundant structural protein in the coronaviruses as we had previously reported.[38] Indeed, cross-reactive antibodies among human betacoronaviruses by IF are well known, and have made large scale surveillance studies and epidemiologic surveys of human coronavirus infections difficult.[39] On the other hand, cross-reactive neutralizing antibodies among betacoronaviruses have rarely been reported except between the closely related human and palm civet SARS-CoVs.[40] The significant neutralizing antibody titers against HCoV-EMC in SARS patients' sera in this study were surprising because neutralization is generally considered as the most specific serological test. Our previous surveillance study showed that anti-SARS-CoV neutralizing antibody in our population was extremely low despite a high seroprevalence of anti-HCoV-OC43 and anti-HCoV-HKU1 antibodies.[12] Zaki and colleagues also failed to detect cross-reactive anti-HCoV-EMC antibodies among 2400 patients in the Kingdom of Saudi Arabia who likely also had serum anti-HCoV-OC43 and/or anti-HCoV-HKU1 antibodies. Furthermore, none of the 152 healthy blood donors in the present study had serum anti-HCoV-EMC antibodies detected by indirect IF and neutralization. Therefore we assessed the structural homologies between these betacoronaviruses for possible explanations of the observed cross-reactive neutralizing antibodies.

Of all the surface proteins, only the ectodomains of S (spike) and Orf3a can induce significant neutralizing antibody with some augmentation from the M (matrix) and E (envelope) proteins.[41,42] Though Orf3a is absent in HCoV-EMC, we cannot completely exclude the possibility that similar Orf3a-like proteins are being coded by the accessory protein gene but homology search does not reveal the presence of similar protein. All betacoronaviruses use the S protein for attachment and fusion of the virion with the host cell membrane. Trimers of the S protein form the peplomers that radiate from the lipid envelope and give the virus a characteristic corona solis-like appearance under the electron microscope. The spike protein ectodomain

Table 2 Titers of anti-HCoV-EMC and anti-SARS-CoV antibodies by immunofluorescence and neutralization among animal handlers and SARS patients with positive immunofluorescent anti-HCoV-EMC antibodies.

	HCoV-EMC IF	HCoV-EMC NT	SARS-CoV IF	SARS-CoV NT	HCoV-OC43 IF	HCoV-OC43 NT
Animal handlers ($n = 2$)						
Case 1	1:20	<1:10	1:40	<1:10	1:640	Not available[c]
Case 2	1:40	<1:10	1:40	<1:10	1:320	1:80
SARS patients ($n = 17$)						
Case 1	1:20	1:20	1:160	1:80	1:320	1:160
Case 2	1:40	<1:10	1:320	1:80	1:640	1:160
Case 3[a]	1:40	<1:10	1:640	1:80	1:640	1:80
Case 4	1:40	<1:10	1:1280	1:80	1:320	1:160
Case 5	1:80	1:10	1:320	1:80	1:320	1:80
Case 6	1:80	1:10	1:640	1:160	1:320	1:40
Case 7	1:80	1:10	1:640	1:160	1:640	1:320
Case 8	1:80	<1:10	1:640	1:80	1:640	1:160
Case 9	1:80	1:10	1:1280	1:320	1:640	1:320
Case 10	1:80	<1:10	1:1280	1:80	1:320	1:80
Case 11	1:160	<1:10	1:320	1:80	1:320	1:80
Case 12	1:160	1:20	1:640	1:160	1:1280	1:160
Case 13	1:160	1:10	1:1280	1:160	1:640	1:160
Case 14	1:160	<1:10	1:1280	1:160	1:640	1:80
Case 15	1:160	<1:10	1:2560	1:160	1:1280	1:80
Case 16	1:160	<1:10	1:2560	1:160	1:2560	1:320
Case 17[b]	1:320	<1:10	1:160	1:80	1:1280	1:160

