Seroepidemiology of Middle East respiratory syndrome (MERS) coronavirus in Saudi Arabia (1993) and Australia (2014) and characterisation of assay specificity

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The pseudoparticle virus neutralisation test (ppNT) and a conventional microneutralisation (MN) assay are specific for detecting antibodies to Middle East respiratory syndrome coronavirus (MERS-CoV) when used in seroepidemiological studies in animals. Genetically diverse MERS-CoV appear antigenically similar in MN tests. We confirm that MERS-CoV was circulating in dromedaries in Saudi Arabia in 1993. Preliminary data suggest that feral Australian dromedaries may be free of MERS-CoV but larger confirmatory studies are needed.

Introduction

Middle East respiratory syndrome (MERS) is an emerging respiratory disease of global public health concern. As of 9 May 2014, 536 confirmed human cases have been reported to the World Health Organization (WHO) with 145 deaths [1]. The current epidemiology of MERS is one of zoonotic transmission, sometimes followed by chains of limited human-to-human transmission for limited periods of time within families or healthcare facilities. This is reminiscent of the emergence of severe acute respiratory syndrome (SARS) in late 2002 [2]. It is therefore critically important to identify the sources of zoonotic transmission, so that evidence-based interventions to minimise such infections can be implemented. Such an approach has for example been used to minimise the human health risk from highly pathogenic avian influenza A(H5N1) and SARS [3,4].

Seroepidemiology is an invaluable tool in such investigations. Many seroepidemiological studies on domestic livestock have reported high MERS seroprevalence in dromedary camels in the Arabian Peninsula and Africa [5-8]. The detection of MERS coronavirus (MERS-CoV) by reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation in such animals supports these seroepidemiological findings and the contention that dromedary camels are a natural host for MERS-CoV [9-11]. But it is not clear if dromedaries are the main source of human infection.

We had previously reported a MERS-CoV pseudoparticle neutralisation test (ppNT) that can be used to detect antibody to MERS-CoV without the need for Biosafety Level-3 (BSL-3) containment that is required for conventional MERS-CoV microneutralisation (MN) tests [6]. In this study, we systematically investigate potential cross-reactions that may confound the use of these two assays in seroepidemiological studies in animals. Sera obtained from dromedary camels in Australia (2014) and different provinces of Saudi Arabia (1993) are included in this study.

Methods

Viruses

MERS-CoV EMC strain was provided by Dr Ron Fouchier, Erasmus Medical Centre, Rotterdam. The virus strains dromedary MERS-CoV Al-Hasa KFU-HKU13 2013 (Al-Hasa 13) and dromedary MERS-CoV Egypt NRCE-HKU270 2013 (Egypt 270) were isolated in our laboratory as previously described [10,12]. The viruses were cultured and titrated in Vero cells (ATCC CCL-81).

Table 1

Cross-neutralisation antibody titres for Middle East respiratory syndrome coronavirus (MERS-CoV) and bovine coronavirus (BCoV) in antisera raised against different coronaviruses

