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RESEARCH

Further studies on MERS-Coronavirus in imported and local breeds of camels in Egypt

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ABSTRACT

Background: Middle East respiratory syndrome (MERS) is a newly emerging viral disease of coronaviridae family which appeared firstly in Kingdom of Saudi Arabia. MERS affects the lower respiratory tract of human. There are many researches which emphasized that camels are carrier for MERS-CoV.

Objective: This study aim to know the extent of prevalence of new Exotic diseases like MERS among imported camels from some endemic countries in Africa, and to check our local breeds of camels in different governorates of Egypt for presence of MERS-CoV among them.

Methods: During the period of March 2016 to December 2017, 1500 nasal swabs were collected from imported camels in Birqash veterinary quarantine, 195 nasal swab and Serum samples collected from imported breeds in abattoirs of different governorates in Egypt, In addition to 98 nasal swabs and serum samples were collected from local breeds presented in some camel farms. All nasal swabs tested using real time RT-PCR while serum samples were tested using indirect ELISA technique.

Results: From 1500 nasal swabs of imported camel breeds collected in Birqash veterinary quarantine, ten samples (0.66%) were positive for MERS-CoV by real time RT-PCR. Out of 195 serum samples which collected from imported camels in abattoirs of some governorates, 110 samples (56.4%) were positive for the presence of specific antibodies against MERS-CoV while the nasal swabs of these tested animals of the abattoirs were negative for detection of MERS-CoV. All 98 nasal swabs and serum samples which collected from local breeds of camel's farms were negative for MERS-CoV or its specific antibodies. Phylogenetic analysis of coronavirus ORF1a partial sequences in some positive nasal swab samples of imported breeds (with accession number on gen bank; MH184776, MH184776 and MH184777) confirmed presence of different branch of MERS-CoV from which isolated in Middle East.

Conclusion: MERS-CoV is amphixnoses in which both human and camels are source of infection and cause mild to severe respiratory disease in human while camels consider carrier of MERS-CoV.

Key words: MERS-CoV, Birqash veterinary quarantine, Egypt.

BACKGROUND

Middle East respiratory syndrome (MERS) is an emerging viral disease of Coronaviridae family which appeared firstly in Middle East (WHO, 2017). MERS affects the lower respiratory tract of humans and cause respiratory signs and may be complicated in people with low immunity causing pneumonia and frequently gastrointestinal symptoms (Assiri *et al*., 2013).

From date of appearance of first infected case with MERS-CoV to last global update of world health organization (WHO) at July 2017, there were 2040 laboratory confirmed cases of MERS-CoV infection including 199 laboratory confirmed cases from last update in December 2016 to July 2017 with 58 death cases (29.2%). Of these 199 infected cases, 47 cases reported direct or indirect contact with dromedary camels (WHO, 2017). This indicate MERS-CoV is still circulating to date with high zoonotic importance.

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Many researches emphasized that camels are carrier for MERS-CoV due to close contact of some infected cases with infected camels of Arabian Peninsula that was proved by isolation and sequencing of MERS-CoV from these infected camels and detection of antibodies specific for MERS-CoV in the serum (Azhar *et al.*, 2014). Until now, Egypt imports large numbers of camels from countries of Africa for purpose of meat consumption. Egypt had the largest camel market in Africa "Birqash market" which receive imported camels from the largest African countries for camel populations (Somalia, Sudan, Kenya, Ethiopia and Eritrea) (Younan *et al.*, 2016).

This study aimed to investigate the prevalence of MERS-CoV in either imported breeds of camels which presented in veterinary quarantine in Birqash and in abattoirs of governorates of high population density or local breeds which presented in camel farms and abattoirs in Egypt. Some RT-PCR positive samples of MERS-CoV where investigated for partial sequence of ORF1a gene for knowledge the phylogenetic relationship between the circulating MERS-CoV in both Africa and Arabian Peninsula. This study act as a safe guard for prevalence of new exotic diseases like MERS through the importation of camels from some endemic countries in Africa, and to check our local breeds of camels in different governorates of Egypt for presence of MERS-CoV among our local breeds.

