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MVSc THESIS

CROSS SECTIONAL STUDY OF MIDDLE EAST RESPIRATORY SYNDROME (MERS-CoV INFECTION) IN CAMELS AT SELECTED SITES OF AMIBARA DISTRICT, AFAR REGION, ETHIOPIA

BY

DEMEKE SIBHATU LOBAGO

JUNE 2019

BISHUFTU ETHIOPIA

CROSS SECTIONAL STUDY OF MIDDLE EAST RESPIRATORY SYNDROME (MERS CoV INFECTION_) IN CAMELS IN SELECTED SITES OF AMIBARA DISTRICT, AFAR REGION, ETHIOPIA

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

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STATEMENT OF THE AUTHOR

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ABREVATIONS

BCoVs	Betacoronavirus
BtCoV	Bat corona virus
C.I.	Confidence Interval
Cov	Corona virus
DNA	Deoxy ribonucleic acid
DPP	Dipeptidyl peptidase
E	Envelope protein
ELISA	Enzyme Linked ImmunoSorbent Assay
EMPRESS	Emergency prevention system for Animal health
HKU	Hong Kong University
iELISA	indirect ELISA
IFA	Immuno Florescent Antibody
KSA	Kingdom of Saudi Arabia
LRT	Lower Respiratory Tract
M	Matrix protein
MERS	Middle East Respiratory Syndrome
N	Nucleocapsid protein
NAHDIC	National Animal Health Disease Investigation Centre
NSP	Non-structural protein
OR	Odd ratio
ORF	Open Reading Frame
P	Protein
PpNT	Pseudo particle Neutralisation Test
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-rtPCR	Reverse transcriptase real-time polymerase chain reaction
RT-PCR	Real time Polymerase Chain Reaction
S	Spike-(surface glycoprotein)
SARS	Severe Acute Respiratory Syndrome
SP	Structural protein
UpE	Upstream Envelope
URT	Upper Respiratory Tract
VTM	Viral transport medium

ABSTRACT

A cross sectional study of MERS CoV in camel was conducted between February 2018 to April 2019 in three selected sites of Amibara district of Afar Region, Northeast Ethiopia. The study was aimed to observe the current sero-prevalence status of MERS, assess *the presence of active cases through detection RNA viral particle using RT -PCR and investigate possible risk factors for the MERS-CoV in camels. A total of 589 sera were collected and tested with indirect Enzyme linked Immuno Sorbent Assay (iELISA).The overall seroprevalance of MERS-CoV with this test was 87.3% (n=514/589, 95% CI: 84.5-89.9). Out of the total sera samples, 198 sera were retested by pseudo particle neutralization test (ppNT)at Hong Kong University (HKU). A total of 857 nasal swab samples were collected for the detection of MERS CoV RNA particle using RT-PCR. Association of different risk factors with seroprevalance revealed that origin (X^2=13.39, P=0.001), sex (X^2=4.5 P=0.034), age ((X^2=185.7, P=0.001) season (X^2=41.7, P=0.000) and reproduction status (X^2=96.1, P=0.001) showed a statistical significant difference for MERS CoV antibody detection among the groups (P<0.05) while herd size did not show a statistically significant difference among groups (p>0.05). In multivariable logistic regression analysis, age (OR=7.39, 95% CI:3.43-15.91), season (OR=4.83, 95% CI:-2.14-10.90), and in adult female camel reproduction status (OR=7.39, 95% CI:3.43-15.91) showed statistically significant difference among the groups for MERS CoV antibody detection while risk factors of origin, animal sex and herd size difference were statistically insignificant. Out of the total 198 sera samples tested using ppNT, 197 (99.5%; 95%CI: 98.4-100) were positive where as in indirect ELISA of 198 sera sample only 182 (91.9%; 95% CI: 88-95.7) were seropositive indicating ppNT is more sensitive than iELISA for MERS CoV antibody detection. Despite the presence of high seropositivity for MERS CoV antibody, all 857 camel nasal swabs samples tested by Real-time reverse transcription polymerase chain reaction (RT-PCR) technique for detection MERS – CoV RNA viral particle were Negative after testing at NAHDIC and H.K.U. School Public Health laboratories .In conclusion, the present study revealed a high seroprevalance of MERS CoV in adult camels. However, in spite of high seroprevalance the lack of any RNA viral particle in the present study suggests the need for further in depth longitudinal study to detect the circulating virus focusing on juveniles and young camels whereby seroprevalance of antibody is*

low when compared with adult camel in order to get the active virus before the camel develop antibody. Moreover, the zoonotic significance and potential transmission routes of MERS CoV to pastoral communities should also be investigated and design strategy for the preparedness in control of the diseases in Ethiopia.

***Key words**: Amibara, Camel, Cross sectional, MERS-CoV, Sero prevalence*

1. INTRODUCTION

The one-humped camel (*Camelus dromedarius*) is an important livestock species exceptionally adapted to hot, dry and harsh environment due to heat and water deprivation tolerance. These tolerances in camels appear to be due to behavioral response that reduces heat absorption, a relatively efficient sweating mechanism for heat dissipation, an ability to reduce fecal and urine water loss and the ability to vary body temperature substantially. It is used for milk and meat production, transportation, and draught power (Payne, 1990).Camels are widely distributed in Ethiopian lowlands especially in Afar, Somali and Oromia region where by pastoralism is the dominant mode of life and mobility is an inherent strategy to efficiently utilize the spatially and temporally distributed pasture and water resources. Usually, large numbers of camels and other domestic animals from many different herds/flocks congregate at watering sites, and this may create a perfect condition for disease transmission and spread among animals. The same water sources are also shared by multitudes of wild animals (Tadele *et al*., 2018). According to CSA 2016/17 report, the camel population of dromedaries in Ethiopia is estimated to be about 1,209,321. Afar region has 474,146 camels (CSA 2016/17).

Pathogens and diseases related to camelids are less well known than those of other domesticated species, but have attracted growing attention recently. For instance, several unusual disease incidents caused by Trypanosoma *evansi* and Morbillivirus infection, causing high morbidity and/or mortality rates in camels, were reported (Miguel *et al*., 2016). There is an increasing need to determine whether camels are clinically susceptible and act as potential reservoirs and maintenance or bridge hosts to viral pathogens affecting other livestock and/or humans. Overall, dromedaries seem to be more resistant hosts for bovine, ovine or caprine viral diseases such as foot-and-mouth disease or rinderpest (Miguel *et al*., 2016).

Middle East Respiratory Syndrome (MERS) is a viral respiratory diseases within the largest group of Coronaviruses (CoVs) belonging to Nidovirale order which includes Coronaviridae,Arteriviridae and Ronaviridae families. The coronavirinae are further divide into four groups the alpha, beta, gamma and delta coronaviruses. MERS CoV is within beta corona virus group (Anthony *et al*., 2015).

Dromedary camels are sturdily suspected of acting as a zoonotic source for human cases of MERS-CoV, by either direct contact through droplet infection via mucous membranes or indirect contact through milk, meat or urine. According to, Miguel *et al*., (2016) five major accounts that suggest dromedary camels can play an important role in the epidemiology of MERS-CoV, possibly as a reservoir host: (1) coronaviruses are widespread in the animal kingdom (in bats and livestock), but MERS-CoV does not infect many of the hosts (e.g. sheep, goats, cattle, chickens, water buffaloes, birds, horses and) whereas high levels of sero positivity have been observed in dromedary camelids, ranging from 0% in Asia to as much as 100% in Africa and the Arabian Peninsula (with mean of 79%); (2) the MERS-CoV isolated from dromedaries are genetically and phenotypically very similar to those infecting humans; (3) retrospective serological studies in Africa going back more than 30 years indicate long-term circulation of the virus in dromedary camels; (4) infection in dromedaries causes no or only mild respiratory symptoms, making it difficult to detect; (5) MERS-CoV genome has likely undergone numerous recent recombination's, which suggests frequent co-infection, probably in camels, with distinct lineages of MERS-CoV (Miguel *et al*., 2016).

Studies have demonstrated that dromedary camels can act as a source of human MERS-CoV infection. Indeed, the current state of knowledge indicates that dromedary camels are the only animal species for which there is convincing evidence that they act as host species for MERS-CoV and hence a potential source of human infections (Hemida *et al.,*2015).Nonetheless, the route of infection of MERS CoV and types of exposures remain largely unknown, and only a small proportion of the primary cases have reported contact with camels. Other possible sources and vehicles of infection include food-borne transmission such as unpasteurized camel milk and raw meat, and medicinal use of camel

urine (Memish *et al*., 2014). Clearly, transmission from camels to humans does take place, and camel exposure is a risk factor for human infection, but such transmission is not efficient and infection is not directly proportional to exposure while in the other hand, many patients with clinically diagnosed MERS did not have an obvious history of direct exposure to camels or their products (Hemida *et al*., 2015).

Reusken *et al.,(*2014) found high percentages of animals sampled from Nigeria and Ethiopia being seropositive for MERS-CoV with an overall seropositivity of 94% in adult dromedaries in Nigeria and 93% and 97% for juvenile and adult animals, respectively, in Ethiopia. More recently, Miguel *et al*., (2017) found a high seropositivity of 99.4% in camel of Ethiopia and also relatively higher MERS-CoV RNA detection in Ethiopia (15.7%) than in Burkina Faso (12.2%) and Morocco (7.6%). Also 10.6% virus detection rate observed by a study in Ethiopia as described by (Chu *et al.,* 2014).

Fekadu *et al.,* (2017) also reported 93% seropositivity and 7% (n=7/100) MERS CoV RNA detection in Ethiopia ,Afar region camels. However, data from experimental camel infections conducted in the Middle East suggest that MERS-CoV causes only mild respiratory infection in camels (Adney *et al.,* 2014). Also study in Ethiopia between 2010-2011 reported 93-97% seropositivity (Reusken *et al.,* 2014).

In Ethiopia, in spite of the high prevalence of MERS-CoV antibodies in camel as indicated in different studies, no human case has been reported to date, and only few ongoing studies have been carried out to investigate public health significance of MERS in highly exposed pastoralist community of Ethiopia who have close contact with camels requires serious attentions for further surveillance both for camel and exposed human population. So based on the mentioned points the objectives of the study were:

- To determine the current seroprevalance of MERS-CoV in camels with in selected sites of Amibara district, Afar Region.
- To identify the potential risk factors for MERS CoV in camels in order to control the disease.
- To detect and characterize MERS CoV from nasal swab of camels in the study sites

2. LITERATURE REVIEW

2.1 History of MERS CoV

Middle East Respiratory Syndrome Coronavirus infection (MERS-CoV) became an evolving worldwide health concern. MERS-CoV originally reported in 2012 in Saudi Arabia (Zaki *et al*., 2012). So far, 27 countries have reported human laboratory confirmed cases in four continents since 2012. At the end of September 2018, a total of 2260 laboratory-confirmed cases of Middle East respiratory syndrome (MERS), including 803 associated deaths (case–fatality rate: 35.5%) were reported globally; the majority of these cases were reported from Saudi Arabia (1882 cases, including 729 related deaths with a case–fatality rate of 38.7%) (WHO, 2018). In Middle East, Saudi Arabia is reflected as the epicenter of "MERS-CoV" infection. Saudi Arabia has unique cultural and religious practices; millions of Muslims come across from the globe travel to Saudi Arabia to perform Hajj and this could be the potential risk for global spread of the disease (Zaki *et al*., 2012).

