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Quantitative Real-Time RT-PCR Detection of Flaviviruses Associated with Camel Ticks in Egypt

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Abstract: Tick-borne flaviviruses are among the most important arboviruses. Their high genetic diversity and immunological relatedness makes them extremely challenging to diagnose. The present study aimed to assess their infection risks, with special regards to Alkhurma hemorrhagic fever virus (AHFV), applying one-step quantitative real-time RT-PCR assay (qReal-Time RT-PCR) as analytical tool for differential diagnosis and the current epidemiological surveillance of these diseases. A total of 279 ixodid ticks were collected from the main market of imported camels from Sudan and Somalia into Giza Governorate in Egypt during July to October 2011. All samples were identified into 7 species including females, males and nymphs. A combination employing qReal-Time RT-PCR with broad-spectrum degenerate primers amplify a 269 to 272 bp fragments from the highly conserved enzymatic motifs at the N-terminal end of nonstructural protein 5 (NS5) gene of genus Flavivirus were used. Four flavivirus-positive females within *Hyalomma dromedarii* and *Hyalomma marginatum rufipe s*ticks species were detected by qReal-Time RT-PCR, then were inoculated into Vero cell cultures for the cytopathic effects (CPE) monitoring during 4 successive blind passages. CPE was observed on cultures at day 4 post inoculation (p.i.) after 2 blind passages. CPE was characterized by a general lysis of the monolayer that reached 90% at 5-7 days p.i. RNA of flaviviruses was re-detected byqReal-Time RT-PCR in culture supernatants of 3rd passage. Unfortunately, these supernatants were found flaviviruses virions-free by Transmission electron microscopy (TEM) and negative for AHFV by traditional RT-PCR assay.The failure to increase virions titers to the threshold detection levels required by TEM and conventional RT-PCR is a cell line-type dependent. Thus, further propagation on tick-origin cell lines is mandatory for further antigenic and genomic characterization and/or isolation of flaviviruses detected during the present study. This is necessitating efforts to be directed to enhance the diagnostic and preventive protocols to the challenge of emerging AHFV that is thought exist in the country, though undetected yet.

Key words: qReal-Time RT-PCR · Alkhurma hemorrhagic fever virus · Transmission electron microscopy · *Hyalomma dromedarii* · *Hyalomma marginatum rufipes* · Vero cells

INTRODUCTION

Genus Flavivirus comprises 70 viruses within more than 50 recognized species, a large number of which are arthropod-borne that infect humans and animals and several other viruses yet to be documented as species [1]. Flaviviruses are single-stranded, positive-sense, RNA viruses classified in within family Flaviviridae [2]. Genomes analysis has demonstrated three major groups of flaviviruses; taking into account their known vectors and natural vertebrate hosts, which are the mosquito-borne clade (27 species belonging to seven different groups) [3], tick-borne clade (12 species belonging to three different groups) [4] and un-known-vector clade (12 species of mammalian viruses belonging to three different groups) for which arthropod vectors have not been identified [2]. Tick-borne clade (TBEV); assigned as encephalitic viruses, are significant human pathogens in various parts of the world [5, 6]. There are several other tick-borne flaviviruses which may be of local medical importance and which are less known, but may be important as differential diagnosis [7]. Among them are