Genus	Antisera – BEI-Resources catalogue number is provided for sera obtained from BEI-Resources	Homologous Ab titre by ELISA unless otherwise specified	MERS-CoV MN titre	MERS-CoV ppNT titre	BCoV MN titre
Alpha-coronavirus	Gnotobiotic pig antiserum to porcine respiratory coronavirus – NR-460	1:1,200[a]	‹1:10	‹1:10	‹1:10
	Guinea pig antiserum to feline infectious peritonitis virus – NR-2518	1:2,000[a]	‹1:10	‹1:10	‹1:10
	Guinea pig antiserum to canine coronavirus – NR-2727	1:4,094[b]	‹1:10	‹1:10	‹1:10
	Gnotobiotic pig antiserum to porcine transmissible gastroenteritis virus – NR-458	1:1,400[a]	‹1:10	‹1:10	‹1:10
Beta-coronavirus	Guinea pig anti-SARS-CoV – NR-10361	1:2,560	‹1:10	‹1:10	‹1:10
	Rabbit antiserum for SARS-CoV S protein (zero titre) – NRC-769	‹1:10	‹1:10	‹1:10	‹1:10
	Rabbit antiserum for SARS-CoV S protein (low titre) – NRC-770	1:80	‹1:10	‹1:10	‹1:10
	Rabbit antiserum for SARS-CoV S protein (medium titre) – NRC-771	1:160	‹1:10	‹1:10	‹1:10
	Rabbit antiserum for SARS-CoV S protein (high titre) – NRC-772	1:640	‹1:10	‹1:10	‹1:10
	Mouse hepatitis virus (JHM strain) hyper-immunised mouse dam 1	1:1,778[c] neutralisation titre	‹1:10	‹1:10	‹1:10
	Mouse hepatitis virus (JHM strain) hyper-immunised mouse dam 2	1:363[c] neutralisation titre	‹1:10	‹1:10	‹1:10
	Mouse hepatitis virus (A59 strain) infected mouse	1:1,000[c] neutralisation titre	‹1:10	‹1:10	‹1:10
	BCoV antisera from guinea pig	1:20,480[b]	‹1:10	‹1:10	1:160
	BCoV antisera from germfree bovine calf – NR-456	1:10,000[a]	‹1:10	‹1:10	1:40
	BCoV antisera from germfree bovine calf	1:580[b] neutralisation titre	‹1:10	‹1:10	1:640
Gamma-coronavirus	Guinea pig antiserum to infectious bronchitis virus – NR-2515	1:50,000[a]	‹1:10	‹1:10	‹1:10

Ab: Antibody; ELISA: enzyme-linked immunosorbent assay; MN: microneutralisation test; ppNT: pseudoparticle neutralisation test; SARS-CoV: severe acute respiratory syndrome coronavirus.

Except if otherwise specified, antibody titres are obtained as part of this study. All homologous antibody titres are ELISA titres except for antisera to mouse hepatitis virus and one BCoV antiserum from germfree bovine calf, which are neutralising antibody titres.

[a] Homologous antibody titre data obtained from BEI-Resources.

[b] Homologous antibody titre data obtained from Linda Saif.

[c] Homologous antibody titre data obtained from Stanly Perlman.

Sera

Immune sera specific for alpha-coronaviruses (porcine respiratory coronavirus, feline infectious peritonitis virus, canine coronavirus and porcine transmissible gastroenteritis virus), beta-coronaviruses (mouse hepatitis virus strains JHM and A59, SARS coronavirus, bovine coronavirus (BCoV)) and gamma-coronavirus (infectious bronchitis virus) were obtained from BEI-Resources (animal CoV reagents supplied to BEI by Dr Linda Saif (http://www.beiresources.org/About/BEIResources.aspx) or generated by Dr Linda Saif or Dr Stanley Perlman, as indicated in Table 1). The homologous antibody titres to the immunising virus were also obtained from the respective sources supplying these antisera (Table 1).

Sera from 25 adult (≥2 year-old) dromedary camels were collected in 2014 in Australia, 17 being from feral camels from central Australia gathered and transported to an abattoir in Caboolture, Queensland, while the other eight sera originated from a camel farm in Coominya, Queensland. Dromedary sera from Egypt were collected from abattoirs in Egypt in 2014. Archived dromedary sera collected in 1993 from Al Hasa, Eastern Province (n=27), As Sulayyil, Ar Riyad province (n=30), Hafar Al-Batin, Eastern Province (n=45) and Medina, Al Medinah province (n=29) were retrieved from the serum archive at the Department of Microbiology and Parasitology, College of Veterinary Medicine, King Faisal University, Saudi Arabia. Paired acute and convalescent sera from three dromedary calves (‹2 years-old), which had RT-PCR confirmed MERS-CoV infection in a dromedary farm in Al-Hasa, Saudi Arabia in December 2013 are included in this study. The epidemiological and virological data on these three animals as well as the serological responses to MERS-CoV have been reported previously [12].

Serological tests

The methods for the ppNT and MN neutralisation test for MERS-CoV, and for the MN test for BCoV have been previously reported [6,13]. We used serial two-fold dilutions of heat inactivated (56°C for 30 minutes) sera with an entry dilution of 1:10. Titres of ≥1:40 are reported as positive and those 1:10–1:20 regarded as indeterminate.

