Serologic evidence of exposure to Rift Valley fever virus detected in Tunisia

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Abstract

Rift Valley fever virus (RVFv) is capable of causing dramatic outbreaks amongst economically important animal species and is capable of causing severe symptoms and mortality in humans. RVFv is known to circulate widely throughout East Africa; serologic evidence of exposure has also been found in some northern African countries, including Mauritania. This study aimed to ascertain whether RVFv is circulating in regions beyond its known geographic range. Samples from febrile patients ($n = 181$) and nonfebrile healthy agricultural and slaughterhouse workers ($n = 38$) were collected during the summer of 2014 and surveyed for exposure to RVFv by both serologic tests and PCR. Of the 219 samples tested, 7.8% of nonfebrile participants showed immunoglobulin G reactivity to RVFv nucleoprotein and 8.3% of febrile patients showed immunoglobulin M reactivity, with the latter samples indicating recent exposure to the virus. Our results suggest an active circulation of RVFv and evidence of human exposure in the population of Tunisia.
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Introduction

Rift Valley fever virus (RVFv) is an arthropod-borne *Phlebovirus* in the family *Bunyaviridae*, with widespread epidemiology throughout sub-Saharan African regions first reported in the early 1900s by a veterinary surgeon in Kenya [1]. The virus is maintained and transmitted by mosquitoes, mainly *Aedes* and *Culex* species [1,2]. Though the virus has been shown to be maintained in mosquito populations through vertical and horizontal transmission [3], there has also been evidence of its maintenance in mammalian reservoirs, and there remains the possibility that there are other uncharacterized wildlife reservoirs which allow transmission at the wildlife/domesticated livestock interface *via* mosquito [4].

The epidemiologic range of the virus has been increasing, with reported outbreaks in West Africa, Saudi Arabia, North Africa and southern Africa [5–9]. There is evidence that RVFv is capable of transmission through a varied repertoire of diverse anthropophilic mosquito species, primarily *Aedes* and *Culex* species [10]. Indications of increasing diversity of vector competence may lead to rapid expansions in the future, which may pose a risk to countries geographically farther from traditional areas considered endemic [11].

Increasing circulation of RVFv in competent mosquito populations is a common observation in endemic areas after increased rainfall; it is usually followed by increased transmission to domesticated animals, where it is capable of causing severe animal health problems such as abortion [11–13]. This

can result in a significant economic burden on the affected communities, particularly in arid areas that rely on geographically limited agriculture. An increased prevalence in human disease is also observed, particularly amongst agricultural workers, who are at a higher risk of exposure *via* mosquito bite, and abattoir workers or butchers, who are at risk of exposure *via* contaminated blood when preparing meat from infected animals [13]. Exposure *via* the bodily fluids of infected animals and also *via* aerosolization of the virus during butchering has been previously suggested as a major route of exposure for high-risk groups [14] and may put an individual at higher risk of developing more severe clinical symptoms, such as haemorrhagic manifestations due to exposure to higher viral titres.

Rift Valley fever outbreaks are regularly reported in East Africa and have also been reported in Mauritania [8]. One recent study identified Tunisia as a high-risk country; the authors inferred that environmental conditions and the presence of the vector meant that an epizootic occurrence was possible if the virus was present—a constant and considerable risk throughout the year, with a particularly high probability in July, after the wettest months [15]. Previous studies looking for serologic evidence of exposure to Rift Valley fever in 2006–2007 found no positivity in the studied population [16]. Indeed, as a result of anthropogenic influences such as irrigation and well drilling, surface water levels in several arid regions have increased over the years, allowing the emergence of vector-borne diseases due to the wider distribution of competent mosquitos throughout the country [17], thus increasing potential risk since previous studies were performed in both human and animal populations. On the basis of mathematical modelling, Tunisia is considered at risk for RVFv [15]. In this study, we performed a seroepidemiologic survey to demonstrate circulation of RVFv in Tunisia.

Materials and Methods

Study sites

Tunisia covers a wide climatic range, from a Mediterranean climate, with its rainy winter, in the north to a Saharan climate in the south (Fig. 1). The northern part of the country is separated from the south by the Tunisian Ridge, a range of hills running northeast to southwest for approximately 220 km, marking the climatic boundary between the Mediterranean Sea to the north and the dry steppes of central Tunisia. Between the northern slopes of the Tunisian Ridge and the chains of hills bounding it on the south are extensive plateaus, called the High Tell. The Sahara is separated from the central steppe land by a series of salted areas called chotts.