The various regional appearances have provided favorable conditions for speedily transmitting viruses to appear. MERS-CoV triggered an occurrence of respiratory illness in the Middle East with secondary spread to Europe, Africa, Asia, and North America. The disease occurred mainly in the Middle East states with a highest cases of 88% followed by Asia 11%" "Europe 0.8%" and "USA 0.2 (Alaenazi *et al* ., 2018).

MERS-CoV infection is transmitted from animals to humans and human to human. Phylogenetic analysis shows a close genetic relatedness between MERS-CoV and the group C Beta corona virus detected in insectivorous bats (Woo *et al*., 2012). This Evidence shows that bats aided as the original host species of MERS-CoV The interaction of humans with bats or their secretion is an occasional transitional to MERS-CoV, and transmission from bat to human is unlikely. Available evidences showed that

camels are the possible source of the virus to human infections. Once it establishes, human-to-human transmission has resulted in clusters of cases, some associated with multiple rounds of human-to-human transmission (Milne *et al*., 2014).

2.2 Epidemiology of the disease

Since March 2012, autochthonous MERS cases have been detected only in the Middle East (Saudi Arabia, United Arab Emirates, Jordan, Qatar, Oman, Kuwait, Yemen, Lebanon and Iran). MERS cases have also been detected in other geographic areas with primary cases having travel connections to the Arabian Peninsula: in Europe (United Kingdom, Germany, France, Italy, Greece and the Netherlands), in Africa (Tunisia and Algeria), in Asia (Malaysia and Philippines) and in the U.S.A as illustrated in figure one (Chantal et *al*., 2014).

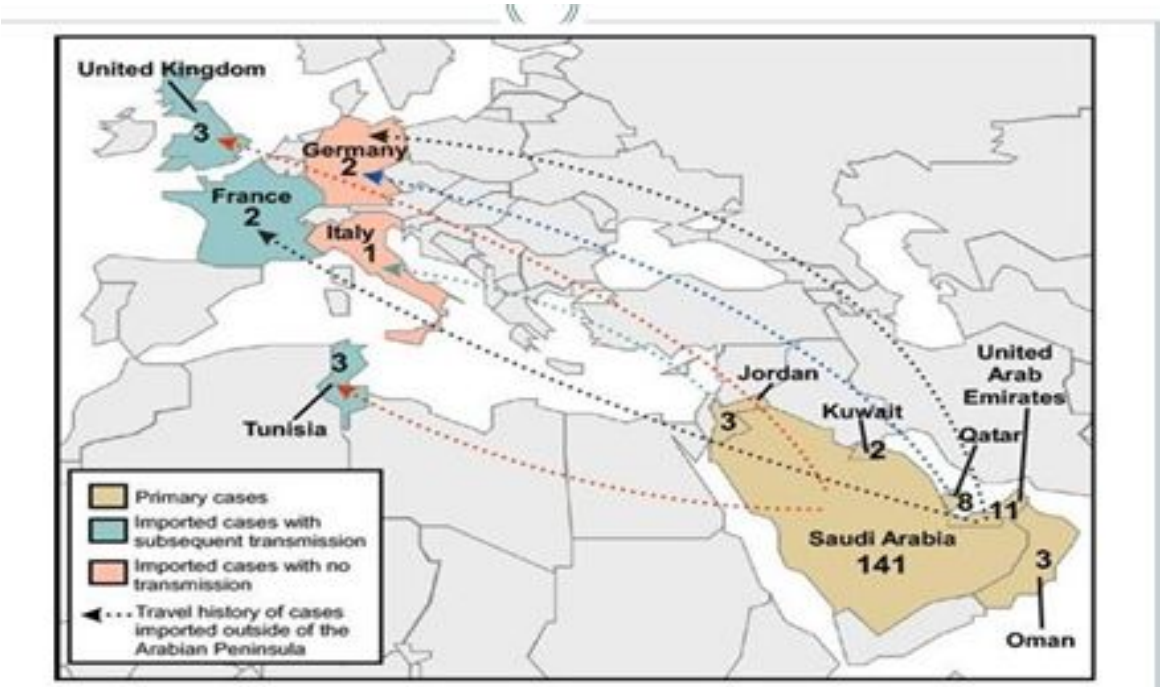
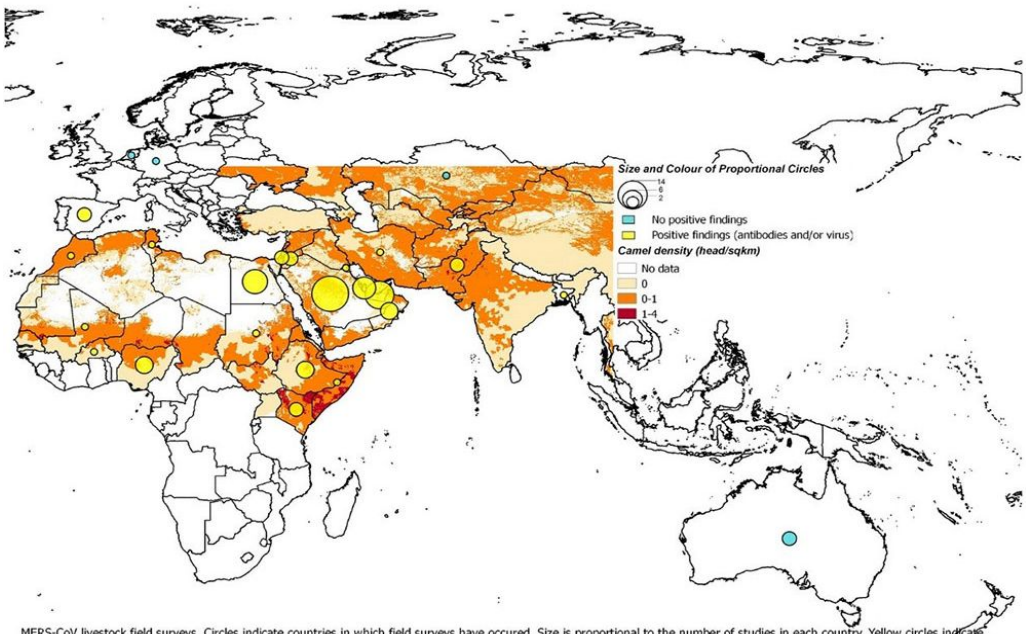


Figure 1 : Travel connection incidence of MERS CoV from Middle East

Source: Pathogens and diseases (Milne *et al*., 2014)

High percentages of animals seropositive for MERS-CoV were observed in Nigeria and Ethiopia; the overall seropositivity was 94% in adult dromedaries in Nigeria and 93% and 97% for juvenile and adult animals, respectively, in Ethiopia (Chantal *et al.,* 2014).

Seropositivity of 36% and 40% was observed in Sidi Bouzid and Sousse Provinces, respectively, and 100% of the dromedaries in the southern province of Kebili were seropositive. Array results were confirmed on a selection of positive and negative serum samples (n = 14 per country) in MERS-CoV neutralization tests performed as described by Reusken *et al.*(2013). Serum samples from 72%, 82%, and 67% of the dromedaries from Nigeria, Ethiopia, and Tunisia, respectively, reacted with the OC43 antigen, confirming common circulation of BCoV in camelids (Chantal *et al* .,2014).



MERS-CoV livestock field surveys. Circles indicate countries in which field surveys have occured. Size is proportional to the number of studies in each country. Yellow circles indicate positive findings in livestock (antibodies or antigen), while turquoise circles represent the lack of positive findings. Please note that the circle in Spain indicates positive findings from the Canary Islands. The density map includes dromedary and bactrian camel distribution, and is an unpublished model based on the methodology described in Robinson et al. (2014) (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0150424)

Figure 2 : Camel MERS CoV findings globally.

Source: FAO EMPRESS MERS CoV situation update 17 April 2019

2.3 MERS CoV transmission and Susceptible hosts

Many uncertainties still exist on the source of MERS-CoV and on the mode of transmission. This is typical of an emerging disease where there are often simultaneous possibilities, including environmental, animal and human exposures. The continued detection of new MERS-CoV cases, the low estimated basic reproduction number of the infection (R0), and the detection of multiple distinct MERS-CoV genotypes suggest the existence of a persistent possible zoonotic source This is corroborated by the growing serological and molecular evidence that dromedary camels (Camelus dromedarius) are a host species for MERS-CoV (Aisha *et al.,*17).

Studies carried out in many Middle Eastern countries, including Saudi Arabia, Qatar, Egypt, United Arab Emirates, and Oman, using samples from lung, nasal, and rectal swabs. Positivity for MERS-CoV by RT-PCR for the RdRp was observed in 1.6–61.5% of samples, mostly lung and nasal swabs . Analyses using anti-MERS-CoV antibodies have shown that 98–100% of camels are positive for MERS-CoV; consistent with this, the incidence of MERS-CoV in humans is 15 times higher in camel shepherds and 23 times higher in slaughterhouse workers than in the general population. The data supported that the main route of transmission from camels to humans is through the respiratory system (Hemida *et al*., 2014).

2.3.1 The route of transmission

It is suspected that nasal mucous, sputum, saliva, milk or uncooked meats of infected camels are the main sources of transmission. However, the secondary infection can be through droplets or direct contact, and the virus may spread through the air or fomites. (Zumila *et al.,* 2015). Animals playing role in the transmission of Mers Cov are the following.

2.3.2 MERS-CoV in humans

Middle East respiratory syndrome in human was first identified in 2012, in Saudi Arabia and more than 1000 infection cases of the disease have been reported in May, 2015 and about 40% of those who were infected died due to the disease (Zaki *et al*., 2012),

accordingly, most cases have occurred in the Arabian Peninsula (Zumla *et al.,* 2015). HCoV-EMC/2012 is a strain of MERS-CoV that is detected in the first infected person in London in 2012, which was found to have a 100% identical viral sequencing to the strain identified in Egypt from tomb bats (Zaki *et al*., 2012).

2.3.3 Dromedary camels

Camels have been confirmed by several studies to be the reservoir of the MERS-CoV infection in humans. Zoonotic transmissions of MERS-CoV from dromedary camels to humans were reported in multiple occasions. MERS-CoV has never been reported as a disease in camels though in experimental infections MERS-CoV has been associated with mild upper respiratory signs. Positive PCR results for MERS-CoV or isolation of the virus from camels is notifiable to the OIE because MERS is an emerging disease with a significant public health impact (Kenneth *et al.,* 2018).

Camels as noted above, it is likely that it serve as hosts for MERS-CoV. The strongest evidence of camel-to-human transmission of MERS-CoV comes from a study in Saudi Arabia in which MERS-CoV was isolated from a man with fatal infection and from one of his camels; full-genome sequencing demonstrated that the viruses isolated from the man and the camel were identical (Azhar *et a*l., 2014).

