QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCE

SEROPREVALENCE OF ANTI-MERS-CoV IgG IN TWO GROUPS OF QATAR POPULATION: BLOOD DONORS AND CASE CONTACTS

BY

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ABSTRACT

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Title: Seroprevelance of Anti-MERS-CoV IgG Among Two Groups of Qatar Population: Blood Donors and Case Contacts

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Since its first isolation in September 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) has diffused across 27 countries infecting more than 1910 individuals with a high case fatality rate. However, MERS-CoV has also been reported to be asymptomatic or to cause influenza-like mild illnesses . In the absence of clear epidemiological view, cross-sectional MERS-CoV antibody surveillances in human populations are of global concern . In this study, we present a comparative serological screening of 4719 blood donors, 135 baseline case contacts and 4 MERS-CoV confirmed patients for the presence of anti-MERS-CoV IgG. **Methods:** Samples were initially screened using MERS-CoV recombinant spike protein Enzyme linked immunoassay (rELISA) from *Euroimmune, Germany*. To confirm rELISA results, farther serological testing has been performed for borderline and reactive anti-MERS-CoV IgG samples by indirect immunofluorescent test (full virus IIFT) IgG/M, recombinant spike protein indirect immunofluorescent assay IgG (rIIFA) and pseudovirus neutralizing assay (ppNT). To access cross reactivity, borderline and reactive samples were also tested for presence of IgG to other human coronaviruses (HCoV) using IIFT, rIIFA and/ or in house rELISA. **Results:** rELISA yielded 3 borderlines (all donors) and 12 reactive (7 donors, 1 case contact and 4 samples collected from 3 MERS-CoV confirmed patients) anti-MERS-

CoV IgG results. However, IIFT IgG confirmed only 5 reactive rELISA results (2 blood donors and 3 patients; the reactive case contact was not sufficient to be tested by IIFT IgG). Yet, r-IIFA and ppNT only confirmed the presence of specific anti-MERS-CoV antibodies in patients' samples. Interestingly, all borderline and reactive tested samples showed reactive titers against recombinant spike proteins of other HCoV. **Conclusion:** Our findings suggest that MERS-CoV is not heavily circulated among the population of Qatar. This study provides an insight about the epidemiological view for MERS-CoV in Qatar population. It also provides a performance evaluation for the available serologic tests for MERS-CoV in a view of serologic status to other human coronaviruses.

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CHAPTER 1: INTRODUCTION

Middle East respiratory syndrome coronavirus (MERS-CoV), originally called "Novel Coronavirus", is a human coronavirus (HCoV) identified in 2012 (1). It is responsible to cause severe acute respiratory syndrome cases and case clusters across the Arabian Peninsula (2) . Exported infections are linked to countries in the Middle East. Majority of the cases acquired the infection in the Middle East, and then exported abroad (2). To date, approximately more than 1,910 laboratory-confirmed cases have been identified with more than 680 fatalities (3). MERS-CoV–specific antibodies are widely found in dromedaries (Camelus dromedarius) (4-7) along with viral shedding of similar viruses detected in human at same region suggesting that MERS-CoV epidemiology may be centrally ruled by Camel (8-10). Though serological surveys are widely spread to explore the role of Dromedary Camels in MERS-CoV transmission (11), studies of the viral distribution among human population are infrequent (8, 11).

The uncertain epidemiology stresses the need for cross-sectional surveillance of anti-MERS specific antibodies among human populations (12). Representative serological surveillances provide data to evaluate antibodies prevalence to a novel pathogen in a population. This estimation plays a vital role toward a better understanding of the extent of novel viral infection in a population (13). As infection with MERS-CoV continues to evolve, a matter of best infection control and management will arise, and studies to better define its prevalence are needed for the reason that infection control and management recommendations might be reviewed & updated as additional data become available (14, 15).

Due to the uncertain epidemiological view of MER-CoV among Qatar population

-in particular-, we designed a staged serologic surveillance study for MERS-CoV among 2 groups of Qatar population (blood donors and case contacts) in the period of 2012-2016. Surveillance started with initial screening for the presence of anti-MERS-CoV IgG followed by confirmation and evaluation of reactive samples in view of other human coronaviruses.

Hypothesis

Seroprevalence of anti-MERS-CoV IgG among healthy individual could be high as those of high risk group.

Objectives

We conduct serological studies that are designed to collect data to evaluate the seroprevalence of anti-MERS-CoV IgG in two groups of Qatar population (i) high risk group: those in close contact with confirmed patients, (ii) low risk group: normal healthy population. Eventually the long-term objective is to provide insight into a better understanding of the epidemiology of the disease and the risk of infection in the population of Qatar.

CHAPTER 2: BACKGROUND

Coronaviruses comprise a large family of enveloped RNA viruses (Figure 1) that have the potential to infect wide spectrum of animals and human (16). Coronaviruses Infections may range from the common cold to severe acute respiratory syndrome (SARS) (16). Various novel strains have been evolved or recognized in animal and human in the last decade (17). The most two recent coronavirus emergence incidence in the twenty-first century include the extremely pathogenic severe acute respiratory syndrome coronavirus (SARS-CoV) (18) and MERS-CoV (1) strains that cause notable mortality and morbidity in affected individuals, particularly the elderly patients (19, 20).

Human Coronaviruses

In 1960s two coronaviruses (HCoV-229E and HCoV-OC43) were identified as potential human pathogens (Figure 2) causing relatively mild common colds with a potential of more severe symptoms in immunocompromised, elderly and infants (21-31).

However, in 2003 (Figure 2), worldwide Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) epidemic were developed resulted in 8,000 cases with almost 800 deaths. It was initially originated in Guangdong Province, China. (17, 32-35). Further investigations denoted that this virus have passed to humans from zoonotic origin i.e. bats (Rhinolophus: horseshoe bats) (36), raccoon dogs (Nyctereutes procyonoides) and Himalayan palm civets (Paguma larvata) (37, 38). Finally, global public health control and extensive containment efforts break this epidemic (33, 39).

Following SARS-CoV epidemic, more two coronaviruses strains (HCoV-NL63 and HCoV-HKU-1) with potential pathogenicity to humans, were recognized (Figure 2) from archived respiratory samples (40-42). The two viruses were result in mild to severe

lower respiratory tract illnesses. However, the prevalence and severity of the disease particularly in the very young are not yet fully covered (43). Despite that the identification of HCoV-NL63 and HCoV-HKU-1 was covered recently, molecular clock analyses show that HCoV-NL63 may evolved from HCoV-229E "closest relative", about 500–800 years back; however, such estimated periods of time could be, greatly under or overvalued due to mutation masking and purifying selection rate changes as with all other molecular clock analyses (44-46).

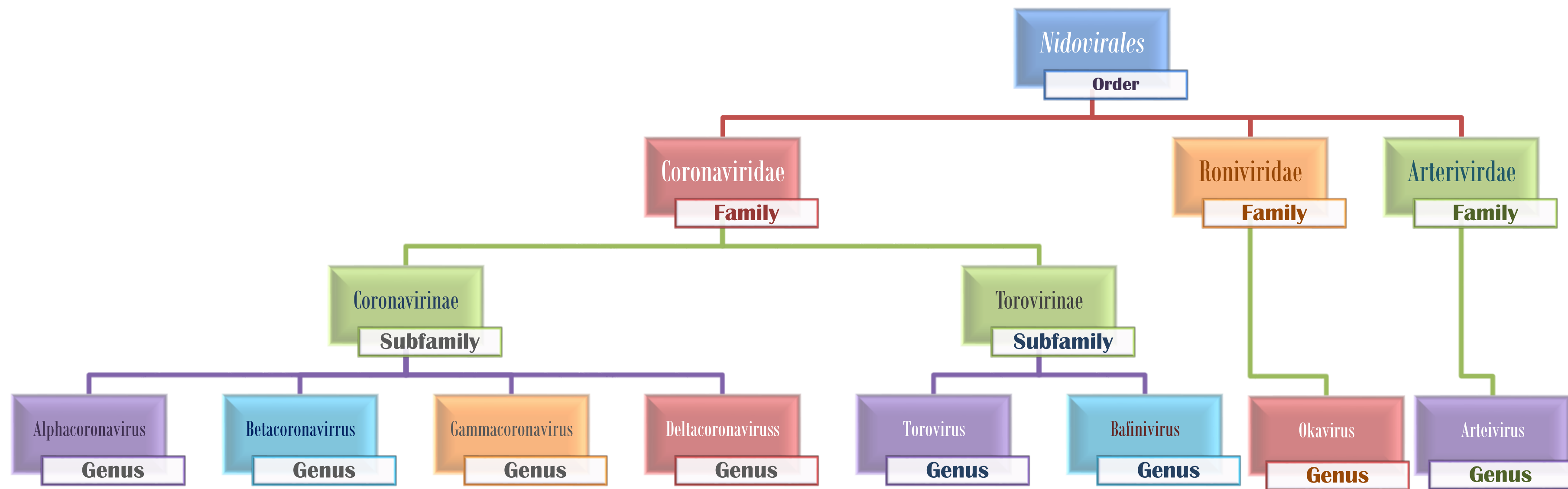


Figure 1. International *Coronaviridae* taxonomy. *Courtesy of G. Whittaker and R. Collins, Cornell University*. Adapted from Chan et al. 2015 (47).

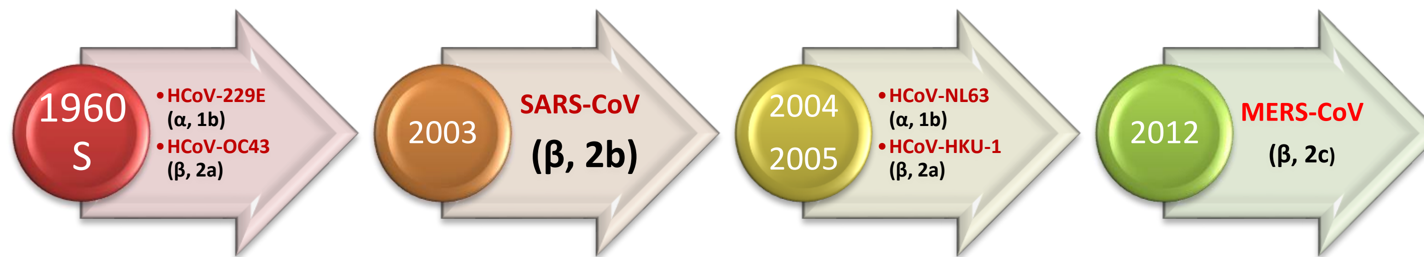


Figure 2. Human Coronaviruses Classification and TimeLine of Identification. α (Alpha), β (Beta): phylogenetic groups; 1b, 2a,2b and 2c: subgroups.

MERS-CoV

Emergence

The library of known coronaviruses has been greatly expanded following bat reservoir searching of SARS-CoV. Coronaviruses are found in various animal species including different bats, birds, whales, dogs, mice, pigs, cats and horses (48). Moreover, several animal coronaviruses have similar phylogenetic with known pathogenic human coronaviruses (36, 49-57). Such findings suggest the highly potential for new coronaviruses emergence. This was approved by the emergence of a novel strain, defined as middle east respiratory syndrome coronavirus (MERS-CoV) (17).

In April 2012, The Health Ministry of Jordan has reported an outbreak of unknown origin of severe lower respiratory tract infection in an intensive care unit of a public hospital in Zarqa city (58). The outbreak formed of 11 cases including 10 health care workers (58, 59). No etiological source could be identified after epidemiological surveillance along with laboratory testing performed immediately following the outbreak. (58).

Around June-mid 2012, a 60-year old Saudi man had been admitted to a private hospital in Jeddah, Saudi Arabia, presented with severe respiratory illnesses that rapidly progressed to acute pneumonia and renal failure followed by death.(1, 60). During hospitalization time, his sputum sample along with infected cell cultures tested negative against known antibodies of a group of commonly known respiratory viral pathogens (influenza A and B, parainfluenza viruses' types 1 to 3, respiratory syncytial virus, and adenovirus). However, suggestion of virus replication has been indicated from cytopathic changes for a sputum sample obtained on admission, inoculated in LLC-MK2 and Vero

cells (1). Ten to eleven days following his admission, his serum samples reacted strongly for immunofluorescence assay specific for IgG antibody at dilutions of 1:20. In contrast around 2400 control serum samples collected from 2010 through 2012 from individual referred to the same Hospital tested negative in this analysis. This contrast suggested that the patient had developed antibodies to an unknown virus that were not recognized in the public through the past 2 years (1). Further Real-time PCR assay on nucleic acids extracted from the patient inoculated cell-culture supernatants resulted negative for enterovirus, human metapneumovirus, and human herpesvirus types 1 to 3 (1).The patient had died elven days following his admission. However, attempts were continued to identify causative pathogen. At the mid of July, wide spectrum PCR assays of all known paramyxoviruses also resulted negative. Later, a positive result had been yielded from family-wide real time PCR assays for broad-spectrum "pan-coronavirus", but it was not SARS-CoV. As consequence, patients sample has been sent to Erasmus Medical Centre (EMC) in The Netherlands and a novel coronavirus was firstly identified and initially called "human coronavirus-EMC/2012" (61).

In 22-9-2012, three months following the admission of the above-mentioned patient in Jeddah, a second infected case with the same virus has been reported from 49-year old Qatari man with a previous traveling history to Saudi Arabia. He was transferred from the intensive care unit (ICU) in Qatar and was diagnosed and treated in the United Kingdom (62).