MATERIALS AND METHODS

Sample collection

The samples were collected from different places. There were 1500 nasal swabs which collected from Birqash veterinary quarantine, in addition to nasal swabs and serum samples were collected from different governorates which characterized by the most consumed for camel's meat and high population density. The samples were 195 samples for both nasal swabs and serum samples of the same camels from the abattoirs and 98 nasal swabs and serum samples from local breeds in camel's farms as shown in table 1. Sample collection, transportation and storage of samples was according to (Haagmans *et al.*, 2014). All samples were transported on ice to Animal Health Research Institute (AHRI) and serum samples were separated from blood clot after arrival to AHRI. All nasal swabs and serum samples were stored at – 80^{o}C until testing.

Sample processing and RNA extraction

Swab specimens in transport media were mixed and then clarified by centrifugation at 350 × *g* for 10 minutes; the supernatants were obtained for extraction. The supernatants were ready for extraction by using the Patho Gene-spinTM DNA/RNA Extraction Kit (iNtRon biotechnology, Korea) according to manufacturer's instructions of the kit.

Table 1: Nasal swabs and serum samples collected from veterinary quarantine and different governorates in Egypt:

Governorate	Place	No. of samples	Types of samples		Type of breed	
			N. swabs	Serum	Local	Imported
Cairo	Abattoirs	42	42	42	-	42
Giza	Veterinary quarantine	1500	1500	-	-	1500
	Abattoirs	100	100	100	4	96
Qaluibiah	Abattoirs	34	34	34	6	28
Sharkyia	Abattoirs	29	29	29	-	29
Alexandria	Camel farm	40	40	40	40	-
Matrouh	Camel farm	48	48	48	48	-
Total	V. quarantine	1500	1500	-	-	1500
	Abattoirs & farm	293	293	293	98	195

Molecular detection of MERS-CoV by real time RT-PCR (qRT-PCR)

Using PrimerDesign genesig® pathogen detection kit (PrimerDesign, United Kingdom) as closed kit. The kit contains two Human Coronavirus 2012 (MERS) primer and probe sets. The UpE regions of ORF5 and ORF1ab primer and probe sets are designed to be specific to the MERS strain regardless of the species of origin. The ORF5E assay was used for detection of MERS-CoV in this study as screening test. The kit was supplied by Primerdesign, Park House, Winship Road, Milton, Cambridge, United Kingdom for rapid detection of MERS-CoV infection.

Using Forward and reverse primers and probe were designed to amplify an upstream region of E gen (Up-E gen) of MERS-CoV (Biosearch technology, USA) as confirmatory test according to (Corman *et al.,* 2012). One-step qRT-PCR Master mix is an optimized complete system for use in one step real-time PCR using QuantiNova Probe RT-PCR Kit (Qiagen, Germany) for both two sets of primers according to the manufacturer's protocol with annealing temperature of 60^{o}C/60 sec. The kit was supplied by QIAGEN GmbH, QIAGEN Str. 1, 40724 Hilden, Germany.

Conventional RT-PCR

Using one step RT-PCR PreMix Kit (iNtRon biotechnology) according to manufacturer's instructions of the kit. Using forward and reverse primer of ORF1a of region 1767 to 2615 (Biosearch technology, USA) according to (Nowotny *et al.*, 2014). Briefly, reactions were performed from one step RT-PCR PreMix kit (iNtRon biotechnology, Korea) in a total volume of 20µl containing one step RT-PCR Premix (10µl), 20 pmole forward primer (2µl), 20 pmole forward primer (2µl), RNA sample (5µl) and RNAse/DNAse free water (1µl). All reactions were carried out using a conventional PCR machine (Bio-Rad, USA) with the following cycling parameters; 30 sec at 45°C (Enzyme activation), 5 mints at 94^{o}C (initial denaturation), 40 cycles of 30s at 94°C (denaturation), 60s at 55°C (Annealing), 1 min at 72^{o}C (extension) and 10 min at 72^{o}C for final extension.