2.3.4 Bats

Corona virus as known to be a zoonotic virus; however, the MERS-CoV is a novel virus, and whether zoonotic transmission occurs is not clear yet. International studies carried out from 2012 to 2014 in Mexico, European countries (i.e., Germany, Ukraine, the Netherlands, and Romania), Ghana, and South Africa have examined whether bats may be carriers of MERS-CoV. These studies have tested bats mainly for the 329-bp fragment of RdRp using blood, fecal, and oral samples. The bat species that were tested in the study included *Pipistrellus*, *P. nathusii*, *P. pygmaeus*, Nycteris, and Neoromiciazuluensis, and 5.3–24.9% were found to be positive for MERS-CoV, with most positive results (> 70%) being identified in fecal samples with high viral loads (Memish. *et .,al* 2013).Thus, it may be possible for transmission to occur via bats; however, in Saudi Arabia,

the species of bats that patients may have come in contact with are different from those tested. Thus, although there was a positive association between bats and corona virus infection, there was no association between bats and MERS-CoV. Therefore, these data have suggested that MERS-CoV is not transmitted through bats (Rachael *et al*., 2013).

On the other hand, the virus that causes Middle East Respiratory Syndrome (MERS) has been found in bats in Saudi Arabia, suggesting a potential origin for the disease .Researchers tested samples from bats living about 7 miles away from the home of the first person known to be infected with MERS in Saudi Arabia (Ian *et al.,* 2015).

Reservoirs and transmission of MERS CoV cycle

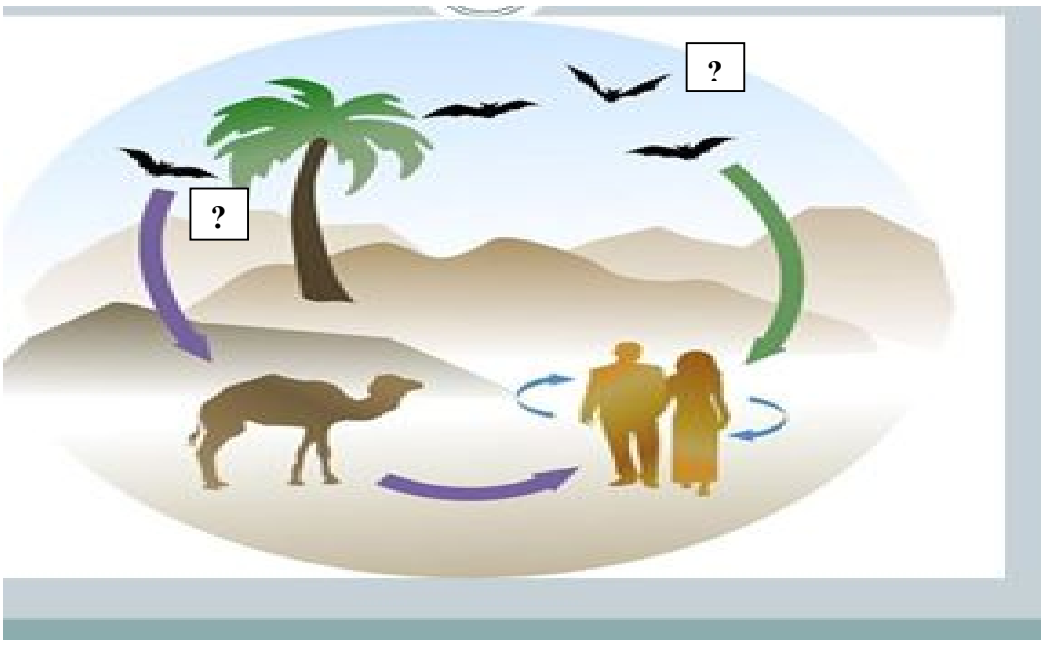


Figure 3 : Cycle transmission of MERS CoV.

Source: Kerri *et al*., (2014).

The putative transmission cycle for MERS-CoV, likely originated from bats, acting as the natural reservoir. From the natural reservoir, MERS-CoV spilled either directly over to humans or via an intermediate host dromedary camels, the exact route of zoonotic

transmission of MERS-CoV into the human population remains unknown although the presence of MERS-CoV neutralizing antibodies and the detection of MERS-CoV in dromedary camels suggest that this species is likely to play a major role in the emergence of MERS-CoV. Phylogenetic analysis suggests that multiple introductions of MERS-CoV into the human population have occurred and both zoonotic transmission events and human-to-human transmission drive the current MERS-CoV outbreak (Kerri *et al.,* 2014).

2.3.5 Other animals

In some countries of the Arabian Peninsula goats, cattle, sheep, water buffalo, swine, chicken and wild birds have been tested for antibodies to MERS-CoV, with no positive results. This indicates that the role of these animals in transmitting the disease or acting as a reservoir is not evitable. However it should be further studied to get clue information about the susceptibility. Because people often don't come in contact with bats, the researchers suspect that bats may infect other animals, which in turn, infect people. (Li, Shi, *et al.,* 2005).

Corona virus as known to be a zoonotic virus; however, the MERS-CoV is a novel virus, and whether zoonotic transmission occurs is not clear yet. International studies carried out from 2012 to 2014 in Mexico, European countries (i.e., Germany, Ukraine, the Netherlands, and Romania), Ghana, and South Africa have examined whether bats may be carriers of MERS-CoV.

2.4 Shedding of the virus

MERS-CoV has been detected in camels in several countries but not in other livestock. It has been detected in camel meat, organs, milk and urine; infected camels may not be observably ill. The continued detection of new MERS-CoV cases, the low estimated basic reproduction number of the infection, and the detection of multiple distinct MERS-CoV genotypes suggest the existence of a persistent possible zoonotic source This is corroborated by the growing serological and molecular evidence that dromedary camels

(Camelus dromedarius) are a host species for MERS-CoV. Transmission from camel to camel is not well being described (Cauchemez et *al.,* 013).

The hypothesis that dromedary camels are hosts of MERS CoV has been proven by the viral RNA detection in different specimens collected from these animals in Qatar, Saudi Arabia, Oman and Egypt and the isolation of the virus from nasal and fecal samples .MERS-CoV RNA has also been detected in the milk of camels actively shedding the virus. Whether infected camels excrete MERS-CoV directly into the milk or the milk is cross-contaminated during milking is unclear .Infection in dromedary camels has been reported to be either asymptomatic or associated with only mild respiratory signs with nasal discharge (Mohammed *et al*., 2018).

The respective roles of human to human and zoonotic transmission in the current MERS CoV outbreak are not well understood . Conclusive evidence of human to human transmission of MERS CoV was first reported in a cluster of MERS CoV cases in the United Kingdom, when an adult male who had travelled to Saudi Arabia transmitted the virus to two of his family members (Health Protection Agency, 2013). Overall, MERS CoV human to human transmission chains have been self limiting and irregular, and more than half of secondary MERS CoV cases have originated in a healthcare setting (WHO, 2014).

2.5 Pathogenesis

The pathogenesis of Middle East respiratory syndrome coronavirus (MERS-CoV) infection is not well understood (Kenneth *et al.,* 2018).

2.5.1 Molecular Virulence

Both MERS-CoV and SARS-CoV have similar ss positive strand RNA in their genome. This ssRNA in MERS-CoV encodes structural proteins (SP), membrane protein (M), envelope (E), nucleocapsid (N), two non structural, replicase polyproteins (ORF1a & ORF1b) and spike (S) . The two non-structural proteins (NSP) initiate genomic replication and RNA synthesis. The huge replicase gene surrounds 5’ proximal side of

RNA genome. Translation of ORF 1a give rise to polyprotein 1 a (pp1a), ribosomal frame shifting makes translation of ORF 1b to pp1b. MERS-CoV utilizes one or two papain like protease to release NSPs. It is established that MERS-CoV possess 16 NSP9.Viral components like NSPs, SPs, M, E, N, S and other proteins, glycoprotein and enzymes play a crucial role in virulence, in establishing and exaggerating the disease .MERS-CoV's E protein is responsible for attachment of virus particles to host cell receptors. S protein is in charge of fusion and entry in to the respiratory epithelia10.Viral proteases help the spread to lower parts of the lungs, thus enhancing the severity of the infection. It results in inflammation and suppression of anti viral interferon's (IFN). At this point of the infection, there is excessive anti viral immune responses like interleukins (IL-6, IL-8 and TNF-α), humoral IgG and IgM are inefficient to control the viral spread and replication in the lungs. The very nature of MERS-CoV virus ability to overcome and suppress host immune challenge is due to its +ssRNA, viral structural (SPs) and non structural proteins (Kannan *et al.,* 2016).

2.6 Virology of MERS CoV

Coronaviruses are a group of enveloped RNA viruses of the family *Coronaviridae*. Their surface appearance resembles that of a crown under the electron microscopy, which has given rise to their scientific name (Latin "corona" meaning "crown" or "halo"). Coronaviruses able to infect humans are shown to emerge via cross-host transmission from animals. MERS-CoV is a lineage C betacorona virus that is found in humans and camels. This virus is different from the other human beta coronaviruses (such as SARS-CoV). It is closely linked to some bat coronaviruses (such as BtCoV-HKU4 or BtCoV-HKU5). This is why it is believed that MERS-CoV (like a plethora of other corona viruses) originated in bats (Aisha *et al*., 2017).

2.7 The virion

MERS-CoV is an envelope, single-stranded, positive-sense RNA virus. The genome is approximately 30.1kb long and contains at least 10 predicted open reading frames (ORF), which are expressed from seven sub genomic mRNAs. These ORFs mainly include ORF 1a/1ab, which encode for large replicase polyproteins containing conserved functional

domains and several non-structural (NS) proteins of CoV, the spike-surface glycoprotein (S), the small-envelope (E) protein, the matrix (M) protein, and the nucleocapsid (N) protein (Ian .*et al.,* 2015).

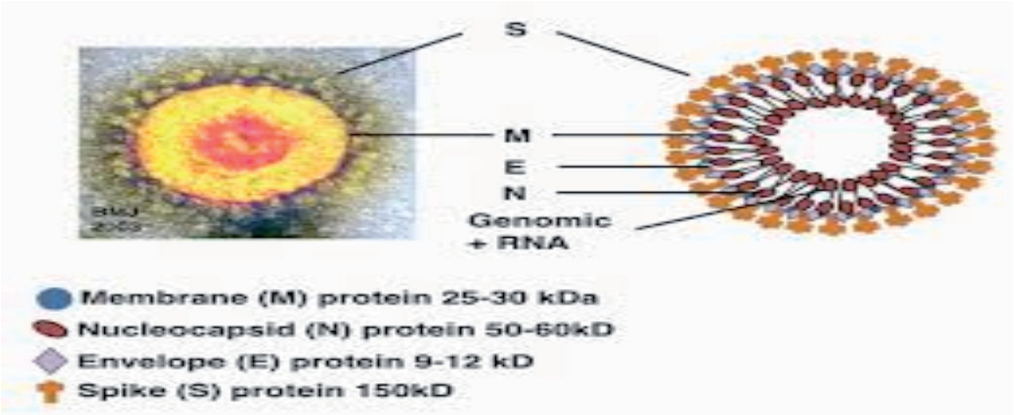


Figure 4 : Molecular structure of MERS-CoV.