Sample collection

Samples were collected from patients seeking care at hospitals who had reported unexplained acute fever (*n* = 181). Samples were also actively taken from abattoir workers (*n* = 38) in order to survey evidence of previous exposure amongst groups designated to be at high risk of infection. Most participants originated from the governorates of Sousse, Sfax and Mahdia (Fig. 1), and samples were collected during the summer of 2014. All participants were asked for history of tick and/or mosquito bites. Samples were collected by Vacutainer, and plasma was separated, frozen at −20°C and transported to Public Health England Porton Down, UK, for analysis. The study was conducted after receipt of ethical approval from the ethical committee (HHS-IRB 00008931; Farhat Hached University Hospital, protocol reference date 8 April 2013).

RNA extraction and PCR

Nucleic acid extraction was performed using a QiaAmp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Purified RNA was stored at −20°C until required. Amplification was performed using a previously published assay [18] as follows: forward: 5′-AAAGGAACAATGGACTCT GGTCA-3′; reverse: 5′-CACTTCTTACTACCATGTCCTC-CAAT-3′, probe 5′-6FAM AAAGCTTTGATATCTCTCAGTG CCCCAA BHQ1-3′. Reverse transcriptase (RT)-PCR assays were conducted in one step using the Superscript III quantitative real-time PCR (qRT-PCR) kit (Life Technologies) according to the manufacturer's instructions with cycling conditions of 50°C for 10 minutes and 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 40 seconds, followed by holding at 40°C for 2 minutes. Data acquisition was performed using the ABI 7500 Real-Time PCR machine (Applied Biosystems) with 45 analysed cycles with a threshold of 0.05 and data analysed using the ABI 7500 on-board software.

Indirect immunofluorescence assay

Sera were tested for the presence of antibodies reactive to RVFv using commercially available indirect immunofluorescence testing kits (Euroimmun). All serum samples were tested for the presence of RVFv by qRT-PCR before serologic testing under Containment Level 2 conditions. In brief, sera samples were diluted 1/100 and incubated on irradiated/fixed RVFv infected and noninfected Vero cells for 60 minutes at room temperature before washing five times for 5 minutes in sample buffer including 0.1% Tween 20. Antibodies binding to the infected cells were detected and measured through a secondary antibody labelled with fluorescein isothiocyanate; 1/100 was set as the minimum cutoff. For immunoglobulin (Ig) M measurement, the process was modified by initial dilution of sera in EUROSORB IgG removal
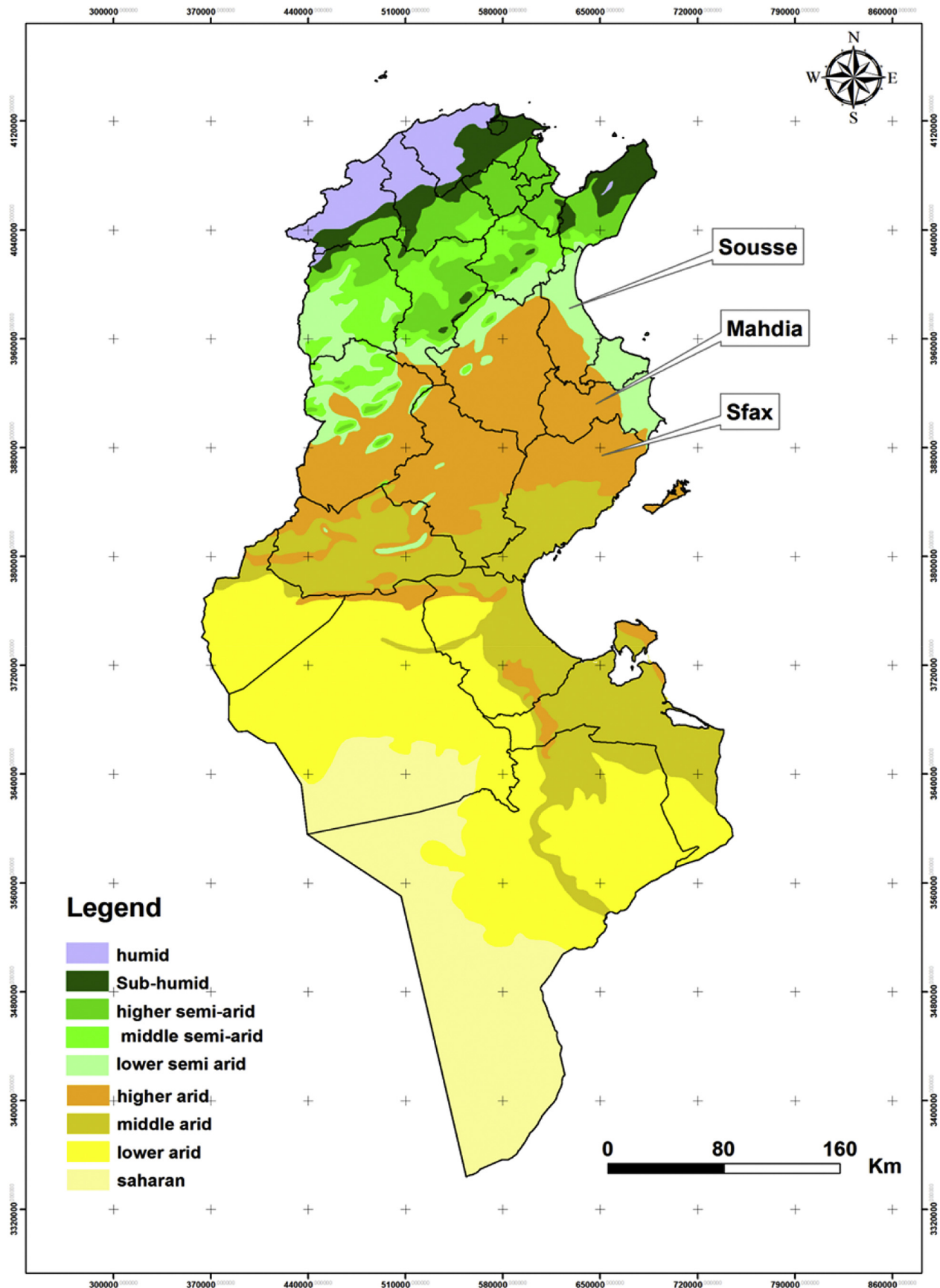
FIG. 1. Bioclimatic map of Tunisia showing sampling sites, with governorates from which samples were derived for this study highlighted.

