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# Investigation of hemorrhagic fever viruses inside wild populations of ticks: One of the pioneer studies in Saudi Arabia

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ABSTRACT

**Objective:** To screen hemorrhagic fever viruses inside wild populations of ticks collected from Riyadh, Saudi Arabia between January and March 2016.

**Methods:** Ticks were identified depending on their morphological features using classical keys then grouped into pools. Ticks in each pool were processed separately using the sterile pestles and mortars. Viral RNA was extracted using Qiagen RNeasy Mini Kit and Qiagen RNAeasy Columns (Qiagen, Hilden, Germany) according to the instructions of manufacturers. A total number of 1 282 hard ticks were collected, and 582 of them were precisely identified then screened for the presence of arboviruses using quantitative real-time PCR. The four species were screened for six viruses: Rift Valley fever virus (RVFV), Chikungunya virus (CHIKV), Crimean-Congo hemorrhagic fever virus (CCHFV), Alkhurma virus (INKV), Sindbis virus (SINV), and Pan Hanta virus (HANTA). CT value for the negative control (RNA free water) was zero. Negative and positive controls were tested for each test to confirm the specificity of the selected primer pairs. SYBR Green One step RT-PCR Master Mix (KAPA Biosystems, Boston, MA) was tested along with primers.

**Results:** Ticks identification resulted into four species: *Hyalomma schulzei*, *Hyalomma onatoli*, *Boophilus kdhlsi*, and *Hyalomm dromedarii*. All the ticks' species (except *Boophilus kdhlsi*) were positive for the following viruses: SINV, RVFV, CHIKV, and CCHFV. While HANTA viruses have been detected in a single species (*Hyalomm dromedarii*).

**Conclusions:** According to our knowledge this research may be one of the pioneer studies in Kingdom of Saudi Arabia. Incrimination of the above mentioned ticks species as well as their vectorial capacity are highly recommended for investigation in the upcoming researches.

## 1. Introduction

Ticks are small arachnids in the order Parasitiformes. In addition to mites, they compose the subclass Acari. Ticks are also ectoparasites (external parasites) that live by hematophagy on the blood of mammals, birds, and sometimes reptiles and amphibians. After mosquitoes, ticks are considered the second biggest important vectors for many pathogens of human and animal diseases[1].

World widely ticks are the most common arthropod vectors for human and animal pathogens. Some species like *Ixodes ricinus* can transmit a wide spectrum of pathogens from bacteria and viruses to parasites. These species of ticks may also be co-infected by more than one type of pathogens, with a subsequent high likelihood of co-transmission to humans or animals[2]. Feeding bites of some species of ticks can also cause paralysis or various forms of toxicosis in their hosts[3].

Ticks act as vectors for number of human and animal diseases. In spite of their poor reputation among human communities, ticks have ecological role through culling infirm animals and preventing overgrazing of plant resources. The risk of viral transmission by ticks, mainly *Ixodes* sp. is the circulation of the viruses between small mammals such as rodents which serve as virus amplifying host. Human is infected after ticks bite as well as consumption of infected milk or milk product[4-7].

Some publications recorded transmission of the causative agents of Rocky Mountain spotted fever, human monocytic ehrlichiosis,

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human granulocytic anaplasmosis, Lyme disease, Q-fever, tularemia, Colorado tick fever, and other several vector-borne diseases, to vertebrates hosts in North America alone[5]. Besides, tick-borne encephalitis (TBE) represents increasing public health concern and it is caused by tick-borne encephalitis virus (TBEV) [5,7]. Infection with this virus in human results in neurological symptoms and the virus rapidly expands to new geographical areas[8].