Following the identification and reporting of the novel coronavirus (MERS-CoV), The Health Ministry of Jordan shipped the stored samples of the previous reported lower respiratory tract infection outbreak to United States Naval Medical Research Unit-3 (US

NAMRU-3) in Cairo. A total of 2 MERS-CoV laboratory-confirmed and 11 probable cases were identified from this outbreak of whom 10 were health care workers and 2 were family members of cases (58).

Since then, Subsequent cases and clusters of infections continue, particularly in Saudi Arabia (63).

Epidemiology & Geographic Distribution

Since April 2012, more than 1910 laboratory-confirmed cases of infection with MERS-CoV have been reported from 27 countries (Table.1) including more than 650 deaths (crude Case Fatality Rate (CFR) 35%)(3, 20).Cases have been reported primarily from Arabian Peninsula with the majority of cases from Saudi Arabia (about 80%), including clusters. Moreover, cases reported from countries outside of the Arabian Peninsula, have noted either a history of traveling to Arabian Peninsula or a close contact to MERS-CoV infected patients (20).

The reported MERS cases of the Middle East showed a small increasing of the cases during March and April 2013. However, it increased dramatically in March and April 2014 followed by sharped declined in mid-May 2014 (20). In May and June 2015, an outbreak of more than 180 cases reported in South Korea; the index case had a recent traveled to different countries in the Arabian Peninsula (64, 65).

The median age among laboratory-confirmed cases is 52 and 65.6% of cases are male (20). About 48% of laboratory-confirmed cases were reported with severe illnesses or death, while 19.9% had moderate symptoms. However, 20.6% of cases had no or mild symptoms. About 20% of cases were reported among health care workers (20).

Table 1

Number of Laboratory-confirmed MERS-CoV Cases Reported by Countries/ Year (2012 - Dec.2016). Exported From WHO (20).

Country	Number of Reported Laboratory Confirmed MERS-CoV Cases
Algeria	2
Austria	2
Bahrain	1
China	1
Egypt	1
France	2
Germany	3
Greece	1
Iran	6
Italy	1
Jordan	28
Kuwait	4
Lebanon	1
Malaysia	1
Netherlands	2
Oman	7
Philippines	2
Qatar	16
Republic of Korea	185
Saudi Arabia	1482
Thailand	3
Tunisia	3
Turkey	1
United Kingdom	4
United Arab Emirates	79
United States of America	2
Yemen	1
Total	1841

Overview of MERS-CoV Status in Qatar

The second identified case with MERS-CoV infection has been reported in September 22nd, 2012 from 49-year old Qatari man with a previous traveling history to Saudi Arabia. He was diagnosed and treated in the United Kingdom (62). Since then, several cases have been reported from Qatar with a total of 20 cases up to April 2017 including 2 deaths (Table 2).

The Ministry of Health along with the Ministry of Environment in Qatar in collaboration with international support investigated the slaughterhouse, linked to two previous MERS cases in Qatar (case # 6 & 7, Table 2) for the circulation rate of MERS-CoV in dromedaries (66, 67). This investigation indicated that a high rate of dromedaries shed MERS-CoV RNA at slaughterhouse with a multiple MERS-CoV variants reflecting introductions of multiple viruses among trade of new animals and a rich site for MERS-CoV circulation with a high-risk for human exposure (66). This exposure risk has been assessed in a compensative study by serological investigation for individuals in Qatar with and without daily occupational exposure to dromedaries (8). The study reported the detection of MERS-CoV neutralizing antibodies in healthy individuals with daily occupational dromedaries contact but not in whom without camel contact (8). Those MERS-CoV–seropositive individuals did not report severe illnesses, suggesting a potential of frequent unrecognized human infections (8). That's could reflect an overestimated death rate of 37.1% associated with MERS-CoV infection (8). However, only few reports are available for the presence of MERS-CoV antibodies among a general population or specific groups with a major concern of the potential contribution from asymptomatic cases to the MERS-CoV transmission (8, 12).

Table 2

List of reported confirmed MERS-CoV infection-Qatar (67).

Case Serial	SEX	Age	Nationality	Month/ Year	Exposure/ Travel history/ Camel Contact	Mortality Status
1	Male	49	Qatari	Sep.2012	Travel history to Saudi Arabia	No reported Death
2				Nov.2012		No reported Death
3 & 4	Male Male	59 29		Aug.2013	Travel history to Medina, Saudi Arabia 6 days before illness onset	No reported Death
5	Female	56		Aug.2013		Died 13 days following onset of illness.
6	Male	61		Oct 2013	• A farm Owner • Significant contact with the animals, including camels	No reported Death
7	Male	23		Oct 2013	• Close contact for case # 6 (epidemiological investigation). • Worker in the animal barn of Case # 6	Mild Symptoms
8	Male	48		Oct.2013	Frequent visits to animal barns.	No reported Death
9	Male	61		Nov.2013	Exposure to farms of livestock.	No reported Death
10	Male	71		Oct.2014	• Symptoms developed during his road traveling from Qatar to the Al-Hasa, Sudia Arabia • Camel barn owner • Consumed raw camel milk.	No reported Death
11	Male	43		Oct.2010	Frequent camel barn visits within 14 days prior the onset of illnesses.	No reported Death
12	Male	55	Non-Qatari	Jan.2015	Frequent contact with camels and goats.	No reported Death
13	Male	69		March.2015	• Frequent contact with camels • Regular raw camel milk consumption	No reported Death
14	Male	29	Non-Qatari	May.2015	• Frequent camels contact • No raw camel milk consumption.	No reported Death
15	Male	73		May 2015	• No direct contact with camels • Family owns a camel barn with Family camel contact history and raw camel milk consumption.	No reported Death
16	Male	66	Qatari	Feb.2016	• Symptoms developed in Saudi Arabia, • A camel barn owner in Saudi Arabia with frequent visit. • Frequent camels contact • Raw Camel milk consumption	Died around 17 days following onset of symptoms.
17	Male	40	Non -Qatari	May. 2016	Frequent occupational exposure to dromedaries.	No reported Death
18	Male	23	Non -Qatari	June 2016	• Frequent camels contact • Camel raw milk consumption. • History of travel to Saudi Arabia on 28 May.	No reported Death

19	Male	62	March 2017	• No travel history for last seven months • No camel or patient contact	No reported Death
20	Male	25	April. 2017	• Frequent contact with dromedary camels	No reported Death

Data extracted from WHO Middle East Respiratory Syndrome, Emergencies preparedness, response- Qatar. Any blank cell indicates unclear or unavailable data from the source.

Virology

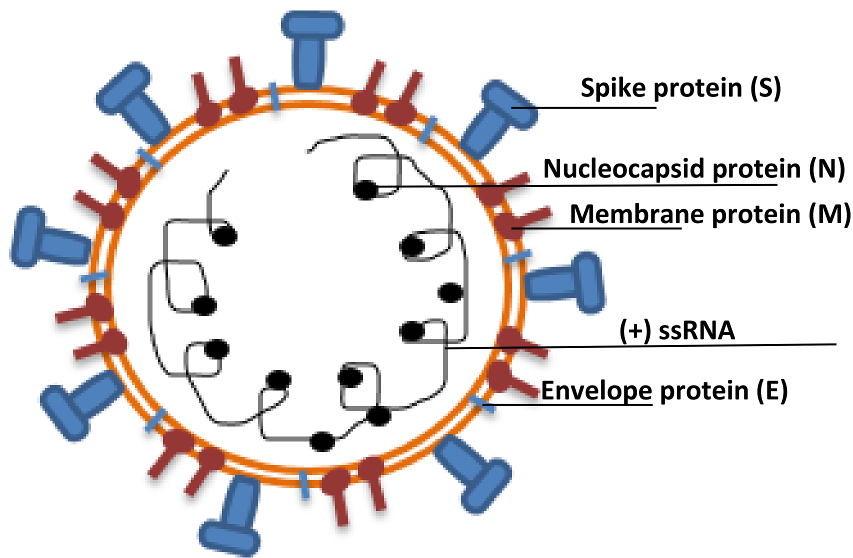
MERS-CoV is an enveloped single-stranded RNA (30 119 nucleotides) virus that belongs to lineage C of the β-coronavirus genus within the Coronavirinae (Figure 1) subfamily (68, 69). MERS-CoV is more relevant -with 90% sequence homology- to bat coronavirus HKU4 and HKU5 (lineage 2C bat coronaviruses) than it is to SARS-CoV (lineage 2B) (69-71). Additionally, MERS-CoV sequence comparisons demonstrated high homology (94%) with coronavirus of Pipistrellus bats (71, 72) .

The genome of MERS-CoV encodes four essential structural proteins: envelope (E), membrane(M), nucleocapsid (N) and spike (S) (Figure 3) along with other accessory proteins with unidentified origins and roles. (16, 73).

MERS-CoV, as other coronavirus, gains entry to the host cell through its S protein, a large surface transmembrane glycoprotein that exists as a trimer on the viral virion surface (74-79). The process of MERS-CoV entry to the host cell is mediated upon binding of the S glycoprotein, through its receptor binding domain (RBD), to the dipeptidyl peptidase 4 human receptors (77-79). The MERS-CoV S protein is cleaved during the infection process into 2 subunits (Figure 3): S1 (receptor-binding) and S2 (membrane-fusion) (75, 80).

Both the N and S proteins constitute major components for the immunogenicity of coronaviruses and are formed in huge amount during infection. However, the protective immunity to coronavirus is mainly determinate by the S protein (81). In a study of SARS-CoV vaccine efficiency, it was indicated that the specific immune responses to the N protein may provide limited protection particularly for challenges of low doses (82). Moreover, various studies showed that neutralization of coronavirus isolates is mediated

by antibodies to the S protein whereas no neutralization observed by antibody specific to the N protein (82-84).



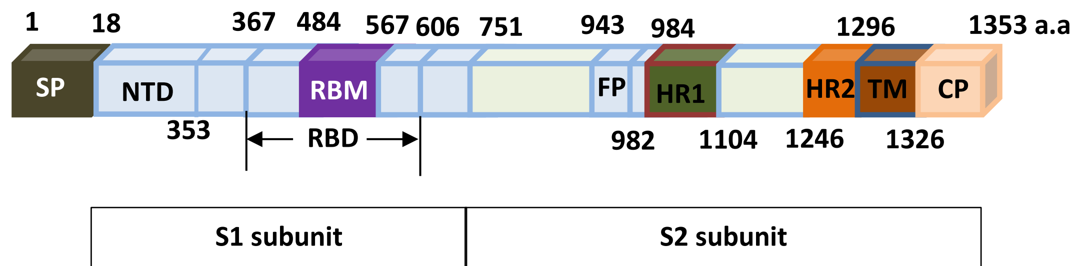


Figure 3. Schematic structures of **A:** Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and **B:** MERS-CoV spike protein. (+) ssRNA: positive, single-stranded RNA. a.a: amino acid, CP: cytoplasmic tail, TM: transmembrane domain, HR1 and HR2: heptad repeats 1 and 2, FP: fusion peptide, RBM: receptor-binding motif within receptor-binding domain (RBD), NTD: N-terminal domain, SP: signal peptide. Adapted from Zhang N et al. 2015 (85, 86).

Antigenic & Serologic Characterization

Serologic and antigenic studies across coronaviruses indicate strain specificity of the S protein. Coronaviruses within or across subgroups demonstrated very low-level cross-reactivity for the S protein and limited if any preservation of cross-neutralizing epitopes. Whereas coronaviruses within same subgroup share cross-reactive epitopes for the N protein (83, 87). Consistently, Agnihothram et al. study revealed no cross-reactivity between N and S proteins of SARS-CoV (β, 2b) and human MERS-CoV (β, 2c) isolates (83). Only high concentrations of mouse antisera to SARS-CoV showed low levels of cross-neutralization of MERS-CoV human isolate (Table 3A). Additionally, human antisera collected from a patient infected with MERS-CoV did not react with SARS-CoV S antigen apart from sera collected in specific one day (Table 3B) (83). The human antisera of a MERS-CoV infected patient only cross-reacted with N protein of the bat coronavirus (BtCoV) HKU 5.5 (β, 2c) with limited if any cross-detection was reported outside the subgroup, apart from transient very low cross-binding of BtCoV 279 N (β, 2b) (Table 3B). Furthermore, their results showed that MERS-CoV human isolate and SARS-CoV reacted- in Enzyme-Linked Immunosorbent Assay (ELISA) - only with mouse antisera to the N but not the S protein of other coronaviruses of the same subgroup for each (Table 3A). The cross-reactive epitope(s) of the S protein among betacoronaviruses was referred -based on bioinformatics- to the S2 domain, which is more highly preserved among coronaviruses than the S1 domain (88, 89).