(Source Infect Dis Trop Med 2016; 2 (4)

In the Properties of the virus protein Dipeptidyl peptidase 4 (or DPP4) is a functional protein receptor for MERS-CoV that enables the infection, and it is found on the surfaces of non-ciliated epithelial cells in human bronchi. This receptor shows high level of amino acid sequence conservation between different species, including the bat cells (Aisha *et al.*, 2017).

2.7.1 Viability of the virus

MERS-CoV remains viable at 48 hours at 20 °C and 40% relative humidity, comparable to an indoor environment on plastic and metal surfaces. The virions are sensitive to heat, lipid solvents, non-ionic detergents, oxidizing agents and ultraviolet light (Van Doremalen *et al*., 2013) In aerosol experiments, MERS-CoV retains most of its viability at 20 °C and 40% relative humidity. Viability decreases at higher temperatures or higher levels of relative humidity In unpasteurised camel milk, MERS-CoV remains infectious beyond 72 hours after introduction to the milk but infectious viruses could not be found after pasteurization (Van Doremalen *et al*., 2013).

2.7.2 *Genome Structure and Gene Functions*

MERS-CoV, a lineage C Betacoronavirus (βCoVs), has a positive-sense single-stranded RNA (ssRNA) genome about 30-kb in size. As of 2016, phylogenetic analysis of MERS-CoV has been done on 182 full-length genomes or multiple concatenated genome fragments, including 94 from humans and 88 from dromedary camels. The MERS-CoV genomes share more than 99% sequence identity, indicating a low mutation rate and low variance among the genomes. MERS-CoV genomes are roughly divided into two clades: clade A, which contains only a few strains, and clade B, to which most strains belong (Al-Ta *et al.,* 2014).

As with other CoV genomes, the first 5′ two-thirds of the MERS-CoV genome consist of the replicase complex (ORF1a and ORF1b). The remaining 3′ one-third encodes the structural proteins spike (S), envelope (E), membrane (M), and nucleo capsid (N), as well as five accessory proteins (ORF3, ORF4a, ORF4b, ORF5 and ORF8b) that are not required for genome replication but are likely involved in pathogenesis . The flanking regions of the genome contain the 5′ and 3′ untranslated regions (UTR) . Typical of the coronaviruses, the MERS-CoV accessory proteins do not share homology with any known host or virus protein, other than those of its closely related lineage C βCoVs.

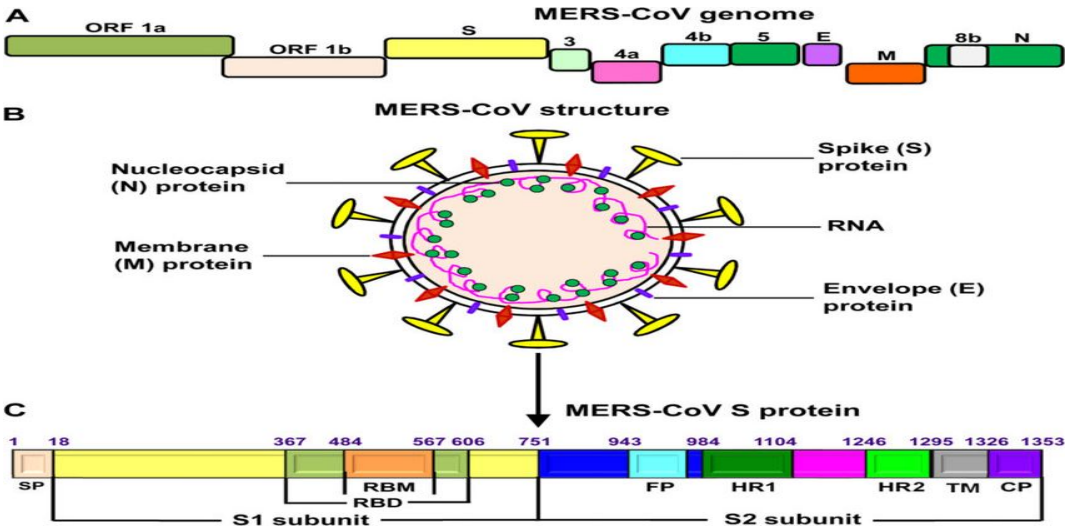


Figure 5: MERS CoV genome structure (Source Anthony and Stanley (2015)

2.7.3 Genetic characterization

Nasal swabs samples collected from Morocco, Burkina Faso, Ethiopia and Nigeria camels positive with RT-PCR with high viral load were selected and attempted to maximize diversity in geography and sampling dates ,for full viral genome sequencing directly from the clinical specimen and for virus Isolation(Dataset S1).Three viruses from Burkina Faso, one from Morocco, nine from Nigeria and three from Ethiopia were fully sequenced. And an additional virus from Ethiopia was sequenced from S2 gene region to the 3′ end of the genome (5,126nt). Genetic nucleotide identity was 99.17% within African camel virus genome,>99.26% with in human and camel MERS-CoV from the Middle East and 99.18-99.58% between viruses from the Middle East and Africa (GenBank accession numbers: MG923465-MG923481) (Chu *et al.,* 2018*).*

According to figure 6 viruses from Africa (clade C) are phylogenetically distinct from contemporary viruses from the Arabian Peninsula (clades A and B) but remain antigenically similar in micro neutralization tests. Viruses from West (Nigeria, Burkina Faso) and North (Morocco) Africa form a subclade, C1, that shares clade-defining genetic signatures including deletions in the accessory gene *ORF4b* (Chu *et al.,* 2018*).*

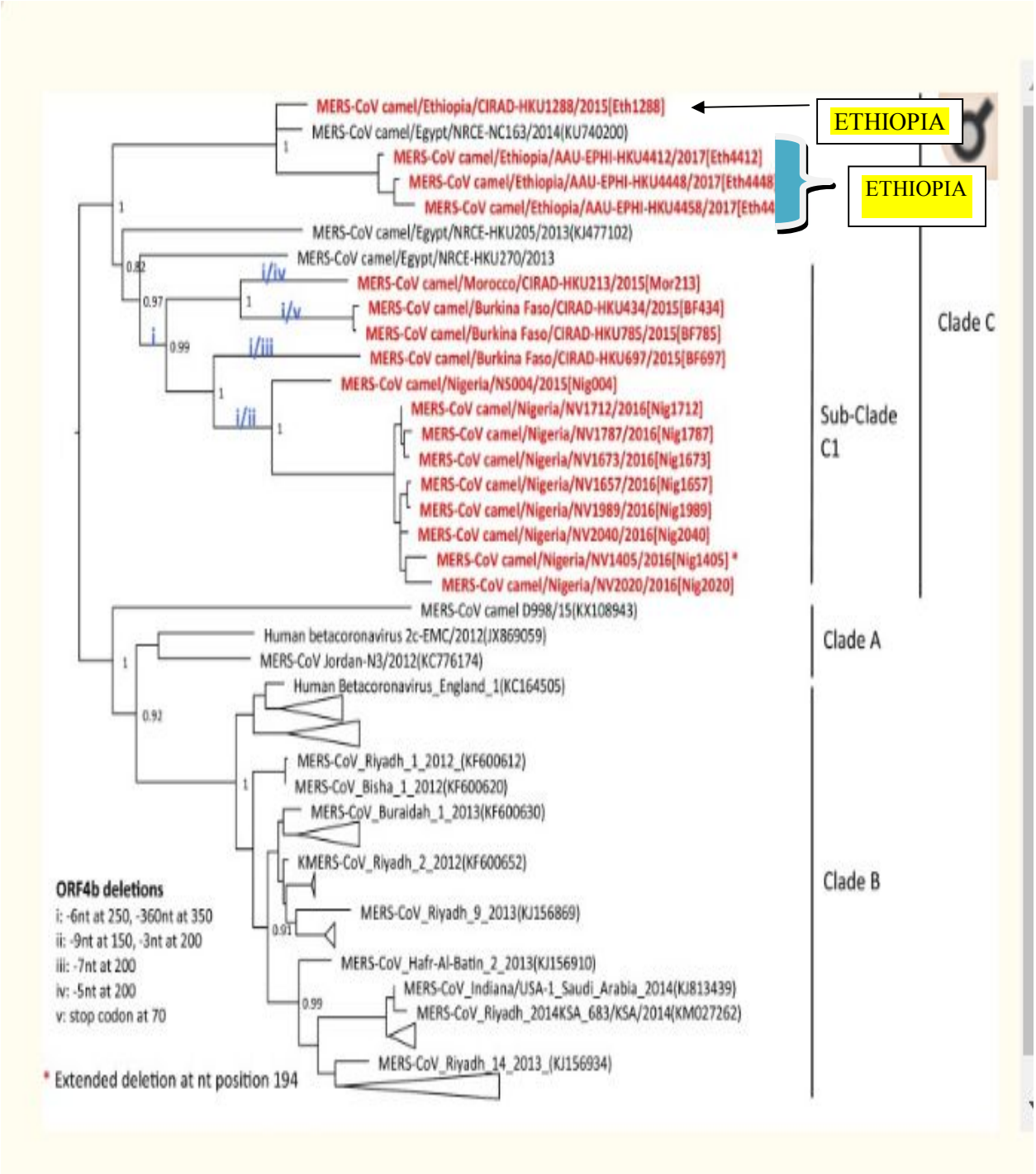


Figure 6 : Figure MERS CoV Phylogenetic data tree. Source (Chu *et al.,* 2018*).*

2.8 Prevention and control

No vaccination or treatment is currently available for MERS-CoV. As dromedaries are contemplate as the source of transmission of MERS-CoV to humans particularly in the Arabian Peninsula, travelers are advised to follow basic safety precautions, including proper hand washing prior and post touching an animal, and to refrain from consuming raw meat and milk and direct contact with camels. Despite all this information the transmission of the disease from camel to human and human to human is not recorded in Africa as a whole. However globally Controlling of MERS-CoV requires interdisciplinary efforts with the One Health concept focusing on human, animal and environmental health, intimately in order to understand the exact dynamics of MERS-CoV.(Nour and Houssam, 2019).

3 MATERIALS AND METHOD

3.1 Study localities

A cross sectional study was carried out in Amibara districts of Afar region, Ethiopia. The district is located at latitude: 9° 39' N. Longitude 40°19'E within Administrative Zone three of Afar region bordered to the south by Awash Fentale district, to the west by Awash River which separates it from Dulecha, on the northwest by the Zone five administrative, to the north by Gewane, to the east by the Somali Region, and to the south east by Oromia Region. Amibara district has an average altitude of 867 m.a.s.l. Within the district, three study sites (Angelele, Melkasedi, and Andido) were selected based on camel population density and being not previously studied.