Besides *Ixodes* ticks, other species such as *Hyalomma* spp. are very risky to humans and animals due to their abundance, wide distribution, and vectorial capacity[3]. *Hyalomma dromedarii* (*H. dromedarii*) and *Hyalomma schulzei* (*H. schulzei*) are species closely associated with camels[9]. Camels are the main hosts of the adults even though they can parasitize other domestic animals[10]. *H. dromedarii* is a cosmopolitan species since it's widely distributed in North Africa, the northern regions of West, Central, and East Africa, the Far and Middle East as well as South Asia[4,6,11,12]. *H. schulzei* has shown more limited geographical distribution to Asia and Egypt than *H. dromedarii*. It is uncommon ticks except in Southeastern Iran. Another species belonged to the same genus has a medical importance, namely *Hyalomma anatolicum* (*H. anatolicum*) which maintains a range of economically important infections transmitted to domestic animals and humans[13,14].

The above mentioned information revealed that wherever present, ticks pose a threat to humans and animals. This makes the understanding of tick species identification, hosts, and geographical distributions constitute an important issue. Investigations on the infectivity of ticks' species with infectious agents such as viruses are very rare in the Middle East specifically in the Kingdom Saudi Arabia (KSA) where the number of published articles are limited. This gave the rationale to conduct this study which aimed to investigate the presence of viral RNA inside wild populations of ticks collected from rural areas proximal to Riyadh, capital of the KSA. More importantly, this study gave a clue on the habitats of ticks in Riyadh city.

## 2. Materials and methods

### 2.1. Study setting

The capital of Saudi Arabia and Riyadh Province is Riyadh city which is located in the center of the Arabian Peninsula on a large plateau and divided into 15 branch municipalities. Riyadh region is generally characterized by a hot dry climate. Riyadh city is considered hot during summer with 43.6 ℃ as an average temperature and warm with cool and windy nights in winter, but the overall weather is arid with dust storms which sometimes affects the visibility at a distance of 10 m. Domesticated animals include Arabian camels or dromedaries (the most dominant domestic animals), sheep, goats, and donkeys. Noticeably, Riyadh has high rates of population growth since the population in 1935 was 40 000 inhabitants and increased to 83 000 in 1949. Recently, 65% of the populations are Saudi families and 35% are non-Saudi.

### 2.2. Population study

Wild populations of hard ticks are thought to be potential vectors of arboviruses. Ticks specimens were collected from Riyadh, the capital of Saudi Arabia between January and March 2016 from three different locations (East, West and South Riyadh).

### 2.3. Study design

Baseline surveys were conducted between January and March 2016 to provide an information base against which to monitor and assess the existence of hemorrhagic fever viruses inside wild ticks collected from Riyadh, the capital of KSA.

The main task of this study was to collate the data and ensure that it can be updated in the longer term. So it was important to find out what information is already available. In KSA, there is no existing data where all available data are incomplete, or it needs to be supplemented or broken out into categories that are relevant for the epidemiology of vector- borne hemorrhagic fevers.

### 2.4. Sample size

From the 1 282 collected hard ticks samples, only 582 samples were precisely identified and screened for the presence of arboviruses using real-time PCR technique. Detection of low levels of arthropods infections requires large samples (between 500 and 1 600 individual) for high probability (0.8) of detection. Assuming the test was perfect (*e.g.* the sensitivity and specificity of the test are 100%), and the number of infected and non-infected ticks are assumed to be equally captured by the sampling method, the probability of detection (P) of any infected individuals in a sample of (N) mosquitoes can be calculated based on the binomial distribution: $P = 1 - (1 - r)^N$[15]. Then, we illustrated the identification of ticks and the places form which it could be collected.

### 2.5. Ticks identification

Ticks were collected manually from camels and domestic animals then transferred to the research laboratory, Department of Biology, Faculty of Science, Princess Norah Bint Abdul-Rahman University to perform morphological identification.