Interestingly, earlier study reported significant immunofluorescent MERS-CoV antibody titers in 60.7% (17/28) of SARS-CoV infected patients with 25% (7/28) having low titers of MERS-CoV neutralizing antibodies (88). They suggested that “the Virulence of

SARS-CoV over other betacoronaviruses may boost cross-reactive neutralizing antibodies against other betacoronaviruses" (88). Agnihothram et al. reported that SARS-CoV infected cell lysates of ELISA assay reacted with mouse anti-N -but not S- sera to betacoronaviruses viruses (BtCoV HKU 279 and HKU 3) within the same subgroup (2b) and did not react with mouse anti-N sera to coronaviruses viruses (BtCoV HKU 4.2 and HKU 5.5) of other group or/and subgroups (83). Consistently, HCoV-OC43 (β, 2a) infected cell lysates of ELISA assay did not reacted with mouse anti-N sera to SARS-CoV (β, 2b) (83). Furthermore, human anti- SARS-CoV serum recognized the N protein of betacoronaviruses infected cell lysates of ELISA assay (BtCoV HKU 279 and HKU 3) within the same subgroup (2b) but did not react with N proteins from other subgroups (BtCoV HKU 4.2 and HKU 5.5) or with the S protein of betacoronaviruses (BtCoV HKU 3) within the same subgroup (2b) (83). Similarly, earlier established capture ELISA for detection of SARS-CoV N antigen showed that neither rabbit polyclonal nor mice specific monoclonal antibodies to the SARS-CoV N protein cross reacted with HCoV-OC43 (β, 2a) or HCoV-229E (α, 1b) (90). However, other investigators reported antigenic cross reactivity between SARS-CoV (β, 2b) N protein and HCoV-OC43 (β, 2a) and 229E (α, 1b) antibodies using recombinant SARS-CoV N protein-based ELISA (91).

Table 3.

A, *Summary of Agnihothram et al. 2013 findings for cross reactivity of MERS-Cov Antigens and anti-mice sera of other coronaviruses.* ***B***, *Summary of Agnihothram et al. 2013 findings for cross reactivity of anti MERS-Cov antibodies and Venezuelan equine encephalitis virus replicons(VR) expressed proteins of other coronaviruses.*

A

		Mice anti-N Sera of					Mice anti-S Sera of						
		BtCoV (β, 2c)		HCoV (β, 2b)	BtCoV (β, 2b)		BtCoV (β, 2c)		HCoV (β, 2b)	BtCoV (β, 2b)		BtCoV (α, 1b)	
		HKU 4.2	**HKU 5.5**	**SARS**	**HKU 3**	**279**	**HKU 4.2**	**HKU 5.5**	**SARS**	**HKU 3**	**279**	**HKU 2**	**1A**
MERS-CoV (β, 2c) Antigènes	**N Protein (VRP)**	Cross reaction (Western Blot, ELISA and IFA		No cross reaction (Western Blot)									
	S Protein (VRP)						No cross reaction (Western Blot)	Cross reaction (Western Blot)	No cross reaction (Western Blot)			Little if any Cross reaction (Western Blot)	
	Patient Isolates	Cross Reaction (ELISA)		No Cross Reaction (ELISA)			No cross reaction (ELISA) & No cross neutralization ($PRNT_{50}$)	No cross neutralization ($PRNT_{50}$)	Low level cross neutralization ($PRNT_{50}$) with high concentration of sera.	No cross neutralization ($PRNT_{50}$)			

B		VRP Expressed N Protein for					VRP Expressed S Protein for						
		BtCoV (β, 2c)		HCoV (β, 2b)	BtCoV (β, 2b)		BtCoV (β, 2c)		HCoV (β, 2b)	BtCoV (β, 2b)		BtCoV (α, 1b)	
		HKU 4.2	HKU 5.5	SARS	HKU 3	279	HKU 4.2	HKU 5.5	SARS	HKU 3	279	HKU 2	1A
Anti-MERS-CoV (β, 2c) Antibodies	Anti-N Mice Sera	Cross reaction (Western Blot, ELISA and IFA		No cross reaction (Western Blot)									
	Anti-S Mice Sera						Little if any cross reaction (Western Blot)		No cross reaction (Western Blot) & No cross neutralization ($PRNT_{50}$)	No cross reaction (Western Blot)			
	Patient Sera	Cross reaction (ELISA)		Transient minimal cross reaction (ELISA)	No cross reaction (ELISA)	Transient very low cross reaction (ELISA)	No cross neutralization		Transient minimal cross reaction (ELISA)	No cross neutralization ($PRNT_{50}$)			

BtCoV: bat coronaviruses. HCoV: human coronaviruses, α & β: phylogenetic groups; 1b, 2b and 2c.: subgroup.

Viral Reservoir & Transmission Mode

The origin of the MERS virus is not yet fully understood. However, several MERS-CoV viral genomes analysis suggests a zoonotic reservoir; originated in bats (72, 92-94) and transmitted to camels' long time ago (5, 6, 95). Bats constitute a diverse and abundant group of mammalian species with extensive geographic distribution and capability of fly (96). Multiple novel coronaviruses have been identified in a various bat species throughout America, Africa, Europe and Asia (97). Various screening studies among multiple bat species within different geographical areas i.e. Ghana, European, South Africa and Saudi Arabia identified bat derived coronaviruses that genetically are very closely related to MERS-CoV (Table 4) (72, 98, 99). Furthermore, an experimental study reported that MERS-CoV replicated in Jamaican fruit bat (Artibeus jamaicensis) without clinical signs of bats illness (100). Collectively, it indicates that MERS-CoV is most likely originated in bats which proposed to serve as an optimal reservoir (101). Yet, no direct contact between bats and humans were reported particularly in Saudi Arabia (101) whereas multiple MERS cases reported contact history with dromedary camels (9, 63). Additionally, genomic sequences showed high similarities (>99 %) for MERS-CoV detected in camels and human isolates (7, 9, 102, 103). Serologic surveillances further confirmed that most of the African and Middle East camels were seropositive for MERS-CoV while other species as goats, cows and sheep were negative suggesting the potential introduction of MERS-CoV to the humans through dromedary camels (4, 5, 9, 95, 104-109). Interestingly, neutralization antibodies of MERS-CoV detected in camels from Sudan and Somalia- main camel exporting countries- could be traced back to more than 30 years (since1983) (6).

The transmission mode is not yet clarified, but is proposed that MERS-CoV could

transmit to human during direct contact with camel (8, 110, 111) through saliva(112) or through consumption of milk (113, 114) or contaminated meat (115). However, the potential of another intermediate host for MERS-CoV transmission to humans may exist (112).

Despite the potential introduction of MERS-CoV to the human beings through infected Dromedary Camels and perhaps other intermediate host yet to be recognized, the vast majority of MERS-CoV infections has evolved via viral transmission from infected to uninfected individuals in prolonged and close contact through poor infection control settings in health care facilities (20). These reported cases or clusters a cross healthcare facilities or household setting, suggest the potential of secondary infection (human-to-human transmission) which may be through air or fomites droplets (116). However, the number of infected individuals through case contact seems to be limited indicating that human-to-human transmission is not sustained (20, 116).

Table 4.

Summary of beta-coronaviruses and MERS-CoV isolated from bats. Adapted from Mohd et al.2016 (101)

Bat Species	Source	Year	No. screened	Type of sample	Virus	% of positive Bats
Nycteris cf. gambiensis	Ghana	2009-2011	185	Fecal	2c betacoronaviruses, (closely related to MERS-CoV)	24.9 %
Coleura afra, Hipposiderosabae, H. cf. gigas, H. fuliginosus, H. jonesi, H. cf. ruber, Rhinolophus alcyone, R. landeri, Taphozous perforates	Ghana	2009-2011	4573	Fecal	2c betacoronaviruses, (closely related to MERS-CoV))	0%
Pipistrellus kuhlii, P. nathusii, P. pipistrellus, P. pygmaeus	Europe: Germany, Netherland, Romania, Ukraine	2009-2012	272	Fecal	2c betacoronaviruses, (closely related to MERS-CoV)	14.70%
Chaerephonpumilus, Mops condylurus, Tadaridaaegyptiaca, H. caffer, Miniopterus natalensis, Nycteristhebaica, R. clivosus, R. darlingi, Neoromicia capensis, N. nana, N. cf. zuluensis, Scotophilus viridis, Rousettus aegyptiacus	South Africa	2011–2012	62	Fecal pellets	bat related-alphacoronaviruses betacoronavirus	6.4 % 1.6 %
Rhinopomahardwickii, R. microphyllum, Taphozous perforatus, P. kuhlii, Eptesicus bottae, Eidolon helvum, and Rosettus aegyptiacus	Saudi Arabia	2012	96	Throat swab, serum, urine, rectal swab or fecal pellets	MERS-CoV	1 %
R. hardwickii, T. perforates, P. kuhlii	Saudi Arabia	2013	14	Throat swabs, roost feces	MERS-CoV	0 %
T. perforatus			82			
P. deserti	Egypt	2013-2015	31	Serum/rectal (alive)	MERS-CoV	0 %
R. aegyptiacus			257			
R. hipposideros			4			
Miniopterus schribersii			6			
	Lebanon	2013-2015		Homogenized lung and liver material (if died or euthanized upon capture)	MERS-CoV	0%
R. ferrumequinm			3			
R. aegyptiacus			438			

Laboratory Diagnosis & Detection

Following first isolation of MERS-CoV, several laboratory tests have been published (117-120). These include several in-house real times reverse transcriptase polymerase chain reaction (RT-rtPCR) assays as well as virus culture (1, 117, 118). But, cell culture is a slow, insensitive and specialized method (121) while specific MERS-CoV RNA sequence real-time reverse transcription polymerase chain reactions (rRT-PCR) are the preferred method for MERS-CoV detection (122). Three rRT-PCR assays for detection of MERS-CoV have been released (117, 118, 122). These assays target one of the following: upstream of the E protein gene (*upE*) (118) , open reading frame 1b (*ORF 1b*) (118) or *ORF 1a* (117). The *upE* assay is recommended for screening (Figure 4) as a highly sensitive assay (118). The *ORF 1a* assay provide an appropriate complement of equal sensitivity to the *upE* assay and thus recommended as confirmatory test (Figure 4) (117), whereas the *ORF 1b* assay showed slightly lower sensitivity than the *upE* assay (117). Another rRT-PCR assay has developed by the United States Centers for Disease Control and Prevention (US CDC). It targets the gene of MERS-CoV N protein and can provide a complement screening and confirmation test to the *upE* and *ORF 1a* assays (123). Earlier as of june.2015, the World Health Organization (WHO) reported that "To date, these rRT-PCR assays have shown no cross-reactivity with other respiratory viruses including human coronaviruses" (122). Later, Kim et al. reported no cross-reactivity with other respiratory viruses among six different commercial rRT-PCR MERS-CoV RNA detection kits: Ultrafast kits (Nanobiosys, Korea, LightMix (Roche Molecular Diagnostics, Switzerland), AccuPower (Bioneer, Korea), Anyplex (Seegene, Korea), DiaPlexQ (SolGent, Korea) and PowerChek (Kogene Biotech, Korea) (124).

The routine Detection of MERS-CoV infection is based on the above mentioned rRT-PCR assays followed by nucleic acid sequencing (Figure 4) for confirmation if needed (122). However, MERS-CoV seroconversion in samples ideally withdrawn at least 2 weeks apart, thorough at least one screening test (i.e. ELISA, indirect fluorescent antibody (IFA) followed with confirmation by a neutralization test meets the current WHO case definition.(122). In another view, The US CDC limited the use of MERS serology tests for investigational or surveillance settings and not for diagnosis (125). They established two-phase serological approach to detect anti-MERS antibodies through one screening test i.e. ELISA followed by a full virus IFA test for confirmation. If the presence of anti-MERS antibodies is not confirmed (indeterminate or negative) by IFA, a microneutralization assay has to be conducted considering it as highly specific confirmatory test (125).

CASE UNDER INVESTIGATION

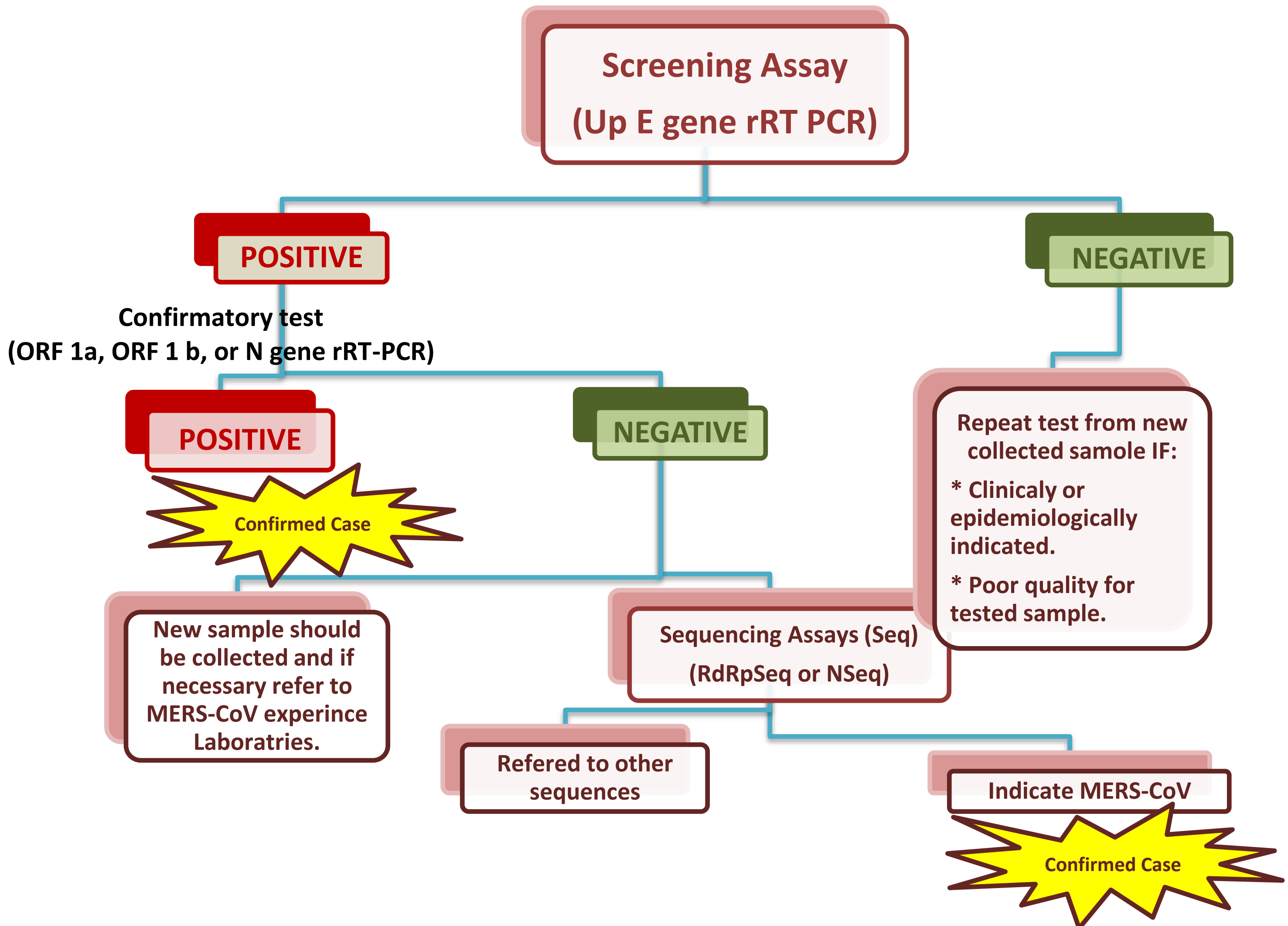


Figure 4. Algorithm for MERS-CoV diagnosis by reverse transcriptase real time polymerase chain reaction (rRT-PCR). ORF: open reading frame, N: nucleoprotein gene, RdRp: RNA-dependent RNA polymerase genes. Adapted from WHO, June 2015 (122).

