EPIDEMIOLOGICAL INVESTIGATION OF MIDDLE EAST RESPIRATORY SYNDROME CORONA VIRUS (MERS-CoV) AMONG DROMEDARY CAMELS IN SELECTED AREAS OF AFAR AND OROMIA REGION, ETHIOPIA

MSc Thesis

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June, 2015

Bishoftu, Ethiopia

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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 Science in Tropical Veterinary Epidemiology

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As members of the examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by **Getnet Fekadu Demissie** entitled**: Epidemiological Investigation of Middle East Respiratory Syndrome Corona Virus (MERS-CoV) Among Dromedary Camels in Selected Areas of Afar and Oromia Region, Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Tropical Veterinary Epidemiology.

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Date of Submission ____________________

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LIST OF ABBREVATIONS

AIIR	Airborne Infection Isolation Room
ARDS	Adult Respiratory Distress Syndrome
BAL	Bronchoalveolar Lavage
CSG	Coronavirus Study Group
Ct	Cycle threshold
HCW	Healthcare Worker
HEPA	High Efficiency Particulate Air
IFN	Interferon
IRF	Interferon Regulatory Factor
MCP	Monocyte Chemotactic Protein
MDAS	Melanoma Differentiation Associated Protein
MERS	Middle East Respiratory Syndrome
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
NCoV	Novel Coronavirus
NVI	National Veterinary Institute
ORF	Open Reading Frame
RIG	Retionic Inducible-acid Gene
SARS	Severe Acute Respiratory Syndrome
TGF	Transforming Growth Factor
WHO	World Health Organization

ABSTRACT

A cross-sectional study was conducted from November 2014 to May 2015 to determine the seroprevalence, assess the presence of active cases through isolation and identify possible risk factors for the MERS-CoV in camels in selected areas of Oromia and Afar regional state of Ethiopia. A total of 472 Dromedary Camel sera were collected and screened with two serological tests: (Pseudoparticle neutralization for screening and Microneutralization test for confirmation. The overall prevalence of MERS-CoV in the study area was (91.5%) (n=432/472). Higher prevalence (92.9%) was recorded in Female Dromedary camel when compared to Male (88.8%) result of multivariate logistic regression shows female camels (OR=2.408) in the study area are 2.4 times at risk of MERS-CoV than male dromedary camel. From Zones included in the study Borena zone recorded the least prevalence (82.2%) (n= 60/73).Multivariate logistic regression shows camels living in Borena zone (OR=0.055) were relatively protected from MERS-CoV when compared to zone 4 of Afar .From the kebeles included camels living in Galifage (OR= 0.018) of Ayssaita district were relatively protected when compared to Urmaytu kebele. Out of 100 swabs collected from four districts (Yabelo, Fentale, Amibara and Dubti) and undergo Real Time PCR , 7 of them evident circulation of MERS-CoV in the study area. Generally this study showed the existence of high seroprevalence of MERS-CoV among Ethiopian dromedary camels and also confirmed with RT-PCR, therefore further study is required to determine its significance from both animal and public health perspectives.

Keywords: *Dromedary Camel, MERS-CoV, Prevalence, Sera, Swab, RT-PCR, Ethiopia.*

1. INTRODUCTION

MERS-CoV is a coronavirus that can cause severe acute respiratory infection in humans. Confirmed cases of MERS-CoV originated in the Arabian Peninsula and surrounding areas (Medicine net, 2015).

In June of 2012, a novel beta corona virus, associated with severe respiratory disease in humans emerged in the Middle East (Zaki *et al.,* 2012), which is closely related to beta corona viruses circulating in bats (Anthony *et al*., 2013). The first isolate of Middle East respiratory corona virus (MERS-CoV), HCoV-EMC/2012, was obtained from a patient with a fatal pneumonia and acute renal failure. To date, 107 additional human cases have been identified, of which 49 were fatal (WHO, 2013). Aside from cases in Saudi Arabia, Qatar, Jordan, and the United Arab Emirates, imported cases have been identified in the United Kingdom, Germany, France, Tunisia, and Italy. Although no information is available on the source or route of primary transmission of MERS-CoV, human-to-human transmission has been recorded (Assiri *et al*., 2013).

Clinical data on human cases of MERS-CoV infection are currently sparse, but it appears that this virus mainly causes severe lower respiratory tract disease, occasionally accompanied by renal disease. Until 23 May 2013, MERS COV had frequently referred to as a SARS-like virus (van Doremalen *et al*., 2013), or simply the novel corona virus.

A study by Ziad *et al* (2012) suggests that the virus arose some time on June 2012, with perhaps as many as 7 separate zoonotic transmissions. It has been determined through molecular clock analysis, that viruses from the EMC/ 2012 and England / Qatar /2012 date to early 2011 suggesting that these cases are descended from a single zoonotic event. It would appear the MERS - COV has been circulating in the human populations for greater than one year without detection and suggests independent transmission from unknown source (Lau *et al*., 2011).

MERS COV is more closely related to the bat corona viruses HKU4 AND HKU5 (lineage 2C) than it is SARS –CoV (lineage 2B) (Pro MED –mail, 2012 and Lau *et al*., 2011), sharing more than 90% sequence identity with their closest relationships, bat corona viruses HKU4 and HKU5

and therefore considered to belong to the same species by the International Committee on Taxonomy of Viruses.

The severity of disease distinguishes MERS-CoV from other corona viruses circulating in the human population, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV HKU1, which are generally associated with upper respiratory tract infections. Instead, MERS-CoV appears to be more similar to the severe respiratory disease caused by severe acute respiratory syndrome (SARS)-CoV. In vitro studies have shown that MERS-CoV replicates efficiently in non-ciliated cells in the primary human airway epithelium (Kindler *et al*.,2013), and in ex vivo human lung cultures MERS-CoV replicated in bronchial, bronchiolar, and alveolar epithelial cells (Chan *et al*., 2013).

The virus has been demonstrated to grow well in cell lines that in the past have commonly been used for diagnostic viral cultures. Early comparisons with other known coronaviruses suggest a genetic similarity to viruses previously described in bats. However, even if an animal reservoir is identified, it is critical to identify the types of exposures that result in infection and the mode of transmission (Hijawi *et al*., 2013).

The MERS-CoV virus is thought to be an animal virus that has sporadically resulted in human infections, with subsequent limited transmission between humans. The evidence for the animal origin of the virus is circumstantial. Nevertheless, the alternative explanation to explain the sporadic appearance of severe human cases with long periods of time between them, and the wide geographical area over which the virus was apparently distributed, is unrecognized ongoing transmission in people. Surveillance efforts since the discovery of the virus and retrospective testing of stored respiratory specimens suggest this is not the case (Gautret *et al.,* 2013).

In August, 2013, the possible source of the MERS-COV virus was traced to an Egyptian tomb bat found in a building in close proximity to the patient home. The virus genome fragment isolated from the bat was a 100% genetic match to the virus isolated from the index patient (Jobs, 2013). There had been speculation that bats might be the source of the virus (Abedine and Saad, 2013). On 9 August 2013, a report in the journal “The Lancet Infectious Disease” showed that 50

out of 50 (100%) blood serum from Omani camels and 15 out of 105 (14%) from Spanish camels had protein specific antibodies against the MERS- COV spike protein and also Egypt (between 94% and 98% of a total of 110 camels tested positive). A further study on dromedary camels from Saudi Arabia published in December 2013, revealed the presence of MERS –COV in 90% of the evaluated dromedary camels (310), suggesting that dromedary camels not only could be the main reservoir of MERS –COV, but also the animal source of MERS –COV (Hemida *et al*, 2013).

Although the Arabian Peninsula remains the site of primary human infection from the zoonotic source of human cases reported so far. Dromedary camels in other parts of North, West and East Africa appear to have serological evidence of MERS infection. The full geographic range of MERS CoV circulation has not been established so far in the above sites including Ethiopia. Seroprevalence of MERS-CoV in Ethiopia was 97% in adult camels, 93% in young camels and overall prevalence of 96% (Reusken *et. al*., 2014).

Therefore the objectives of this study were:-

- ✓ To determine the seroprevalence of MERS-CoV.
- ✓ To assess the presence of active cases through isolation of MERS-CoV.
- ✓ To identify possible risk factors for the MERS-CoV in the study areas.

2. LITRATURE REVIEW

2.1 Definition of MERS

MERS-CoV is a coronavirus that can cause severe acute respiratory infection in humans. Confirmed cases of MERS-CoV originated in the Arabian Peninsula and surrounding areas (Medicine net, 2015).

2.2 Etiology

Since the initial discovery, isolates of the virus have been described in the scientific literature, databases, and popular press under various names (e.g., human betacoronavirus 2c EMC, human betacoronavirus 2c England-Qatar, human betacoronavirus 2C Jordan-N3, betacoronavirus England 1) with novel coronavirus (NCoV) as the one used most often. As this lack of uniformity in virus nomenclature complicates communication both in the research field and with health care authorities, governments, and the general public, the Coronavirus Study Group (CSG) of the International Committee on Taxonomy of Viruses (http://ictvonline.org/index.asp?bhcp=1) took the lead to address this issue. After careful consideration and broad consultation, the CSG has decided to call the new coronavirus Middle East respiratory syndrome coronavirus (MERS-CoV). This name is endorsed by the discoverers of the virus and other researchers that pioneered MERS-CoV studies, by the World Health Organization, and by the Saudi Ministry of Health (Groot and members of ICTV, 2013).

New MERS-CoV isolates or variants detected by reverse transcription (RT)-PCR may be provided with an affix, analogous to convention in influenza virus nomenclature (the host/country of origin plus the strain identification number/year; e.g., MERS-CoV Hu/Jordan-N3/2012). The knowledge of the epidemiology and host preference of this virus is still incomplete (Groot and members of ICTV, 2013).