Nucleotide sequencing and phylogenetic analysis

Gel product of 848 bp was purified by the Thermo Scientific GeneJET Gel Extraction Kit (EU, Lithuania) according to manufacturer's instructions of the kit then sequencing reaction was performed using a BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, USA) according to the manufacturer's instructions. Products were purified by CENTRI-SEP kit (Applied Biosystem, USA) then the samples were loaded in capillary electrophoresis of Applied Biosystems 3500 Genetic analyzer (Applied Biosystem, USA) immediately after heat shock at 100^{o}C for 5 mints.

Homology search and phylogenetic analysis

Obtained sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis to search the GenBank for homologous nucleotide sequences**.** Related sequences were downloaded from GenBank for the phylogenetic analysis, multiple sequence alignments were generated using the default settings of Clustal W using BioEdit version 7.2.5 software to make analysis for the obtained sequences of ORF1a gene.

A phylogenetic tree was performed using the Neighbor-joining (ML) method and the reliability of each tree branch was estimated by preforming 1,000 bootstrap replicates, in the MEGA version 5 software (Tamura *et al.*, 2013)

MERS-CoV Serodiagnosis by ELISA for detection of antibodies against MERS-CoV.

The test was performed by Medizinische Labordiagnostika AG Elisa kit (EUROIMMUN, Germany) according to the manufacturer's protocol. Wells of ELISA plate were coated by purified specific S1 protein for MERS-CoV only for detection of specific IgG antibodies against MERS-CoV.

RESULTS

Results of molecular detection of MERS-CoV using one step real time RT-PCR.

There were 10 nasal swabs which were positive for MERS-CoV RNA using one step real time RT-PCR from total tested nasal swabs of imported breeds (1500 samples) with percentage of 0.66% of total tested samples as shown in figure 1. While all nasal swabs samples which taken from camels in abattoirs and local breeds were negative for MERS-CoV detection by real time RT-PCR as shown in table 1.