reagent (Euroimmun) before commencing the protocol as before. Slides were mounted in mounting media and viewed under a fluorescent microscope at excitation/emission peaks of 495/517 nm, respectively. Positive and negative controls provided with the commercial kit were tested to provide a benchmark to which samples were compared.

Results

Samples collected from patients with acute fever were initially tested according to local protocols for serologic reactivity to *Brucella, Rickettsia* and West Nile virus and the presence of these agents through blood and urine cultures. All samples in this study were shown to be negative during this testing phase and were sent to Public Health England Porton Down to be analysed for Rift Valley fever by both molecular and serologic testing.

In total 219 samples were tested by qRT-PCR and indirect immunofluorescence to ascertain the level of exposure in the study population. Commercial immunofluorescence controls were tested to provide a benchmark to which samples could be compared. The commercial immunofluorescence assay test kit utilized in this study is European Community (CE) marked as a diagnostic kit and has been validated in house for diagnostic use

against a panel of positive and negative samples. The presence of RVFv was not detected by qRT-PCR in either cohort; however, 7.8% of the tested nonfebrile cohort showed IgG serologic reactivity (*n* = 3) to RVFv at the screening dilution, a reaction shown to be repeatable on retesting (Fig. 2). Furthermore, 8.3% of febrile patients were found to have circulating IgM reactive to RVFv (*n* = 15). Samples which tested positive by IgG and IgM were titrated to 1/10 000 dilutions in phosphate-buffered saline to evaluate the abundance of reactive antibody.

The 18 serologically reactive samples were derived from patients and participants in the governorates of Sousse, Mahdia and Sfax, areas highlighted in the bioclimatic map comprising Fig. 1. Fifteen samples drawn from febrile patients showed only IgM reactivity. In contrast, three seropositive samples drawn from afebrile farmers and abattoir workers showed only IgG reactivity, with IgM absent (Table 1). Clinical details were taken for all samples collected during this study, in line with normal clinical practice in Tunisia, including travel history.