Comparison of MERS-CoV Serologic Assays

Validated serologic assays play a vital role for MERS seroepidemiology and detection to estimate risk factors and prevalence (111, 122). A wide variety of MERS-CoV serologic assays has been developed including enzyme-linked immunosorbent assay (ELISA), immune-fluorescence assay (IFA), micro-neutralization (MN), plaque reduction neutralization (PRNT) and MERS-spike pseudoparticle neutralization tests (ppNT) (106, 126).

ELISA assays are based on solid surfaces coated with inactivated MERS-CoV or recombinant viral structural antigens: nucleoprotein (NP), full-length spike protein S, S1, receptor-binding domain (RBD), N-terminal domain (NTD, amino acids 18–353 of S protein) (86). Whereas, IFA are relied on cell culture infected with MERS-CoV or expressing recombinant viral structural antigens (i.e. S or N protein) for the detection of anti-MERS-CoV antibodies in human or animal samples (1, 4, 5, 117, 127). Several commercial ELISA kits, and IFA slides/ kits have been released by various companies i.e., *mybiosource* (USA), *komabiotech* (Korea), *Alpha Diagnostic International* (USA). *Euroimmune* (Germany) commercial recombinant S1-based ELISA and whole virus IFA kits were applied in various studies (12, 126, 128-132).

Neutralization assays confirm the reactivity to MERS-CoV by the detection of anti-antibodies capable to neutralize MERS-CoV (126, 133). The PRNT assays determine the neutralizing antibody titers at the highest serum dilutions that resulted in ≥ 50% ($PRNT_{50}$) or ≥ 90% ($PRNT_{90}$) reduction in the number of viral plaques within stained culture plates of cell line incubated earlier with mixture of serum dilutions and viral plaque-forming units (126). Quite like PRNT, the MN assays measure neutralizing antibody titers at the highest

serum dilution that suppressed virus cytopathic effect in microtiter plate containing cell line following infection with a mixture of serum dilutions and infectious viral dose (106, 126).

The development of ppNT offers a safe alternative for the live MERS-CoV (biosafety level 3) neutralization assay (133). The ppNT determine the highest serum dilution showing a 90% reduction for the activity of the luciferase enzyme following lysis of cell line incubated earlier with mixture of serum dilutions and pseudotyped MERS-CoV, a recombinant lentivirus encoding luciferase and MERS-CoV S protein and has power of one-cycle infection but can't undergo multiple replication cycles. (106, 133, 134).

Data comparing serological assays for detecting anti-MERS-CoV antibody in humans are limited (86, 126). Recently, Wang et al. compare the detection of MERS-CoV antibodies in a series of sera from a MERS-CoV confirmed patient using ELISA based on inactivated MERS-CoV or alternative recombinant antigens (NTD, RBD, S, S1 and NP) and ppNT assays (86). For S1- based ELISA, they purchased the commercial Anti-S1 MERS-CoV IgG ELISA Kit from EUROIMMUNE (Luebeck, Germany) whereas for other recombinant antigens they used inhouse ELISA assays (86). The S1, S and RBD ELISAs reported more sensitivity than the whole virus ELISA for detecting anti-MERs-CoV antibodies with highest sensitivity reported for MERS-CoV S-based ELISA (86). Further, the study illustrated a strong correlation of S ELISA with the ppNT (0.9319 Pearson's correlation coefficients, Figure 5). Though, S1, RBD, NTD and inactivated MERS-CoV ELISAs were also correlated significantly ($P<0.001$) with the ppNT (0.71– 0.8439 Pearson's correlation coefficients, Figure 5) (86). Excellent correlations were reported for the MERS-CoV ELISA with the S1, NP and RBD based ELISAs (0.9292–0.9488 Pearson's

correlation coefficients, Figure 5). Whereas, NTD and S ELISAs showed lower correlation (Figure 5) with the MERS-CoV ELISA (0.8122–0.8420) (86). Subsequently, S1 ELISA showed excellent correlations (Figure 5) with the RBD and the S ELISAs (0.9234–0.9701). However, the NTD ELISA was less strongly correlated (Figure 5) with the S1 ELISA (0.8807) (86).

Earlier to Wang et al. study, Park et al. conducted a serologic assays comparison on 95 sera from 17 confirmed MERS-CoV patients using S1-ELISA from EUROIMMUNE, MN, $PRNT_{50,}$ $PRNT_{90}$, and ppNT (126). MERS neutralization assays ($PRNT_{50,}$ $PRNT_{90}$, MN, & ppNT) showed excellent correlation (≥ 0.94) (126). The $PRNT_{50}$ was the most sensitive tests and could be the only one that can detect anti-MERS antibodies at early stage of the infection and in cases with weak antibody responses (126).However, the ppNT test had good correlation with MN and $PRNT_{90}$ assay and unlike other neutralization assays it does not include handling live virus, therefor no biosafety level 3 containment required (126). The specificity of spike ppNT was previously confirmed when sera from healthy humans from Saudi Arabia, Hong Kong and Egypt tested negative with ppNT (126). In the other hand, MERS S1 ELISA gave lower, but acceptable, correlations (0.86–0.87) with the different neutralization tests, regarding the seroconversion time in MERS patients. This lower correlation is not surprising because S1 ELISA assay is detecting IgG only and is not an active neutralizing test (126).

Moreover, Park et.al had demonstrated in various studies that the virus clade is not affecting antibody titers. In other word, “Genetically diverse MERS-CoV are antigenically homogenous” (126).

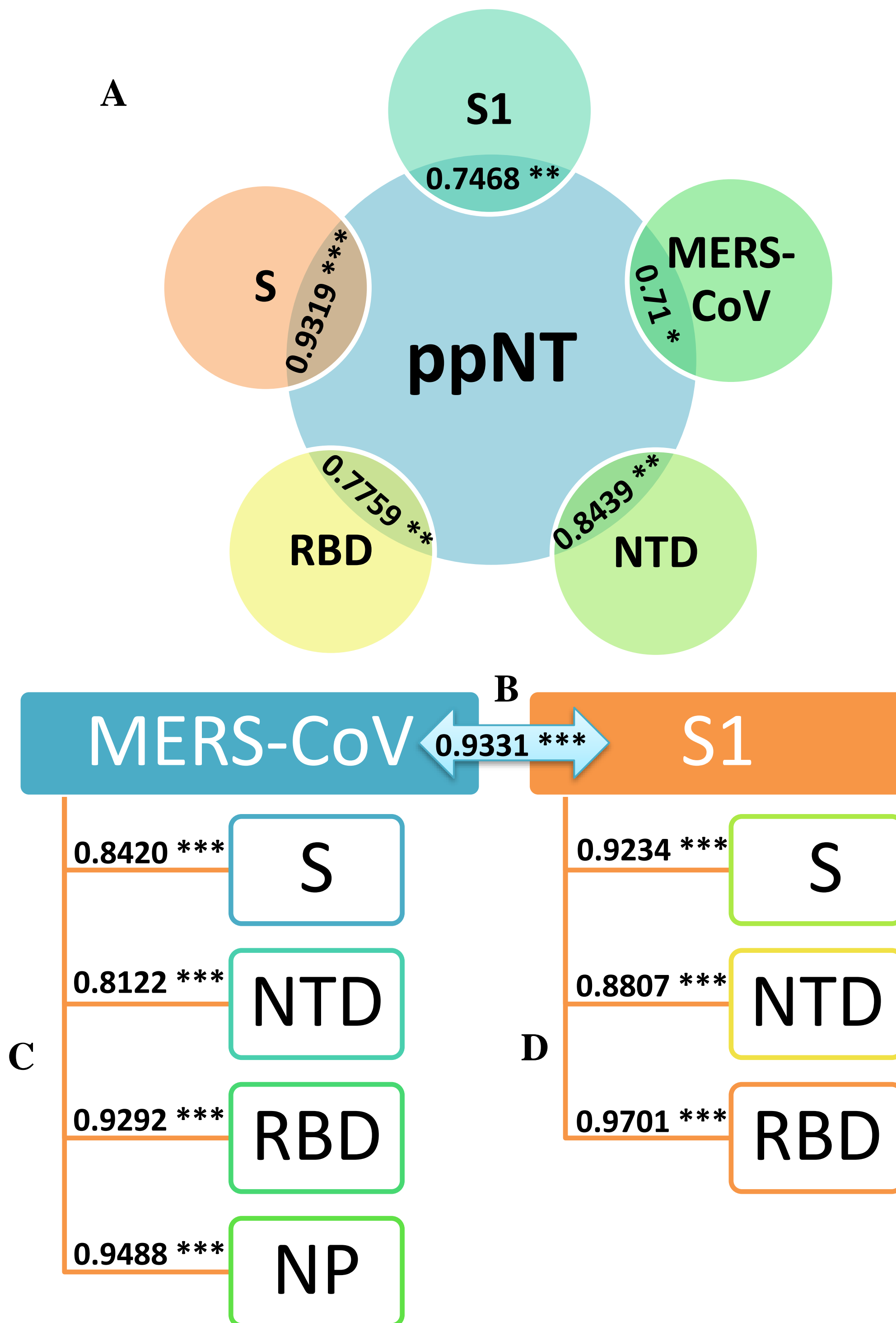


Figure 5. Correlations (Pearson's correlation coefficients) among **A**. MERS-CoV ELISAs (IgG) and the ppNT assay, **B.** the Euroimmune anti-S1 MERS-CoV Kit and the inactivated MERS-CoV ELISA (IgG), **C.** the inactivated MERS-CoV based and recombinant protein based (S, NTD, RBD and NP) ELISAs (IgG), **D.** the Euroimmune anti-S1 MERS-CoV Kit with other recombinant MERS-CoV protein-based IgG ELISAs (S, NTD and RBD). **P*<0.05; ***P*<0.01; ****P*<0.001. ELISA: Enzyme-linked immunosorbent assay, MERS-CoV: Middle East respiratory syndrome coronavirus, NP: nucleoprotein, NTD: N-terminal domain, RBD: receptor-binding domain, S: recombinant MERS-CoV full-length spike protein. S1: recombinant MERS-CoV spike protein subunit 1. Summarized from Wang et al. 2016 (86).

Human Antibody Response

Interpretation of sero-epidemiologic data in defining prevalence and risk factors for MERS-CoV infection is based on the understanding of the kinetic serologic response to the infection (130). However, limited studies addressed this issue. These studies showed that the severity of the infection is reduced by early MERS-CoV antibody response (130). It was demonstrated that MERS antibodies developed during the second to the third week after the onset of illnesses, resembling that of SARS-CoV (132), with a strong anti-S1 IgG -detected by ELISA- and neutralizing antibody responses at the third week of onset in most of studied patients (86, 130). Nevertheless, few patients failed to develop a strong response. Thus, serologic assay may fail in detecting infections in such subjects (130).

Corman et al. showed that there was no earlier detection of IgM than IgG using anti-MERS-CoV Indirect immunofluorescent test (IIFT-IgM) and recombinant enzyme-linked immunosorbent assay (ELISA; Anti-MERS-CoV ELISA IgG, Euroimmun, Germany) (132). They suggested that this finding, particularly when suspecting a cross-reacting IgM for more prevalent human coronaviruses, restricts the diagnostic utility of IgM, using same methodology, to cases when an overcome and recent MERS-CoV infection must be approved (132). Interestingly, Wang et al. recent study showed that IgG antibodies to specific MRS-CoV antigens were detectable earlier or in parallel with the IgM (86). They referred this either to the sensitivities variations of the immunoglobulin class-specific ELISAs or later development of IgM than IgG antibodies (86).