Ticks were examined under the light stereomicroscope (STEMI SR-ZEISS® Germany) through related taxonomic keys of Kaiser and Hoogstraal[16] and Estrada-Peña and de la Fuente García[17]. The representative specimens of each species were photographed then drawn by using a drawing tube connected to stereomicroscope. The common names related to each taxon were derived from the names authored by Hoogstraal (Robbins and Carpenter)[18]. Finally, the specimens were identified into four species depending on the morphological characteristics, namely, *H. schulzei*, *Hyalomma onatoli*, *Boophilus kdhlsi* (*B. kdhlsi*), and *H. dromedarii* (Table 1).

**Table 1**
Ticks pools screened for viruses using RT-PCR.

| RNA viruses | Species of ticks | | | |
|---|---|---|---|---|
| | *H. schulzei* | *H. onatoli* | *B. kdhlsi* | *H. dromedarii* |
| SNBS virus | 1 | 2 | 3 | 4 |
| CCHFV | 5 | 6 | 7 | 8 |
| RVFV | 9 | 10 | 11 | 12 |
| Alkhurma virus | 13 | 14 | 15 | 16 |
| Hanta virus | 17 | 18 | 19 | 20 |
| Chikengunya virus | 21 | 22 | 23 | 24 |

*H. onatoli*: *Hyalomma onatoli.*

### 2.6. Samples processing

Sample size was determined according to the availability of ticks during the surveys. Each pool was triturated separately with a grinder with pestles in varying volumes of virus transport medium (VTM) according to the size of each group as follows: 10 – 50 specimens (2.0 mL), 100 specimens (3.0 mL), 150 specimens (4.0 mL), 200 specimens (5.0 mL), and 300 specimens (8.0 mL) (Table 1). The medium components were Leibovitz medium besides antibiotics, fungicide, and 10% fetal calf serum. The supernatants

were filtered then centrifuged at 3000 r/min for 30 min then re-filtered using 0.45 μm syringe filters. The supernatants were used for the RNA extraction.

### 2.7. Primers (synthetic genes) preparation, RNA extraction and cDNA synthesis

Synthetic genes were prepared according to the instructions of manufacturers as mentioned in Table 1. The total RNA was mined and the isolation was done as stated by the constructor with Qiagen RNeasy Mini Kit and Qiagen RNAeasy Columns (Qiagen, Hilden, Germany) and recovered in 40 μL of free water-nuclease. It was stored at 70 ˚C for further usage. The final RNA was approximately folded, increasing the sensitivity of the whole method.

Preferred QPCR method was conducted in 20 μL reaction medium having RNA template (1 μL), each primer (25 μmol/L), and KAPA master mixture (10 mL) (KAPA SYBR® FAST qPCR Kits). Samples were initially incubated for 30 min at 42 ˚C for RT step and for 3 min at 95 ˚C for the denaturation step. 40 cycles in total were applied, each containing 3 s in 95 ˚C for denaturation step and 58 ˚C and 56 ˚C as an annealing temperature for RVFV (CHIKV, CCHFV and Hanta virus), and SINBV, respectively. For each test the positive and negative controls were considered. For specificity confirmation of the selected primer pairs, the following set of negative control samples were tested under the same conditions as described above.

### 2.8. RT-PCR procedures

Different real-time PCR instruments were used for the evaluation of QRT-PCR procedures at two different locations; Sequence Detection System 2.0 (ABI Prism 7900HT); Applied Biosystems and Mastercycler® ep realplex, real-time thermal cycler (Eppendorf AG, Germany). Two regularly used in-house PCR kits (Power SYBR Green One step RT-PCR Master Mix) and (KAPA Biosystems, Boston, MA) were evaluated along with primers (Table 2) which obtained from DNA Technology (Denmark). For the assessment, plasmid DNA of the synthetic genes was isolated by the standard procedures at indicated dilutions and used as templates for the above primer sets. For the PCR kits the basic conditions considered as commended by the constructor. Furthermore, using temperature gradient PCR's, 52 ˚C and at 62 ˚C were used as starting and ending temperatures with 2 ˚C intervals as annealing temperatures.