2.2.1 Taxonomy and Phylogeny

Generally Coronaviruses Classified in to four groups: The first group is Alpha Coronavirus (Human examples: HCoV-229E, HCoV-NL63), Pig, dog, and cat Coronavirus, Beta (HCoV-OC43, HCoV-HKU1, HCoV-SARS MHV, rat, pig and cow CoVs and MERS-CoV), Gamma (Chicken and turkey CoVs) and the final group is Delta Coronavirus (Bird CoVs) *(Roos,2013 and Bermingham et al.,2012).*

MERS-CoV is more closely related to the bat coronaviruses HKU4 and HKU5 (lineage 2C) than it is to SARS-CoV (lineage 2B) (2, 9), sharing more than 90% sequence identity with their closest relationships, bat coronaviruses HKU4 and HKU5 and therefore considered to belong to the same species by the International Committee on Taxonomy of Viruses (ICTV).Scientific name of the virus is Middle East respiratory syndrome coronavirus and Common used name: MERS-CoV. Other names used are novel coronavirus (nCoV), London1 novel CoV 2012 and Human Coronavirus Erasmus Medical Center/2012 (HCoV-EMC/2012).

The virus lineage is Group: IV; positive-sense, single-stranded RNA viruses ,Order: *Nidovirales,* Family: *Coronaviridae* , Subfamily: *Coronavirinae* ,Genus: *Betacoronavirus and* Species: *Betacoronavirus 1* (commonly called *Human coronavirus OC43*), *Human coronavirus HKU1*, *Murine coronavirus*, *Pipistrellus bat coronavirus HKU5*, *Rousettus bat coronavirus HKU9*, *Severe acute respiratory syndrome-related coronavirus*, *Tylonycteris bat coronavirus HKU4*, *MERS-CoV(Roos,2013 and Bermingham et al.,2012).*

Alignment of 56 complete or near complete MERS-CoV genomes and an Egyptian divergent variant from a camel. Alignment made using Geneious v6.1.7. The PHYML v2.2.0 plug-in was added to make this tree, using 1000 bootstraps. Red starsindicate virus which is reportedly from the same patient (seems doubtful). Vertical bars to the right indicate Clade A (dark blue) and B (pale blue). Sequences from the 2013 Al-Ahsa hospital outbreak are boxed in pink; from the Jeddah 2014 hospital outbreak in blue; from the Hafr Al-Batin community cluster in green. Camel icons indicate genomes from camel variants for MERS-CoV.

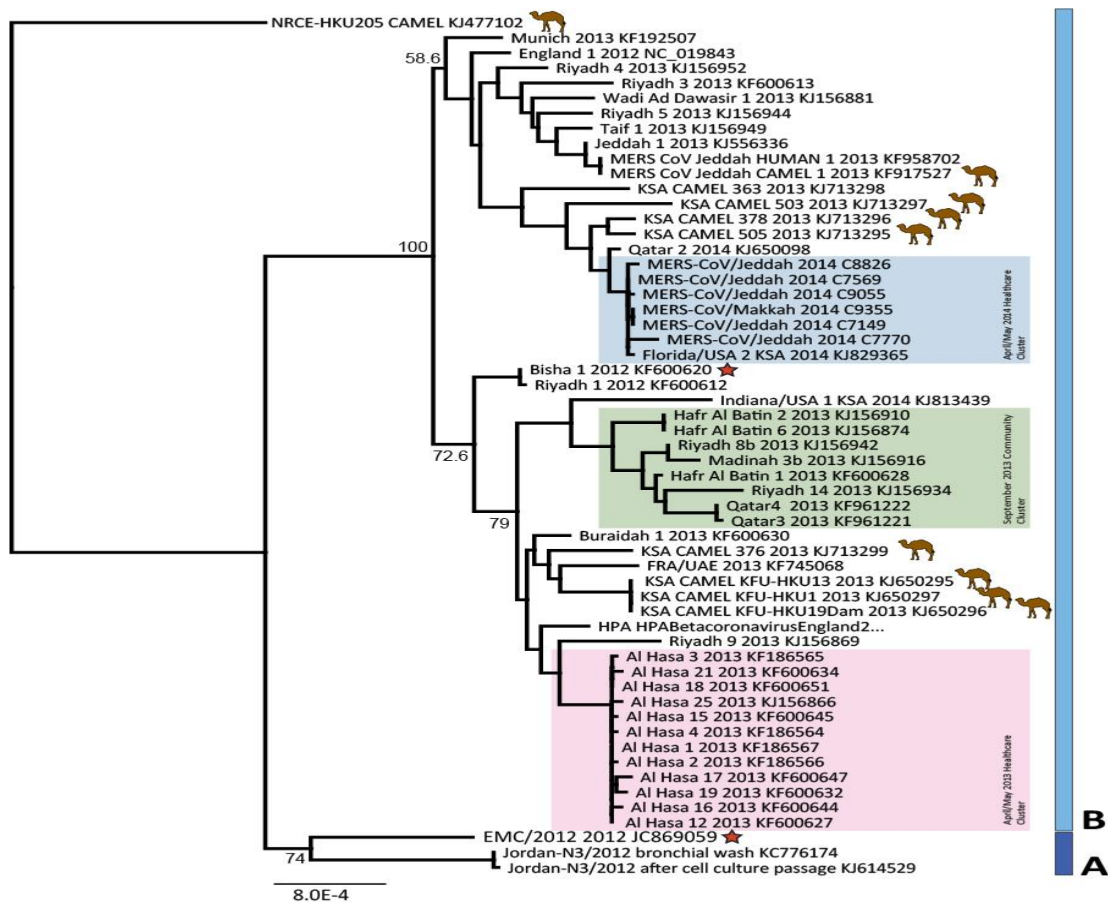
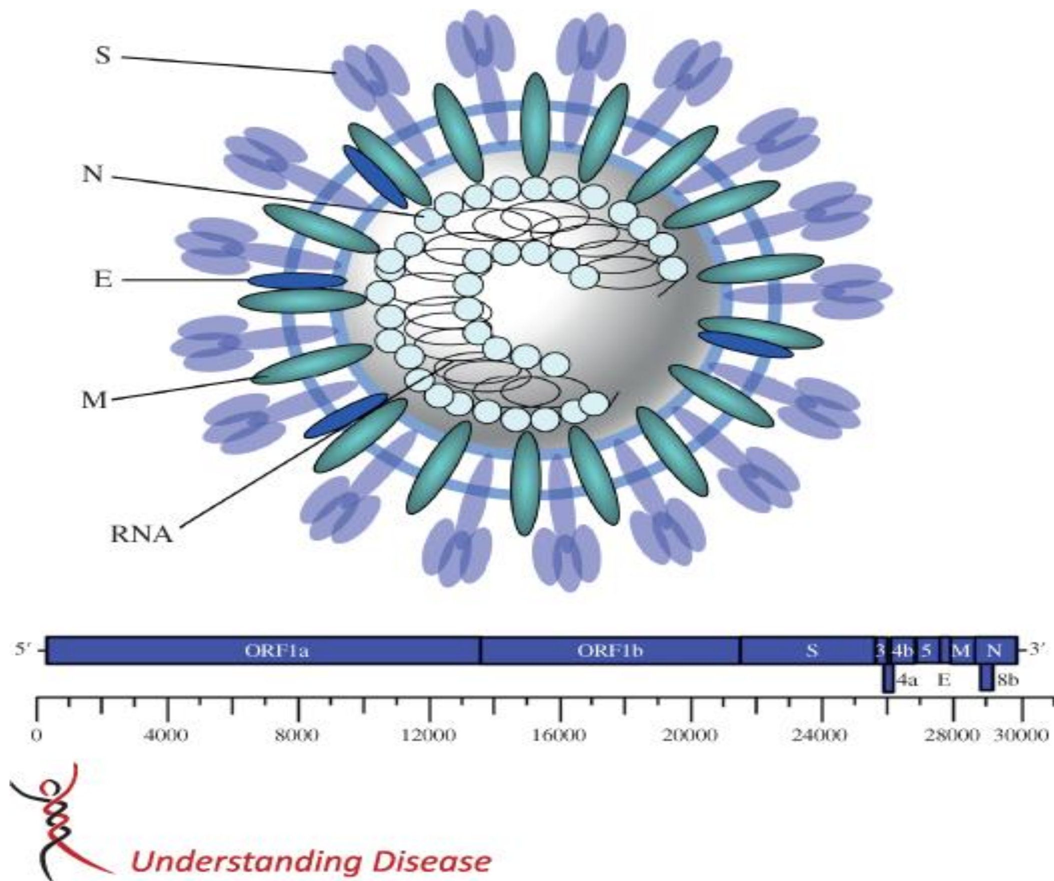


Figure1. Different Variant of MERS-CoV identified in Human and Dromedary camel

Source :(Mackay, 2015)

2.2.2 Virus Structure and Tropism



S= spike protein, M= Membrane protein, E= Envelop protein & N= Nucleocapsid protein

Figure 2. Structure of MERS-CoV and its spike protein

Source :(Van den Brand, 2015)

In humans, the virus has a strong tropism for nonciliated bronchial epithelial cells, and it has been shown to effectively evade the innate immune responses and antagonize interferon (IFN) production in these cells. This tropism is unique in that most respiratory viruses target ciliated cells (Kindler et al., 2013 and Raj et al., 2013).

Due to the clinical similarity between MERS-CoV and SARS-CoV, it was proposed that they may use the same cellular receptor; the exopeptidase, angiotensin converting enzyme 2 (ACE2). However, it was later discovered that neutralization of ACE2 by recombinant antibodies does not prevent MERS-CoV infection. Further research identified dipeptyl peptidase 4 (DPP4; also known as CD26) as a functional cellular receptor for MERS-CoV. Unlike other known coronavirus receptors, the enzymatic activity of DPP4 is not required for infection. As would be expected, the amino acid sequence of DPP4 is highly conserved across species and is expressed in the human bronchial epithelium and kidneys. Bat DPP4 genes appear to have been subject to a high degree of adaptive evolution as a response to coronavirus infections, so the lineage leading to MERS-CoV may have circulated in bat populations for a long period of time before being transmitted to people(Kindler et al ., 2013 and Raj et al.,2013) .

2.3 Epidemiology of MERS-CoV

2.3.1 Host Range

Early research suggested the virus is related to one found in the Egyptian tomb bat. In September 2012 Ron Fouchier speculated that the virus might have originated in bats (Doucleff, 2012). Work by epidemiologist Ian Lipkin of Columbia University in New York showed that the virus isolated from a bat looked to be a match to the virus found in humans (Abedine, 2013) 2c beta corona viruses were detected in *Nycteris* bats in Ghana and *Pipistrellus* bats in Europe that are phylogenetically related to the MERS-CoV virus (Augustina *et al* .,2013).