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louping-ill disease which is present mainly on the British Islands, Omsk hemorrhagic fever, which is prevalent in parts of Russia, Kyasanur Forest Disease, which is distributed in parts of India, Alkhumra hemorrhagic fever, occurring in Saudi Arabia, Powassan encephalitis, which is known to be the only tick-borne flavivirus of human pathogenicity so far detected in North America and some other mosquito-borne flaviviruses [8]. Most of these viruses are also more or less important for veterinary medicine [2]. Several other tick-borne flaviviruses so far have neither been associated with human nor animal diseases and their potential pathogenicity for humans and animals is unknown [1]. Additional sub classification within the genus ranked Alkhurma hemorrhagic fever virus (AHFV) under the mammalian group of tick-borne virus clade which is denoted as emerging hemorrhagic virus with a high potential for global dispersion [9]. This concept is totally consistent with the isolation of Alkhurma hemorrhagic fever virus from a butcher in Holy Makah, Saudi Arabia in 1995 which found associated with camel ticks [9-11] and recently in Italian traveler had come back from southeastern Egypt [12, 13]. Indeed, its subsequent discovery in southern China [8] suggested that migratory birds might carry the infected ticks to or from different regions of the world [9, 13]. There was no clear explanation for its apparent isolation in Saudi Arabia except movements of thousands of animals annually from Africa and other countries to Holy Makah to meet the human demand for food and transport during the Hajj (pilgrimage) rites [14]. Many of these animals, including camels, are infested by ticks that may carry AHFV and thus provide the source of this human infectious agent. There is also the fear of the virus being exported elsewhere by home-bound pilgrims returning from the yearly Hajj (pilgrimage) rites [9]. Phylogenetic evidence based on the genealogical relationships data analysis had previously suggested that the tick-borne encephalitic flavivirus serocomplex originated in Africa and gradually evolved and dispersed across the Northern Hemisphere of the Old World [4]. On the other hand, several other studies showed that tick-borne flaviviruses probably originated in Central Asia [2]. Thus, the possibility of an African evolutionary origin for these viruses should be investigated on humans, animals and ticks level that cause unspecific, unusual, or totally unknown clinical signs especially in animals [15].

Flaviviruses have an average genome size of around 11 kb. Virions contain three structural proteins, the capsid (C), membrane (M) and envelope (E) and infected cells contain seven nonstructural (NS) proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [15]. Development of broad-range flavivirus diagnostic assays has been problematic largely because of the high degree of genetic diversity and immunological cross-reactivity among these viruses [16]. During the past decade, broad range of universal flavivirus molecular amplification systems employing highly conserved enzymatic motifs of the nonstructural proteins 3 (NS3) and 5 (NS5) genes were optimized [17-21]. This is evident that they will continue to constitute valuable tools for the discovery of classical and atypical flaviviruses [15]. In the past, such systems have been described [22-26]. However, their ability to detect a large number of species was, in most cases, based only on sequence analysis [4, 16] or analysis by restriction digestion of the amplicons [15] then the virus identification has been experimentally confirmed only for a few species [27]. Nevertheless, their use in large screening analyses was hampered by the traditional coasty reverse transcriptase polymerase chain reaction (RT- PCR) procedures and/or false-positive results arising from PCR contamination during nested PCR [17-19]. In continence, to overcome these reported trouble shootings, the detection system in the present study dependent on pan flaviviruses degenerated primers complementary to highly conserved enzymatic motifs of the genome utilizing quantitative real-time RT-PCR assay; a modern, high-performance molecular amplification protocol for the identification of flaviviruses and the discovery of new flavivirus species, which had proved its capability to detect different flavivirus species in threshold copy number during a convenient one-step [20, 21].

The present study aimed to detect the naturally occurring flaviviruses infection with special concern to Alkhurma hemorrhagic fever virus in ticks infesting camels in Giza Governorate, Egypt. Since, the increased geographic distribution and severity of induced disease symptoms thus detection is critical for both vector surveillance efforts, updating the epidemiological status of a neglected virus, better clinical diagnosis of human undiagnosed fever and increases the awareness to pathogen that causes unspecific, unusual, or totally unknown clinical signs.

MATERIALS AND METHODS

Ticks Specimens: A total of 279 ixodid ticks were collected from hides of 50 camels from the main market of imported camels from Sudan and Somalia into Giza Governorate in Egypt. Trips for collection were carried out

from July to October 2010. After morphological identification to tick species [28-30], individuals were washed in 75% ethanol and rinsed twice with deionized distilled water, dried on filter paper and immediately stored at -80°C until further processing.