Primers were prepared according to the aim of (1). Primers having almost equal Tm; (2). Minimizing primer-primer and primer-primer interactions; (3). Validity since they were still working with a conserved stretch. For primer-primer annealing and self-annealing loops, all oligonucleotides were assessed using a cutoff of –5 k cal/mol for the Gibbs free energy (ΔG). Predicted Tm and oligonucleotide interactions were checked and the primer-primer interactions were tested using Oligo Analyzer (http://207.32.43.70/biotools/oligocalc/oligocalc.asp; http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/)[19-21]. Primers were bought from DNA Technology A/S (Risskov, Denmark).

## 3. Results

### 3.1. Screening of SINBV in the four groups of ticks

SINBV was detected inside all the species of ticks except Group 3 (*B. kdhlsi*) as shown in Figure 1. CT values for the positive samples were 23, 24 and 25 for Groups 1, 2 and 4, respectively. CT value for the negative control (RNA free water) was zero.

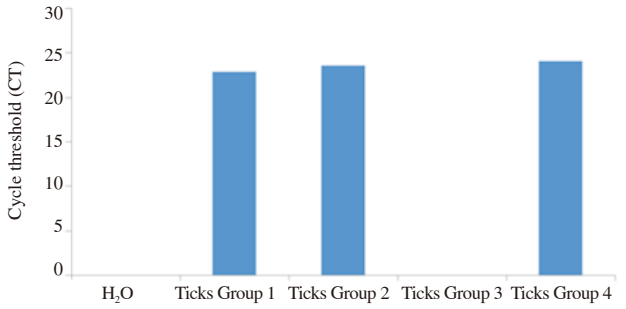


**Figure 1.** SINBV-positive ticks groups detected by RT- PCR.

### 3.2. Screening of CHIKV in the three groups of ticks

As represented in Figure 2, CHIKV was detected inside all the species of ticks except Group 3 (*B. kdhlsi*). CT value for the positive samples was 28. CT value for the negative control (RNA free water) was zero.

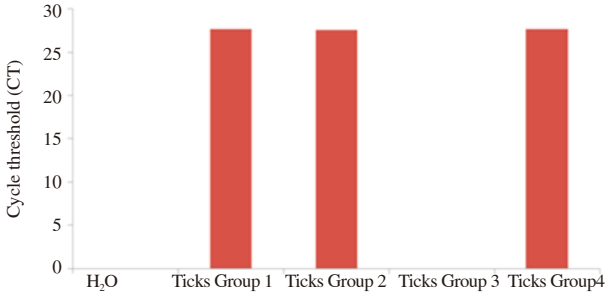


**Figure 2.** CHIKV-positive ticks groups detected by RT-PCR.

### 3.3. Screening of CCHFV in the three groups of ticks

CCHFV was detected inside all the species of ticks except Group

**Table 2**
Primers (synthetic genes) preparation.

| No. | Oligo name | Sequence (5"->3) | Vol for 100 pmol/μL | Vol for 100 pmol/μL |
|---|---|---|---|---|
| 1 | RVFV 233F | (AAGGCAAAGCAACTGTGGAG) 20 | 187 | 187 |
| 2 | RVFV 388R | (TGAGTGGCTTCCTGTCACTG) 20 | 207 | 207 |
| 3 | CHIK E1 FW | (CATGCAAAACAGAATTTGC) 19 | 200 | 200 |
| 4 | CHIK E1 RV | (TAGGCAGTTACAGTGATG) 18 | 187 | 187 |
| 5 | CCHFV (S-gene) fw | (AGGTTTCCGTGTCAATGCAA ) 21 | 212 | 212 |
| 6 | CCHFV(S-gene) rv | (TTGACAAACTCCCTGCACGAGT) 22 | 231 | 231 |
| 7 | SINV FW | (ACAAGATCTTCTTTGAGTAGTCCAGC) 2 | 365 | 365 |
| 8 | SINF RV | SINF RV | 201 | 201 |
| 9 | PANHANTA-F2 | (TGCWGATGZACRAAATGGTC) 21 | 280 | 280 |
| 10 | PANHANTA-R2 | (GCATCATCWGARTGATGZGCAA) 22 | 199 | 199 |

3 (*B. kdhlsi*) (Figure 3). CT values for the positive samples were 27, 28 and 29 for ticks Groups 1, 2, and 4, respectively. CT value for the negative control (RNA free water) was zero.