Seroconversion determined by Wang et al. study using S based ELISAs were detected as early as neutralizing antibodies with the ppNT assay (86). First, anti-S IgG

antibodies were detected by ELISA (IgG on day 5 following admission). Next, anti-S IgM antibodies (day 6) and followed by anti-S1 and anti- RBD antibodies, then antibodies against inactivated MERS-CoV and NTD. Finally, anti- NP antibodies occurred last (days 12–13) (Figure 6) (86).

Viral Shedding

Studies of the Virus shedding showed that the concentrations of RNA found in lower respiratory tract samples are at least two orders of magnitude higher than the RNA concentration in the upper tract samples, stool or serum (132). The Viral RNA loads gradually decline in the lower respiratory tract secretions (132). However, shedding usually persist for 3 weeks or more (132). The severity of disease is proportional to the viral RNA load detected in higher respiratory tract (132).

In the other hand, asymptomatic subjects express prolonged viral shedding of nearly 6 weeks detected by polymerase chain reaction (PCR) (135). This finding raise a concern for the potential of viral transmission from asymptomatic subjects (136). However, it is not clear how a detected RNA by PCR is refer to an infectious virus (136, 137).

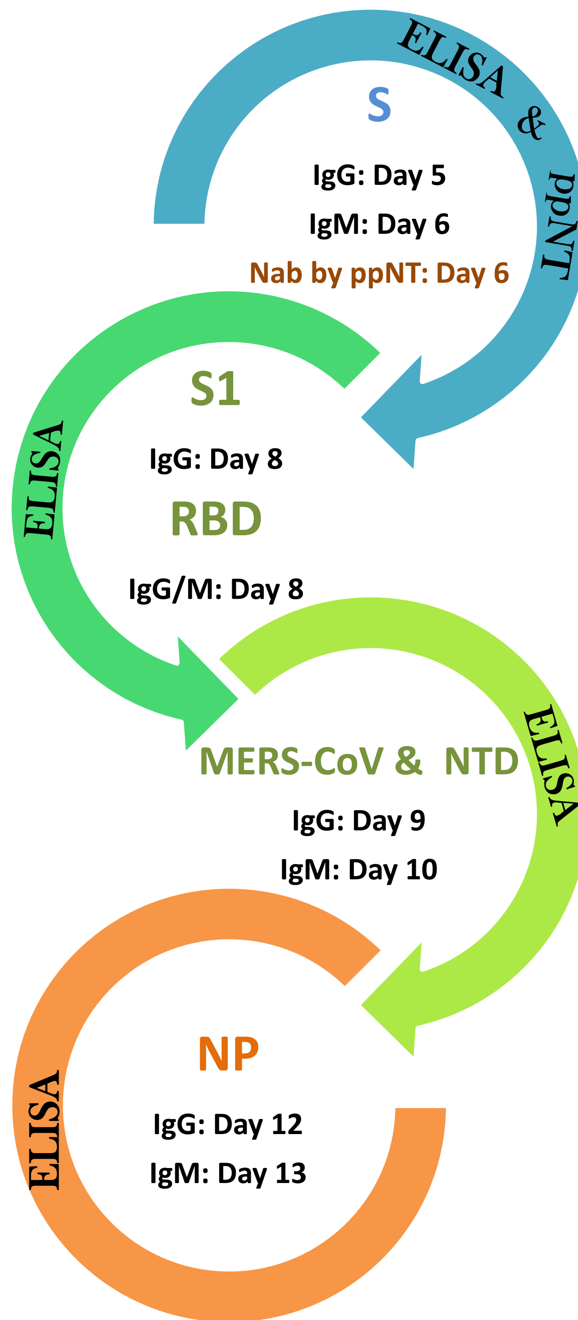


Figure 6. Timeline (days after hospitalization) of detection of IgG, IgM and neutralization antibodies in the first MERS-CoV case in China (May.2015) determined by recombinant antigens (S, S1, RBD, NTD and NP) based enzyme-linked immunosorbent assay (ELISA) and the pseudovirus particle neutralization test (ppNT). The patient was hospitalized 8 days after the onset of illness. S: full-length spike protein, Nab: neutralizing antibody, S1: Subunit 1 of spike protein, RBD: receptor-binding domain, MERS-CoV: inactivated Middle East respiratory syndrome coronavirus, NTD: N-terminal domain, NP: nucleoprotein. Summarized from Wang et al. 2016 (86)

CHAPTER 3: MATERIAL AND METHODS

Study Design, Sample Collection and Ethical Compliance

This cross sectional MERS-CoV IgG sero-surveillance study was conducted among two groups of Qatar population: the low risk group or the blood donors group and high-risk group or the case contacts. Anonymized sampling for blood donors was accomplished through existing arrangement between Biomedical Research Center (BRC) at Qatar University and Blood Donation Center at Hamad Medical Corporation. For case contacts, samples were sent to BRC through the Ministry of health of Qatar. This study was approved by Qatar University-Institutional Review Board (QU-I 622-E/16 RB).

Sample Size

As per review literature there is no precise information and estimates available on the primary seroprevalence of MERS-CoV among healthy individuals in Qatar. To the best of our understanding this is the first study of its kind to be conducted in Qatar and hence due to non- availability of precise information related to primary outcome, no prospective statistical power had been planned for specimen collection.

In total, this study included 4858 subjects: 4719 blood donors collected over five year periods (2012- 2016), 135 baseline case contacts collected from individual that were in close contact to three positive confirmed cases (case no. 14, 16, and 18, Table 2) and four confirmed MERS-CoV patients (case no. 13,14, 16 and 17, Table 2). The demographic profiles for all subject included in the study are shown in Table 5,6 & 7.

Table 5

Characteristic profile of study population/ blood donors

	2012	2013	2014	2015	2016	Total
Qatari	28	6	111	660	123	**928**
Male	16	5	107	656	122	906
Female	12	1	4	4	1	22
Non-Qatari	92	22	500	2723	454	**3791**
Male	77	22	489	2694	454	3736
Female	15	0	11	29	0	55
Age Range (Mean)	27-65 (37)	21-56 (38)	18-64 (36)	17-88 (37)	19-66 (37)	19-88 (37)
Total	120	28	611	3383	577	**4719**

Table 6

Characteristic profile of study population/ case contacts

	Case Index			
	May. 2015 (No.14)	**Feb. 2016 (No.16)**	**Jun. 2016 (No.18)**	**Total**
Qatari	8	6	0	14
Male	6	5	0	11
Female	2	1	0	3
Non-Qatari	92	4	25	121
Male	64	4	25	93
Female	28	0	0	28
Age Range (Mean)	20-49 (35)	14-48 (32)	20-37 (26)	14-49 (31)
Family contact	31	6	--	37
Health Care Worker	69	4	--	75
Farm Camel Contact	--	--	25	25
Total	100	10	25	135

Table 7

Characteristic profile of study population/ MERS-CoV confirmed patients

	Case Index				
	March.2015 (No.13')	**May. 2015 (No.14')**	**Feb. 2016 (No.16')**	**May. 2016 (No.17')**	**Total**
Qatari Male	1	--	1	--	2
Non-Qatari Male	--	1	--	1	2
Total	1	1	1	1	4
Age	69	29	66	40	29-69
Exposure	Frequent camels contact	Frequent camels contact	A camel barn owner in Saudi Arabia	occupational exposure to Camels	
Total	1	1	1	1	4

Serological Testing

MERS-CoV Serologic Assays

Recombinant S Protein Enzyme Linked Immunosorbent Assay IgG (rELISA)

Initially all blood samples were screened for the presence of anti-MERS IgG using ELISA. The ELISA plate is coated with purified S1 MERS-CoV antigens, developed by the invitro diagnostic company "*Euroimmune*" and provided in a ready-to use kit format (cat no. EI 2604-9601 G). The test has been performed following manufacture instructions. Later, endpoint rELISA titration has been performed for all borderline and reactive anti-MERS-CoV IgG samples.

Full Virus Indirect Immunofluorescence Test IgG (IIFT IgG)

To confirm the presence of anti-MERS-CoV IgG, we tested borderline and reactive anti-MERS-CoV IgG rELISA samples with anti-MERS-CoV IgG IIFT. The test has been performed using Vero cells infected with MERS-CoV; spotted and fixed on glass slide (117) (Figure 7) and provided within a reagent kit set (cat no. FI 2604-1005 G / M) by an invitro diagnostic company (Euroimmune, Germany). Initially samples were diluted with sample buffer at titers of 1:100 (Figure 8). Next, 30 µl of diluted samples along with ready to use negative and positive controls were transferred into reaction fields of the reagent tray (Figure 9). Subsequently, BIOCHIP slides were fixed into the corresponding recesses of the reagent tray. Following 30-minutes incubation at room temperature, the BIOCHIP slides were rinsed with a flush of washer solution (phosphate buffer saline with tween 20: PBS Tween) and then immediately immersed in a beaker containing the washer solution for 5 minutes. Thereafter, BIOCHIP slides were removed from PBS Tween, blotted at the back with a paper towel and fixed on the recesses of the reagent tray containing 25 µl of

fluorescein labelled anti-human globulin at the reaction fields. Following 30 minutes incubation at room temperature away from direct light, the BIOCHIP slides were rinsed and washed in the same manner described above. Finally, cover glasses containing 10 µl / reaction field of embedding medium were applied on the washed BIOCHIP slides. Slides were evaluated for fluorescence reaction under a fluorescent microscope. MERS-CoV antibodies cause fine to coarse granular fluorescence pattern mainly in the cytoplasmic area of the infected cells (Figure 10).

Full Virus Indirect Immunofluorescence Test IgM (IIFT IgM)

Borderline and reactive anti-MERS-CoV IgG rELISA samples have been screened for the presence of anti-MERS-CoV IgM using anti-MERS-CoV IgM IIFT. The test has been performed using reagent kit set (cat no. FI 2604-1005 G / M) from Euroimmune, Germany in the same manner describe above for IIFT IgG (Figure 9). The only difference is that apart from sample dilution, here samples was diluted (Figure 8) by EUROSORB reagent (goat anti-human IgG antibody) rather than sample buffer to deplete IgG antibodies by immunoadsorption.

Along with the borderline and reactive anti-MERS-CoV IgG rELISA samples, we tested 13 randomly selected (12 blood donors and 1 case contact) negative samples with anti-MERS-CoV IgG/ M IIFT.

Recombinant S Protein Indirect Immunofluorescence Assay IgG (rIIFA)

To reduce the possibility of cross reactivity of human sera with the full MERS virus antigen presented by Vero cells in the virus IIFT IgG test kit, we screened borderline and reactive anti-MERS-CoV IgG rELISA samples along with the 13 selected negative ones using recombinant S protein IIFA IgG kit. This test was performed in institute for Virology,

University Medical Center Bonn, Germany using cells verso cells expressing the MERS S protein. Vero cells were transfected as follow: First, 2.5 µg of plasmid DNA harboring the gene encoded for the S protein was added to 500 µl Opti-Pro (serum-free medium). Following vortex and centrifugation, 7.5 µl FuGene HD (transfection reagent Promega Cat# E2311) was applied and incubated for 15 minutes at room temperature. Transfected Vero cells were harvested and seeded (1x10^6 cells/well) into a 6-well plate with 2.5 ml Dulbecco's Modified Eagle Medium (DMEM) 10% Fetal Calf Serum (FCS) without antibiotics. Then the transfection mix were added on the cells. After one day incubation in a cell culture incubator at 37°C, transfected Vero cells were harvested using trypsin-treatment and then re-suspended in 5 ml DMEM. Following centrifugation at 300 x g for 5 min, 50 µl of cells (density: 2.5x10^5 cells/ml of DMEM 10% FCS without antibiotics) were applied on the spot area of multi-test cover slides in a humid chamber. After 6-hours incubation at 37°C in a cell culture incubator, slides were twice washed with PBS and cells were fixed with ice-cold 1:1 acetone/methanol for 10 min. Slides were dried at room temperature. Further, the same protocol described above (under IIFT-IgG) was applied for the detection of human anti-MERS-CoV S IgG.

Pseudoparticle neutralization tests (ppNT)

Regardless of the confirmatory results of IIFT IgG, borderline and reactive anti-MERS-CoV IgG rELISA samples along with the 13 selected negative ones were further tested by ppNT. In this test, two MERS-CoV strains were used: EMC (GenBank JX869059) and Jordan N3 (GenBank KC776174) strains. The test was performed at in Virology Laboratory -NIH, USA as follow (138).

Pseudovirus Generation

293T cells were cultured in 15-cm plate using DMEM containing 2 mM glutamine, 1X penicillin/streptomycin (D10) and 10% bovine serum albumin (BSA) in a 37 °C 5% CO2 incubator. Cell were then transfected at 90% confluency using calcium phosphate transfection solution (Invitrogen) with the following plasmids: pCMVΔR8.2 (packaging plasmid ,17.5 μg), pHR' CMV-Luc (transducing plasmid, 17.5 μg) and CMV/R-MERS-CoV S plasmid (1 μg). Following overnight incubation, old medium was discarded and replaced with a fresh D10. After 48 hours, the cell supernatants were collected and filtered with 45 μm filters and subsequently stored at −80 °C.

Pseudovirus Titeration

10^6 Huh7.5 cells were seeded into a 96-well white/black Isoplate (PerkinElmer, Waltham, MA) using same condition described above. After overnight incubation, old medium was removed and 50 μl of twofold serial dilutions of pseudovirus were applied to the cells for 1 hour and additional 100 μl of fresh media was then added.