IF, immunofluorescence; NT, neutralization.
[a] Case 3 in Table 2 and Case C (convalescent) in Table 3 were the same specimens.
[b] Case 17 in Table 2 and Case D (convalescent) in Table 3 were the same specimens.
[c] Test was not performed due to insufficient quantity of archived sera.

consists of the S1 and S2 domains. The S1 domain contains the receptor binding domain and is responsible for recognition and binding to the host cell receptor. The S1 fragment between amino acids 318 and 510 is the receptor binding domain for ACE2 in the case of SARS-CoV. However, the homology of S1 between SARS-CoV and HCoV-EMC is low with only 16.6% amino acid identity. Indeed, this region is generally more divergent relative to the S2 region for coronaviruses. Hence, while the S1 region induces the majority of the neutralizing antibody in convalescent sera of SARS patients,[43,44] it would be unlikely to result in antibodies with significant cross-neutralizing activity.

The S2 domain, responsible for fusion, contains the putative fusion peptide and the heptad repeat HR1 and HR2. The binding of S1 to the cellular receptor will trigger conformational changes which collocates the fusion peptide upstream of the two heptad repeats of S2 to the transmembrane domain, and, finally, fusion of the viral and cellular lipid envelopes. An epitope situated between amino acids 1055 to 1192 and around heptad repeat 2 of the S2 subunit is likely to have induced the cross-reactivity of neutralizing antibody against HCoV-EMC and SARS-CoV.[63] Our phylogenetic and antigenic epitope analysis suggested that this area is highly conserved among these 4

Table 3 Titers of anti-human-coronaviruses antibodies by immunofluorescence and/or neutralization in SARS patients with available paired acute and convalescent serum samples.

	HCoV-EMC IF	HCoV-EMC NT	SARS-CoV IF	SARS-CoV NT	HCoV-OC43 IF	HCoV-229E IF	HCoV-NL63 IF
SARS patients with paired sera ($n = 4$)							
Case A (acute)	<1:20	<1:10	<1:20	<1:10	1:80	1:80	<1:20
Case A (convalescent)	<1:20	<1:10	1:160	1:20	1:160	1:40	<1:20
Case B (acute)	1:20	<1:10	<1:20	<1:10	1:80	1:20	1:40
Case B (convalescent)	<1:20	<1:10	1:160	1:160	1:640	1:20	1:20
Case C (acute)	<1:20	<1:10	<1:20	<1:10	1:160	1:40	<1:20
Case C[a] (convalescent)	1:40	<1:10	1:640	1:80	1:640	1:20	<1:20
Case D (acute)	<1:20	<1:10	<1:20	<1:10	1:160	1:20	<1:20
Case D[b] (convalescent)	1:320	<1:10	1:160	1:80	1:1280	1:80	1:20

IF, immunofluorescence; NT, neutralization.
[a] Case C (convalescent) in Table 3 and Case 3 in Table 2 were the same specimens.
[b] Case D (convalescent) in Table 3 and Case 17 in Table 2 were the same specimens.