Middle East respiratory syndrome coronavirus spike pseudoparticle neutralisation test (ppNT)

A codon optimised spike gene was designed based on MERS-CoV genome sequence (GenBank accession number: JX869059.1), synthesised in Genecust (Luxembourg) and subcloned into pcDNA3.1+ vector to generate pcDNA-S. To produce human immunodeficiency virus (HIV)/MERS spike pseudoparticles, 10 µg pNL Luc E- R- and 10 µg pcDNA-S were co-transfected into 4×10^6 293T cells. Supernatants of transfected cells were harvested 48h later and quantified for HIV p24 viral protein using a p24 enzyme-linked immunosorbent assay (ELISA) Kit (Cell Biolabs, INC, San Diego, CA, USA) [6].

HIV/MERS pseudoparticles containing 5ng HIV p24 was used to infect Vero E6 cells (ATCC CRL-1586) in a single well (96 well plate format; 1×10^4 cells/well). Infected cells were lysed in 20 µl lysis buffer and 100 µl of luciferase substrate at two days post-infection (Promega Corporation, Madison, WI, USA). Luciferase activity was measured in a Microbeta luminometer (PerkinElmer, Waltham, MA, USA). For the ppNT assay, HIV/MERS pseudoparticles (5ng of p24) were pre-incubated with serially diluted sera for 30 min at 4°C and then added to cells in triplicate. Residual virus infection of the cells was assayed at two days post-infection, as described above. The highest serum dilution giving a 90% reduction of luciferase activity was regarded as the ppNT antibody titre.

Microneutralisation (MN) tests

MERS-CoV (strain: EMC) and BCoV (ATCC BRCV-OK-0514-2) were used. Vero cells (ATCC CCL-81) were used for MERS-CoV and HRT-18G cells (obtained from ATCC) for BCoV. Serum dilutions were mixed with equal volumes of 200 tissue culture infective dose $(TCID)_{50}$ of virus and incubated for one hour at 37°C. The virus–serum mixture was then added in quadruplicate to cell monolayers in 96-well microtitre plates. After one hour of adsorption, the virus–serum mixture was removed and 150µl of fresh culture medium was added to each well and the plates incubated at 37°C in 5% CO2 in a humidified incubator. A virus back-titration was performed without immune serum to assess input virus dose. Cytopathic effect (CPE) was read at three days post-infection for MERS-CoV and four days post-infection for BCoV. The highest serum dilution that completely protected the cells from CPE in half of the wells was defined as the neutralising antibody titre. Positive and negative control sera were included in each assay [13].

Table 2

Serological reactions to Middle East respiratory syndrome coronavirus (MERS-CoV) and bovine coronavirus (BCoV) in selected sera collected from dromedary camels in Egypt (2014), Australia (2014) and Saudi Arabia (1993)

Location of serum collection (year)	Serum identity number	MERS-CoV MN	MERS-CoV ppNT	BCoV MN
Egypt (2014)	E2	1:320	1:640	1:40
	E4	1:160	1:320	1:40
	E5	1:40	1:160	1:40
	E6	1:40	1:160	1:40
	E7	1:80	1:320	<1:10
	E8	1:40	1:80	1:40
	E9	1:320	1:640	1:160
Australia (2014)[a]	A1	<1:10	<1:10	<1:10
	A2	<1:10	<1:10	1:160
	A3	<1:10	<1:10	1:160
	A4	<1:10	<1:10	1:160
	A5	<1:10	<1:10	1:320
	A6	<1:10	<1:10	1:320
	A13	<1:10	<1:10	1:320
	A24	<1:10	<1:10	1:160
Saudi Arabia (1993)[b]	S1	1:320	1:1,280	1:80
	S2	1:320	1:2,560	1:40
	S3	1:640	1:1,280	1:160
	S4	>1:1,280	>1:5,120	1:160
	S5	1:40	1:160	<1:10
	S7	1:80	1:80	<1:10
	S8	1:640	1:1,280	1:320
	S9	<1:10	<1:10	<1:10

MN: microneutralisation test; ppNT: pseudoparticle neutralisation test.

[a] Results for eight sera selected from 25 are shown.

[b] Results from eight sera selected from 131 are shown.

Results

We tested immune sera to a range of animal alpha-, beta- and gamma- coronaviruses and found no cross-reaction to MERS-CoV in either the MERS-CoV ppNT or MN assays (Table 1). Specifically, we demonstrated that bovine calf and guinea pig immune sera to BCoV do not cross-react in the MERS-CoV ppNT or MN assays.