Neutralization Assay

Serial dilutions of human samples were mixed with 10^6 relative fluorescence unit (RFU) of two strains (EMC and Jordan N3 viral MERS-CoV). After 30 minutes of incubation at room temperature, mixtures were added in triplicate to Huh7.5 cells cultured in 96 wells as described above. Following 2 hours of incubation, plates were refilled with 100 μl/ well of fresh media. 72 hours later, cells were lysed using luciferase cell culture lysis (Promega, USA) and 50 μl of luciferase substrate (Promega, USA) was applied to each well. Microbeta luminescence counter (PerkinElmer) was used to measure luciferase activity in relative luciferase unit and neutralizing activity was reported as the dilution of each sample

required to inhibit 50%, 80% and 90 % (IC50, IC 80 and IC 90) of infection.

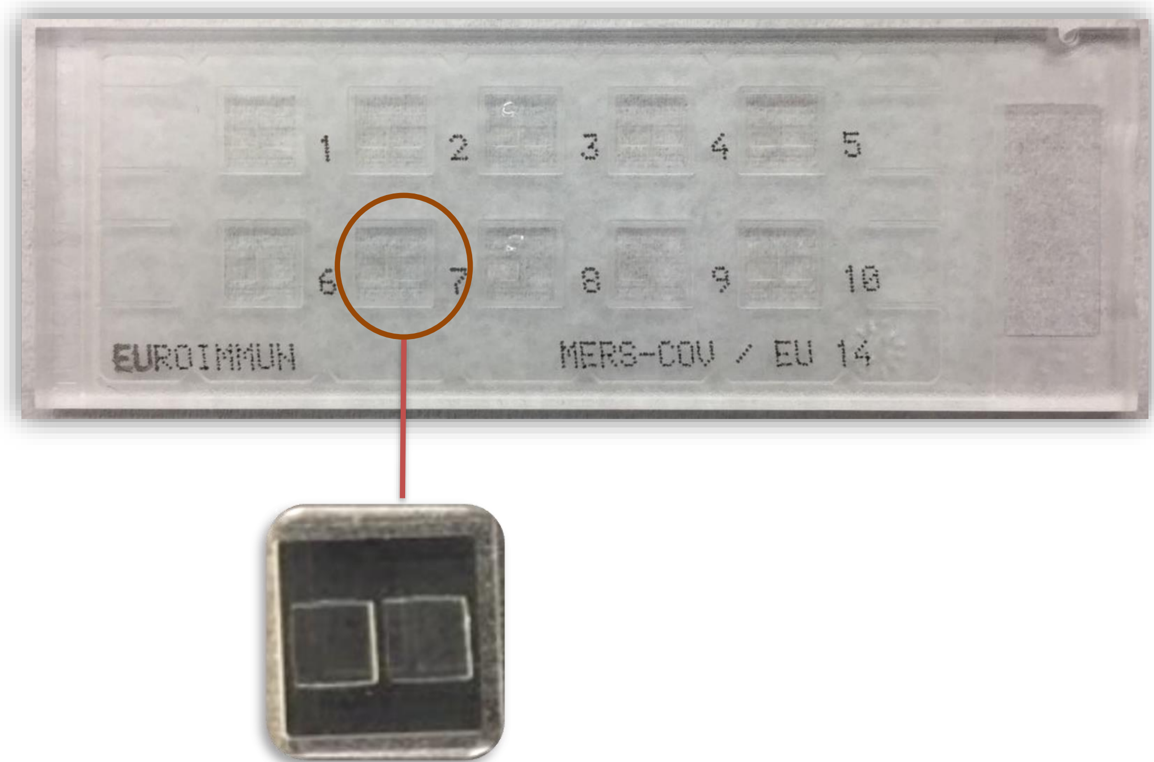
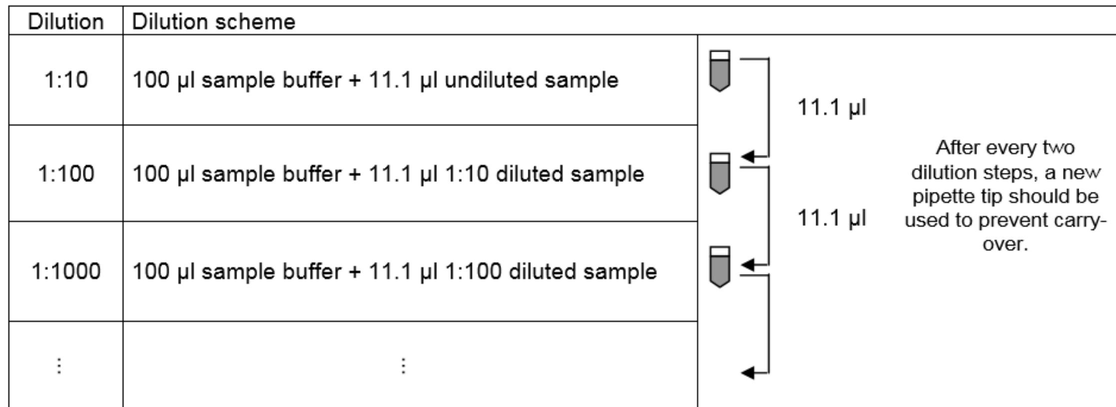


Figure 7. Anti-MERS-CoV IIFT BIOCHIP slide. Each slide contains 5 X 2 Biochips: one chip coated with MERS-CoV infected cells and the other coated with non-infected cells.



Dilution	Dilution scheme
1:10	100 µl sample buffer + 11.1 µl undiluted sample
1:100	100 µl sample buffer + 11.1 µl 1:10 diluted sample
1:1000	100 µl sample buffer + 11.1 µl 1:100 diluted sample
⋮	⋮

Figure 8. **Samples dilution scheme for indirect immunofluorescent test.** Sample dilution for semi-quantitative evaluation of antibodies of class IgG: apply 100 µl of sample buffer to each tube and mix with 11.1 µl of the next highest concentration then vortex for 2 seconds. Incubate samples from 1:100 dilution.

Sample dilution for semi-quantitative evaluation of class IgM: dilute the patient samples 1:10 with EUROSORB (i.e.: 11.1 µl sample to 100 µl EUROSORB). Incubate the mixture for 15 minutes at room temperature or centrifuge the mixture (5 minutes, 2000 rpm, room temperature). Incubate samples from 1:10 dilution. Exported from Euroimmune.

EUROIMMUN Medizinische Labordiagnostika AG

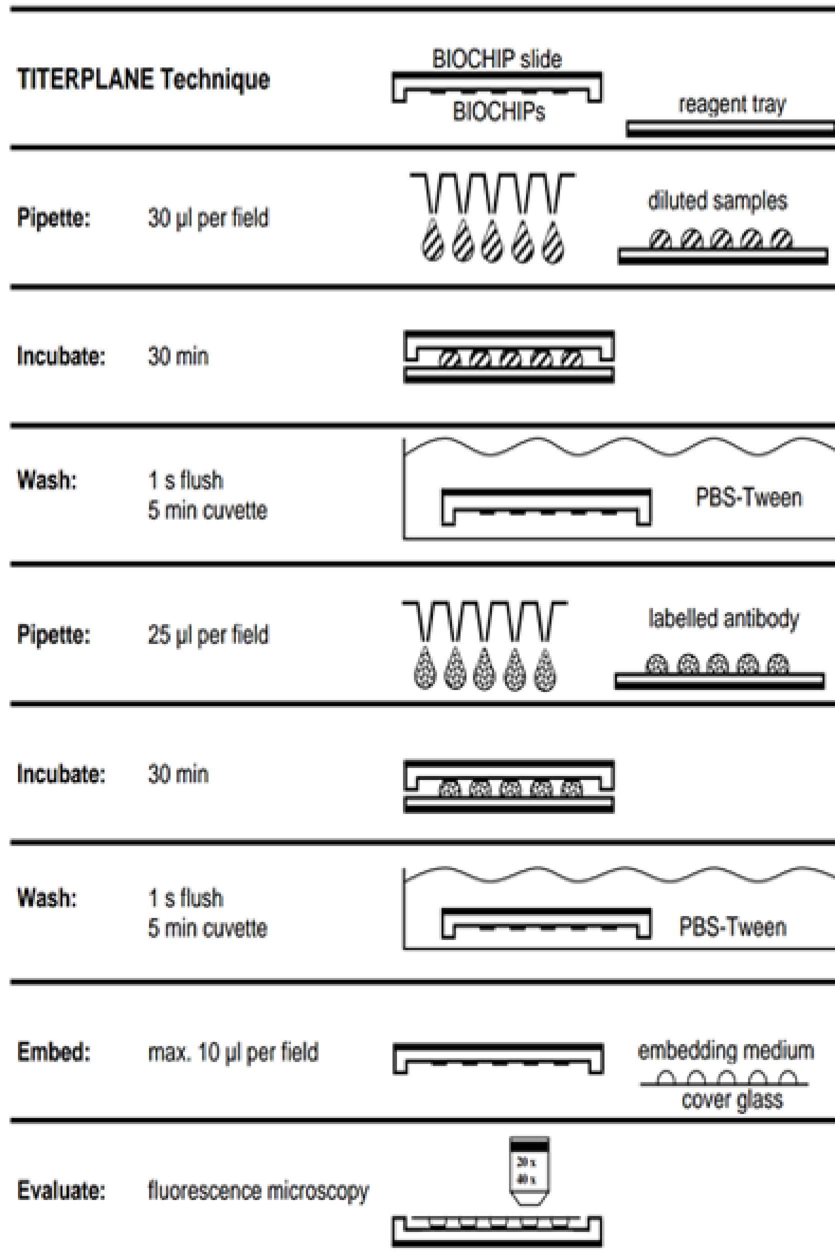


Figure 9. Indirect immunofluorescent test layout. Exported from Euroimmune.

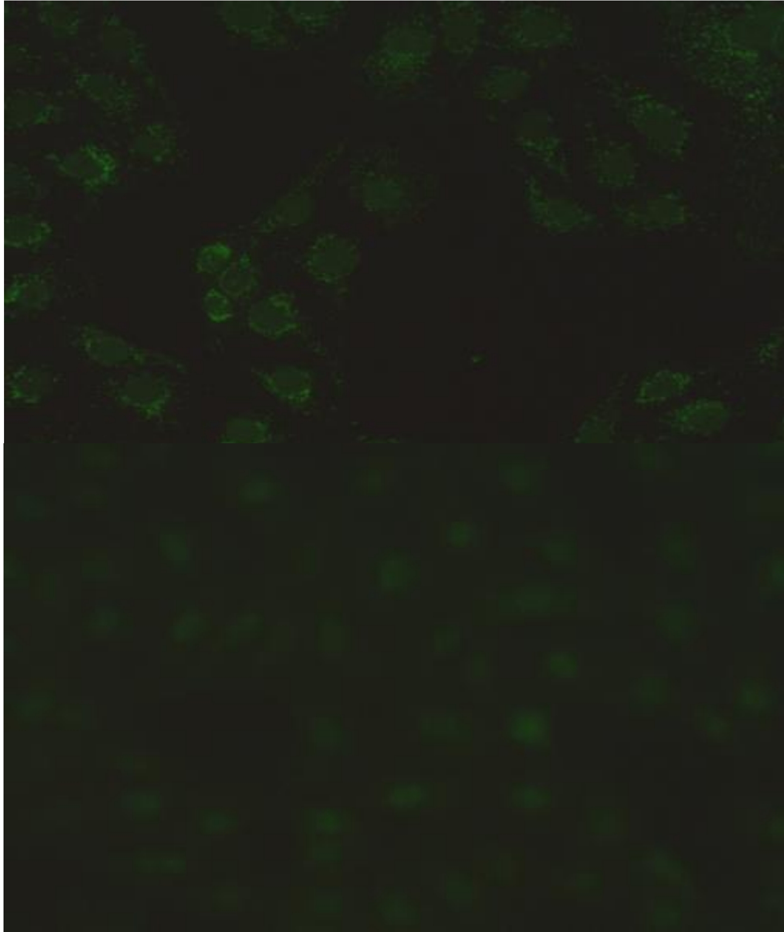


Figure 10. **Anti-MERS IgG indirect immunofluorescence control results view**
1. Right chip for Reactive control. (Antibody against Mitochondria (AMA, IgG).
2. Right chip for Negative control.

Right chip coated with MERS Coronavirus infected cells and the left Chip coated with non-infected cells. Anti-MERS antibodies cause fluorescence of fine to coarse granular structure coating viral material mainly in the cytoplasm of the infected cells.

Known Pathogenic Human Coronaviruses Serologic Assays

To access cross reactivity among both rELISA and IFA for anti-MERS-CoV antibody with other family member of pathogenic human coronaviruses, we initially tested borderline and reactive samples for the presence of the of **IgG** (and IgM for specific samples) antibody of **SARS-CoV** and **HCoV-229E** using the Euroimmune **Full Virus IIFT** (as described above under MERS-coV Full Virus IIFT IgG).