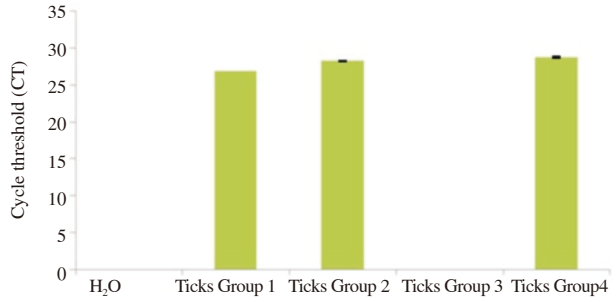


**Figure 3.** CCHFV-positive ticks groups detected by RT-PCR.

### *3.4. Screening of HANTV in the three groups of ticks*

HANTV was detected inside all the species of ticks except Group 3 (*B. kdhlsi*) (Figure 4). CT value for the positive samples was 36, 38 and 37 for ticks Groups 1, 2, and 4, respectively. CT value for the negative control (RNA free water) was zero.

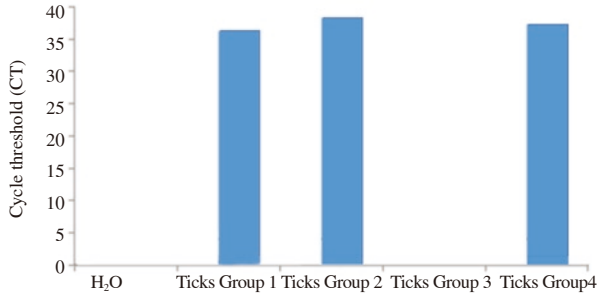


**Figure 4.** HANTV-positive ticks groups detected by RT-PCR.

### *3.5. Screening of RVFV in the three groups of ticks*

Figure 5 shows that RVFV was detected inside all the species of ticks except Group 3 (*B. kdhlsi*). CT value for the positive samples was 23, 24 and 25 for Groups 1, 2 and 4 respectively. CT value for the negative control (RNA free water) was zero.

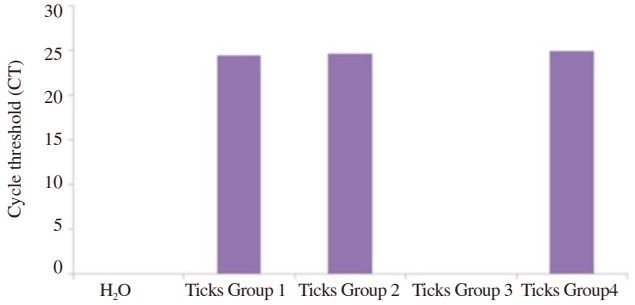


**Figure 5.** RVFV-positive ticks groups detected by RT-PCR.

### *3.6. Screening of ALKV in the three groups of ticks*

Figure 6 demonstrates that ALKV was not detected inside any of the species of ticks except Group 4 (*H. dromedarii*) which was positive to the virus. CT value for the positive samples was 25. CT value for the negative control (RNA free water) was zero.