Of the archived dromedary sera collected in 1993, 26 of 27 sera from Al Hasa, 22 of 30 sera from As Sulayyil, 43 of 45 sera from Hafar Al-Batin and 27 of 29 sera from Medina had detectable (≥1:40) ppNT antibody titres to MERS-CoV, with antibody titres ranging from 1:40 to ≥1:5,120. Data from representative sera are shown in

Table 3

Comparative antibody titres of dromedary camel sera to different isolates of Middle East respiratory syndrome coronavirus (MERS-CoV) and to an isolate of bovine coronavirus (BCoV)

Dromedary camel sera	Reciprocal microneutralisation (MN) antibody titres			
	MERS-CoV			BCoV
	Al-Hasa 13/2013	Egypt 270/2013	EMC/2012	
Calf pre-infection[a]	<1:10	<1:10	<1:10	<1:10
Calf post-infection[a]	1:80	1:40	1:80	<1:10
Adult 1, Saudi Arabia[b]	1:640	1:320	1:320	1:80
Adult 2, Saudi Arabia[b]	1:640	1:640	1:640	1:40
Adult 1, Egypt[b]	1:640	1:320	1:640	<1:10
Adult 2, Egypt[b]	1:640	1:640	1:1,280	1:40

MN: microneutralisation; ppNT: pseudoparticle neutralisation test.

[a] Acute and convalescent serum from a dromedary calf infected with Al-Hasa 13/2013 MERS-CoV (described in reference [12]. Note that titres in reference [12] were ppNT titres and the ppNT assay is more sensitive than MN assays).

[b] Adult sera were selected dromedary camel sera from Saudi Arabia and Egypt known to be seropositive to MERS-CoV.

Table 2. Many, but not all of the MERS-CoV antibody positive sera were also positive for BCoV antibody in MN tests.

Sixteen of the 25 dromedary sera collected in Australia in 2014 had BCoV antibody titres ranging from 1:40 –1:320 but none of them had any antibody reactivity to MERS-CoV in either ppNT or MN assays. Representative results are shown in Table 2.

Comparative MN tests were carried out using a clade B dromedary MERS-CoV isolate from Al Hasa (Al-Hasa 13), a clade A human MERS-CoV isolate from Saudi Arabia (EMC) and a genetically divergent MERS-CoV isolate from Egypt (Egypt 270) using an acute and convalescent serum from the dromedary calf 13 from which Al-Hasa 13 MERS-CoV was isolated [12]. Sera from two other adult dromedaries from Saudi Arabia and two from Egypt were included. All three MERS-CoV were neutralised to comparable titres by the convalescent sera from calf 13 and the four adult dromedaries. The paired sera from calf 13 did not show an antibody response to BCoV (Table 3) and two other calves (numbers 15 and 19 (reported in reference [12]), which seroconverted to MERS-CoV also failed to seroconvert to BCoV (data not shown).

Discussion

Antisera to alpha-, beta- or gamma- coronaviruses (other than MERS-CoV) had high homologous antibody titres but failed to cross-react with MERS-CoV in MN or ppNT tests. Amongst the studied serum panel, the lack of cross-reaction with SARS coronavirus is of note since this virus is phylogenetically more closely related to MERS-CoV.

Many dromedary camel sera have antibodies to both MERS-CoV and BCoV and it is important to establish whether this represents separate infections with the two viruses or serological cross-reactions. Some previous studies have addressed this problem by testing for multiple viruses in parallel and demonstrating some sera with MERS-CoV reactivity in the absence of BCoV (or closely related human coronavirus OC43) reactivity [5,13-16]. In the present study, the lack of MERS-CoV ppNT or MN antibody reactivity in BCoV immune bovine calf or guinea pig sera (Table 1) confirms the specificity of these two serological assays to discriminate between these two viruses. However, dromedaries have unusual single heavy chain immunoglobulins [17] and it is conceivable that these single-chain Ig sera may have unusually broad cross-reactivity, although there is no direct evidence for this hypothesis. The observation that 18 of 25 dromedary sera from Australia have antibodies to BCoV (titres up to 1:320) without any cross-reactivity to MERS-CoV in the ppNT and MN assays is an important confirmation that these assays discriminate between the two viruses in dromedaries as well. Finally, we had three acute and convalescent sera from dromedary calves, which had RT-PCR confirmed MERS-CoV infection and they showed significant (more than four-fold) increases in antibody to MERS-CoV without any change in titre to BCoV. Collectively, these data conclusively demonstrate that ppNT or MN positive antibody titres to MERS-CoV in any animal species are strongly suggestive of MERS-CoV infection. This does not exclude the hypothetical possibility that a hitherto unknown coronavirus more closely related to, but distinct from MERS-CoV, may give cross-reactive antibodies in serosurveillance studies.