Recent work links camels to the virus. An ahead-of-print dispatch for the journal *Emerging Infectious Diseases* records research showing the coronavirus infection in dromedary camel calves and adults. That’s 99.9% matching to the genomes of human clade B MERS-CoV (Hemida *et al*., 2014).

At least one person who has fallen sick with MERS was known to have come into contact with camels or recently drank camel milk (Roos, 2014).

Countries like Saudi Arabia and the United Arab Emirates produce and consume large amounts of camel meat. The possibility exists that African or Australian bats harbor the virus and transmit it to camels. Imported camels from these regions might have carried the virus to the Middle East.

In 2013 MERS-CoV was identified in three members of a dromedary camel herd held in a Qatar barn, which was linked to two confirmed human cases who have since recovered. The presence of MERS-CoV in the camels was confirmed by the National Institute of Public Health and Environment of the Ministry of Health and the Erasmus Medical Center (WHO Collaborating Center), the Netherlands. None of the camels showed any sign of disease when the samples were collected. The Qatar Supreme Council of Health advised in November 2013 that people with underlying health conditions, such as heart disease, diabetes, kidney disease, respiratory disease, the immunosuppressed, and the elderly, avoid any close animal contacts when visiting farms and markets, and to practice good hygiene, such as washing hands (Qatar Supreme Council of Health, 2013).

According to the 27 March 2014 MERS-CoV summary update, recent studies support that camels serve as the primary source of the MERS-CoV infecting humans, while bats may be the ultimate reservoir of the virus. Evidence includes the frequency with which the virus has been found in camels to which human cases have been exposed, serological data which shows widespread transmission in camels and the similarity of the camel CoV to the human CoV (MERS-CoV update, 2014).

On 6 June 2014, the *Arab News* newspaper highlighted the latest research findings in the New England Journal of Medicine in which a 44-year-old Saudi man who kept a herd of nine camels died of MERS in November 2013. His friends said they witnessed him applying a topical medicine to the nose of one of his ill camels four of them reportedly sick with nasal discharge seven days before he himself became stricken with MERS.

Researchers sequenced the virus found in one of the sick camels and the virus that killed the man, and found that their genomes were identical. In that same article, the *Arab News* reported that as of 6 June 2014, there have been 689 cases of MERS reported within the Kingdom of Saudi Arabia with 283 deaths (Mohammed, 2014).

2.3.2. Transmission

On 13 February 2013, the World Health Organization stated "the risk of sustained person-to-person transmission appears to be very low. The cells MERS-CoV infects in the lungs only account for 20% of respiratory epithelial cells, so a large number of virions are likely needed to be inhaled to cause infection (WHO, 2013).

As of 29 May 2013, the WHO is now warning that the MERS-CoV virus is a "threat to entire world (WHO, 2013). However, Dr. Anthony S. Fauci of the National Institutes of Health in Bethesda, Maryland, stated that as of now MERS-CoV "does not spread in a sustained person to person way at all." Dr. Fauci stated that there is potential danger in that it is possible for the virus to mutate into a strain that does transmit from person to person (Fauci, 2012).The infection of healthcare workers (HCW) has lead to concerns of human to human transmission (Knickmeyer, 2014).

The Centers for Disease Control and Prevention (CDC) list MERS as transmissible from human-to-human. From their FAQ, in answer to the question "Does MERS-CoV spread from person to person?", they answer "MERS-CoV has been shown to spread between people who are in close contact. Transmission from infected patients to healthcare personnel has also been observed. Clusters of cases in several countries are being investigated (CDC FAQ) There is also a *New York Times* article which provides some correlative context for this (Denise, 2013).

However on the 28th of May, the CDC revealed that the Illinois man who was originally thought to have been the first incidence of person to person spread (from the Indiana man at a business meeting), had in fact tested negative for MERS-CoV. After completing additional and more definitive tests using a neutralising antibody assay, experts at the CDC have concluded that the Indiana patient did not spread the virus to the Illinois patient. Tests concluded that the Illinois man had not been previously infected. It is possible for silent MERS to occur; this is when the patient does not develop symptoms. Early research has shown that up to 20% of cases show no signs of active infection but have MERS-CoV antibodies in their blood (Jonrl, 2014).

MERS-CoV transmitted through three main routes Ingestion, Aerosols and contact.

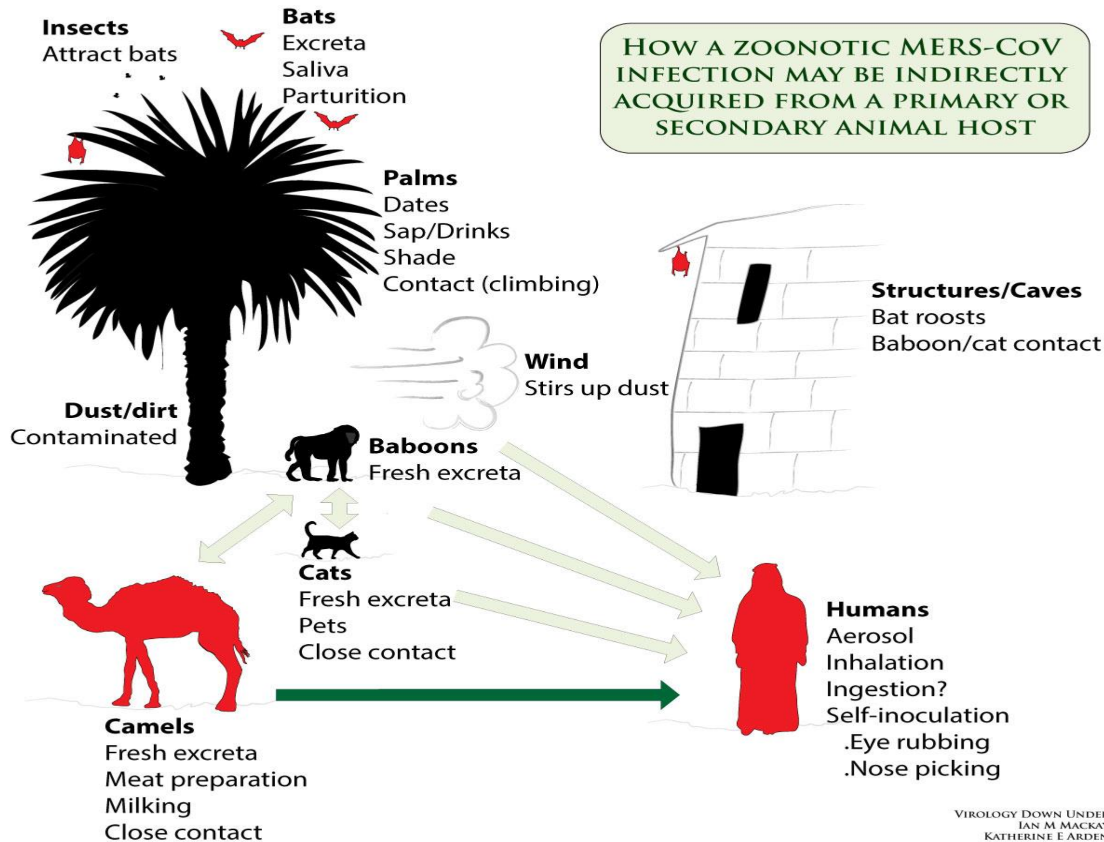


Figure 3. How MERS-CoV transmitted from Animal to human being.

Source (Mackay, 2015)

2.3.3 Geographical Distribution of MERS-CoV

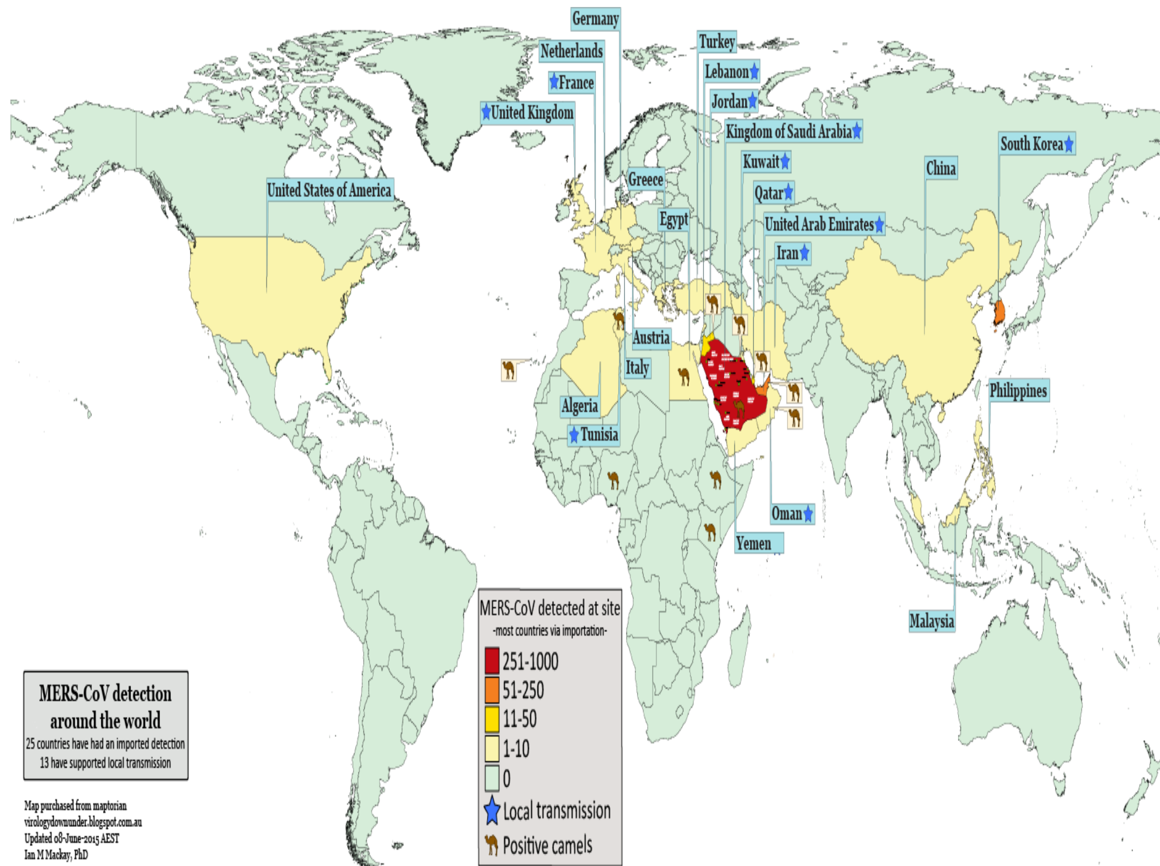


Figure 4. Geographical Distribution of MERS-CoV in Dromedary camel and human being.