Homogenization of Ticks: Samples were prepared from frozen tick pools of 5 nymphs or individual adult female or male ticks. Two hundred fifty microliters of shelled 10mM PBS buffer (10mM di-sodium hydrogen phosphate, 137mM sodium chloride, 2mM potassium di-hydrogen phosphate and 2.7mM potassium chloride, pH 7.2, Sigma-Aldrich) containing antibiotics (penicillin 100µg/ml and streptomycin 100µg/ml, Invitrogen) and complete protease inhibitor cocktail (one tablet/10 ml; Roche Diagnostics, Rotkreuz, Switzerland) were added to each specimen then were immediately homogenized using the Tissue Lyser system (Qiagen) for 5min/sample at 30Hz [31]. Second individual homogenization step/sample was done manually using liquid nitrogen in mortem and piston/sample. After a short centrifugation step (13000rpm/4min), the supernatants were collected in separate collection microtubes, stored at -80°C for further use.

RNA Extraction Procedure: According to biosafety level requirement to comply with flaviviruses handling safety regulation, total RNAs were efficiently extracted from 100ml of each supernatant using Trizol®Reagent; modified guanidinium thiocyanate-phenol-chloroform extraction procedure, (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA was eluted in a final volume of 60µl of RNA carrier solution (Poly-A, 30µg/mL, Qiagen) and either directly used for downstream applications or stored at -80°C for further use.

Internal Quality Control Measure for RNA Extraction and RT-PCR Assays

Ixodid Ticks Calreticulin Gene (CRT) Detection: To monitor false-negative results in flaviviruses infection analysis, a highly specific RT-PCR assay was applied as a qualitative control for the total RNA extraction from ixodid tick species. This assay target amplification of the intron-exon structure of the tick calreticulin (CRT) gene [32, 33], which is highly conserved in all hard ticks using primers; CRT-F1 (5`-CTTGTGTGCTTGTTGCTGCTTC-3`) and CRT-R3 (5`-GATGTTCTGCTCGTGCTTCA-3`). Primers were synthesized by (Operon Technologies, Germany). RT-PCR assays were performed using Reverse-iT™ One Step RT-PCR Kit (AB gene thermophilus); according to the manufacturer's instructions, in total volum 25µl/reaction that included 5µl RNA with carrier, 50µM Randum hexamers, Reverse-iT® Reverse Transcriptase enzyme blend of 50U/µl, 20U/µlRNase inhibitors, 10mM dNTP, 25mM $MgCl_2$, 5U/reaction Taq DNA polymerase, 50pM/µl of primers pair and nuclease free water to complete the volume. The mixtures were incubated at 25°C for 10min and at 48°C for 60min and inactivated at 95°C for 3min, Then reactions were subjected to 35 cycles of 94°C for 1min, 53°C for 1min and 72°C for 1min, followed by 10min extension at 72°C, finally the reactions were cooled down to 4°C. Amplification step was performed in a PTC-100™ thermal cycler (MJ Research Inc., USA). A reagent blank was run as control negative simultaneous with every RT-PCR. Amplified DNA were visualized on a 2% agarose gel in TBE buffer (45mMTris-borate, 1mM EDTA, pH 8.3, Sigma Aldrich) and stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Alliance Bio, USA) was used with each gel. The differences observed in size of the amplified products were RNA and/or DNA -dependent in each sample. Specifically, RT-PCR products detected were 313 bp and 468 bp for the mRNA and the DNA of ticks origin, respectively. The analysis of the type, subsequently, the significance of the obtained products was dependent on previous publication [31, 32].

DNA Digestion by Deoxyribonuclease I: A second RT-PCRs under the same conditions; using the same samples, with an additional incubation step with deoxyribonuclease I, amplification grade (Invitrogen) prior to RT-PCRs were applied [33]. Distilled water was included in each reaction as a negative control. Amplification step was performed in a PTC-100™ thermal cycler (MJ Research Inc., USA). Amplified DNA were visualized on a 2% agarose gel in TBE buffer (Sigma Aldrich) and stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Alliance Bio, USA) was used with each gel.