Some closely related coronaviruses are antigenically diverse and show limited cross-reactivity in serological assays, as has been reported, for example, for two serotypes of feline coronaviruses [18]. Given that MERS-CoV from different geographical regions (Saudi Arabia and Egypt) are genetically diverse [10], the question arises as to whether the MERS-CoV ppNT and MN assays using one MERS-CoV will detect antibodies to these genetically diverse MERS-CoV viruses. We find that genetically diverse MERS-CoV strains (clade A EMC, clade B Al-Hasa 13 and genetically distant Egypt 270) give comparable MN antibody titres in a dromedary calf seroconverting to Al-Has 13 clade B virus. Similarly adult dromedaries from Saudi Arabia and Egypt each give comparable (within twofold) titres to all three MERS-CoV. The data provide a-priori evidence that a single MERS-CoV isolate is likely to be sufficiently representative for MERS-CoV seroepidemiological studies. It also suggests that genetically diverse MERS-CoV may be antigenically conserved.

Although we have not carried out studies using specific immune sera to exclude cross-reactivity to currently endemic human 229E, OC43, HKU-1 and NL63 coronaviruses, we have so far tested human sera from Egypt and Hong Kong by the MERS-CoV MN tests (n=1,343) and ppNT (n=394) [6,10] with negative results. Since these human coronaviruses are ubiquitous with high seroprevalence in human adults worldwide [19,20], it is very likely that antibodies to 229E, OC43, NL63 and HKU-1 do not cross-react with MERS-CoV in these assays.

Serological evidence of MERS-CoV in dromedaries has been previously reported in archived sera dating back over past decades [7,11,16]. Our data with serological assays that have been demonstrated to be free of cross-reaction with BCoV and other coronaviruses reconfirms that MERS-CoV was circulating in dromedaries in Saudi Arabia as early as 1993.

Although adult dromedaries in the Arabian peninsula and in North and East Africa (e.g. Egypt, Nigeria, Tunisia, Ethiopia, Kenya) have very high seroprevalence to MERS-CoV (>90%) [6,8,21], we found that the sera from adult dromedary camels in Australia were uniformly seronegative. Given the small number of sera tested in this study, a larger seroepidemiological study would be needed to confirm that Australia is indeed MERS-CoV free. On the other hand, the BCoV-like virus so common in the Middle East is also prevalent in Australia. Dromedaries were imported into Australia between 1840 and 1907 to serve as means of transport but are now largely found as feral animals [22]. The dromedary population in Australia is now estimated to be around 450,000 (Al Jassim – data not shown).

We conclude that the MERS-CoV ppNT and MN tests reported here do not detect cross-reactive antibodies to other animal coronaviruses including the BCoV-like virus that is common in dromedaries. Thus these two serological assays can be used with confidence in seroepidemiological studies to identify animal species that may serve as reservoirs or vectors of MERS-CoV. We also confirm that MERS-CoV or a very closely related virus has been circulating in dromedaries in Saudi Arabia for at least two decades.

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Conflict of interest

None declared.

Authors' contributions

MH Hemida, RAM Jassim, G Kayali, MA Ali and A Alnaeem carried out field studies in Saudi Arabia, Australia and Egypt to collect the clinical specimens and epidemiological data. RAPM Perera helped plan the study, carried out the serological testing and analysed the data. P Wang and LY Siu developed the MERS-CoV pseudoparticle neutralisation test. DKW Chu isolated and genetically characterised the viruses used in this study. L Saif and S Perlman generated the coronavirus sera used in this study. Y Guan and LLM Poon contributed advice in study design and data analysis. M Peiris conceived and planned the study, analysed the data and wrote the manuscript. All authors critically reviewed the data and the manuscript.

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