Source: (Mackay, 2015)

2.4. Pathogenesis of MERS-CoV

Although MERS and SARS resemble each other clinically, *in vitro* studies have highlighted remarkable differences between these viruses with respect to their growth characteristics, receptor usage and host responses, suggesting that their pathogenesis may be quite different. One way to predict the changes in the lungs after MERS-CoV infection is to use human tissue that has been infected *ex* vivo. Hocke *et al* demonstrated widespread MERS-CoV antigen expression in type I and II alveolar cells, ciliated bronchial epithelium and unciliated cuboid cells of terminal bronchioles, using spectral confocal microscopy. Virus antigen was also found in endothelial cells of pulmonary vessels and rarely in alveolar macrophages. Electron microscopy revealed alveolar epithelial damage, consisting of detachment of type II alveolar epithelial cells and associated disruption of tight junctions, chromatin condensation, nuclear fragmentation and membrane blebbing, the latter suggesting apoptosis .Although this *ex vivo* model does not fully mimic the situation *in vivo*, these changes are in line with observations in cell lines infected with MERS-CoV. Severe cytopathic effects were observed in human hepatoma cells infected with MERS-CoV; these were more severe than those due to SARS-CoV infection, although the *in vivo* relevance of this observation remains unclear (Van den Brand et al., 2014).

DPP4 (also named CD26) has been identified as the receptor for MERS-CoV .All HCoV receptors identified to date are exopeptidases, although their proteolytic activity is not necessary for the virus to bind to the receptors, nor for them to enter the host cell .A comparative analysis of HCoV receptor expression across the respiratory tract of humans may provide clues regarding differences in pathobiology between HCoVs. In cell lines and *ex vivo* lung cultures, DPP4 is expressed in type I and II alveolar cells, ciliated and non-ciliated bronchial epithelium, bronchial submucosal glands, endothelium, alveolar macrophages and leukocytes .This largely corresponds with viral tropism in *ex vivo* human lung cultures, which show infection of non-ciliated cells in bronchi, bronchioles, endothelial cells and type I and II pneumocytes, but rarely in alveolar macrophages.

Remarkably, the binding site of DPP4 is different in different species, explaining why not all animals can be infected with MERS-CoV (Van den Brand et al., 2014).

SARS-CoV and NL63 use a different receptor for entry into cells, angiotensin-converting enzyme 2 (ACE2) , which shows in part a similar cell-type tropism as is seen for DPP4. In humans, ACE2 is expressed in ciliated bronchial epithelial cells, type I and II pneumocytes and arterial and venous endothelial cells, but not in tissues of the upper respiratory tract, suggesting that these tissues are not the primary site of entrance for SARS-CoV or NL63 .SARS-CoV infects some ACE2-positive cells – ciliated bronchial epithelial cells, bronchioles and type I and II pneumocytes, but not endothelial cells(Van den Brand et al., 2014).

Comparative genomics provides a way to study the molecular basis for the host response against different but related viral pathogens, as was demonstrated previously in SARS-CoV infection of different primate host species .At present, only one comparative *in vitro*study using MERS-CoV has been performed .Calu3 cells, differentiated into polarized ciliated cells, permit replication of both SARS-CoV and MERS-CoV at similar levels. However, MERS-CoV induces a substantial cytopathic effect, starting 18–24hr after infection, whereas SARS-CoV maintains steady replication and cell viability until 72hr after infection .On the other hand, MERS-CoV seems more sensitive to prophylactic and therapeutic treatment of infection *in vitro* than SARS-CoV .In addition, MERS-CoV induces much greater dysregulation of the host response to infection than SARS-CoV. MERS-CoV specifically down-regulates genes involved in the antigen-presentation pathway, which could have substantial implications for the development of adaptive immune responses .With respect to the analysis of the immune response in humans infected with MERS-CoV, limited data from two patients with different disease outcomes are available to date. In BAL and serum from the patient with a poor outcome, there was a decrease in interferon (IFN)α as well as decreased expression of retinoic inducible-acid

gene (*RIG*)-*1*, melanoma differentiation-associated protein 5 (MDA5) and interferon regulatory factors (IRF)3 and IRF7, which are involved in the recognition of viruses by the innate immune system. In the patient with poor outcome there were also high levels of CXC-motif chemokines ligand (CXCL) 10 and interleukin (IL)-10, which may have resulted in lower IFN*γ* expression and higher levels of IL-17A and IL-23 .In the patient who survived, rapid clearance of the virus with increased levels of IL-12 and IFN*γ* was noted (Van den Brand et al., 2014).

In SARS, the development of severe lower respiratory tract disease correlates partly with aberrant immune responses, with unbalanced cytokine and chemokine profiles .The levels of both cytokines and chemokines in the blood are elevated: IL-1, IL-6, IL-8, IL-12, IFNγ, monocyte chemotactic protein (MCP)-1 (or CC-motif ligand 2, CCL2), monokine induced by IFNγ (MIG), IFN-inducible protein (IP-10, or CXCL10), and transforming growth factor (TGF)*β* .Some of these chemokines are important for chemotaxis and activation of neutrophils and monocytes ,which corresponds with the infiltration of these cells in the respiratory tract of human SARS cases .It remains to be determined which host responses dominate MERS-CoV infection *in vivo* but, based on the results obtained thus far, MERS-CoV and SARS-CoV may induce different pathways (Van den Brand et al., 2014).

2.5 Clinical Findings of MERS

Initially, the illness resembles influenza with fever and a mild cough. The breathing disorder often progresses to severe shortness of breath (dyspnea) and inability to maintain oxygenation (hypoxia). Progression may be rapid, or it may take several days. Severely affected people develop a potentially fatal form of respiratory failure, known as adult respiratory distress syndrome (ARD or ARDS). In addition to attacking the alveoli in the lungs, the virus also infects other organs in the body, causing kidney failure, inflammation of the heart sac (pericarditis), or severe systemic bleeding from disruption of the clotting system (disseminated intravascular coagulation).

People with compromised immune systems such as severe rheumatoid arthritis or organ transplantation may not experience respiratory symptoms but can have fever or diarrhea (Medicine net, 2015).

2.6. Diagnosis of MERS

2.6.1. Serological testes for MERS-CoV

Details of two immunofluorescence assays to detect antibodies to MERS CoV have been published, and these assays, along with a serum neutralization test, were used to screen contacts of a case treated in a German hospital with apparently good sensitivity and specificity. An assay for detection of MERS-CoV antibodies using protein microarray technology has also been developed. It appears to be highly specific. US CDC has developed a two- stage approach for detecting antibodies to MERS-CoV, based on a screening test using a recombinant nucleocapsid (N) protein-based indirect enzyme-linked immunosorbent assay (ELISA), and followed by a confirmatory test using a whole-virus indirect fluorescent antibody (IFA) test or microneutralization test. All serological tests developed thus far have been validated only against a small number of convalescent sera from MERS CoV cases and it has not yet been possible to compare the performance of the different assays. No kits are currently available for serological testing (WHO, 2013).

It is recommended that any positive result by a single serological assay should be confirmed with a neutralization assay. Although techniques such as ELISA used for screening samples could eventually become widely available in laboratories in many countries, the confirmatory neutralization assays will need to be performed in laboratories with specialized facilities and highly trained staff (WHO, 2013).

At present, there is no clear consensus on interpretation of serological test results in individual patients. Given that currently available assays have been validated using only a limited number of convalescent sera, it is prudent to take a cautious approach when confirming cases based solely on serological testing.

Thus, for the time being, cases where the testing laboratory has reported positive serological test results in the absence of PCR testing or sequencing are considered probable cases of MERS-CoV infection, if they meet the other conditions of that case definition. Final classification of cases will depend on clinical and epidemiological information combined with laboratory results (WHO, 2013).

However, serological testing can provide valuable information on rates of infection in populations, and serological surveys, particularly among known risk groups or populations, are encouraged. Member States and laboratories wishing to conduct serological testing for MERS-CoV should contact the institutions that have developed the assays mentioned above to discuss the most appropriate way to meet their requirements. WHO can facilitate communication between Member States and specialized laboratories if needed. The continued collection and testing of serum from confirmed cases and those potentially exposed to the virus is encouraged as it adds to the overall understanding of MERS-CoV infection and also to the performance of the assays thus far developed (WHO, 2013).

2.6.2. PCR and sequencing for detecting MERS-CoV

Routine confirmation of cases of MERS-CoV infection is based on detection of unique sequences of viral RNA by real-time reverse-transcription polymerase chain reaction (rRT-PCR) with confirmation by nucleic acid sequencing when necessary(WHO, 2013). In certain circumstances, but not for routine diagnosis, laboratories with the appropriate experience and containment facilities may attempt to isolate the virus in cell culture. The current version of these recommendations does not cover virus isolation procedures. Any testing for the presence of this virus should be performed in appropriately equipped laboratories by staff trained in the relevant technical and safety procedures.

Individual Member States will decide which, if any, of their laboratories should perform these tests (WHO, 2013).

Three rRT-PCR assays for routine detection of MERS-CoV have been developed and their details published. Currently described tests are an assay targeting upstream of the E protein gene (upE) and assays targeting the open reading frame 1b (*ORF 1b*) and the open reading frame 1a (*ORF 1a*). The assay for the upE target is considered highly sensitive and is recommended for screening, with the *ORF 1a* assay considered of equal sensitivity. The *ORF 1b* assay is considered less sensitive than the *ORF 1a* assay. . In addition, the US Centers for Disease Control and Prevention (US CDC) has developed rRT-PCR assays targeting the MERS-CoV nucleocapsid (N) protein gene, which can complement upE and *ORF 1a* assays for screening and confirmation. Two target sites on the novel coronavirus genome suitable for sequencing to aid confirmation have been identified. These are in the RNA-dependent RNA polymerase (*RdRp*) and (*N*) genes (WHO, 2013).