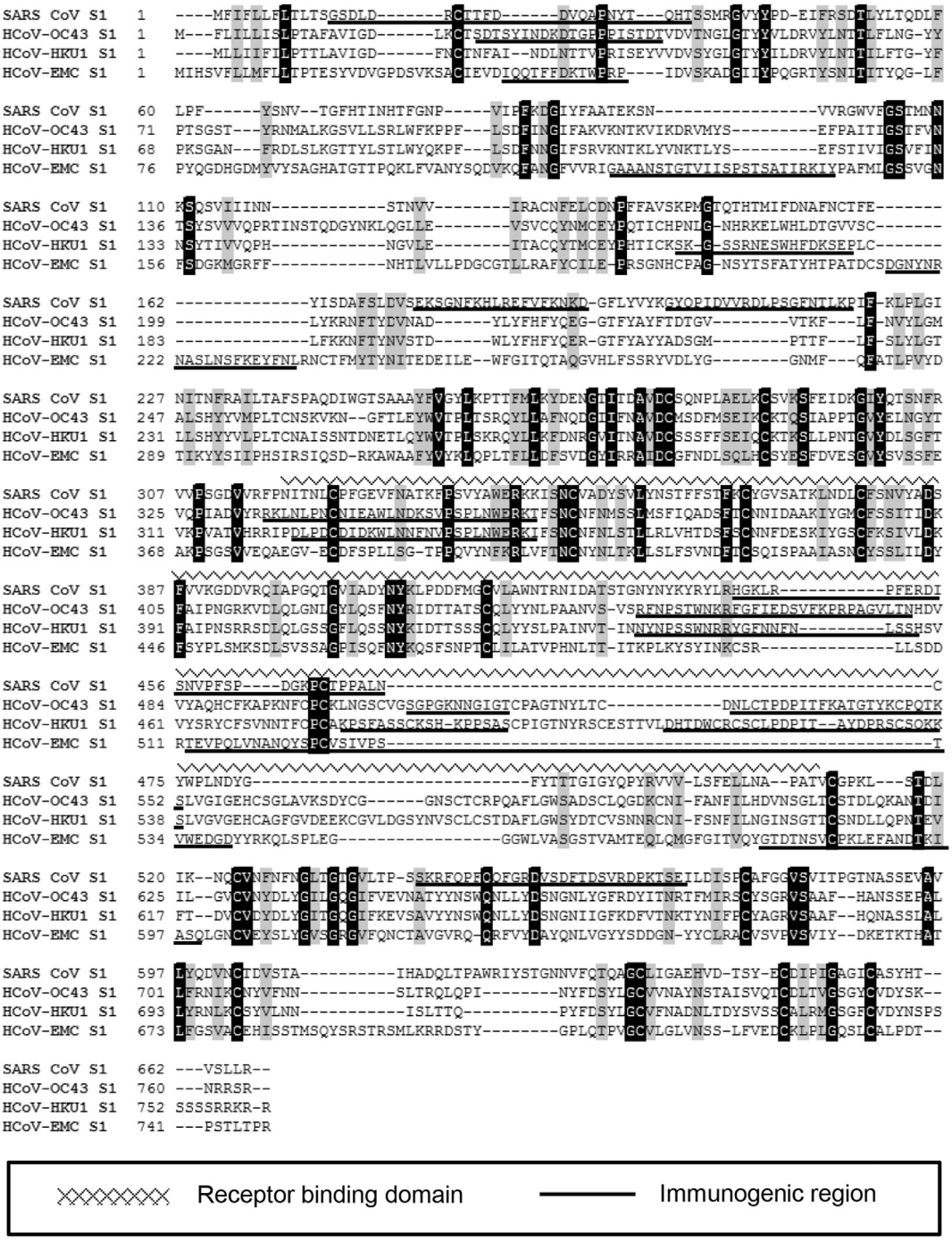

Figure 2 Structure-based protein sequence alignment of the S1 region of HCoV-EMC, SARS-CoV, HCoV-OC43 and HCoV-HKU1, constructed using PROMALS3D (http://prodata.swmed.edu/promals3d/). The receptor binding domain is highlighted. Identical and similar residues are shaded in black and grey respectively. Immunogenic regions predicted by Epitopia of at least 10 residues in length are highlighted by a black line. Only 1 representative sequence from each virus is used to improve clarity of presentation.

betacoronaviruses and therefore could not completely explain the presence of cross-reactive anti-HCoV-EMC neutralizing antibodies among SARS patients but not the general population. We postulate that in addition to the structural homologies between HCoV-EMC, SARS-CoV, HCoV-OC43 and HCoV-HKU1, the different clinical manifestations and subsequent host immunological response of these infections may account for this pattern of neutralizing antibody cross-reactivity. While SARS-CoV causes severe infection with viremia,[45] HCoV-OC43 and HCoV-HKU1 predominantly cause superficial mucosal infections of the upper respiratory tract which is self-limiting. Therefore unlike the highly virulent SARS-CoV or HCoV-EMC which can induce a solid humoral immune response, an insufficient B cell maturation process with failure to induce high avidity antibodies is more likely to occur with