Flaviviruses Oligonucleotide Primers Design: Three degenerated primers applied in two pairs had been used wherethe design of which was based on the yellow fever virus strain 17 Dopen reading frame (Gen Bank accession number NC-002031) [17-19], in addition to, the alignment-dependent modifications done to take into account the variability of the sequence among a large number of genus *Flavivirus* and to allow a strong hybridization at the 3`-termini

(the 3`-terminal nucleotide is either at the first or second position of the corresponding codon) [20, 21]. The PF1S-PF 2R primers were originally degenerated. The first pair of primers included primers PF1S [5`-TGYRTBTAYAACATGATGGG-3`, positions 8869-8888] [17-19] and PF2R-bis [5`-GTGTCCCAICCNGCNGTRTC-3`, positions 9, 121-9,140] [20] which was designed to amplify a 269-272 bp fragment. The region amplified is located in the N-terminal end of the RNA-dependent RNA polymerase domain, between Motif 2 of the methyl transferase and Motif A of the polymerase gene of genus *Flavivirus*. The additional PF3S primer [5`-ATHTGG TWYATGTGGYTDGG-3`, position 8941-8960] [21] was designed to amplify a second PCR fragment with the aid of PF2R-bis of 197 bp molecular size. Primers sets were synthesized by (Operon Technologies, Germany).

Nested RT-PCR Protocol: Two step cycling protocol were performed according to the procedures described by Moureau *et al*. [20, 21]. RT-PCR assays were performed using Reverse-iT™ One Step RT-PCR Kit (ABgene); according to the manufacturer's instructions, in total volum 50µl/reaction utilizing 50pM/µl of first primers pair (PF1S and PF2R-bis) and nuclease free water to complete the volume. The mixtures were incubated at 25°C for 10min, 48°C for 60min and inactivated at 95°C for 3min, then reactions were subjected to 40 cycles of 94°C for 1min, 50°C for 1.5min and 72°C for 1min, followed by 10min extension at 72°C, finally the reactions were cooled down to 4°C. Amplification step was performed in a PTC-100™ thermal cycler (MJ Research Inc., USA). The second PCR reaction utilized 25µl total volume of 2X ReddyMix™ PCR Master Mix (ABgene), 1µl of first RT-PCR product, 50pM/µl of PF3S and PF2R-bis primers pair and amplification reaction was developed using the same cycling protocol. All amplification steps were performed in a PTC-100™ thermal cycler (MJ Research Inc., USA). A reagent blank was run as control negative simultaneous with every PCR. Amplified products from the PCRs were visualized in 2% agarose gels in TBE buffer (Sigma Aldrich) and stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Alliance Bio, USA) was used with each gel.

Quantitative Real-time RT-PCR Protocol: Aliquots (5ml) of each extract were screened for tick-borne RNA of flaviviruses by one-step RT- PCR assay performed in a real-time PCR format, utilizing PF1S and PF2R-bis primers pair [20], using the QuantiTect®SYBR®Green RT- PCR (Qiagen) onto an ABI Prism 7000 Sequence Detection System (Applied Biosystems) was the second step in molecular detection and/or characterization of different tick-borne flaviviruses. The optimized protocol was synthesized in a 25µl final volume, where each reaction mixture contained 5µl of RNA with carriers, 12.5µl of 2X QuantiTect® RT- PCR Master Mix (Qiagen), 0.25µl of QuantiTect® RT Mix (Qiagen), 0.55µM of each primer. The cycling program was 50°C for 30min, 95°C for 15min, followed by 40 cycles consisting of 94°C for 15sec, 50°C for 30sec and 72°C for 45sec[21]. Analysis of the melting curve of specific PCR products was performed by slowly raising the temperature from 60°C to 95°C by means of regular fluorescence measurements, which should be distinguished from primer dimmers (dissociation temperature <74°C) [20, 34]. PF1S/ PF2R-bis primers pair resulted in earlier detection signals as evaluated by the comparison of Ct values. Distilled water served as a negative control.

Viruses and Cell Culture: All real-time RT-PCR flaviviruses-positive samples were handled and propagated according to their biosafety level requirement to comply with safety regulation [8]. About 0.5ml of the tick suspensions were inoculated in monolayers of Green monkey (Vero) cells in p25 culture flasks (Coaster) then maintained at 37°C under 5% CO_2 (Haraeus) in Eagle's minimum essential medium (Sigma Aldrich) with 3% fetal calf serum (Invitrogen) and penicillin G, streptomycin, at 100IU/mL and 100µg/mL (Invitrogen), respectively [35]. Negative controls were inoculated with fetal calf serum free-MEM. The inoculate of each real-time RT-PCR positive sample was removed and 1ml of MEM supplemented with 2% FCS was added. Cells were incubated at 37°C and examined during 8 days every 24h for the occurrence of cytopathic effects (CPE) [11]. From cultures with no visible CPE, 3 sub passages were carried out. Then all passages were screened by one-step duplex real-time RT-PCR assay to detect the increase in viruses titer which is ultimate for further antigenic and genomic characterization and/or isolation of each virus type.