2.7 Treatment of MERS-CoV

Because MERS-CoV is caused by a similar virus as SARS, the management of MERS-CoV has been extrapolated from experience with the 2002 SARS outbreak and some limited experimental data. Like SARS, patients with MERS-CoV often require oxygen supplementation, and severe cases require mechanical ventilation and intensive-care-unit support. No medication has been proven to treat MERS-CoV, and treatment is based upon the patient's medical condition. Several medications have been tried in both SARS and MERS-CoV without conclusive benefits, and further research is to be done. Management of the individual with MERS is aided by infectious disease, pulmonary and critical-care specialists. Middle East Respiratory Syndrome is associated with a rapidly progressive severe respiratory illness, and mortality compared to the SARS-CoV outbreak of 2003 is very high. Since 2012, the MERS outbreak in Saudi Arabia has slowly continued with a total of 933 individuals diagnosed with 401 deaths as of March 2015.

2.8 Prevention and Control of MERS

Fortunately, MERS-CoV does not appear to spread as easily or rapidly from person to person as the SARS-CoV outbreak of 2003. Travelers to the Arabian Peninsula and surrounding countries, and their close contacts, may protect themselves by taking simple measures that help to prevent the spread of germs. Frequent hand hygiene using soap and water, or an alcohol-based hand sanitizer, avoidance of close contact with sick people, and avoidance of touching one's eyes, nose, and mouth can prevent the spread of viruses. Caregivers of patients who are not hospitalized should perform frequent hand hygiene as stated above and wear a face mask for direct care until the patient has recovered (Medicine net, 2015).

In the Arabian Peninsula and surrounding countries where MERS-CoV has been found, the World Health Organization (WHO) recommends precautions in handling live camels or their raw meat and dairy products. Anyone who does so, should frequently wash hands and consider using respiratory protection, as well as barriers to protect skin and clothing. Clothing worn during handling of camels or their products should be kept away from household contacts and washed daily. All products intended for human consumption should be cooked thoroughly or pasteurized (Medicine net, 2015).

In the healthcare setting, suspected cases of MERS should be placed in airborne infection isolation rooms (AIIR) in which room exhaust is recirculated under high-efficiency particulate air (HEPA) filtration. If not available, the patient should be given a face mask and should be isolated in a single-patient room with the door closed. Staff assigned to the patient, and the patient's movements outside of the isolation area, should be minimized. Before entering the isolation room, health-care workers should wear a gown, gloves, eye shield, and a fit-tested NIOSH-certified disposable N95 filtering respirator; if an N95 mask or respirator is unavailable, a surgical mask should be worn.

Before exiting the room, personal protective equipment should be discarded in the room. Hand hygiene must be performed with soap and water or an alcohol-based hand sanitizer after exiting (Medicine net, 2015).

Local public-health authorities should be consulted promptly when a MERS-CoV diagnosis is considered. Preventing an outbreak requires identification of the first infected patients as soon as possible before the infection spreads further. Those who have been exposed to an infected person should be carefully monitored for fever or respiratory symptoms. Exposure is defined as living with or caring for an infected person, being within 3 feet of the infected person, and exposure to bodily fluids or direct physical contact with an infected person. The Centers for Disease Control and Prevention does not mandate quarantine measures for exposed individuals who are otherwise healthy (Medicine net, 2015).

With respect to controlling MERS, rapid isolation and rigorous infection control practices may in the end suffice to limit the outbreak. These include standard contact and airborne precautions, such as wearing a surgical mask, gloves and a gown on entering the room of infected or suspect patients, and removing them on leaving .Dromedary camels infected with MERS-CoV may not show disease but still may excrete MERS-CoV through nasal fluids, faeces and, potentially, in their milk and urine .Therefore, the WHO recommends avoiding contact with camels, not drinking raw camel milk or camel urine and not eating meat that has not been thoroughly cooked. Also, people who work, or come into contact with, dromedary camels, such as people working at camel farms, slaughterhouses, markets and camel-racing facilities, and also veterinarians, are at risk and should practice good personal hygiene and wear facial protection and protective clothing where feasible (Medicine net, 2015).

2.9 Current Outbreak of MERS-CoV in South Korea

South Korea has reported seven new cases of MERS, bringing the total number of infection to 145. The deadly virus has killed 14 people so far, as world health organization team, which is now in the country to assess the situation, said the outbreak was large and complex. The group warned the government to remain vigilant because it expects the number of cases to rise further. The expert says although the MERS virus is not spreading outside hospitals, the outbreak is not yet over (Aljazeera English, 2015).

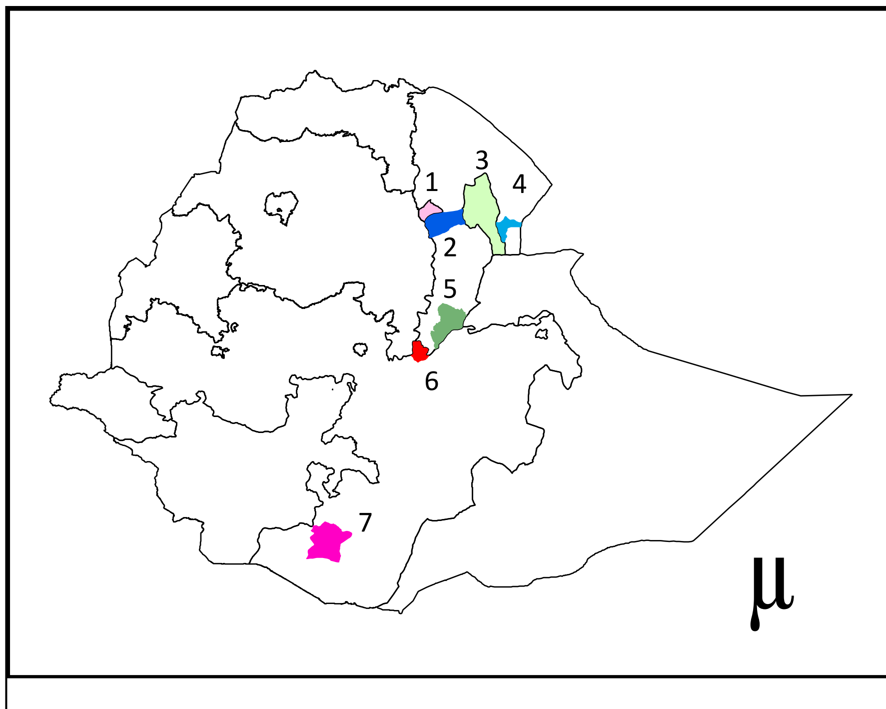
Two hospitals which treated MERS patient have been closed as a precaution while thousands of schools remain shut. All of the cases are believed to be linked to hospital settings and traced to a business man who had returned from a trip to the Middle East. All the deaths involve elderly patients or people who had been suffering serious ailments (Aljazeera English, 2015).

The outbreak in South Korea is the largest outside the Arabian Peninsula, where the disease was first identified in humans in 2012, and has stirred fears in Asia of a repeat of a 2002-2003 scare when Severe Acute Respiratory Syndrome (SARS) killed about 800 people worldwide (Aljazeera English, 2015).

3. MATERIALS AND METHODS

3.1 Study area and Animals

This study was conducted from November 2014 to May 2015 in seven pastoral districts namely Dubti, Ayssaita, Chifra, Ewa and Amibara districts of Afar and Yabello and Fentale districts of Oromia regions where camels are populated. Camels of all age and both sexes was randomly selected and included in the study for determination of antibody prevalence against MERS-COV. For the purpose of virus isolation and characterization nasal swabs were taken from 100 camels (Yabelo, Amibara, Fentale and Dubti) 25 from each district.



Study areas

1: Ewa, 2: Chifra, 3 Dubti, 4: Ayssaita, 5Amibara, 6Fentale and 7Yabelo

Figure 5. GIS map indicating the study area

According to Wikipedia 2015 the regions and districts included are described as follows

Afar Region

Afar Regional State is one of the nine regional states of Ethiopia, and is the homeland of the Afar people.The CSA estimated in that farmers in the Afar Regional State had a total of 327,370 cattle (representing 0.84% of Ethiopia's total cattle), 196,390 sheep (1.13%), 483,780 goats (3.73%), 200 mules (0.14%), 12,270 asses (0.49%), 99,830 camels (21.85%), 38,320 poultry of all species (0.12%), and 810 beehives (less than 0.1%).Like other Regions in Ethiopia, Afar is subdivided into administrative zones and one special woreda. These are Zone 1: Awsi Rasu , Zone 2: Kilbet Rasu, Zone 3: Gabi Rasu, Zone 4: Fantena Rasu, Zone 5: Hari Rasu and Argobba special woreda.

Amibara

Amibara is one of the woredas in the Afar Region of Ethiopia. Part of the Administrative Zone 3, Amibara is bordered on the south by Awash Fentale, on the west by the Awash River which separates it from Dulecha, on the northwest by the Administrative Zone 5, on the north by Gewane, on the east by the Somali Region, and on the southeast by Oromia Region. Towns in Amibara include Awash Arba, Awash Sheleko, Melka Sedi and Melka Werer.

Ayssaita (Asaita)

Asaita is a town in northeast Ethiopia, and before 2007 was the capital of the afar regional state of Ethiopia. Located in Afambo, part of the regions administrative zone1the town has elevation of 300 meters. The town has estimated population of 22,718.

Chifra

Chifra is one of the woredas in the Afar Region of Ethiopia. Part of the Administrative Zone 1, Chifra is located near the base of the eastern escarpment of the Ethiopian highlands and bordered on the south by Mille, on the west by the Amhara Region, on the north by the Administrative Zone 4, and on the east by Dubti; the Logiya River defines part of the boundary with Zone 4. The administrative center of Chifra is Chifra.

Dubti

Dubti is one of the woredas in the Afar Region of Ethiopia. Part of the Administrative Zone 1, Dubti is bordered on the south by the Somali Region, on the southwest by Mille, on the west by Chifra, on the northwest by the Administrative Zone 4, on the north by Kori, on the northeast by Elidar, on the east by Asayita, and on the southeast by Afambo. Towns in Dubti include Date Bahri, Dubti, Logiya, Sardo/Serdo and Semera. Kori woreda was separated from Dubti.