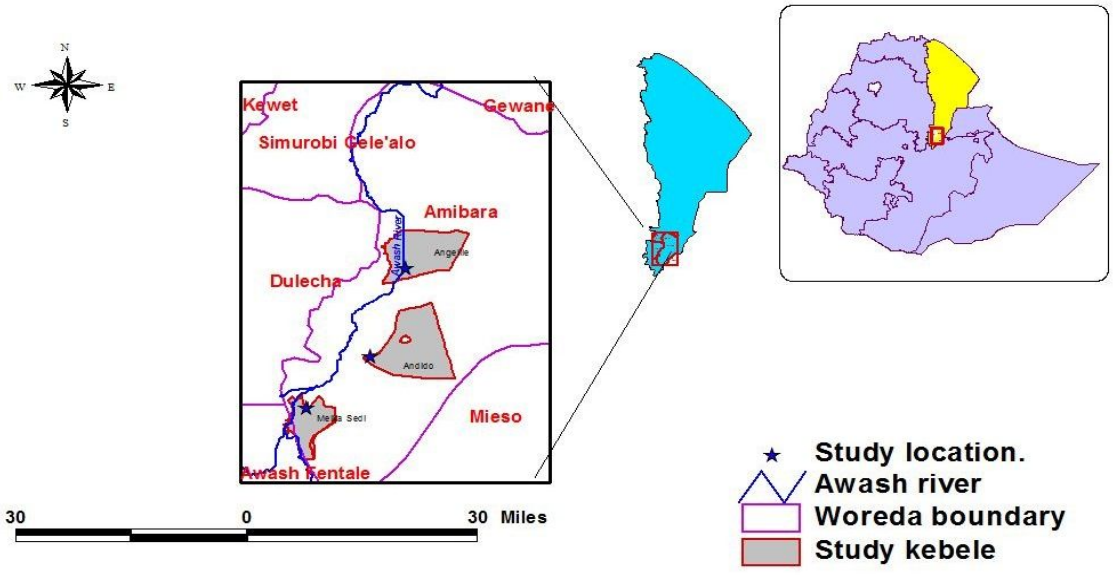


Figure 7 : Study map for Amibara sites

3.2 Study design and population

A cross sectional study design was used to assess the seroprevalance of MERS-CoV in Amibara.

The target populations for the study were dromedary camel of all age groups, (juvenile, young and adult) and both sexes (male and female). Camel population in Amibara district was 148,769 (Yosef *et al*., 2013). The herd size of study population was composed of, high >30, medium =11-30 and low/small number of camel herds.=1-10 and the age categories is described as Juvenile <1 year, Young 1-3 years, Adult >3years (Yasser *et al*.,2009).

3.3 Sample Size Determination and Sampling

The sample size determined for serological study was calculated by considering previously achieved epidemiological investigation of MERS-CoV with an expected prevalence of (92.3%) in the study area (Fekadu *et al*.,2017). Thus, the calculated sample size using a 95% confidence interval at 5% absolute precision was 95% using the formula described by Thrust field (2007). The total sample size in camels was 110. Increasing the sample size was considered to expand the precision.

$$n = \frac{1.96^2 (PexP)(1-PexP)}{d^2}$$

$$n = \frac{1.96^2 (92.3)(1-0.923)}{0.05^2}$$

$$n = \frac{3.84 (.923) (1-0.923)}{0.0025}$$

n= 110 camels

Increasing of sample size by 5 fold was planned to enhance the precision of sampling. Total sample number was 589.

3.3 Sampling techniques

Camels are restrained in all cases before sampling. Adequate health safety measures like wearing hand gloves, overall and mouth masks had been used at sampling site while sampling.

3.3.1 *Blood sample for sera harvesting*

Blood Samples were taken in duplicate from camels from each study three site. 10 mm of blood sample was collected from jugular vein using sterile needle and plain Vacutainer tube The blood was allowed to clot at room temperature. Serum was separated from the clot by centrifugation at 3000rpm for 3 min and transferred to 2 ml cryo vial with a volume of 1.5-2 ml sera. The separated serum was labeled and kept under refrigeration (–20°C) until transported to NAHDIC for analysis in NAHDIC and HKU. A total 589 sera were collected.

3.3.2 *Nasal Swab sampling for detection of the virus*

A total of 857 nasal swabs samples were collected in duplicate (for NAHDIC and HKU) by using applicator cotton swab (Ihab, 2018). The swab was taken for deep lateral turbinate as illustrated in Figure 8.After taking sample the swabs are immersed into 2 ml cryo vial containing 1.2 ml Viral transport medium (VTM) & preserved in liquid Nitrogen at -196 °C until transported to NAHDIC for keeping at -80°C freezer. Finally the swabs samples belonging to NAHDIC were tested in molecular laboratory and the other swab samples were shipped to HKU laboratory for MERS CoV RNA detection.

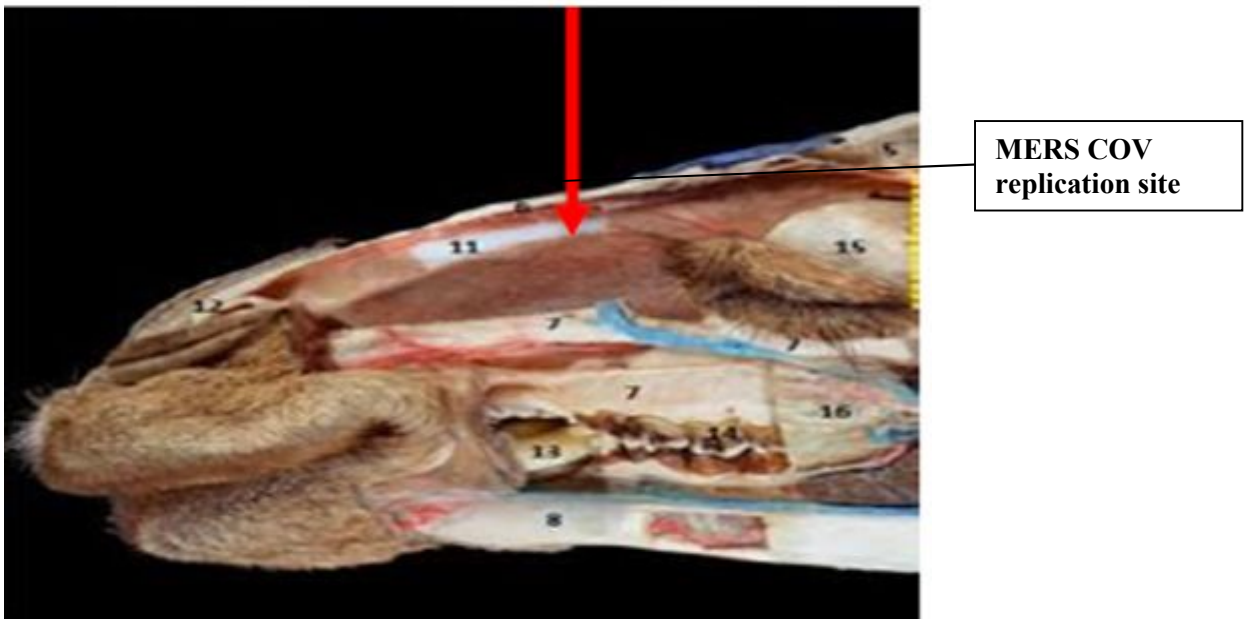


Figure 8 : Highest replication site of MERS CoV (Source: Andey *et al*., (2014).

3.4 Laboratory analysis

3.4.1 MERS CoV antibody detection through indirect ELISA test

The MERS CoV antibody detection through indirect ELISA test was carried out according to the kit manufacturer work instruction which is EUROIMMUN Anti – MERS-CoV ELISA Camel (IgG) kit AG product of Lübeck, Germany product. The test was done both at NAHDIC and HKU. Details of the technique are presented in annex 2.

3.4.2 Antibody detection through Pseudo particle neutralization test (ppNT)

Sera were shipped on dry ice to the University of Hong Kong for testing. A total of 198 sera samples were tested for MERS-CoV antibodies at a screening dilution of 1:20 using an extensively validated MERS-CoV spike pseudo particle neutralisation test (PpNT) as mentioned by Miguel *et al.,* (2015) & Perera *et al*., (2013). Details of the technique are presented in annex 3.

3.4.3 Virus detection through RT –PCR

The Real-time polymerase chain reaction (RT-PCR) was used for detection of RNA of MERS-CoV. RNA extraction was carried out as described previously/according to the manufacturer instruction described at annex 3 and 4 (Sameera and Ali, 2016). Screening of the upstream of envelope gene (UpE) was done using UpE- FWD primer (GCAACGCGCGATTCAGTT and UpE-Rev primer (GCCTCTACACGGGACCCATA) by reverse transcription quantitative PCR (RT-PCRI hydrolysis probe assay. as described by Miguel *et al.,*(2015).

3.5 Data analysis

The Data obtained from the investigations was coded and stored in Excel spread sheets. The data was analyzed using STATA software version 13 software . Logistic regressions reporting the odd ratio at 95% confidence interval were used to determine the level of variation between the sero-prevalence and the independent variable factors. The paired t test was also applicable to compare the result output of ELISA and PPNT. The association of the explanatory and outcome variables was also analyzed by Chi2 test where $p<0.05$ indicates the significance level of the risk factors.

4 RESULT

4.1 Sero prevalence of MERS CoV antibody

Based on Indirect ELISA test result the overall prevalence of MERS CoV in camels at study sites is 87.3% (n=514/589) (95% CI:84.5-89.9%). Association of different risk factors to seropositivity status of camels using X^2 analysis revealed that there was a statstically significant difference in proportion of MERS Cov antibody positivity among the three study sites (X^2 =13.7, p=0.001); Age categories (X^2 = 185.69, p=0.001); sex categories (X^2 = 4.5,p=0.034) and season (X^2 = 41.69, p=0.000); and in reproduction status of adult female (X^2 = 96.13, p=0.001); while no statistical significant difference was observed amongst herd sizes (X^2 = 5.88, p=0.053) as illustrated in table 1.

Table 1 : Association of different risk factors to seropositivity of camels MERS-CoV

Risk factors	**No of tested camel**	**No of Positive**	**Prevalence (%positive)**	**X^2 value**	**p-value**
Origin				13.39	0.001
Andido	289	266	92		
Melka sedi	149	127	85.2		
Angelele	151	121	80.1		
Sex				4.50	0.034
Male	55	43	78.2		
Female	534	471	88.2		
Herd size				5.88	0.053
Small	100	83	83.		
Medium	168	155	92.3		
Large	321	276	86		
Age				185.69	0.001
Juvenile	89	39	43.8		
Young	123	108	87.8		
Adult	377	367	97.3		

Season				41.69	0.001
Winter (December –February)	88	79	89.8		
Autumn (September-November)	272	260	95.6		
Summer (June –August)	229	175	76.4		
Dry	162	157	96.9		
Pregnant	68	64	94.1		
Lactating	146	145	99.3		

In multivariable logistic regression analysis young age (OR=7.39, 95% CI: 3.43-15.91), season from September -November (OR=4.83, 95% CI:2.145-10.90), and in adult female camel lactation status((OR=10.75, 95% CI:1.15-100.08)) showed a statistically significant difference among the groups for MERS CoV antibody detection while risk factors of origin, animal sex and herd size did not show a statistical significant difference in the status of result as indicated in table 2.