Virus Purification and Transmission Electron Microscopy (TEM): The third passages of inoculated Vero cells cultures with real-time RT-PCR positive samples maintained at 37°C under 5% CO_2 for 3 days were subjected to transmission electron microscopy detection/ characterization of viral particles. Cells and supernatants were recovered and centrifuged at 5000rpm for 5min at 4°C (Labofuge- 400R, Haraeus, Germany). Supernatants were

collected and centrifuged at 110000 *xg* using an SW41 rotor for 30min at 4°C. Centrifugation of the supernatant was carried out over a cushion of sucrose 66% (w/w) in 10mMTris-HCl pH 7.5 (Sigma Aldrich). Virions should be collected at the surface of the sucrose cushion and diluted in 100mM PBS, pH 7.2 (Sigma Aldrich). For TEM examination, viral preparations were adsorbed onto carbon-coated grids and stained with 2% phosphotungstic acid. The grids were examined using a Tecnai 12 TEM (FEI Co.).

RESULTS

Ticks Species Infesting Camels: Seven species were identified within collected ticks (Table 1). Ticks were separated according to their sex and developmental stage, where 143 females, 96 males and 40 nymphswere obtained (Table 1).

Internal Quality Control Results for RNA Extraction and RT-PCR Assays

Calreticulin Gene (CRT) Amplification: To check quality of RNA extracted and absence of inhibitory substances that may hamper RT-PCR reactions. The primers design amplified the intron sequences from nt 88 to nt 244 of the complete CRT gene of any ixodid ticks.

DNA Digestion by Deoxyribonuclease I: This second RT-PCR internal control step was used to check false-positive results that may developed due to the presence of genomic DNA since only RNA is the target template to work on during following assays. No false-positive results were observed, implying high specificity for the flavivirus sequences and the purity of extracted RNA from examined tick samples.

Flaviviruses Detection by Nested RT-PCR Assay: The conventional nested-type RT-PCR despite its specificity could not detect viral RNA genomes to amplify the designated fragment of flaviviruses by the universal primers pairs.

Flaviviruses Viral Particles Quantizationby Real-time RT-PCR Assay: Analysis of the melting curve of specific PCR products was performed by slowly raising the temperature from 60°C to 95°C by means of regular