Ewa

Ewa is one of the woredas in the Afar Region of Ethiopia. Part of the Administrative Zone 4, Ewa is located near the base of the eastern escarpment of the Ethiopian highlands, and bordered on the south by the Administrative Zone 1, on the west by the Amhara Region, on the north by Gulina, and on the east by Aura; part of its boundary with Zone 1 is defined by the Logiya River. The major settlement in Ewa is Alele Subula.

Based on the 2007 Census conducted by the Central Statistical Agency of Ethiopia (CSA), this woreda has a total population of 47,203, of whom 26,437 are men and 20,766 women; with an area of 1,463.89 square kilometers, Ewa has a population density of 32.24. While 1,237 or 2.62% are urban inhabitants, a further 26,913 or 57.02% are pastoralists.

Oromia Region

Oromia (spelled **Oromiyaa** in Afan Oromo language) is one of the nine ethnically based regional states of Ethiopia, covering 284,538 square kilometers. It is bordered by the Somali Region to the east; the Amhara Region, the Afar Region and the Benishangul-Gumuz Region to the north; South Sudan, Gambela Region, and Southern Nations, Nationalities, and Peoples' Region to the west; and Kenya to the south. The 2007 census reported Oromia's population at over 27 million, making it the largest state in population and area.

The Oromia is subdivided into administrative zones: Arsi, Bale, Borena, East Hararghe, East Shewa, East Welega, Guji, Horo Gudru Welega, Illubabor, Jimma, Kelem Welega, North Shewa, South West Shewa, West Arsi, West Hararghe, West Shewa, West Welega, Adama (special zone), Jimma (special zone), Oromia-Finfinne (special zone) and Kemise (special zone).

Fentale

Fentale is one of the woredas in the Oromia Region of Ethiopia. Part of the East Shoa Zone located in the Great Rift Valley, Fentale is bordered on the southeast by the Arsi Zone, on the southwest by Boset, on the northwest by the Amhara Region, and on the northeast by the Afar Region. The administrative center of Fentale is Metehara; other towns include Haroo Adii.

Yabelo

Yabelo (also spelled Yabello, Yavello, and Iavello) is a town in southern Ethiopia. An alternative name for this town is Obda, which is also the name of a nearby mountain. Located northwest of Moyale in the Borena Zone of the Oromia Region , this town has a latitude and longitude of 4°53′N 38°5′ECoordinates: 4°53′N 38°5′E and an elevation of 1857 meters above sea level. It has been the administrative center of the Borena Zone since its division from Guji Zone in September 2002, as well as Yabelo district.

3.2 Study Design and Sampling Technique

A cross sectional study was conducted for serological investigation of MERS-CoV among dromedary camels found in the study area. Multi stage cluster sampling technique was used to select specific cluster (district) for all sites found in the study areas. Finally samples were taken from kebele of randomly selected cluster. The least number of sera sample taken from a cluster (district) was 52.

Dromedary camels found in Afar and Oromia region (study area) of both age groups (young 1-3 years and adults > 3 years of age), both sex groups (male and female) and two categories of herd size(large herd size > herd having 50 camels and small herd size = herd having less than or equal to 50 camels) were included in the study.

3.3 Sample Size

Based on Thrusfield (2007) formula, the sample size of study animals was calculated.

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

$$n = \frac{1.96^2 (0.96) (1 - 0.96)}{0.0025}$$

n = 59 and the sample size multiplied by the cluster (district) number to get the final sample size 59 x 7 district = 413 (Total sample size required)

But additional 59 samples were taken to make the final sample size 472.

Where n = required sample size

Pexp = expected prevalence (96%) from (Reskeun *et al*., 2014)
d = desired absolute precision (5%)

3.4 Sample collection, Transportation and storage

The samples were taken from randomly selected Dromedary camels from each cluster (district) of randomly selected Zones and woredas of Afar and Oromia. Then 10 milliliter of blood sample was collected from jugular veins of the camel using needle and plain test tube (without anticoagulants). The blood was allowed to clot for 2 hours at room temperature, stored overnight at 4ºC and then the serum was separated from the clot standing for 20 minutes at room temperature to allow for clot formation. Serum was separated from the clot by centrifugation at 3000rpm for 10 minutes and transferred to crayovial tubes. Then the separated serum was labeled and kept under refrigeration (–20ºC) until transported to National Veterinary Institute (NVI) for storage till their transport to University of Hong Kong for analysis.

Hundred swabs were collected by using cotton swab from randomly selected districts (Yabelo and Fentale) from Oromia region and (Dubti and Amibara) Afar region. Twenty five swabs were taken from each site mentioned above. The swabs were preserved in buffer solution until transported to NVI virology section for deep freezing at -80 ºC. Finally the swabs were transported to Hong Kong University for analysis (Real time Reverse Transcriptase PCR).

3.5 Laboratory Analysis

The blood samples collected were analyzed by two serological tests MERS-CoV spike pseudoparticle neutralization assay (screening test) and Microneutralisation tests (confirmatory test).

The swabs collected undergo extraction of DNA and Polymerase Chain Reaction (PCR) to identify the presence of MERS-CoV in the sample.

3.5.1 Serology

MERS-CoV spike pseudoparticle neutralisation assay

A codon-optimised spike gene was designed according to published MERS-CoV genome sequence (GenBank accession number: JX869059.1), synthesised by GeneCust (Luxembourg) and subcloned into pcDNA3.1+ vector to generate pcDNA-S. To produce MERS spike pseudoparticles, 10 µg pNL Luc E- R- and 10 µg pcDNA-SS were co-transfected into 4x106 293T cells. Supernatants of transfected cells were harvested 48 h later and quantified for p24 viral protein using a p24 ELISA Kit (Cell Biolabs, San Diego, United States). For the ppNT assay, HIV/MERS pseudoparticles containing 5 ng p24 were used to infect Vero E6 cells (ATCC CRL-1586) in a single well (96-well plate format; 1x104 cells/well). Infected cells were lysed in 20 µl lysis buffer and 100 µl of luciferase substrate at two days postinfection (Promega Corporation, Madison, United States). Luciferase activity was measured in a Microbeta luminometer (PerkinElmer, Waltham, United States). For the ppNT, MERS pseudoparticles (5 ng of p24) were pre-incubated with serially diluted sera for 30 min at 4 °C and then added to cells in triplicate. Residual virus replication was assayed at two days post infection, as described above. The highest serum dilution giving a 90% reduction of luciferase activity was regarded as the ppNT antibody titre.

Viruses and virus titration

Virus stock for MERS-CoV was prepared in Vero cell culture (ATCC CCL-81) in minimal essential medium containing 2% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin.Virus aliquots were stored at -80 °C. Virus was titrated in serial half-log10 dilutions (from 0.5 log to 7 log) to obtain 50% tissue culture infectious dose (TCID50) on 96-well tissue culture plates of Vero cells. The plates were observed in a phase contrast microscope for cytopathic effect (CPE) daily for three days. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated by using the Reed Muench method and designated as one TCID50. SARS-CoV was grown and titrated in the same manner with the exception that Vero E6 cells (ATCC CRL-1586) were used.

Microneutralisation tests

Serial two-fold dilutions of heat-inactivated sera (56 °C for 30 minutes) were made, starting with a dilution of 1:10. The serum dilutions were mixed with equal volumes of 200 TCID50 of MERS-CoV or SARS-CoV as indicated. After 1 h of incubation at 37 °C, 35 µL of the virus–serum mixture was added in quadruplicate to Vero or Vero-E6 cell monolayers for MERS-CoV and SARS-CoV, respectively, in 96-well microtiter plates. After 1 h of adsorption, an additional 150 µL of culture medium were added to each well and the plates incubated for three more days at 37 °C in 5% CO_2 in a humidified incubator. A virus back-titration was performed without immune serum to assess input virus dose. CPE was read at three days post infection. The highest serum dilution that completely protected the cells from CPE in half of the wells was taken as the neutralising antibody titre and was estimated using the Reed-Muench method. Positive and negative control sera were included to validate the assay.

3.5.2 Extraction of RNA and Polymerase Chain Reaction (PCR)

Real-time reverse transcription PCR (RT-PCR) targeting upstream of E gene of MERS-CoV was used for screening. The open reading frame (ORF) 1a gene was used for confirmation as recommended by the World Health Organization.

3.6 Data Analysis

Data obtained from the investigations was coded and stored in Excel spread sheets. The independent variables age, sex, herd size and origin (Region, Zone, district and kebele) of the dromedary camels have been compared to evaluate their effect on the magnitude of the disease. The collected data was analyzed using SPSS version 20 software. Descriptive statistics was used to determine the frequency of proportion (prevalence) and chi square and logistic regression was used to check the association between the sero-prevalence and the independent variables. The precision level used was 5% with the level of significance differences set at $p < 0.05$, chi square(X^2) > 3.841 for association and logistic regression with odds ratio >1 for the magnitude of the risk(effect) of exposure variables.

4. RESULTS

Seroprevalence of MERS-CoV

From the total of 472 camel sera tested 91.5% (n=432/472) were positive for MERS-COV specific antibody with MERS-CoV spike pseudoparticle neutralisation assay and Microneutralisation tests. Out of 194 sera tested from young Dromedary camels (n=174/194) (89.7%) were positive and from 278 adult camels (n=258/278) (92.8%) were positive the difference seen was not significant (Pearson Chi-Square=1.429 and P=0.232) (Table 1).

Table 1. Seroprevalence of MERS-CoV in the two age groups (Young and Adult Dromedary camels).

	age		Total
	adult	young	
Negative	20(7.20%)	20(10.30%)	40(8.50%)
Positive	258(92.80%)	174(89.70%)	432(91.50%)
Total	278(100.00%)	194(100.00%)	472(100.00%)
	Chi-Square=1.429	df=1	P=0.232

From 472 dromedaries camel sampled 312 were Female and 160 were Male. (n=290/312) (92.9%) and (n=142/160) (88.8%) of Female and Male were positive respectively. The difference seen between the two sex groups was not significant (Pearson Chi-Square=2.404 and P=0. 121) (Table2.).