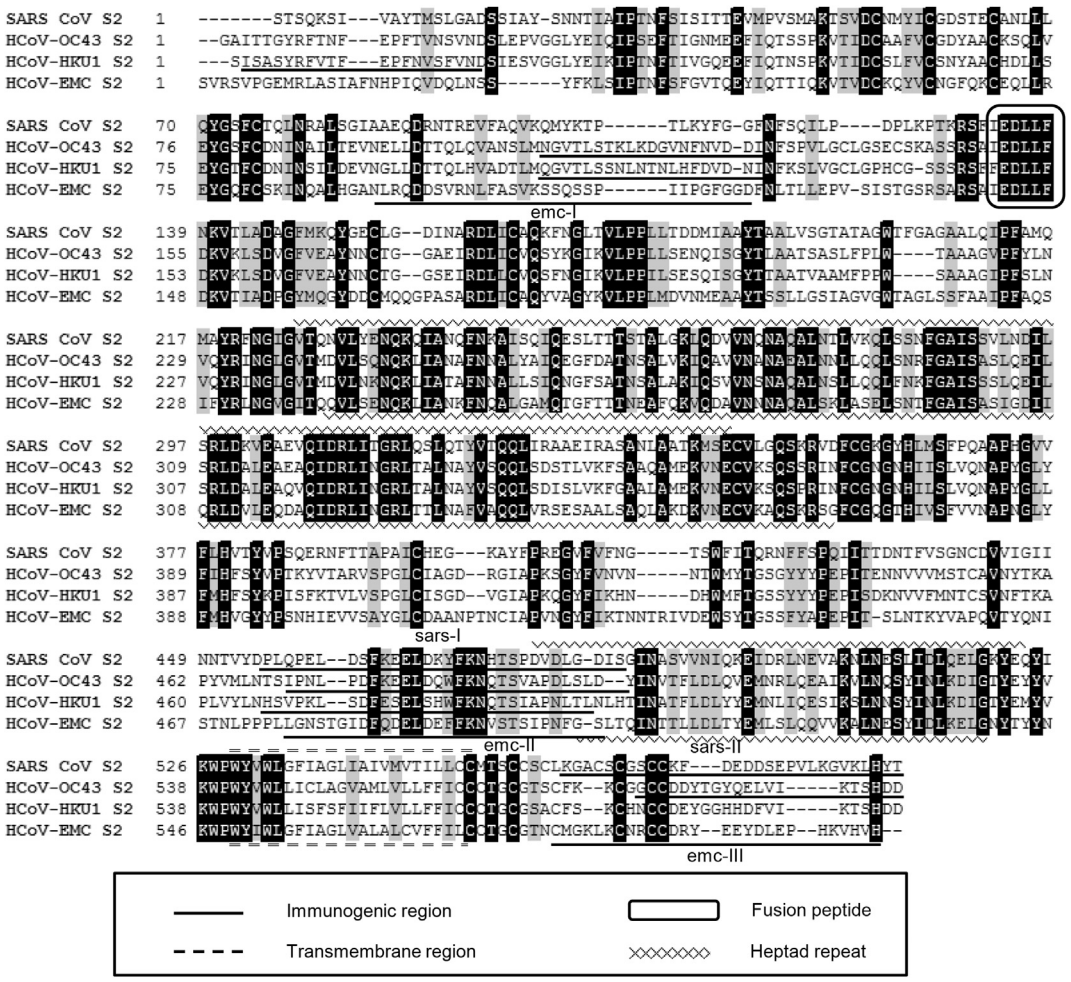

Figure 3 Structure-based protein sequence alignment of the S2 region of HCoV-EMC, SARS-CoV, HCoV-OC43 and HCoV-HKU1 constructed using PROMALS3D (http://prodata.swmed.edu/promals3d/). Identical and similar residues are shaded in black and grey respectively. Immunogenic regions predicted by Epitopia of at least 20 residues in length are highlighted by a black line. The heptad repeat regions are highlighted. Only 1 representative sequence from each virus is used to improve clarity of presentation.