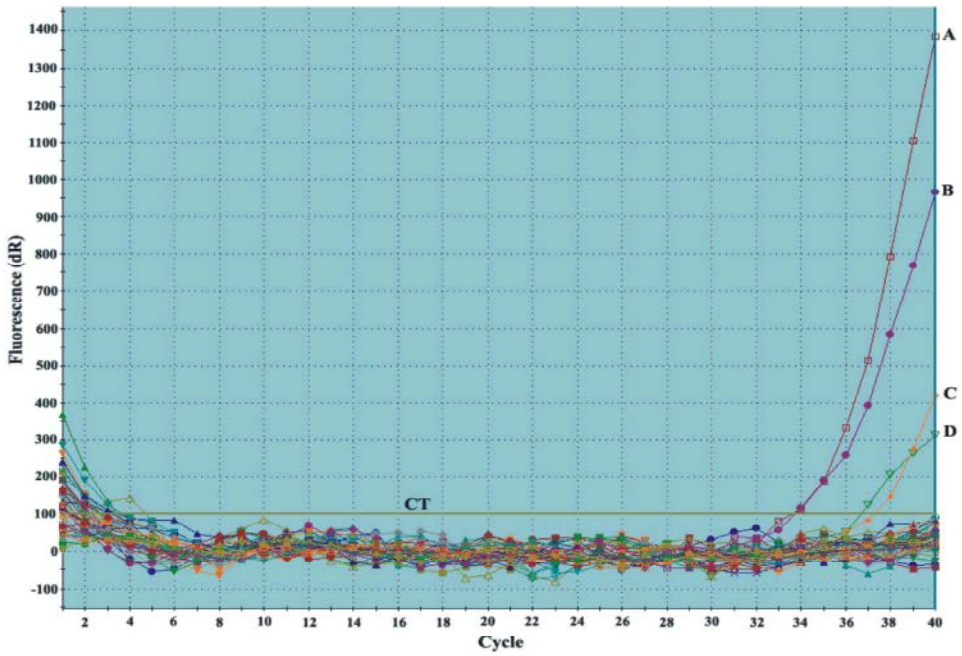

Fig. 1: Amplification plot of the qReal-Time RT-PCR assay showing the cycle threshold (Ct) of the four positive ticks' samples which are reversely proportional to viral particles number/sample

A: Ct = 33.64 for *H. dromedarii* female.
B: Ct = 33.81 for *H. dromedarii* female.
C: Ct = 37.34 for *H. dromedarii* female.
D: Ct = 36.77 for *H. marginatum rufipes* female.

Table 1: Camel tick species collected from the main market of imported camels from Sudan and Somalia into Giza Governorate in Egypt

		Developmental Stages of Ticks		
Tick Species	Total No.	Females	Males	Nymphs
Hyalomma analuticum excavatum	8	3	5	-
Hyalomma dromedarii	217	88	89	40
Hyalomma impressum	1	1	-	-
Hyalomma marginatum marginatum	3	2	1	-
Hyalomma marginatum rufipes	39	38	1	-
Amblyomma lepidum	8	8	-	-
Amblyomma vareigatum	3	3	-	-
Total	279	143	96	40

fluorescence measurements. Flavivirus RNA could be detected in 4 females of *Hyalomma dromedarii* and *Hyalomma marginatum rufipes* ticks species. Analyzing the amplification plot, could indicate slight long cycle threshold (Ct) ranged 33.64, 33.81 and 37.34 for *H. dromedarii* and 36.77 for *H. m. rufipes* (Fig. 1). These results are positive reactions indicative of moderate amounts of targets nucleic acids (Fig. 1), subsequently, moderate to weak viral particles number/sample necessitating samples inoculation into suitable cell cultures.

Virus Inoculation on Vero Cells and Characterization by Transmission Electron Microscropy (TEM): Cytopathic effects (CPE) were observed on cultures at day 4 post inoculation (p.i.) after two blind passages. Which was characterized by a general lysis of the monolayer that reached 90% at days 5-7 p.i. RNA of flaviviruses was re-detected by quantitative real-time RT-PCR in cell-culture supernatants of third passage showing CPE, a slight increase in viruses titers was detected, however, was not enough for transmission electron microscopy examination nor for conventional RT-PCR assay specific for AHFV detection. Then all 4 passages of samples were screened by one-step duplex real-time RT-PCR assay to detect the increase in viruses' titer which is ultimate for further antigenic and genomic characterization and/or isolation of each virus type.

DISCUSSION

Over the past two decades, there has been an increase in the number of AHFV cases reported in many countries including Egypt [9, 12, 13, 36, 37]. Although AHFV has not been detected in animals, camels and sheep have been epidemiologically linked with acute AHF, where these animals may only be acting as hosts for transmission between ticks as well as ticks amplification [37]. Whether other mammals and/or migratory birds are also involved in its life cycle, remains unknown [12], since it has been known so far, the natural hosts of all tick-borne flaviviruses belong either to the groups of rodents/insectivores (mammalian subgroup) or to the group of birds (seabird subgroup) [15].