Table 2.Seroprevalence of MERS-CoV in the two sex groups of dromedary camels

	sex		Total
	female	male	
Negative	22(7.10%)	18(11.20%)	40(8.50%)
Positive	290(92.90%)	142(88.80%)	432(91.50%)
Total	312(100.00%)	160(100.00%)	472(100.00%)
	Chi-Square=2.404	df=1	P=0.121

Out of 186 Camels tested from small herd sized group (n=168/186) (90.3%) were positive and from 286 large herd sized camels (n=264/286) (92.3%) were positive but the difference observed was not statistically significant (Pearson Chi-Square=0.573and P=0.449 (Table3.).

Table 3.Seroprevalence of MERS-CoV in the herd size categories of Dromedary camels

	herd size		Total
	large	small	
Negative	22(7.70%)	18(9.70%)	40(8.50%)
Positive	264(92.30%)	168(90.30%)	432(91.50%)
Total	286(100.00%)	186(100.00%)	472(100.00%)
	Chi-Square=0 .573	df=1	P= 0.449

Out of 153 camels tested from Oromia region (n=139/153) (90.8%) were positive and 319 sera samples from Afar (n=293/319) (91.8%) were positive but the difference was not statistically significant (Pearson Chi-Square =0.133 and P=0.715) (Table4.).

Table 4. Seroprevalence of MERS-CoV in the two regions (Afar and Oromia) Ethiopia

	regions		Total
	Afar	**Oromia**	
	26(8.2%)	14(9.2%)	40(8.5%)
Negative	293(91.8%)	139(90.8%)	432(91.5%)
Positive	319(100.0%)	153(100.0%)	472(100.0 %)
Total	Chi-Square=0.133	df=1	P=0.715

Among Zones included in the study highest prevalence recorded in East Shewa (n=79/80) (98.8%) and the lowest at Borena (n=63/73) (82.2%).The prevalence difference among Zones was highly statistically significant (Pearson Chi-Square=19.535 and P=0.001) (Table5.).

Table 5. Seroprevalence of MERS-CoV in different zones of the study area among Dromedary camels

	zones					Total
	Borena	**East Shewa**	**Zone 1(Afar)**	**Zone 3(Afar)**	**Zone 4(Afar)**	
Negative	13(17.8%)	1(1.2%)	20(10.4%)	1(1.3%)	5(9.6%)	40(8.5 %)
Positive	60(82.2%)	79(98.8%)	172(89.6%)	74(98.7%)	47(90.4 %)	432(91. 5%)
Total	73(100.0%)	80(100.0%)	192(100.0 %)	75(100.0 %)	52(100.0 %)	472(10 0.0%)
	Pearson Chi-Square 19.535	df=4	P=0.001			

Out of 7 districts included in the study the highest prevalence was observed in Fentale (n=79/80) (98.8%) and the lowest in Yabello (n=60/73) (82.2%) with Pearson Chi-Square=21.968 and P=0.001 value) (Table 6.).

Table 6. Seroprevalence of MERS-CoV among Dromedary camels in different districts of Afar and Oromia

	District							Total
	Ayssaita	Chifra	Dubti	Ewa	Amibara	Fentale	Yabelo	
Negative	4(6.20%)	8(13.60%)	8(11.80%)	5(9.60%)	1(1.30%)	1(1.20%)	13(17.80%)	40(8.50%)
Positive	61(93.80%)	51(86.40%)	60(88.20%)	47(90.40%)	74(98.70%)	79(98.80%)	60(82.20%)	432(91.50%)
Total	65(100.00%)	59(100.00%)	68(100.00%)	52(100.00%)	75(100.00%)	80(100.00%)	73(100.00%)	472(100.00%)
		Chi-Square =21.968	df=6	P=0.001				

Univariate logistic regression shows among the risk factors investigated (Age, Sex, herd size and origin of the dromedary camel (region, zone, district and kebele) origin of the camels was responsible for the variations observed in seroprevalence of MERS-CoV. Factors affecting seroprevalence of MERS-CoV are listed in (Table7.) and the whole result of univariate logistic regression found in annex 1.

The references used both in univariate and multivariate logistic regression were Oromia for region, Zone 4 of Afar for Zone, Yabelo for district, Urmaytu for kebele, young for age group, male for age and small herd size for the herd category.

Table7.Risk factors responsible for MERS-CoV identified by Univariate logistic regression

	B	S.E.	Wald	df	Exp(B)	95% C.I.for EXP(B)		Sig.
						Lower	Upper	
Afar	0.127	0.347	0.133	1	1.135	0.575	2.241	0.715
Zone4(Afar)					1			Ref
East Shewa	2.129	1.111	3.672	1	8.404	0.953	74.137	0.055
Yabelo					1			Ref
Ayssaita	1.195	0.6	3.968	1	3.304	1.019	10.71	0.046
Amibara	2.775	1.052	6.954	1	16.033	2.039	126.081	0.008
Fentale	2.84	1.052	7.291	1	17.117	2.178	134.493	0.007
Urmaytu					1			Ref
Surupha	-2.082	1.059	3.866	1	0.125	0.016	0.993	0.049

Final result identified by multivariate logistic regression shows Sex, Zone and kebele were the ultimate factors affecting seroprevalence of MERS-CoV. Where camels living in Borena (OR=0.55) and Galifage (OR=0.18) being protected and Female being risk factor for MERS-CoV. (Table 8.).

Table 8. Factors affecting seroprevalence of MERS-CoV identified by multivariate logistic regression.

Risk factors	B	P value	OR	95% CI for OR
Borena vs zone4(Afar)	-2.901	0.006	0.055	(0.007, 0.436)
Galifage vs Urmaytu	-4.001	0.002	0.018	(0.001, 0.240)
Female vs male	.879	0.023	2.408	(1.127, 5.145)

Molecular identification of MERS-CoV

We detected MERS-CoV RNA in (n=7/100) (7%) of 100 nasal swab specimens from dromedary camels with the upstream of E gene assay (cycle threshold [Ct] 23.2–36.8), confirmed by the ORF1a assay (Ct 23.2–39.1), fulfilling the World Health Organization criteria for diagnosis of MERS-CoV infection. PCR was repeated from a fresh RNA extract to confirm positive results.

From the 100 swab randomly sampled (n=7/100) (7%) of them evident circulation of MERS-CoV in the study area the samples were 2 from Dubti, 1 from Fentale and 4 from Amibara. (Table8.)

Table 9. Isolation and Identification MERS-CoV among dromedary camels from selected sites (Yabelo, Fentale, Dubti and Amibara) of the study areas.

Yabelo	**Fentale**	**Dubti**	**Amibara**	
0(0.00%)	1(4%)	2(8%)	4(16%)	
1.	(33.956,33.929)	(38.268,36.642)WEAK positive	(36.514, 36.945)	**(ct for upE, ORF 1a)**
2.		(29.306, 29.579)	(34.566, 35.303)	
3.			(39.788,39.061) WEAK positive	
4.			(39.727,39.568) WEAK positive	

5. DISCUSSION

The overall high prevalence (n=432/472) (91.5%) evident in this study completely agree with study done in Ethiopia by (Reusken *et al*., 2014). The prevalence recorded in Young Dromedary camel (n=174/194) (89.7%) and Adult (n=258/278) (92.8%) was also similar with work of the same author (93% in young Dromedary and 97% in Adult).The higher prevalence observed in the Adult Dromedary camel may be due to their access to MERS-CoV infected animal at common watering place found in the study area. The overall prevalence of MERS-CoV in this study also agree with study done in Egypt by (Chu *et al*., 2014) (n=48/52) (92.3%) using the same pseudoparticle neutralization assay as the current study.

The slight high prevalence recorded in Female dromedary camels of this study (n=290/312) (92.9%) compared to the Males (n=142/160) (88.8%) may be due frequent interaction of female camels with humans and their calves. The result of multivariate logistic regression shows also Female (OR= 2.408) camels are 2.4 times at risk of MERS-CoV when compared to male camels of the study area.

The difference in seroprevalence of MERS-CoV in Female and Male camels was not significant in the Chi-square test Chi-Square=2.404 and P-value of 0.121 but significant in multivariate logistic regression P-value 0.023 this may be the interaction effect of age to the sex categories(groups).

Highest seroprevalence was observed in East Shewa zone (n= 79/80) (98.8%) and the lowest in Borena (n= 60/73) (82.2%) with Pearson Chi-Square 19.535 and P-value 0.001 both from Oromia regional state of Ethiopia. The difference observed among the zones were also supported by the result of multivariate logistic regression which indicated that camels living in Borena zone (OR= 0.055) are relative protected from MERS-CoV when compared to the reference (Zone 4 of Afar). This difference may be due to the life style of the pastoralist at Borena (owners segregate camels according to their age group , camels of different owners are kept and fed separately and its unlike camels and other domestic animals of different owners to be mixed at watering points).

The lowest seroprevalence was recorded in Surupha kebele of Borena (n= 60/73) (82.2%) and the highest seroprevalence at Adis Ketema of Fentale district (n= 79/80) (98.8%) with Chi-Square =24.145 and P-value of 0.002.This result was also supported by multivariate logistic regression making Urmaytu of Ayssaita reference kebele. The result of multivariate logistic regression indicated that camels living in Galifage (OR= 0.018) have relatively low risk of acquiring MERS-CoV. This may due to the low population of camel found in Galifage and the relative low access of camels to other domestic animals of the area.

Hundred nasal swabs were used to identify MERS-CoV from Yabelo, Fentale, Dubti and Amibara district by RT-PCR. Twenty five swabs from each district. Except Yabelo MERS-CoV was recovered from all the districts sampled. From the overall 7 identified MERS-CoV cases Amibara district account the highest (n=4/100) (4%), this may relate with the highest Seroprevalence recorded in the area (n=74/75) (98.7%).

Chu *et al*.,2014 reported identification of MERS-CoV (n=4/110 swabs) (3.6%) from dromedary camels of Egypt by using RT-PCR the same molecular technique used in the current study except MERS-CoV strain(variant) was not identified in the current study.