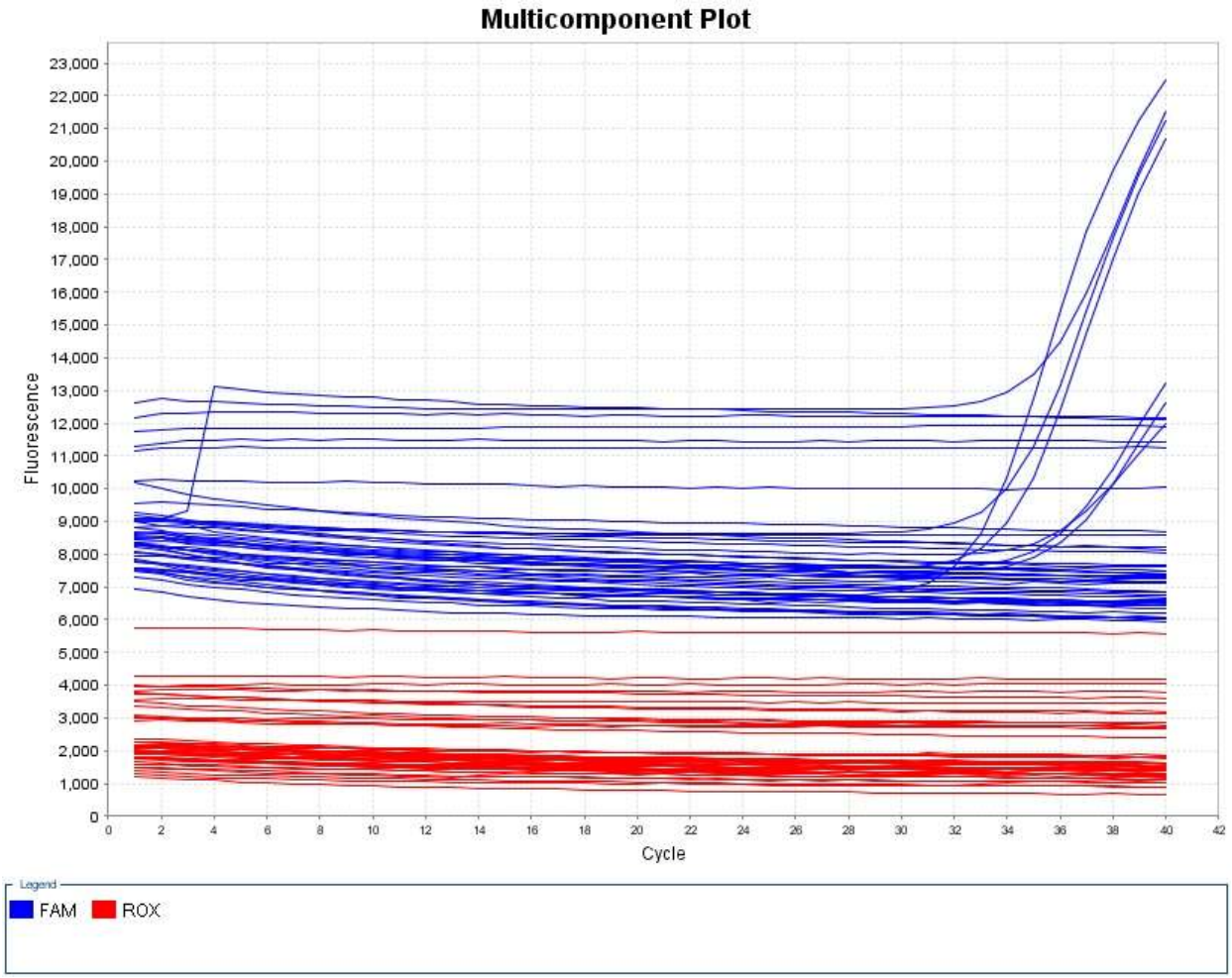

Fig. 1: Results of RT-PCR of UpE gen of MERS-CoV from nasal swabs samples from imported camels. PCR positive control, extraction positive control, while rest of PCR curves were positive samples for MERS-CoV RNA using one step Reverse transcriptase -polymerase Chain reaction (RT-PCR).

Results of MERS-CoV Serodiagnosis by ELISA for detection of Antibodies against MERS-CoV IgG.

There were 110 positive serum samples of total 195 examined samples from imported camels present in abattoirs with percentage of 56.4%. While all 98 tested serum samples of local camels in Egypt were negative for presence of antibodies against MERS-CoV as shown in table (2).

Table 2: Results of detection of RNA in nasal swabs by RT-PCR and IgG antibodies in serum samples by ELISA test collected from different governorates in Egypt

Governo -rate	Imported breeds (abattoirs)						Local breeds (Camels farms)		
	No. tested samples	No. positive					No. tested samples	No. positive	
		Serum samples				Nasal swabs		Serum samples	Nasal swabs
		≤ 3yrs	≤ 5yrs	≤ 8yrs	> 8yrs				
Cairo	42	1	2	12	10	0	-	-	-
Giza	96	3	9	30	15	0	4	0	0
Qaluibiah	28	0	2	6	7	0	6	0	0
Sharkyia	29	2	1	4	6	0	-	-	-
Alexandria	-	-	-	-	-	-	40	0	0
Matrouh	-	-	-	-	-	-	48	0	0
subtotal	195	6	14	52	38	0	98	0	0
Total samples	195	110 (56.4%)				0	98	0	0

Most of positive serum samples were of old camels (more than 5 years age) as shown in table (2). Number of positive serum samples containing specific antibodies against MERS-CoV in both imported and local breeds of camels in some governorates of Egypt according to age of camels is shown in table (2).

Results of conventional RT-PCR

There were 3 samples which gave a clear specific band of MERS-CoV DNA of ORF1a on agarose gel at 848 bp and others failed to continue sequencing due to their low concentration of DNA as shown in figure 2.