Previous virological, entomological, epidemiological and phylogenetic reports had evidenced AHFV to be a tick-borne flavivirus [10, 14, 38]. Based on limited data, vectors/reservoirs of AHFV presently included the soft tick *Ornithodoros savignyi* and the hard tick *Hyalomma dromedarii* [14, 39]. However, *Hyalomma marginatum rufipes*; extensively ectoparasiting camels, which is notorious as vector of crimean-congo hemorrhagic fever virus; an arbo-flavivirues, [30], is proved to be vector of AHFV according to obtained results. Hence, the dynamics of the AHFV and its tick vectors remain poorly understood [2, 13]. The association of studied tick species in the present study with camels further supports the role of camels in AHFV transmission cycle as well as the zoonotic nature of the disease, thus expanding the opportunity for importing/exporting clinical disease by infected ticks [9, 36]. Considering the role played by different Red Sea port cities on both Asian and African coasts which are heavily traveled corridor for humans and animals entering Saudi Arabia; particularly during the annual Hajj pilgrimage. This gives the diversity of potential vectors and their extensive ranges leading to circulation of AHFV as well as other undiscovered arboviruses more broadly throughout Saudi Arabia and beyond in the surrounding countries [14, 40]. Therefore it is thought that AHFV exists in Egypt, though undetected yet. One benefit of the used pan-Flavivirus qReal-Time RT-PCR assay is its ability to identify more than one type of virus in a sample. This characteristic would also have clear benefits for use in vector surveillance where a possibility of two or more flaviviruses is co-circulating within the same geographic region [20, 21]. Extensive studies involving more tick species are needed to better understand AHFV ecology and transmission dynamics.

Investigations to obtain better knowledge of the geographic distribution of AHFV are necessary in near countries, with special regards to other west African countries where camels are imported to Egypt from; Sudan, Somalia and Djibouti. Since the short sequences available from the Shalatin isolate (2010 Egyptian isolate) placed it within Sublineage II of AHFV, possibly suggesting a relatively recent introduction to Egypt from Saudi Arabia [12, 40], but did not exclude effect of African ancestral viruses. Dispersal of the ancestral viruses of AHFV may have been accomplished through the movement of animals, including camels presumably carrying ticks, along Al-Arbaain Road; dispersal corridor of camels to Egypt from Sudan, Somalia and Djibouti.

Both *Hyalomma dromedarii* and *Hyalomma marginatum rufipes* possess more than one host along their life cycles those continue along the year which should reflect a long-term evolutionary relationship between the vectors and AHFV in its epizootic cycle [16, 30]. On the other hand, considering the infrequent feeding (only three blood meals per generation), implying a limited viral replication rate/tick per tick generation [16]. In addition to, the necessity for the virus to further replication in two very distantly related biological systems (animal, human) [32, 33], all justify the low susceptibility and/or adaptive behavior noted, that could be interpreted as replication in a yet non adapted host [15]. Thus moderate to weak viral quantities during qReal-Time RT-PCR assay were detected. Also, scanty replication rate in Vero cell cultures, subsequently, the failure to duplicate viral titers to the threshold detection levels required by TEM and conventional RT-PCR because of its genetic background and cell line dependent [12,40]. Thus, further propagation on tick-origin and/or C6/36 cell lines is mandatory [39]. As well as, inoculation of viral material and/or infected ticks homogenates intracerebral in newly born mice, simultaneous with experimental infection of ticks are required.This may facilitate determination of specific threshold copy number for AHFV detection by qReal-Time RT-PCR and traditional RT-PCR assays that is previously reported for most flaviviruses [20, 21, 39]. Consequently, AHFV growth behavior, pathogenicity and virulence can be studied in vivo [37], which may create a basis for understanding the interactions between AHFV and the immune systems of humans and different animals [2]. Hence, most arboviruses are highly stable both genetically and phenotypically in nature and during laboratory passages [19].

There is no doubt that emergence and re-emergence of human and animal pathogens on a global scale continues, hence, the changes in human behavior, land use, climate, will subsequently, change the actual geographical distribution and transmission intensity, so tick-borne flaviviruses are potential risk of medical and veterinary importance [2, 13].

In conclusion: No commercial tests are available for neither serologic nor molecular detection of many arboviruses or for their differential diagnosis since laboratory diagnosis is often made difficult by antibody cross-reactivity. More research is needed in human and animal health, as well as in entomologic and environmental studies, especially in light of the recent data suggesting a nonexclusive role of ticks as vectors for human infection with Alkhurma virus. Further research is needed in the animal setting because little is known about length and severity of illness, duration of viremia and modes of animal-to-animal and animal-to-human transmission; it is crucial to better understand the role of vectors to limit the spread of the disease.

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