6. CONCLUSION AND RECOMMENDATIONS

There was high seroprevalence of MERS-CoV evident in the study area (Afar=91.8% and Oromia = 90.8%) regional states of Ethiopia. The seroprevalence evidence was supported by identification of the virus by RT-PCR which indicates existence of active infection of MRS-CoV in the study area. Among 472 Dromedary camel sampled highest prevalence was observed in East Shoa, Fentale district and the lowest in Borena, Yabelo district both from Oromia regional state of Ethiopia. The study also implies the probability of circulation of MERS-CoV in human being in the study areas due the zoonotic nature of the virus and is transmissibility between Dromedary camel and human. Based on the findings of this study the following recommendations are forwarded:-

- ✓ Additional study should be done to identify the strain (variant) of MERS-CoV in the area.
- ✓ Awareness creation about the transmission, prevention and control strategies of MERS-CoV should be done for high risk groups (Pastoralist, health care workers and Veterinarians).
- ✓ Serological and Molecular study should be conducted to check status of the disease in human beings in Ethiopia.

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8. ANNEXES

Annexe 1. Result of Univariate logistic regression using SPSS version 20.

	B	S.E.	Wald	df	Exp(B)	95% C.I.for EXP(B)		Sig.
						Lower	Upper	
Afar	.127	.347	.133	1	1.135	.575	2.241	.715
Zone 4(Afar)					1			Ref
Borena	-.711	.561	1.607	1	.491	.163	1.475	.205
East Shewa	2.129	1.111	3.672	1	8.404	.953	74.137	.055
Zone1 (Afar)	-.089	.526	.029	1	.915	.326	2.567	.866
Zone 3(Afar)	2.063	1.111	3.448	1	7.872	.892	69.497	.063
Yabelo					1			Ref
Ayssaita	1.195	.600	3.968	1	3.304	1.019	10.710	.046
Chifra	.323	.488	.438	1	1.381	.531	3.595	.508
Dubti	.486	.485	1.002	1	1.625	.628	4.204	.317
Ewa	.711	.561	1.607	1	2.037	.678	6.117	.205
Amibara	2.775	1.052	6.954	1	16.033	2.039	126.081	.008
Fentale	2.840	1.052	7.291	1	17.117	2.178	134.493	.007
Urmaytu					1			Ref
Surupha	-2.082	1.059	3.866	1	.125	.016	.993	.049
Adis ketema	.759	1.428	.282	1	2.135	.130	35.083	.595
Badule	-1.370	1.117	1.504	1	.254	.028	2.270	.220
Galifage	-1.531	1.184	1.673	1	.216	.021	2.202	.196
Geriro	-2.053	1.153	3.169	1	.128	.013	1.230	.075
Gurmudale	-1.596	1.081	2.180	1	.203	.024	1.687	.140
Helidage	.693	1.428	.235	1	2.000	.122	32.881	.628
Jara kontola	-1.531	1.144	1.793	1	.216	.023	2.035	.181
Adult	.394	.331	1.415	1	1.483	.775	2.837	.234
Female	.513	.334	2.364	1	1.671	.868	3.215	.124
Large herd size	.251	.333	.570	1	1.286	.670	2.468	.450

Annexe 2. Details of the primers and conditions used for forward and reverse PCR amplification and sequencing of MERS-CoV genomes.

Reverse Transcription:

1. In a 1.5-µL Eppendorf tube, add 1-4 µL RNA and 1 µL Random Hexamers.
2. Incubate at 70ºC for 5 min.
3. Place reaction briefly on ice and assemble the reverse transcription reaction using the kit-supplied reagents (a master mix can be made if multiple reactions are being run):

4 µL 5X First-Strand Buffer
2 µL DTT
1 µL SuperScript III reverse transcriptase
1 µL dNTPs
1 µL RNasin (if desired)
x µL H2O to 20 µL

3. Incubate at 55ºC for 45 min to 1 h.
4. Inactivate the reverse transcriptase at 70ºC for 15 min. Place reaction on ice after inactivation.
5. Proceed with PCR setup.

PCR (with Phusion PCR kit):

1. Assemble PCR reactions to generate amplicons (for whole-genome sequencing) or with any combination of forward and reverse primers .
2. PCR reaction setup:

2 µL First-strand template
1 µL Forward Primer
1 µL Reverse Primer
5 µL 10X HF Buffer
1 µL dNTPs
0.5 µL Phusion polymerase
x µL H2O to 50 µL

3. PCR reactions are run under standard PCR conditions:

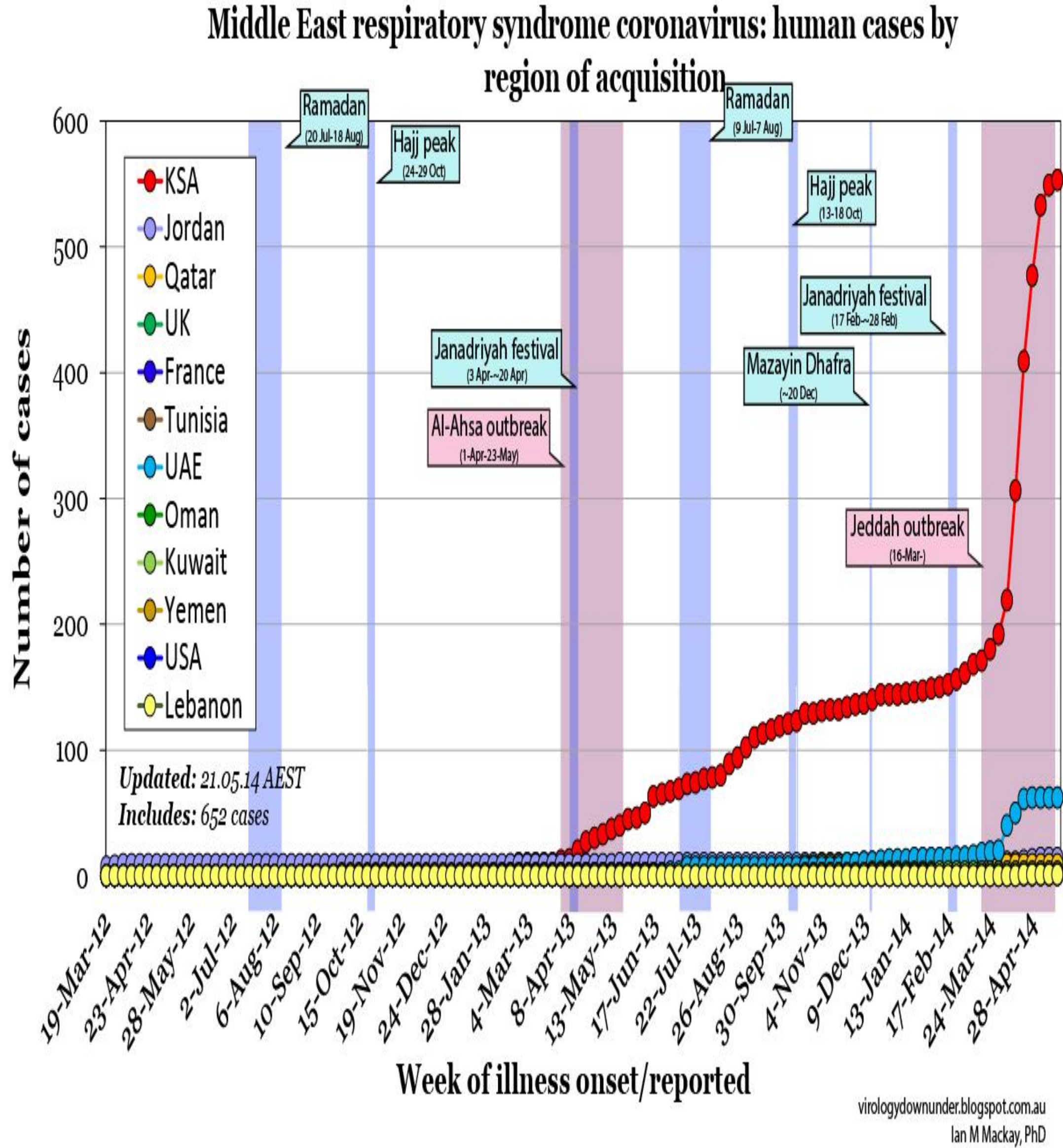
98ºC 5 min
35 cycles of:
98ºC 15 sec
xºC for 30 sec*
72ºC for ~45 sec/kb
72ºC 10 min
8ºC Hold

Annealing temperature is primer-dependent, but for most SARS-CoV primers , annealing temperatures 52-55ºC will work.
Confirmation of PCR products and sequencing:

1. Run PCR products (5 µL/reaction) on a 0.8% agarose/1X TAE gel to verify PCR success.
2. Purify PCR products with PCR purification kit of choice

3. PCR products can be diluted to 150-200 μL/reaction to ensure that enough product is present for assembling sequencing reactions.
4. Assemble sequencing reactions according to the primer/amplicon combinations

Annexe 3. Human cases of MERS-CoV by region of acquisition.



Source: (Mackay, 2015)

9. CURRICULLUM VITAE

A. PERSONAL DATA

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B. EDUCATIONAL BACKGROUND

1. University

   2013- 2015: Addis Ababa University, College of Veterinary Medicine and Agriculture (CVMA), Masters of Science.

   2005-2010: Mekelle University, Faculty of Veterinary Medicine, Mekelle, DVM.

2. High school

   2002-2004: Ada'a Model secondary school

3. Elementary and Junior secondary school

   1996-2002: Funete Selam Elementary and junior secondary school.

C. TRAINING AND PUBLICATIONS

1. Study on seroprevalence of Infectious Bursal Disease in and around Bishoftu town (2012).
2. A cross-sectional study on prevalence of Trypanosomiasis in Amhara region, North West Ethiopia (2012).
3. Study on prevalence, Economic and public health importance of Hydatidosis in Harar city (2014).
4. Training on One Health Approach Special focus on Environmental Health Science, Ohio State University in collaboration with University of Gonder (2014).

D. WORK EXPREINCE

1. Lecturer at Haramaya University since 2010 till now.
2. Summer Education coordinator of college of Veterinary Medicine at Haramaya University (2012).
3. Coordinator of Rabies and Hydatidosis campaign held in Bate, Haramaya, Harar and Dire Dawa (2011).

E. LANGUAGE

	Read	Speak	Write
Amharic	Excellent	Excellent	Excellent
Afan Oromo	Excellent	Good	Good
Tigrigna	Good	Good	Good
English	Excellent	Excellent	Excellent

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