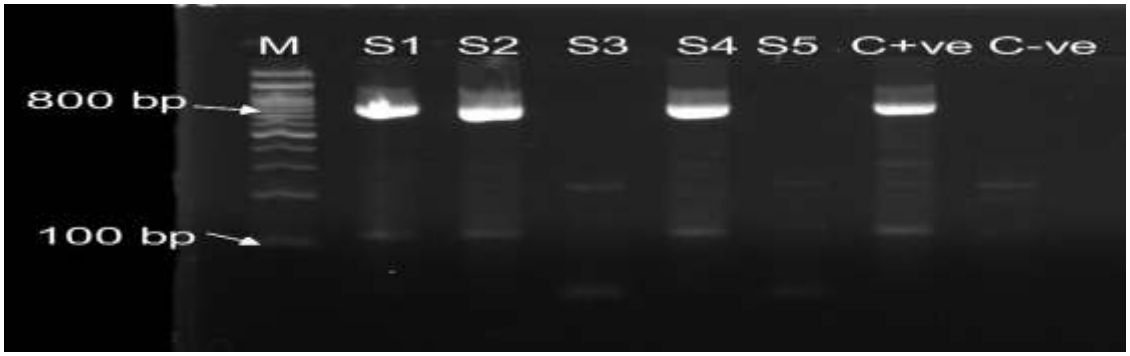

Fig. 2: Result of conventional RT-PCR for MERS-CoV on agarose gel electrophoresis, M mean 100 bp Marker, C+ve mean positive control, C-ve means negative control while S mean Sample of nasal swab. Sample 1, 2 and 4 gave clear specific thick bands of ORF1a gene on agarose gel at 848 bp.

Results of nucleotide sequencing and phylogenetic analysis

Phylogenetic analysis of coronavirus ORF1a sequences showed that three Egyptian viruses of this study called MERS-CoV isolate Egypt/Giza-3/2018-Camel-ORF1a, MERS-CoV isolate

Egypt/Giza-6/2018-Camel-ORF1a and MERS-CoV isolate Egypt/Giza-9/2018-Camel-ORF1a were gathered in the same cluster and closely related to each other and to Egyptian strain called Dromedary/Egypt-NRCE-HKU205/2013. There was another observation that sequences from MERS-CoV strains isolated several years apart were very similar to sequences of our study, indicating that MERS-CoV circulation in camels of Africa is relatively stable. Also, these viruses showed a genetic relation with the African strains from Nigeria and Borkinafaso, but genetically different from the MERS-CoV strains isolated from the Arabian peninsula as shown in figure 3.