Table 2 : Multivariable Logistic regression analysis of MERS CoV prevalence

Risk factor	No of tested	No of positive and prevalence (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Origin				
Angelele	151	121 (80.1)	1	1
Melka sedi	149	127(85.2)	1.43(0.78-2.62)	1.03(0.44-2.41)
Andido	289	266 (92)	2.87(1.60-5.14)	1.00(0.42-2.41)
Sex				
Male	55	43 (78.2)	1	1
Female	534	471 (88.2)	2.09(1.04-4.16)	0.86(0.35-2.14)
Herd size				
Small	100	83 (83)	1	1
Medium	168	155(92.3)	2.44(1.13-5.27)	2.35(0.89-5.15)
Large	321	276 (86)	1.26(0.68-2.31)	1.51(0.71-3.55)

Age				
Juvenile	89	39 (43.8)	1	1
Young	123	108 (87.8)	9.23(4.55-18.28)	7.39(3.43-15.91)*
Adult	377	367 (97.3)	47.05(22.11-100.10)	21.91(0.27-1743.85)
Season				
Summer (June – August)	229	175 (76.4)	1	1
Autumn (September – November)	272	260 (95.6)	5.59(3.47-12.85)	4.83(2.14-10.90)*
Winter (December –February)	88	79 (76.4)	2.71(1.27-5.76)	4.10(1.30-12.86)
Production Status(Females)				
Pregnant	68	64 (94.1)	1	1
Lactating	146	145 (99.3)	9.06(0.99-82.59)	10.75(1.15-100.08)*
Dry	162	157 (96.9)	1.95(0.51-7.54)	2.49(0.53-9.92)

*Note: Risk factors displaying significant difference in Multivariable Logistic regression

4.2 Comparison of PpNT Vs iELISA test result status

The analysis was done using paired t test. Based on the analysis, of the total 198 sera samples tested using ppNT, 197 (99.5%; 95%CI: 98.4-100) were positive where as in iELISA of 198 sera sample only 182 (91.9%; 95% CI: 88-95.7) were seropositive indicating that ,ppNT is more sensitive than iELISA for MERS CoV antibody detection. The difference in between them was about 15(7.57%) samples where ELISA display as a negative was read as positive in ppNT. This implies that, there is a significant sensitivity difference in between the two serological tests 0.0001($p<0.05$) as shown in table 3.

Table 3 : Test result comparison between PpNT and ELISA test

Type of test	No of tested camel	No of positive	% Sensetivity(95% CI)	t value	p-value
Serology				4.0184	0.001
PPNT	198	197	99.49(98.45-100)		
ELISA	198	182	91.92(88.09-95.75)		

4.3 Viral RNA detection

All tested nasal swabs samples were negative for MERS CoV RNA particle by Real time polymerase chain reaction (RT-PCR) both at NAHDIC, Sebeta and Hong Kong University (HKU) which is referral laboratory for corona virus. In each reaction standard controls were included as indicate below in figure 9.

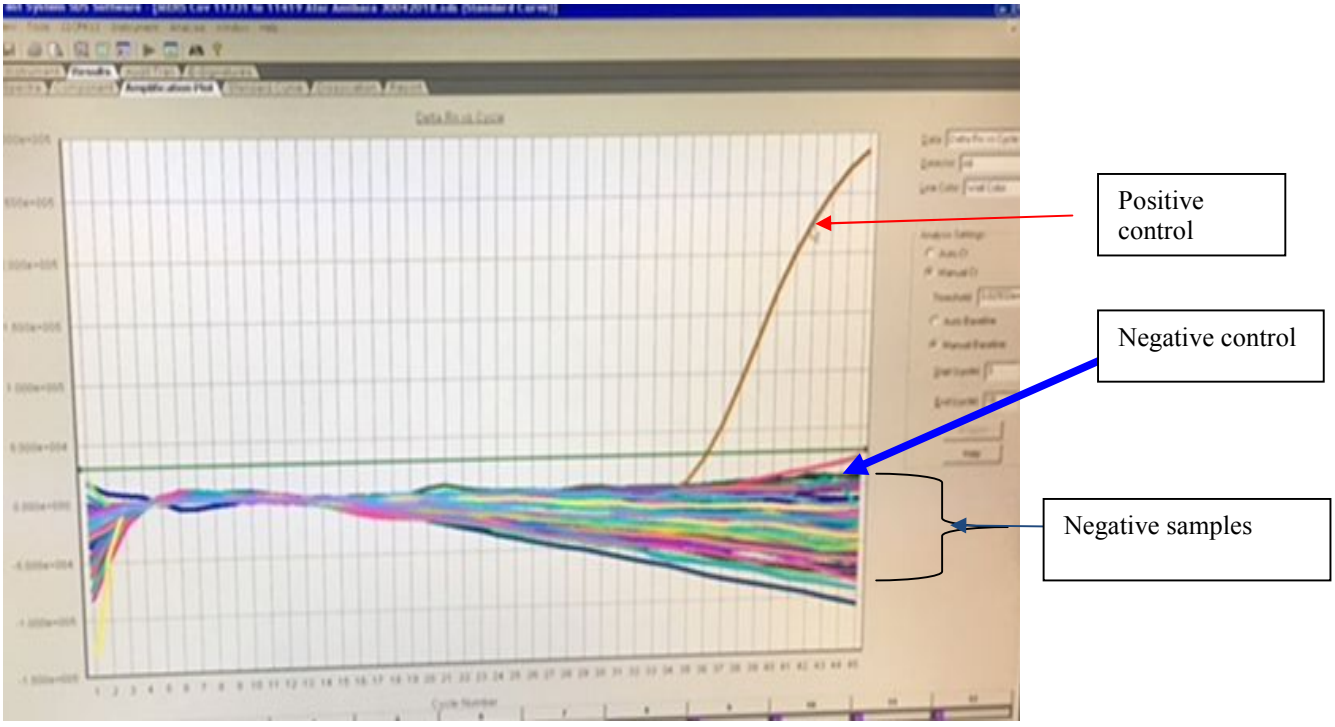


Figure 9 : RT-PCR test result cycle thresh hold (Ct values) graph

5 DISCUSSIONS

Middle East respiratory syndrome (MERS) is a zoonotic disease of global health concern, and dromedary camels are the source of human infection. In Ethiopia, a high seroprevalance of MERS-CoV in camel have been reported ranging from 93-97% in pastoral camel rearing areas of the country (Reusken *et al*., 2014; Fekadu et al 2017). In present study, a high prevalence of MERS-CoV with 87.3*%, (95% CI: 84.5-89.9) a*s observed in camels of Amibara district, Afar Region. This high seroprevalance result was in agreement with previous studies in pastoral areas of Ethiopia who reported 85.1-99.4% in camels of Afar and Oromia (Miguel *et al.* , 2015), 92.3% in Afar (Fekadu et al., 2017), 93-97% in Afar, Somali and Oromia regions (Reusken *et al.,*2014).

In multivariate logistic regression analysis three significant factors were observed in MERS CoV prevalence. Age; OR=7.39 (95%CI 3.43-15.91) with in this factor Adults >3 year are with high prevalence 97.3 %, young camels 1-3 years 87.8 % and Juvenile <1 year age 43.8 %. This study agree with previous study done by Miguel *et al.,* (2015) in which antibodies detection rates were higher in older animals while viral RNA was higher in young camels whereby they were free from antibody.

The reproduction status of female camels showed a considerable variation with OR=10.75(95%CI 1.15-100.08).With this result, pregnant camels were being stated with 94.1%, dry (96.9%) and lactating camels with (99.3 %). From this analysis we observe that high seroprevalance antibodies prevail in lactating camels when comparing with pregnant and dry camels (Miguel *et al.,* 2015).

Seasonal variation observed in this study (OR =4.83) illustrate high sero-prevalence is prevailed 95.6% (n=260/272) in autumn (September, October and November); 76.4% (n= (175/229) in summer (June, July and August) and 89.7% (n=79/88) in winter (December, January and February). The high prevalence in autumn was due to gathering of camels at one place for extended period of time .For this reason camels are getting sufficient

vegetation and grass there had been high probability of infection which induces the development of natural infection antibody. When observed herd size, high seroprevalance was observed in medium herd size 92.3% (n=155/168) subsequently large herd size 86% (n=276/321) and in the last part small herd size 83% (n=83/100). But the difference between the herd's categories was not indicating statistically significant difference as seen in table 1 and 2.

This analysis slightly differs from previous studies as such that the larger herd size have slightly higher prevalence (n=324/347) (93.4%) than the small herd sized 92.3% (n=205/222), (Fekadu *et al*., 2017) but the difference between the herds categories was not statistically significant. When looking at the results for reproduction status, high prevalence was seen in lactating camels (n=145/146) (99.3%) following dry camels (158/163) (96.9%) consequently Pregnant camels (n=62/66) (93.9%) at the last part young and Juveniles sero positivity indicates 69.6% (n=149/214). In general, the result denotes that there is statistically significant difference in sero positivity ratio among different reproduction status of camels which is $P < 0.05$ as indicated in table 7.

Despite high sero positivity of MERS CoV antibody, the virus couldn't be detected in the current study. This has been due to high numbers of camels that developed natural immunity (Miguel *et al*., 2015). However in previous studies at Afar area (Fekadu *et al.,* 2017) (n= 7/100 (7%) of samples had been detected by RT- PCR technique which was an indicative for the existence of circulating virus where it can be an evident for high sero positivity. Higher virus RNA detection rate in young animals compared with older animals could be related to a lack of prior immunity as published in previous studies in Saudi Arabia. Young animals were naïve and more susceptible to virus infection (Miguel *et al*., 2015).

The test for 198 sera in detecting MERS CoV antibody using PPNT with ELISA test is done only to observe and compare the sensitivity of the test methods. In this test PPNT detected 100% seropositivity for MERS-CoV antibody in serum comparing with ELISA which detects only about 92%. The difference in between them was 15 (7.57%) samples

where by iELISA display as a negative was read as positive in PPNT. Even -though that there is no sufficient evidence about the comparism output of the two tests, we can observe from the current study that PPNT is high sensitive than iELISA test in detection of MERS CoV antibody .`However, PPNT test method consumes more than 48 hrs to accomplish the overall test procedure ,while ELISA test consumes less than two hours. ppNT test require biosafety level II cabinet for safety measures while ELISA technique doesn’t require .

6. CONCLUSION AND RECOMMENDATIONS

From the current study, there was clear evidence for overall high sero-positivity of MERS-CoV in the study sites of Amibara district which was 87% (n=514/589). Among the study sites (Andido=45.16%, Melka Sedi 21.56 % and Angellele 20.54 % . Within the risk factors Age, Production status and season have significant difference in multivariate analysis for the prevalence of MERS CoV antibody.