other betacoronavirus infections in the general population but their neutralizing antibody titer against these less virulent betacoronaviruses such as HCoV-OC43 can be boosted with superimposed SARS-CoV or HCoV-EMC infections (Table 2). These viral, clinical and immunological differences may explain the absence of cross-reactive neutralizing antibody against both SARS-CoV and HCoV-EMC in normal blood donors despite that most of them should have been exposed to HCoV-OC43 and HCoV-HKU1 in the past. Our finding has important implications in the serodiagnostic testing, treatment and development of vaccine for the prevention of human infection caused by betacoronaviruses. The possibility of cross-reactive antibodies giving rise to false-positive results concurs with the suggestion of a recent report to use anti-HCoV-EMC IF antibody test only in patients with very clear epidemiological linkage.[46] Besides the possibility of wrong serodiagnosis due to cross-reactivity, this observation would support the use of antiviral peptides in the treatment of this emerging HCoV-EMC infection as antiviral peptides targeting the heptad repeat 2 has been successfully used in neutralizing SARS-CoV in cell culture.[47] Furthermore, this antigenic epitope could be an important vaccine target though the danger of immunopathology must also be considered. The possibility of low level neutralizing antibody leading to immune enhancement should also be considered if SARS convalescent plasma or normal intravenous immunoglobulin are used for the treatment of HCoV-EMC infection.[48]

No definitive evidence of intrusion of HCoV-EMC into at-risk groups was found in the present study. Two out of 94 sera from animal handlers had indirect IF antibody against both HCoV-EMC and SARS-CoV but no specific neutralizing activity toward these 2 viruses. Though this can be due to cross-reactivity with any betacoronaviruses such as HCoV-OC43, the possibility of cross-reactivity to Ty-BatCoV HKU4 and Pi-BatCoV HKU5 remains a distinct possibility which may represent sporadic interspecies jumping in this high risk group. Indeed, coronaviruses are found in many mammalian and avian species,[49–53] and have repeatedly crossed species barriers to cause interspecies transmission throughout history and occasionally caused major zoonotic outbreaks with disastrous consequences.[11,54–56] Phylogenetic analysis showed that the lineage A betacoronavirus HCoV-OC43 might have jumped from a bovine source into

human in the 1890s.[57] The more recent example of interspecies transmission was the jumping of the lineage B betacoronavirus SARS-CoV from bats to civets and then to humans which caused the SARS epidemic in 2003.[11,19,58–62] Though the seroprevalence of anti-HCoV-EMC antibody found no indication of positivity among residents in the Kingdom of Saudi Arabia, their demographic details, particularly the history of animal exposure, were not described.[4] Further studies including seroprevalence studies with more refined serological test should be conducted among at-risk groups in the Middle East to confirm the zoonotic nature of this emerging human coronavirus.

There were a number of limitations in this study. First, only a relatively small number of SARS patients were tested because of the lack of archived sera. However, most of the positive anti-HCoV-EMC IgG titers in this group were of high values between 1:80 to 1:160 which made the results less ambiguous. It would be interesting to test a larger group of laboratory-confirmed SARS patients with different viral strains to substantiate our observation. Second, the low seroprevalence of anti-SARS-CoV in the general population make the possibility of wrong serodiagnostics due to cross-reactivity less important for routine diagnostics. However, the finding is essential for confirmation of serological surveillance studies especially in some Southeast Asian countries including China where the seroprevalence for anti-SARS-CoV may not be well established, as HCoV-EMC may continue to spread and cause an epidemic in this densely populated area in the future.

Conflict of interests

None.

Acknowledgments

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