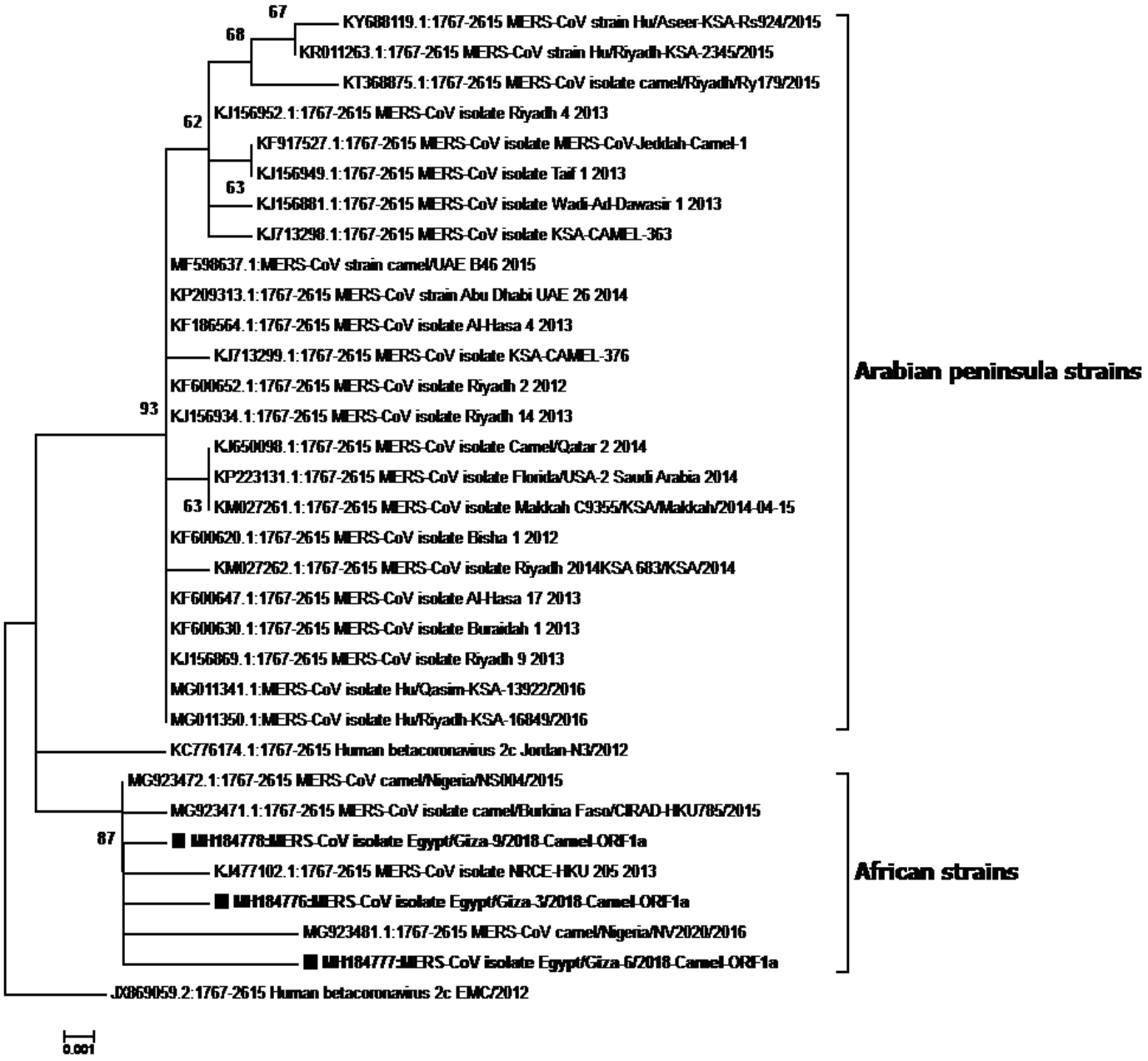

Fig. 3: phylogenetic tree of ORF1a gene of MERS-CoV, Phylogenetic analysis was done using the neighbour-joining, Viruses sequenced for this study are marked with black squares. These viruses showed a genetic relation with the African strains from Nigeria and Borkinafaso, but genetically different from the MERS-CoV strains isolated from the Arabian Peninsula.

DISCUSSION

Middle East respiratory syndrome (MERS) is the last emerging viral disease of Coronaviridae family which appeared firstly in Middle East (WHO, 2017). It is a novel lineage C beta-coronavirus which was characterized by respiratory, enteric, renal and/or neurological disease in humans, many other mammals and birds. In 2002-03 a previously unknown coronavirus emerged from a bat reservoir to cause the SARS pandemic, with about 8,000 human cases and more than 770 deaths (Drosten *et al.*, 2003). Previously, cross-species transmission from coronaviruses of bat and bovine origin caused emerging of human respiratory coronaviruses 229E, NL63 and OC43 to become established in the human population worldwide (Graham *et al.*, 2010).

Infection of dromedary camels with MERS-CoV cause mild respiratory signs (nasal discharges) or silent infection. So, there are two different types of diagnostic methods for MERS-CoV infection in camels, direct method by detection of viral nucleic acids by RT-PCR or indirect method by detection of specific antibodies against previous viral infection by ELISA(Jores, 2015).