The correlation of different risk factors were assessed in this study . In doing so, almost all risk factors were highly associated and were an important determinant for the disease In this study despite high sero prevalence of MERS CoV antibody, the viral RNA is not able to be detected by RT- PCR test both at NAHDIC and HKU referral laboratories as previous studies indicated. This result disagree with the past studies high MERS CoV RNA rate detected in Ethiopia up to 15.7% ;(C.I. 95%, 8.2-28.0) (Miguel *et al*.,2015). In another study MERS CoV RNA with 7% was detected in Ethiopia between October 2014 and May 2015 (Fekadu *et al*., 2017).

The possible causes for not getting /detecting the Viral RNA in the study area would be due to the following factors and challenges

Lack of sufficient information in understanding the viral shedding period or incubation time of the disease and the sample where by the virus is able to be detected, lack of observation for apparent form of clinical sign of MERS CoV on camels as to enable to take the swab sample at early time of the disease, difficulty in deep swab sample taking process due to far distance of posterior turbinate of elongated nasal cavity of camels whereby it is the virus replication site compared to application swab stick length .

Based on the conclusion, the following recommendations are forwarded:-

- Further study on the disease should be conducted in the study area by considering all aspects of the MERS-CoV disease including in identifying other risk factors which will have value in the control of the disease.

- Even though, priority is given for swab sampling from nasal cavity of the camel due to nature of replication site of virus; milk, urine and feces might be an appropriate samples to detect the virus hence these sample should be included at sampling.

- Camel abattoirs /slaughter houses to be included in taking swab samples from Slaughtered camels to get access to the deep of nasal turbinate get the virus.

- A study to be considered by repeated or as longitudinal study sampling and focus should be targeted to well marked and known juvenile and young camels as they are being considered that , most of them are not developing MERS CoV antibody. This intensifies a chance of getting active virus to understand the virus characteristics.

- Since MERS CoV is one of the recently recognized zoonotic disease in Arabian penusilla & camels are the sources of the virus to humans' public awareness about the disease should be created in camel rearing pastoralists area

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8. ANNEXES

Annex 1: Sample collection format

Spp: Came Date: _____District: Amibara Locality (Kebele)_____

Sample collection form (To be filled in the field)

S/N	Animal I.D	Sample code	Sex (M/F)	Age (J/Y/A)	Types of sample	Herd size	reproduction status	Remark

Age : J,Juvanile(<1yr) Y,Young (1-3yrs) ,A ,Adult (>3yrs).Type of sample : Nasal swab (N) ,Serum (S) Herd size ; Large, medium and small ; Production status : Dry, Pregnant and lactating.

Annex 2 : iELISA technique

Principle of the test: The ELISA test kit provides a semi quantitative in vitro assay for antibodies of class IgG against MERS coronavirus in serum or plasma of camels. The test kit contains micro titer strips each with 8 detachable reagent wells coated with purified S1 anti-gen of MERS coronavirus (MERS-CoV S1). In the first reaction step, diluted samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labeled anti-camel IgG (enzyme conjugate) catalyzing a colour reaction.

Plate lay out for MERS CoV test sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Cal**	**S6**	**S14**									
B	**Pos**	**S7**	**S15**									
C	**Neg**	**S8**										
D	**S1**	**S9**										
E	**S2**	**S10**										
F	**S3**	**S11**										
G	**S4**	**S12**										
H	**S5**	**S13**										

***Note Cal: Calibrator, Pos: Positive, Neg: Negative, S: Sample**

Reagents and materials inside the kit

Contents within the kit are Micro plate wells coated with antigen, Calibrator (IgG camel) ready within the kit, Positive control IgG camel /Human ready for use, Negative control, IgG camel and Human ready for use. Conjugate enzyme (Peroxidase –labeled anti came/or Anti human IgG ready for use. Sample buffer Ready to use, Wash buffer 10X concentrate, Substrate solution (TMB/H2O2 ready to use) and Stop solution (0.5M Sulphuric acid) ready to use. Preparation and stability of sample. Samples are diluted 1:101 in sample buffer Samples are diluted 10µl in 1 ml sample buffer. and mix. well by vortexing Calibrator and controls are pre diluted and are ready for use;

- Add 100µl of calibrator to well A1
- Add 100µl of Positive control to B1
- Add 100µl of Negative control to C1
- Add 100µl of sera to wells according to plate lay out.
- Seal with plastic sealant or ASluminum foil and keep in incubator for 30 minutes at 37•C
- Wash 3X using 10 X wash solution leave 30 to 60 seconds.
- Remove the wash solution from the micro plate and then tap it using Absorbent paper.

- Add 100µl of conjugate to all wells and incubate 37 degree Celsius for 30 minutes
- Remove the conjugate from the plate
- Wash 3X using 10 X wash solution leave 30 to 60 seconds.
- Remove the wash solution from the microplate and then tap it using Absrbent paper.
- Add substrate solution of 100 micro liter. Put bthe plate in dark for 15 minutes at room temperature.
- Add stop solution with 100 microliter
- Read with wavelength of 450nm. Using Sphectrophotometer.

Calculation of the results.

The extinction of the calibrator corresponds to the upper limit of the reference range for non infected animals (Cut off) recommended by EURO IMMUN .If the extinction of a sample is found to be above the extinction value of the calibrator ,the samples is to be considered as Positive.

Extinctions below this value are to be interpreted as negative.

Semi quantitative :

Results can be evaluated semi-quantitatively by calculating a ratio of the extinction value of the control or sample over the extinction value of the calibrator.

Calculate the ratio according to the following formula:

$$\frac{\textbf{Extinction of the control or sample}}{\textbf{Extinction of calibrator}}$$

EURO IMMUN Recommends interpreting results as follows

- Ratio < 0.8 : Negative
- Ratio ≥0.8 to <1.1 : Border line
- Ratio ≥1.1 : Positive

For duplicate determinations the mean of the two values should be taken to calculate the ratio. If the two values deviate substantially from one another, EUROIMMUN kit recommends retesting the samples.

At the end all the reagents within the kit are kept $+4^0$C and sample $-80\ ^0$C

This was a Serology test used for the detection of MERS CoV antibody screening . PpNT reagents and other laboratory materials used for the test were done with in BSL II biosafety level II cabinet. Cell used for PpNT test origin of African Green Monkey kidney. DMEM (Media) 10% FBS (Fetal Bovine Serum) into 8 ml of Trypsin containing cultured cell. Trypsin EDTA, Pseudo particle gene spike from MERS virus, PBS, Luceferase, Illuminometer, Inverted type microscope , Incubator with 5% CO_2 and tally counter.

As a principle of PpNT test if MERS CoV antibody in the serum exists it reacts with Pseudo particle Spike gene, so the cell is not attached by the spike gene that means spike is neutralized by Antibody. As a result counted number of Optical Density (OD) is less than 10% of counted control on Column 11(Positive).

If MERS CoV antibody in serum is not existed the Pseudo particle attach the cell. Hence there is no reaction between Antibody and Pseudo particle Spike. So after cells being attached, the PP uses cells machinery such as Ribosome's to produce Luceferase protein. Luceferase substrate uses Luceferase protein produced by infected cells. As a result counted number of OD is greater than 10% of Counted control on column (Negative). Read immediately otherwise the color with substrate will be degraded. If the no of counting cells are <10% of the control say positive. If >10% of control say Negative .

Annex 3 : Psedoparticle neutralization test (ppNT) procedure

Step 1

- There is prepared cell used for PpNT test origin of African Green Monkey kidney.
- Check the cell morphology under inverted type microscope to assure that the cells are intact as normal (spindle) shape.
- Discard the supernatant media fluid from the TCflask using serology pippet by Aspirator.
- Add PBS 8 ml to the TCF to wash old media from TCF cultured cells.Jently mix up on the pBS with the surface of TCF.
- Discard the PBS

- Add 3 ml of Trypsin EDTA To the TCF. This is to cleave or detach the intact cells from the bottom of TCF.
- Put the flask in Incubator at 37 ^{0}C accompanied with 5% CO_2 for 5 minutes.
- Then the cells cleaved or detached from the bottom of flask due to trypsin
- Check the cell morphology (circular) and detachability.
- Add 10 ml DMEM(Media) 10% FBS (Foetal Bovine Serum) into 8 ml of Trypsin containing cultured cell.
- Now the total volume in the flask is 13 ml of Media +Trypsin. Mix it using serology pippet by pippeting up and down for few time disseminating to the surface of the bottom side of TCF.
- Transfer it the 13 ml of media +Trypsin to the falcon tube 0f 50ml volume capacity
- Centrifuge at 453 RCF (relative centrifugal Force) or 1500 rpm for 5 minutes.
- Aspirate the supernatant and discard the DMEM and Trypsin. Then the cell is sediment at the bottom of falcon tube..
- Add 12 ml of 10% Media with FBS in to the Falcon tube containing the sedimented cell.Mix up the cells and the media by serology pippet up and down
- Add 10 ml media 10% FBS(DMEM to the cells New TCF and register the passage no,Name of cell type and date of Passaging for the next passage .
- Take 1 ml from the mixed cultured cells in the falcon tube and add to the new TCF containing 10 ml of new 10% FBS media.(10 +1=11ml)in the new flask.Then put in incubator at 37^{0}C of 5% CO_2 for the next passage as a regular cell culture work. Staying for about 4 days and then the passage process cycle continued.
- Take 1 ml of mixed cultured cells from falcon tube to the microcentrifuge tube .
- Take 10µl of mixed cultured cells from the 1 ml micro centrifuge tubeand add to the cell counting chamber using 10 µl pippet tip through the covered slide.
- Put the slide under microscope and start to count at 4 edges od angles of the slide using tally counter in order to determine the concentration of cells .Take the counted average of 4 sides. Multiply by 10000 cell as standard.

- After counting from 4 angles take the average no of Cells.52+76+62+40=230 /4=57.5 cells
- 57.5x10000cells =575000cells.
- We need 20000 cells per 100microliter in each well
- 575000/1000microliter
- 57500 cells /100 microliter. Because we need 100 microliter in each well
- So 57500/20000cells in 100 microliter (Standard) =2.875 =2.9(More concentrated)
- 2.9 X10ml in the falcon tube =29 which is the final volume.
- 29-10mlin falcon tube =19 ml of media.
- So we need to add 19 ml of 10% FBS to the 10 ml of mixed cultured cells in to the falcon tube.
- Now the total volume is 29ml diluted cells.(concentration that we need)
- Pour on the diluted cells in to the trough.
- Use new dilution plate and white cell culture plate. Make a line of plate lay out by marker at the out side cover of the plate.
- Starting from B2-B11,C2-C11,D2-D11,E2 E11,G2-G11,
- Aspirate 100 µl of diluted cells from the trough using multichannel pippet to the wells of both white and transparent(Dilution) plate. Cover the plates with lid.
- Label the plates and incubate at 37^0C of 5% CO2 for 24 hrs.
- Check the cells morphology under microscope after 24 hrs incubation.