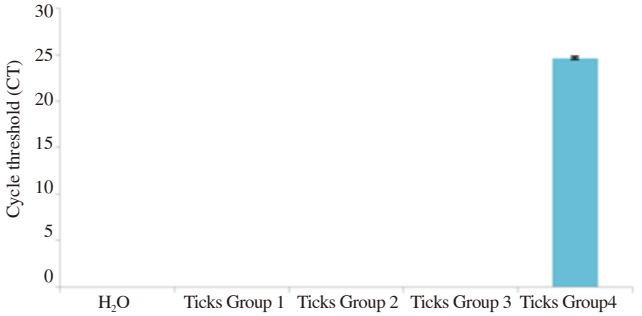


**Figure 6.** ALKV-positive ticks groups detected by RT-PCR.

## 4. Discussions

Ticks (Acari: Ixodidae) are important vectors/reservoirs of more than one arboviral diseases such as Crimean Congo hemorrhagic fever virus (CCHFV). They significantly contribute in the survival of the virus in nature. The parasitic behavior of ticks during their all life stages exhibits excessive blood feeding on hosts[22,23]. Humans may be infected via a tick bite whenever a normal cycle of virus-tick-nonhuman vertebrates is interrupted by the undesirable presence of human (such as herdsmen, tourists, veterinarian researchers) in the nature cycle. Domestic animals are known to have susceptibility to CCHF virus; however, there is no evidence that any symptomatic diseases develop[24,25]. A range of disease from mild to severe clinical illness occurs in human. In severe cases, petechial rash on the trunk and limbs, besides the appearance of hemorrhage from the body cavities are among the common clinical symptoms of the disease[26,27]. The approximate mortality rate of 22% has been reported in CCHF cases infected by a tick bite[28].

Arboviruses constitute a large proportion of the ticks-borne pathogens. In this study, certain viruses have been screened inside whole specimens of ticks collected from stockyard areas 50 km east and west of Riyadh, Capital of Saudi Arabia. Results of this study may reflect the diets of ticks[29].

RT-PCR was used for screening the viruses in the four ticks species because it has completely revolutionized the detection of RNA and DNA viruses. RT-PCR is valuable as a confirmatory test and it is a rapid technique characterized by high sensitivity and specificity. It is, a more sophisticated technique, requires infrastructural support, is expensive but nevertheless, one cannot discount its utilitarian advantages which are many compared to the existing conventional diagnostic methods[20].

Although it remains unclear whether the viruses described in this study are actually transmitted by ticks, the detection of CCHFV, RVFV, CHIKV, SINDBIS, and AlKV using RT-PCR from some species of ticks supports the hypothesis of arboviruses replication in at least three of the four tick species and coincides with previous studies conducted in Asia[26,30,31].

Findings of this study are in agree with results of a previous study conducted in 2007 in the KSA which documented the presence of certain viruses inside some species of ticks collected from Al-Qasim, Jazan, and Riyadh areas[32]. Al-Khalifa *et al.* isolated Sindbis from *H. dromedarii* and *H. impeltatum*, Chick Ross and Kadam from *H. dromedarii*[32]. Kadam virus has also been isolated from *H. anatolicum*. Dhori virus has been isolated from *H. impeltatum* and *H. schulzei*. Other alpha viruses have been isolated from *H. dromedarii*, *H. impeltatum*, *H. anatolicum*, and *Rhipicephalus sanguineus*.

Early in 1990, researchers in the western region of Saudi Arabia

isolated hemorrhagic fever viruses from some species of ticks. High rate of tick infestation (97%) was reported in camels where *H. dromedarii* was the commonest tick (70%).

In our study, we cannot exclude the possibility that the viruses we identified came from common prey. To understand the distribution and host specificity of these viruses and the association of these viruses to humans, further epidemiological studies are required.

Considering the influence of some biases including limited geography and the small size of the population, the host ranges of the identified viruses remain to be determined. Phylogenic studies to investigate the relationship of the detected viruses to human viruses were not conducted but they are essential.

The detection of emerging zoonotic agents from ticks' in a rural community in the KSA highlights the potential risk of human infection that may be caused by these pathogens.

## Conflict of interest statement

We declare that we have no conflict of interest.

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