In the present study, there were 10 nasal swabs which were positive for MERS-CoV RNA using one step real time RT-PCR from total tested nasal swabs (1500 samples) from imported camels which were presented in veterinary quarantine of Birqash in Egypt with percentage of 0.66% of total tested samples only. The low percentage of positive samples may due to most of imported camels herd were of ages more than 5 years while the prevalence of MERS-CoV shedding is higher in young camels (around one year old) than in adult camels (Wernery *et al.*, 2015), (Hemida *et al.*, 2014) due to stress factors as weaning, shipping, handling practice and gradual decrease in maternal antibodies. When these infected young calves were exposed to MERS-CoV again, their immune system will be stimulated to increase antibodies titer (Adney *et al.*, 2016). The increased antibodies titer in infected animals for the second time or contact animals give protection against subsequent MERS-CoV infection without virus shedding in protected camels and low level of virus shedding in the contact camels(Wernery *et al.*, 2015).

There were 195 nasal swabs and serum samples which collected from imported camels in abattoirs when tested by RT-qPCR were negative for MERS-CoV in nasal swabs. While serum samples of these camels were tested for follow up any previous infection. The results were positive by 110 (56.4%) of total tested serum samples. These result may due to previous infection of imported camels with MERS-CoV. The imported camels were grazing in the open desert and were surrounded by caves and trees. This environment was very suitable for presence of bats which were considered a reservoir for MERS-CoV with subsequent exposure of camels for MERS-CoV.

To rule out the fact of cross-reactive antibodies due to crossing the species barrier of coronaviruses especially BoCoV to camels, we used EUROIMMUN ELISA test kit which were very accurate test because it had wells coated with purified S1 antigen of MERS coronavirus (MERS- CoV S1). The quality of the antigen used ensures a high specificity of the ELISA, Thirteen sera from camels which were tested negative for antibodies against MERS-CoV, but positive for bovine coronavirus using protein microarray, were investigated using the anti-MERS CoV ELISA Camel (IgG). Results for all 13 sera were negative, no cross reactivity with anti-BoCoV positive sera was detected.

In previous study, primers and probe were designed for targeting regions upstream of the E gene (Up E) of MERS- CoV because there was no cross-reactivity observed with coronaviruses NL63, 229E, OC43, SARS-CoV, nor with 92 clinical specimens containing common human respiratory viruses (Corman *et al.*, 2012). These primers and probe were used for confirmation

for positive nasal swabs samples by the screening test of MERS- CoV in both imported and local camels in Egypt.

As shown in phylogenetic tree of figure 3, Although there were genetically diverse MERS-CoV(clade A EMC, clade B Al-Hasa 13 and genetically distant Egypt 270) from different geographic regions of Arabian Peninsula and Egypt (Chu *et al.*, 2014). But one MERS-CoV could recognize antibodies to these genetically diverse viruses which indicated they were antigenically conserved (Hemida *et al.*, 2015).

Most of positive serum samples were of old ages (more than 5 years), these support that prevalence of MERS-CoV antibodies is significally higher in older camels compared to with those aged two years or less (Hemida *et al.*, 2014).

Shedding period of infectious virus in camels is about 7 days while non-infectious RNA for up to 35 days (Adney *et al.*, 2014) while in bats is continues shedding with lack of immune response from bats, so MERS-CoV in bats will circulate in the blood with continues replication (Omrani *et al.*, 2015) unlike the case with camels which have well developed unique immune response (Muyldermans, 2001) and can form specific antibodies against MERS-CoV after 14 days from infection. Because dromedary camels weren't sufficient species to maintain MERS-CoV and may and may appear clinical signs during infection (Adney *et al.*, 2014) so there were not confirmation that camels are reservoir species for MERS-CoV.

Partial sequencing was done for 3 positive samples which had the highest RNA concentration of all tested nasal swab samples by using primers of ORF-1a because of most of positive selection target the nonstructural components especially ORF1a. In previous studies, it was found that some of functional protein regions of the ORF1a did several positive selections and contributed to functional mutations in other coronaviruses to make immune evasion or adaptive evolution in a new host (Forni *et al.*, 2016). In addition to, the most susceptible region for recombination in the coronavirus genome is ORF1ab (Lau *et al.*, 2011).

Phylogenetic analysis of coronavirus ORF1a sequence showed presence of genetically different branch of MERS-CoV which mostly related to African strains and genetically different from strains of Arabian Peninsula of zoonotic importance as shown in figure 3. Average sequence identity between sequences of our study and sequences of African strains isolated several years apart was 99.2% which indicate that MERS-CoV circulating in camels of Africa is very stable as shown in table (3).