Step 2

Prepare new dilution plate. , for PpNT test then Label with sample code on the plate lid by making drawing of plate layout.

- Add 144 µl of DMEM (media)containing 2% FBS to rows from of B2-B10
- Add 144 µl of 2% FBS DMEM media to rows of E2-E10
- Add 160 µl of 2% FBS DMEM media to B11 and E11
- Add 16 µl of test serum B2-B10 and E2 –E10 according to the sample ID labeled.Total 18 samples. (9X2).

Note *: Prepare Dilution of PP gene spike from Mers virus with ratio of 1:8

- Take 1ml of PP Gene spike solution and Add to 7 ml of media 2% DMEM in to falcon tube. 1:8 ratio and mix it by serology pippet.
- Pour the prepared diluted gene spike solution + media to new trough.
- Add 160 µl prepared dilution gene spike to the sera 16 µl + 144 µl of 2% DMEM FBS rows B and E from 2-11 and then keep it in + 4Degree celcius for 90 minutes.(Sera +Pseudo particles.)

Step 3

- Take out the white color plate incubated overnight which contain cells.
- Discard all media liquids within the plate. So the cell is sediment at the bottom.
- Take out the dilution plate that incubated at 4 ^{0}C for 90 minutes.
- Aspirate 100 µl using multichannel pippet from dilution plate containing DMEM 2%FBS +sera+diluted PP gene spike to the white plate which has a sediment cell. From B2 –B11 in triplicate and E2-E11 in triplicate. Cover the lid of plate and incubate at 37^{0}C of 5% Co_2 for 72hrs.
- After 24 hrs Add 100 µl of 10% FBS media.
- Again incubate for 48 hrs (Total incubation from starting is 24+48 = 72 hrs.)

Step 4

Power on the BSL II. Disinfect with 70 % Ethanol. Use vericon 10% (50 gram vericon powder to 5 liters of water for virus disinfection. **Additional reagents used for the last procedure.**

- Cell culture lysis which is liquid form 5X concentrate.
- Luciferase Substrate: In liquid form .Use molecular grade water. It is kept in -30^{0}C freezer .When wanted to use it will be thaw by putting in warm water.

A. Luceferase assay buffer (white color).

B. Luceferase assay substrate (Green cup lid) both will be mixed with vortex.

Each plate needs < 5ml solution.

After making solution and used it, put in to -30^{0}C.

Procedure for final reading of the result

- Discard all fluids (DMEM +Sera+Spike) from all wells from incubated white plates(culture plate).
- Dilute the lysis reagent 5X
- Add 2ml cell culture lysis buffer with 8 ml molecular grade water.
- Add 25µl of lysis buffer diluted solution to all labeled wells of culture plates.(white color)
- Add 50µl of Luceferase substrate to all wells with in 25µl.
- Put the plate in the shelf of Microplate counter connected with computer having Microbeta soft ware.
- Read immediately otherwise the color with substrate will be degraded. If the number of counting cells are <10% of the control say positive. If >10% of control say Negative.
- Interpretation of the result
- Take the average of the triplicate samples and also the average of triplicate control for analysis of the result OD value.
- If Antibody in the serum exists it reacts with Psedo particle Spike gene ,so the cell is not attacked by the spike gene that means spike is neutralized by Antibody.As a result counted no of OD is less than 10% of counted control on Column 11.(Positive)
- If Antibody in serum is not existed the Pseudo particle attack the cell . Hence there is no reaction between Antibody and Pseudo particle Spike.So after cells being attacked ,the PP uses cells machinery such as Ribosomes to produse luceferase protein .Luceferase substrate uses Luceferase protein produced by infected cells. As a result counted no of OD is greater than 10% of Counted control on column 11(Negative).

Annex 4 : Molecular diagnostic technique for MERS CoV RNA detection

The test starts from the preparation of sample. In this case we were using extraction of swab sample. Hence no need of preparation of sample.

A. Extraction of Swab sample

- Prepare AVL lysis buffer and carrier RNA
- Dispense 560µl of prepared lysis buffer and carrier RNA to the new microcentrifuge tube of 1.5ml.Label sample ID.
- Add 140 µl to the lysis buffer containing the above tube.
- Pulse vorteing and incubate for 10 minutes at room temperature.
- After 10 minutes short/brief centrifuge 8000rpm/15 seconds.
- Add 560 of Ethanol 96-100% molecular grade
- Pulse vortexing and brief centrifuge as speed of 8000rpm/15 seconds.
- Prepare minispin column
- Label sample ID on the mini spin column lid.
- Transfer 630 µl of the prepared sample+lysis buffer and Ethanol to the mini spin column.
- Centrifuge at 8000rpm/1 minute.
- Transfer the minispin column to the new collection tube .Discard the previous collection tube containing fluids.
- Again add 630 µl of the remaining prepared lysis buffer +sample +ethanol to the mini spin column
- Centrifuge again 8000rpm/1 minute.
- Transfer the minispin column to the new collection tube .Discard the previous collection tube containing fluids.
- Add 500 µl of AW1 or wash 1 to the mini spin column.
- Centrifuge at 8000rpm/1 minute.
- Transfer the minispin column to the new collection tube .Discard the previous collection tube containing fluids.
- Add 500 µl of AW2 or wash 2 to the mini spin column.
- Centrifuge at full speed or 14000rpm for 3 minutes.

- Transfer the minispin column to the new collection tube .Discard the previous collection tube containing fluids.Centrifuge the mini spin column with dry and without adding anything at full speed or 14000 rpm for 1 minute.
- Transfer the minispin column to the new collection tube .Discard the previous collection tube.
- Add 60 µl of AVE or elution buffer .to the minispin column and incubate at room temperature 15-25 ®C for 1 minute.
- Centrifuge at 8000rpm/1 minute.
- Transfer the eluted final RNA product to the micro centrifuge tube having a cap.
- Now we completed extraction of RNA.

B. Master mix preparation

Protocol for UPE QRT PCR (CORMAN et *al.* 2012) for preparation of Master Mix required.

- Supescript III one step qRT PCR system with platinum Taq Polymerase (Invitrogen, containing 0.4mM of each dNTP and 3.2 mM Magnesium sulfate) or other common one step real time RT-PCR kit formulated for application with probes.
- 50mM magnesium sulfate solution (Invitrogen)
- RNase /DNase free water
- Centrifuge
- Real time PCR machine
- Extracted RNA samples
- Positive control UPE (dilutions)
- Negative control
- Filtered pipet tips
- Eppendruf tubes (0.2ml and 0.5ml,compatible with RT-PCR machine)

Per sample, a 20µl reaction prepared in a designated RNA/DNA free area by making a master mix in a separate eppendruf tube and dividing this over the PCr eppendruf (NB calculate sufficient quantities to include the positive and Negative controls). Always wear gloves for the entire procedure and change gloves in case of contamination, use

designated coats for each area to keep potential contamination to a minimum . Keep all the reagents on ice and keep the Taq enzyme in freezer until use.

- 12.5µl of 2Xreaction buffer provided with the superscript III one step RT-PCR system with platinum Taq Polymerase (Invitrogen ; containing 0.4mM of each dNTP and 3.2 mM magnesium sulfate).
- 1 µl of reverse transcriptase/Taq mixture from the kit.
- 0.4 µl of 50mM magnesium sulphate solution (Invitrogen).
- 3.6 µl of RNase/DNase free water
- 1 µl (400nM final concentration) primer UpE-FWD (GCAACGCGCGATTCAGTT).
- 1 µl (400nM final concentration) primer UpE-Rev (GCCTCTACACGGGACCCATA).
- 0.5 µl (200 nM final concentration) of probe uPE-Prb (6-carboxyfluorescein [FAM]-CTCTTCACATAATCGCCCCGAGCTCG-6-carboxy-N,N,N,N'-TETRAMEYHYLRHODAMINE [TAMRA])

Take PCR tubes to a separate area (on ice) and add

- 5 µl of extracted RNA from samples (freeze remaining extracted RNA at -80°C) or 5 µlPositive control (UpE RNA) or 5 µl negative control (bidest).

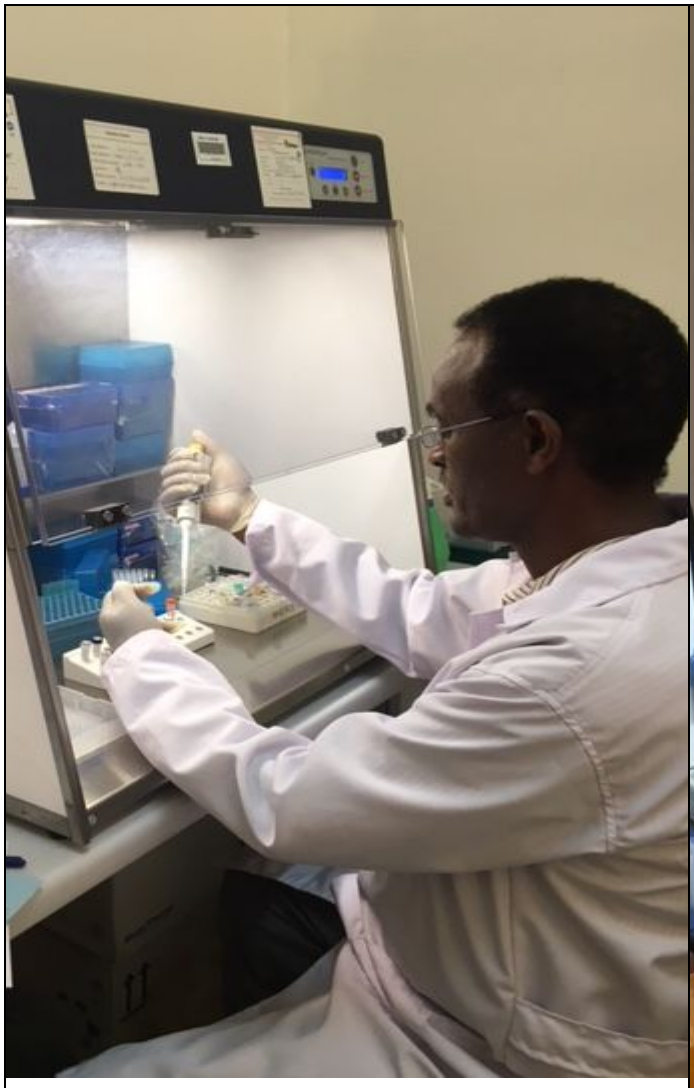
Transfer extracted RNA including the standards to PCR microplate wells according to the lay out plan.

Seal the plate firmly then insert the plate into applied biosystem .Then program the computer with software and run the PCR machine.

C. **Thermal cycling**

- 55°C for 20min
- 95°C for 3 min
- 45 cycles of 95°C for 15 seconds
- 58°C for 30 seconds

Source: (CORMAN et *al.* 2012) : Protocol for UPE QRT PCR

	
Master mix preparation	Dispensing extracted RNA samples and standards

Inserting of dispensed PVR plate into applied biosystem PCR machine | Running the PCR cycle according to the protocol

Figure 10 : Working at NAHDIC Molecular laboratory