Further, we tested borderline and reactive anti-MERS-CoV IgG/M samples along with the 13 randomly selected negative samples for the presence of IgG antibodies to the human coronavirus **HKU-1** using in-house recombinant **S protein anti-IgG ELISA**. Initially, a flat ELISA plate was coated (0.1 ml/ well) with HKU1 spike glycoprotein (Catalog # 40021-V08H, Sino Biological Inc.) at 2 µg/ml in PBS at 4 °C overnight. Following incubation, the coating buffer has been discarded and residue has been absorbed by tapping plate on paper towel. Next, the plate was blocked by incubating with 200-300 µl blocking buffer/ well (PBS with 5% skim milk) for 1-2 hours at room temperature. After that, plate has been washed in triplicate with 300 µl/ well of washing buffer (Bio-Rad, Plaetlia) using THERMO Plate Washer and buffer residue was absorbed by tapping plate on paper towel. Further, 100 µl of blocking buffer was added as a blank at the first well followed by serial samples dilutions of 1:100. Following 1 hour incubation at room temperature at dark, plate was washed in same manner described above. Subsequently, 100 µl / well of peroxidase labelled anti-human IgG monoclonal antibodies (Bio-Rad, Plaetlia) were applied and incubated for 1 hour at room temperature. After washing plate, 100 µl/ well of tetramethylbenzidine/hydrogen peroxide (TMB) Substrate (Bio-Rad, Plaetlia) were added and incubated for 5-7 minutes at room temperature (light protected). Reaction was

stopped by addition of 100 µl (per well) of 0.3 molar Sulphuric acid (Bio-Rad, Plaetlia). Plate was read at 450 nm with 650 nm reference using Epoch 2 Microplate Spectrophotometer, Bio Tek and Endpoint titer has been calculated as the highest dilution of 5 times increase in optical density in referral to background reading (blank).

Finally, we extended our assessment and screened borderline and reactive anti-MERS-CoV IgG/M samples along with the 13 randomly selected negative samples for the presence of IgG antibodies to all known pathogenic human coronaviruses (**HCoV-229E, HCoV OC43, SARS-CoV, HCoV NL63 and HCoV HKU-1**) using **recombinant S protein IIFA** in the same manner described earlier -under MERS-CoV serologic assays/ IIFA-.

Statistical Analysis

Microsoft Excel was used in our cross sectional descriptive study to manipulate data. Several functions were used to express data i.e. sum, mean, max., min., percent. Additionally, "find" feature was applied to calculate the count of study population referred to various demographic features. Yet, more specific statistical analysis i.e. tests correlation; specificity and sensitivity are further planned upon including more reactive samples i.e. patient samples.

CHAPTER 4: RESULTS

Between 2012 and 2016, we obtained 4719 blood samples from the Blood Donor Center in Qatar. Blood donor participants were aged 17-88 years (mean age 37 years). Additionally, we obtained blood samples from 135 MERS-CoV case contacts (aged 14-49 years; mean age 31 years) and from 4 confirmed MERS-CoV cases (aged 30-70 years; mean age 52). The anti-MERS CoV-IgG seroprevalence as jugged by anti-MERS-CoV rELISA-IgG was 0.13% (7/4719) in healthy blood donor or ratio of 1: 674, 0.74% or 1:135 in case contacts (high risk) and 80% (4/5) or 4:5 for patients. No one of the reactive donor samples was confirmed by (Table 9 A & B) ppNT, suggesting that the true ratio of anti-MERS-Cov-IgG antibody in healthy donors is 0: ~5000. However, one confirmed -by ppNT- reactive anti-MERS-CoV-IgM was detected in blood donors (Table 10 C). For the high-risk case contact group and the true patients (confirmed by PCR), the above-mentioned ratios were not affected as of rELISA-IgG results were confirmed in all samples by ppNT assay (Table 9 B).

All blood samples were initially screened for anti-MERS-CoV S IgG by rELISA giving borderline results (Ratio 0.8- <1.1) in 3 samples (all are blood donors) and reactive results (Ratio ≥ 1.1) in 12 samples (7 donors, 1 case contact and 4 samples collected from 3 MERS-CoV confirmed patients (Table 8). Subsequent testing with full MERS-CoV IIFT IgG confirmed only 5 reactive rELISA results (2 blood donors and 3 patients; the reactive case contact was not sufficient to be tested by IIFT IgG, Table 9 A & B). However, none of borderline or reactive IgG blood donors were confirmed by recombinant S MERS-CoV IIFA or ppNT. Both recombinant S IIFA and ppNT only confirmed the presence of specific

anti-MERS-CoV antibodies in patients' samples (Table 9 A & B). The reactive rELISA anti-MERS-CoV IgG case contact was also confirmed by ppNT; however, it was not sufficient for testing with immune-florescent assays (Table 9 B). In our study, one patient sample (May. 2016) screened negative (Ratio < 0.8) for anti-MERS S IgG by rELISA and immunofluorescent assays. Unfortunately, we were not able to screen this sample by ppNT due to difficulties in shipment (Table 10 C).

Further investigation for borderline and reactive anti-MERS-CoV IgG samples with IIFT IgM yielded only one IgM reactive (titer = 100) patient (Feb.2016) sample (Table 10 A). Interestingly, one donor sample (D2015/3379) which screened negative for anti-MERS-CoV IgG by rELISA and immunofluorescent assays (Table 10 C) showed reactive IgM (titer = 320) and was confirmed by ppTN.

Subsequent assessment of reactive specificity of anti-MERS-CoV IgG rELISA and IIF with other human pathogenic coronaviruses (HCoV-229E, HCoV OC43, SARS-CoV, HCoV NL63 and HCoV HKU-1) is summarized in Table 9 A, B & C. Majority of tested samples including the negative control samples (total=28) showed reactive titers against recombinant spike proteins of HCoV-229E (100 %), OC43-CoV (96.55 %), NL63 (85.71 %) and HKU1(89.29 %), but not with the SARS-CoV spike protein. Only two samples from two MERS-CoV infected patients (P/March.2015 and P/Feb.2016- second sample) reacted with SARS-CoV spike protein with titer of 320 and 3200, respectively (Table 10 A, B &C).

In our study, full virus IIFA-IgG assays for HCoV-229E and SARS-CoV showed slightly lower number of reactive samples than rIIFA-IgG (Table 10 A, B & C). Three samples of two MERS-CoV infected patients, which showed negative reaction with full

viral HCoV-229E IIF-IgG reacted with HCoV-229E spike protein in rIIFA (Table 10 A). Consistently, two samples collected from MERS-CoV infected patients reacted with SARS-CoV spike protein, whereas only the one with higher anti-SARS spike protein IgG titer reacted with full SARS-CoV IIF-IgG (Table 10 A). On the other hand, tested samples showed more reactive results with HKU1 IgG rELISA than with IgG rIIFA. Three samples of two MERS-CoV infected patients which reacted with HKU1 spike protein in rELISA yielded negative reaction with HKUI rIIFA (Table 10 A).

Interestingly, one patient sample (P/ May.2015, Table 10 A) initially screened reactive for anti-MERS IgG (optic density (OD) = 0.412, Ratio = 1.37) with rELISA-IgG, later, about one year, the same frozen sample was tested for anti-MERS-IgG using same rELISA-IgG and yield negative result (OD < 0.2) in 4 independent runs. However, we tested this sample along with other borderline and reactive samples by IIFA, rIIF-IgG and yielded reactive result. Other reactive samples of the same batch yielded the same ELISA result when tested after one year.

Table 8

Result of anti MERS-CoV S IgG rELISA for subjects in Qatar.

Sample Source	Year of Collection (No. screened)	No. Borderline / No. Screened (%)	No. reactive/ No. Screened (%)
Blood Donors			
	2012 (120)	1/120 (0.83)	0 (0)
	2013 (28)	0/28 (0)	0 (0)
	2014 (611)	0/611 (0)	1/611 (0.16)
	2015 (3383)	1/3383 (0.03)	5/3383 (0.15)
	2016 (577)	1/577 (0.17)	1/577 (0.17)
	Total (4719)	**3/4719 (0.08)**	**7/4719 (0.13)**
Base Line Case Contacts			
	May.2015 (100)	0/100 (0)	1/100 (1)
	Feb. 2016 (10)	0/10 (0)	0/10 (0)
	June. 2016 (25)	0/25 (0)	0 /25(0)
	Total (135)	**0/ 135 (0)**	**1/ 135 (0.74)**
Patients			
	Mar. 2015 (1)	0/1 (0)	1/1 (100)
	May. 2015 (1)	0/1 (0)	1/1 (100)
	Feb. 2016 (2) *	0/2 (0)	2/2 (100)
	May. 2016 (1)	0/1 (0)	0/1 (0)
	Total (5)	**0/5 (0)**	**4/5 (80)**
All samples	**Total (4859)**	**3/4859 (0.06)**	**12/4859 (0.25)**

* collected in two different dates (admission and discharge).
Total number and (percentage) of reactive anti-MERS-CoV IgG by rELISA over the total no. screened for each group of the study population. MERS-CoV: middle east respiratory syndrome coronaviruses. rELISA: recombinant enzyme linked immunosorbent assay

Table 9

Evaluation of (A: Borderline, B: Reactive) anti-MERS-CoV rELISA-IgG samples with anti-MERS-CoV immunoassays & ppNT

A	**Borderline rELISA-IgG**			
Sample Source Year of Collection: No. Borderline/ No. Screened (%)	**No. reactive/ No. Screened (%)**			
	Full Virus IIFA-IgG	**Full Virus IIFA-IgM**	**rIFA-IgG**	**ppNT**
Blood Donors				
2012: 1/120 (0.83)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
2015: 1/3383 (0.06)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
2016: 1/577 (0.17)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
Total: 3	**0/3 (0)**	**0/3 (0)**	**0/3 (0)**	**0/3 (0)**
B	**Reactive rELISA-IgG**			
Sample Source Year of Collection: No. reactive/ No. Screened (%)	**No. Reactive / No. Screened (%)**			
	Full Virus IIFA-IgG	**Full Virus IIFA-IgM**	**rIIFA-IgG**	**ppNT**
Blood Donors				
2014: 1/611 (0.16)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
2015: 5/3383 (0.15)	2/5 (40)	0/5 (0)	0/5 (0)	0/5 (0)
2016: 1/577 (0.17)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
Total: 7	2/7 (28.57)	0/7 (0)	0/7 (0)	0/7 (0)
Base Line Case Contacts				
May. 2015: 1/100 (1)	Sample quantity Not sufficient			1/1 (100)
Total: 1				1/1 (100)
Patients				
Mar. 2015: 1/1 (100)	1/1 (100)	0/1 (0)	1/1 (100)	1/1 (100)
May. 2015: 1/1 (100)	1/1 (100)	0 /1 (0)	1/1 (100)	1/1 (100)
Feb. 2016: 2/2*	2/2 (100)	1/2 (50)	2/2 (100)	Test not done
Total: 4	4/4 (100)	1/4 (25)	4/4 (100)	2/2 (100)
Total: 12	**6/11 (54.55)**	**1/11 (9.09)**	**4/11 (36.36)**	**3/11 (27.27)**

* 2 samples for same patient collected in two different dates (admission and discharge).

Serological findings for A. Borderline and B. Reactive anti-MERS-CoV IgG rELISA samples expressed by total number and (percentage) of reactive sample with other MERS-CoV serologic assays over the total no. screened for each group of the study population. MERS-CoV: middle east respiratory syndrome coronaviruses. rELISA: recombinant enzyme linked immunosorbent assay, IIF: indirect immunofluorescent, rIFA: recombinant

immunofluorescent assay, ppNT: pseudoparticle neutralization test.

Table 10-A

Serological investigation of reactive anti-MERS IgG rELISA samples

	MERS-CoV					Full Virus IIFA (Titer)		rELISA	rIIFA (Titer)				
	rELISA IgG	Full Virus IIFA		rIIFA	ppNT	229E	SARS	HKU1	229E	OC43	SARS	NL63	HKU1
Sample Identifier	(OD, Ratio, Endpoint Titer)	IgG (Titer)	IgM (Titer)	IgG (Titer)	(Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)
D2014/597	+ (0.905, 2.114, 201)	0	0	0	< 50	+ (≥ 320)	0	+ (≥ 100)	+ (320)	+ (320)	0	+ (3200)	+ (3200)
D2015/1303	+ (0.397, 1.3, 101)	+ (10000)	0	0	< 50	+ (320)	0	+ (≥ 101)	+ (3200)	+ (3200)	0	+ (3200)	+ (320)
D2015/3004	+ (0.439, 1.26, 201)	0	0	0	< 50	+ (≥ 1000)	0	+ (≥ 101)	+ (320)	+ (3200)	0	+ (3200)	+ (3200)
D2015/3119	+ (0.402, 1.06, 401)	0	0	0	< 50	+ (≥ 320)	0	+ (≥ 101)	+ (320)	+ (320)	0	+ (320)	+ (320)
D2015/3380	+ (0.477, 1.1, 101)	+ (10000)	0	0	< 50	+ (320)	0	+ (≥ 101)	+ (3200)	+ (3200)	0	+ (320)	+ (320)
D2015/3513	+ (0.497, 1.39, 401)	0	0	0	< 50	+ (≥ 1000)	0	+ (≥ 101)	+ (3200)	+ (3200)	0	+ (3200)	+ (3200)
D2015/4435	+ (0.661, 1.74, 201)	0	0	0	< 50	+ (≥ 320)	0	+ (≥ 101)	+ (320)	+ (3200)	0	+ (3200)	+ (3200)
C/ May.2015	+ (0.61, 1.5, 101)	Quantity Not Sufficient			**EMC:** 76, IC50 **JordanN3:** 149, IC50	Quantity Not Sufficient							
P/ March.2015	+ (1.084, 2.86, 201)	+ (10000)	0	+ (> 10000)	**EMC** 630, IC50/ 184, IC80/ 93, IC90 **JordanN3**	+ (100)	0	+ (≥ 101)	+ (3200)	+ (320)	+ (320)	+ (320)	+ (320)