Average sequence identity between sequences of our study and sequences of Arabian Peninsula was 98.7%. While sequences identity between sequence of our study and human betacoronavirus 2c EMC/2012 was 99% as shown in table (3), suggesting that different mutation were occurred in Arabian Peninsula strains of the lowest identity with sequences of our study (98.7%) which support the zoonotic importance and higher evolutionary rate of Arabian Peninsula stains as shown in table (3). this percentages of sequence identity support the theory of genetic conservation
and diversity which occurred in coronaviruses to overcome the species barriers without change in skeleton or function of other important components of these coronaviruses (Menachery *et al.*, 2017).

Results of MERS-CoV RNA and antibodies detection in serum samples of local breeds of camels in farms of some governorates were negative which indicate that there is on circulation or shedding of MERS-CoV among the local breeds which supported by no human cases of MERS reported among people of close contact with the local breeds as workers, farmers, veterinarians or tourists. The finding that MERS-CoV not endemic in dromedaries of all countries support the idea of presence of natural reservoir animals like bats necessary for the virus spill over to camels and virus maintenance within camels for varying periods of time.

In conclusion, MERS-CoV is amphixnoses in which both human and camels are source of infection and camels consider carrier of MERS-CoV. We concluded also although there are different strains of MERS-CoV present in Africa which different from that present in Arabian Peninsula causing severe disease in human, but they have cross neutralizing antibodies which can be diagnosed by serological tests as ELISA test. In parallel to appearance of RVF from east Africa to Saudi Arab (Ithete *et al.*, 2013)

Table 3: Sequence identity matrix for nucleotide sequence of ORF1a region of MERS-CoV.

	Hu/beta-CoV 2c EMC/2012	Camel/Qatar_2_2014	Camel/Riyadh/Ry179/2015	Hu/Aseer-KSA-Rs924/2015	Riyadh_2_2012	Camel/Burkina Faso/CIRAD-HKU785/2015	Camel/Nigeria/NV2020/2016	Egypt/Giza-3/2018/Camel	Egypt/Giza-6/2018/Camel	Egypt/Giza-9/2018/Camel
Hu/beta-CoV 2c EMC/2012	ID	0.989	0.985	0.985	0.991	0.991	0.985	0.991	0.988	0.989
Camel/Qatar_2_2014	0.989	ID	0.992	0.992	0.998	0.989	0.983	0.989	0.986	0.988
Camel/Riyadh/Ry179/2015	0.985	0.992	ID	0.994	0.994	0.985	0.979	0.985	0.982	0.983
Hu/Aseer-KSA-Rs924/2015	0.985	0.992	0.994	ID	0.994	0.985	0.979	0.985	0.982	0.983
Riyadh_2_2012	0.991	0.998	0.994	0.994	ID	0.991	0.985	0.991	0.988	0.989
Camel/Burkina Faso/CIRAD-HKU785/2015	0.991	0.989	0.985	0.985	0.991	ID	0.991	0.997	0.994	0.995
Camel/Nigeria/NV2020/2016	0.985	0.983	0.979	0.979	0.985	0.991	ID	0.994	0.988	0.989
Egypt/Giza-3/2018/Camel	0.991	0.989	0.985	0.985	0.991	0.997	0.994	ID	0.994	0.995
Egypt/Giza-6/2018/Camel	0.988	0.986	0.982	0.982	0.988	0.994	0.988	0.994	ID	0.992
Egypt/Giza-9/2018/Camel	0.989	0.988	0.983	0.983	0.989	0.995	0.989	0.995	0.992	ID

we suggested that emergency of MERS-CoV may due to homologous recombination between co-circulation of NRC-HKU270 which present in Africa either with HCoV-229 in human or with camelid α-CoVs in camels which need further investigation especially there was exportation trade of camels from Sudan and Somalia to KSA (Younan *et al.*, 2016).

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