					1707, IC50/ 206, IC80/ 69, IC90								
P/ May.2015	+ (0.412, 1.37, *)	+ (3200)	0	+ (320)	**EMC:** 199, IC50 **JordanN3:** 688, IC50	+ (1000)	0	+ (≥ 101)	+ (320 0)	+ (3200)	0	+ (320)	+ (3200)
P/ Feb.2016 (first sample)	+ (0.42, 1.34, 101)	+ (3200)	0	+ (3200)	Not Done	0	0	+ (≥ 101)	+ (320 0)	+ (> 10000)	0	0	0
P/ Feb.2016 (second sample)	+ (2.229, 6.517, ≥ 3201)	+ (> 3200 0)	+ (100)	+ (> 10000)	Not Done	0	+ (1000)	+ (≥ 101)	+ (320 0)	+ (> 10000)	+ (3200)	0	0

Table 10-B

Serological investigation of borderline anti-MERS IgG rELISA samples

	MERS-CoV					Full Virus IIFA (Titer)		rELISA	r IIFA (Titer)				
	rELISA IgG	**Full Virus IIFA**		**r IIFA**	**ppNT**	**229E**	**SARS**	**HKU1**	**229E**	**OC43**	**SARS**	**NL63**	**HKU1**
Sample Identifier	(OD, Ratio, Endpoint Titer)	IgG (Titer)	IgM (Titer)	IgG (Titer)	**(Titer)**	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)	IgG (Titer)
D2012/2644	± (0.333, 0.83, 101)	0	0	0	< 50	+ (≥ 1000)	0	+ (≥ 101)	+ (320)	+ (320)	0	+ (320)	+ (320)
D2015/1816	± (0.456, 0.823,201)	0	0	0	< 50	+ (≥ 320)	0	+ (≥ 101)	+ (3200)	+ (3200)	0	+ (3200)	+ (3200)
D2015/4708	± (0.408, 1.07, 101)	0	0	0	< 50	Not Done	Not Done	+ (≥ 101)	+ (3200)	+ (3200)	0	+ (320)	+ (3200)

Table 10-C

Serological investigation of selected negative anti-MERS IgG rELISA samples

	MERS-CoV					Full Virus IIFA (Titer)		rELISA	r IIFA (Titer)				
	rELISA IgG	**Full Virus IIFA**		**r IIFA**	**ppNT**	**229E**	**SARS**	**HKU1**	**229E**	**OC43**	**SARS**	**NL63**	**HKU1**
Sample Identifier	**(OD, Ratio, Endpoint Titer)**	**IgG (Titer)**	**IgM (Titer)**	**IgG (Titer)**	**(Titer)**	**IgG (Titer)**	**IgG (Titer)**	**IgG (Titer)**	**IgG (Titer)**	**IgG (Titer)**	**IgG (Titer)**	**IgG (Titer)**	**IgG (Titer)**
D2014/469	**-** (0.027, 0.069, < 101)	0	0	0	< 50	Not Done	Not Done	+ (≥ 101)	+ (320)	+ (3200)	0	+ (3200)	+ (320)
D2015/1190	**-** (0.033, 0.078, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	0	0	+ (320)	+ (3200)
D2015/1229	**-** (0.03, 0.07, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	+ (3200)	0	+ (3200)	+ (320)
D2015/1616	**-** (0.026, 0.059, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (3200)	+ (3200)	0	+ (3200)	+ (320)
D2015/2859	**-** (0.034, 0. 076, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (3200)	+ (3200)	0	+ (3200)	+ (3200)
D2015/2988	**-** (0.039, 0.112, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	+ (320)	0	0	+ (320)
D2015/3379	**-** (0.065, 0. 16, < 101)	0	+ (320)	0	**EMC :** 531, IC50/ 76, IC80 **JordanN3 :** 502, IC50	+ (320), IgM= 0	0, IgM= 0	Not Done	+ (3200)	+ (3200)	0	+ (320)	+ (320)
D2015/3581	**-** (0.186, 0. 51, < 101)	0	0	0	< 50	Not Done	Not Done	+ (≥ 101)	+ (320)	+ (320)	0	+ (320)	+ (3200)
D2015/3746	**-** (0.031, 0.101, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	+ (3200)	0	+ (3200)	+ (3200)
D2015/3899	**-** (0.037, 0. 083, <	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	+ (320)	0	+ (320)	+ (320)

	101)												
D2015/3962	- (0.06, 0. 134, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	+ (320)	0	+ (320)	+ (320)
D2015/4472	- (0.189, 0. 409, < 101)	0	0	0	< 50	Not Done	Not Done	+ (≥ 101)	+ (3200)	+ (3200)	0	+ (3200)	+ (3200)
C/ May.2015	- (0.039, 0. 096, < 101)	0	0	0	< 50	Not Done	Not Done	Not Done	+ (320)	+ (320)	0	0	+ (320)
P/ May.2016	- (0.131, 0. 38, < 101)	0	0	0	Not Done	0	0	Not Done	+ (320)	+ (3200)	0	+ (320)	0

Serological findings for A. Reactive, B. Borderline and C. Negative (randomly selected) anti-MERS-CoV IgG rELISA samples with other serologic method for detection of anti-MERS-CoV along with serological findings for antibodies to other human coronaviruses. Interpretation of rELISA result as follow Ratio: < 0.8 Negative, ≥ 0.8 - < 1.1 Borderline, ≥ 1.1 Positive.

Identifier= D: Donor followed by year of collection then identifier No, C: case Contact, P: patient. MERS: Middle East Respiratory Syndrome, rELISA: recombinant enzyme linked immunosorbent assay, IIF: indirect immunofluorescent rIIFA: recombinant indirect immunofluorescent assay, ppNT: pseudoparticle neutralization tests, OD: Optic Density, +: Reactive, ±: borderline, -: Negative, 229Em OC43, SARS, NL63 ang HKU1: Human coronavirus. EMC (GenBank JX869059) and Jordan N3 (GenBank KC776174): viral MERS-CoV strains.

CHAPTER 5: DISCUSSION

As infection with MERS-CoV continues to evolve, a matter of best infection control and management will arise, and studies to better define its prevalence are needed because infection control and management recommendations might be reviewed & updated as additional data become available. In the absence of clear epidemiological view of MER-CoV, our study provides an insight about the epidemiological status among Qatar population. It also provides a performance evaluation for the available serologic tests for MERS-CoV in a view of serologic status to other human coronaviruses.

In our investigation, we did not found a significant rate of anti-MERS antibodies in samples from blood donors in Qatar through 2012-2016. The actual prevalence of anti-MERS IgG among Qatar population is difficult to estimate, because our study sampled 4719 from blood donors only in which the focus on adult male group and few females (77 vs 4642 male) were included. However, the known predominance of overall reported MERS-CoV infection was among male (139) and the low prevalence of anti-MERS-IgG among blood donors (0.02 %, 1/4719) detected in our study –confirmed by ppNT- may refer to a similar low prevalence among general population in Qatar, which is also indicated by the low number (20 cases) of detected confirmed MERS-CoV infections. Interestingly, the low prevalence reported in our study is slightly like a reported prevalence from nationwide cross-sectional study in Saudi Arabia. They reported 15 (0.2 %) reactive anti-MERS-CoV sera collected from 10,009 healthy individuals in 13 provinces in 2012- 2013 (111). They initially screened sera by rELISA (EUROIMMUN, Germany) and MERS-CoV seropositivity were confirmed by rIFA and $PRNT_{50}$ & $PRNT_{90}$.

Furthermore, the detection of anti-MERS IgM among one of the screened blood donors stress the need for further investigation of the possibility of transmission of MERS CoV through blood transfusion from asymptomatic patients, though no cases has been reported so far with evidence of blood transfusion as source of infection. Additionally, the detection of probable (cross reactivity cannot be ruled out) subclinical infections in our study supports the believe that the current estimated overall fatality rate associated with MERS-CoV infection is most likely overestimated (8). Furthermore, it adds to the concern that those subclinical infections may role as an unknown source for spread of infection.

In term of serological view, our study showed similar results to Drosten C et al. of excess anti-MERS rELISA IgG reactive samples that were not confirmed with IIF-IgG assay (129) (8 samples in our study :3 borderline and 5 reactive). These findings suggest low specificity of the rELISA IgG test. Further investigations for these potential positive samples by rELISA-IgG showed reactive titers against other common human pathogenic coronaviruses, further suggesting lower specificity of rELISA test than immunofluorescence assay.

Our results indicate a higher specific reactivity of anti-MERS CoV rIIFA than full virus IIFA assay, when compared to the gold standard ppNT assay. Aburizaiza et al. study indicates that HKU1-CoV could act as a cause of cross-reactive antigen in the full virus anti-MERS IF assay (12) They stated that "despite that immunofluorescence assay is considered a highly robust test, the use of whole viral-infected cells immunofluorescence assay alone, without a panel of analyses, could result in a portion of false-reactive MERS-CoV antibody results" (12). Ours study results suggest that not HKU1-CoV antibody could be the source of cross reactivity but also other antibodies to other coronavirus families as

our study showed that > 80 % reactive or negative anti-MERS-CoV samples were reactive to other human coronaviruses (HCoV-229E, OC43-CoV, NL63) along with HKU1-CoV. Thus, crosses reactivity with those coronaviruses cannot be ruled out, though these coronaviruses are from different phylogenic groups or/and subgroups than MERS-CoV. Our study, collectively with the above discussed studies highlight the importance of following the US CDC and WHO strategy of two-phase serological approach of detecting anti-MERS antibodies through one screening test such as rELISA, followed by a neutralizing confirmatory assay or rIIF (122).

We reported a failure in detecting anti-MERS CoV-IgG in one of patient (confirmed by PCR test) sample included in our study using all rELISA-IgG, IIFT-IgG and rIIFA-IgG. However, this sample was not screened with ppNT due to shipping difficulties. Yet, reported failure for detection of anti-MERS CoV-IgG is in consistent with previous report, where few patients failed to develop a strong antibody response (130). Such findings may restrict the use of serologic screening in diagnostic settings as infection may not be able to be detected by any of screening serological assays. Furthermore, previous studies open a debate of later development and or detection of anti-MERS-IgM than IgG (86, 132). In our study, we reported two opposite status. First one was a detection of anti-MERS-IgM in blood donor sample (D2015/3379) without detection of anti-MERS-IgG, which may indicate earlier development IgM than IgG. Second, two samples were drawn from one of our patients (P/ Feb.2016), was drawn at the admission time, and the second one was taken just before patient discharge (Table 9 A). Anti-MERS IgG was detected in both samples (P /Feb.2016 first and second samples), however, anti-MERS-COV-IgM was detected only the second sample. Thus, the debate is still opened waiting for more concerned study for

timeline development of anti-MERS-IgG/IgM and variations of the immunoglobulin class-specific serologic assays.

The detection of anti-SARS- CoV IgG in 2 samples collected from 2 confirmed MERS-CoV patients by IIFT and/ or r-IIFA is consistent with previous reports (83, 88).

Additionally, this study questions the ability of rELISA-IgG for detection of anti-MERS IgG in serum samples stored frozen (-70 ^{0}C) for one year. The Negative anti-MERS-IgG rELISA patient sample after one year of reactive result for the same sample may restrict the screening utility using the same ELISA method used in our study for long shelf (> one year) stored samples. However, one sample result conflict is not enough to implement such restriction and further studies should be conducting before draw such conclusion particularly, when keeping in consideration that the conflict in the result with period may resulted from improper handling or storage specifically for that sample.

The base line investigation of case contacts in our study is of limited value as we were not able to collect further samples after 2 weeks of exposure to check for seroconversion. However, detecting of one reactive anti-MERS-IgG among one of the traced case contact, value the WHO recommendations of active survey of case contacts following confirmation of MERS CoV human infection. Yet, our reactive anti-MERS IgG samples, which was confirmed by ppNT, need a follow up investigations i.e. anti-MERS IgM and PCR, which may indicate the current stage of the subject and rule out cross reactivity. Additionally, the presence of anti-MERS-CoV IgG and neutralizing antibodies at the time of baseline surveillance rise the question that this subject could be the case index rather than case contact.

CHAPTER 6: CONCLUSION

Our findings suggest that MERS-CoV is not heavily circulated among the population of Qatar. Additionally, the presence of antibody of other pathogenic human coronavirus may cause false positive results of both rELISA and IIFT (full virus), which stress the need for more evaluation studies for the available serological assays. This study provides an insight about the epidemiological view for MERS-CoV in Qatar population. It also provides a performance evaluation for the available serologic tests for MERS-CoV in a view of serologic status to other human coronaviruses.

FUTURE PERSPECTIVES

Though our knowledge about MERS-CoV has been expanded over the last years, yet, the human serologic response to MERS-CoV infection and seroconversion of infected individuals is not fully covered and understood. Few studies addressed the antibody response to MERS-CoV infection and its correlate to the severity of the disease (127, 130, 132). Additionally, limited studies followed the longevity of the antibodies response and its correlate to the initial severity of the infection (129, 140, 141). The findings of studies following patients' antibody response may impact the understanding of the pathogenesis of MERS-CoV infection and hold the promise for a better treatment and management of the diseases. Thus, further studies of human antibody response to MERS-CoV infection are of our priority for research studies.

Though our study mainly aimed to provide an insight for the epidemiological view for MERS-CoV in Qatar population, the enriched results of various serologic assays in our study trigger us to fatherly correlate serologic assays –used in this study- and compare it with published data. This perspective will be conducted upon including more reactive samples.

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