Two samples were titrated beyond the screening dilution (Fig. 3). Sample 48 showed some fluorescence at 1/1000 dilution; however, on retest, it failed to titrate beyond 1/100. Sample 42 titrated beyond this, to 1/10 000, making this the strongest IgM reaction detected in this study.
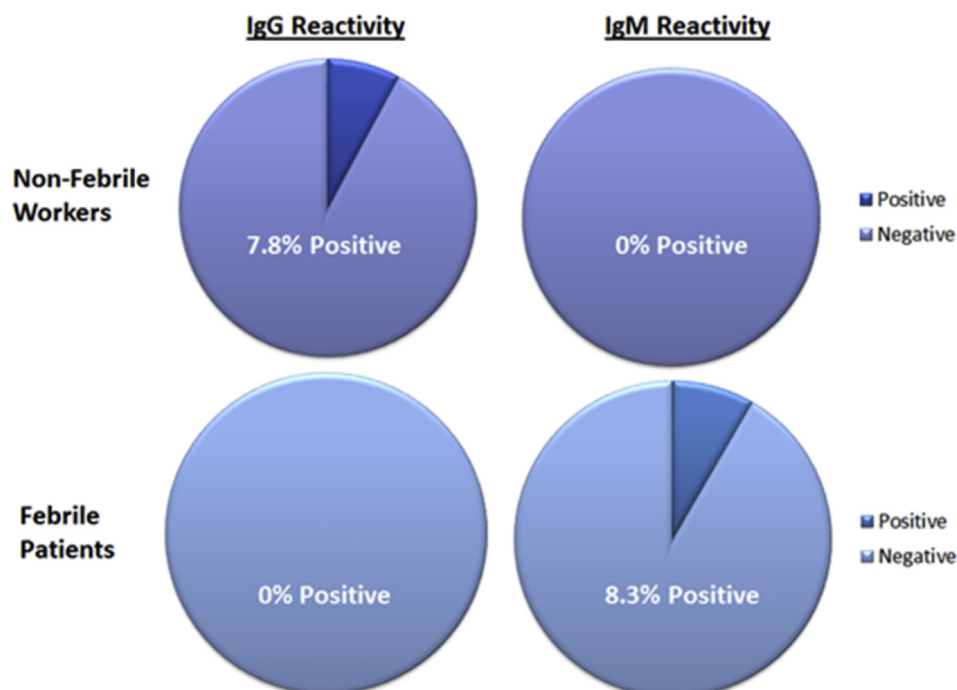

FIG. 2. Representation of percentage of immunoglobulin (Ig) G−positive and IgM-positive samples detected in febrile patient cohort collected at presentation to hospital, and nonfebrile worker cohort collected actively from volunteers in rural areas. Data show similar levels of IgG positivity amongst nonfebrile patients compared to IgM in febrile patients, 7.8% IgG and 8.3% IgM.

TABLE 1. **Positive samples, cohort and associated results**

Sample ID	Type	IgG	IgM	Confirmation	PCR	Outcome	Origin
TUN/008	Farmer Sera	1:100	Negative	IgG Positive	Negative	RVF IgG Positive	Sfax
TUN/026	Farmer Sera	1:100	Negative	IgG Positive	Negative	RVF IgG Positive	Sousse
TUN/028	Farmer Sera	1:100	Negative	IgG Positive	Negative	RVF IgG Positive	Sousse
TUN/042	Febrile Patient	Negative	1:10000	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/048	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/053	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sfax
TUN/078	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sfax
TUN/083	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/086	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/089	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/094	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sfax
TUN/102	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Mahdia
TUN/107	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/120	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/123	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/129	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/135	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse
TUN/188	Febrile Patient	Negative	1:100	IgM Positive	Negative	RVF IgM Positive	Sousse

Discussion

Our results strongly suggest the presence of active RVFv circulation in Tunisia, confirming our expectation from a recent appraisal of North Africa for the relative risk of harbouring circulating RVFv that was recently undertaken [15]. This finding is not unexpected, with RVFv also being found in northwestern Africa [8,11]. Tunisia is notable for its close proximity to European Mediterranean countries and the migratory patterns of birds through the country leading northwards into Europe [11]. There is also evidence of RVFv exposure in bird populations [19], which are a potential zoonotic risk for vector-borne disease. For example, birds infested with *Hyalomma marginatum,* the principal vector of Crimean-Congo haemorrhagic fever virus, have been detected as far north as the United Kingdom

| 1/100 | 1/1000 | 1/10000 |

Sample 42

Sample 48

Negative Control
(Commercial Control)
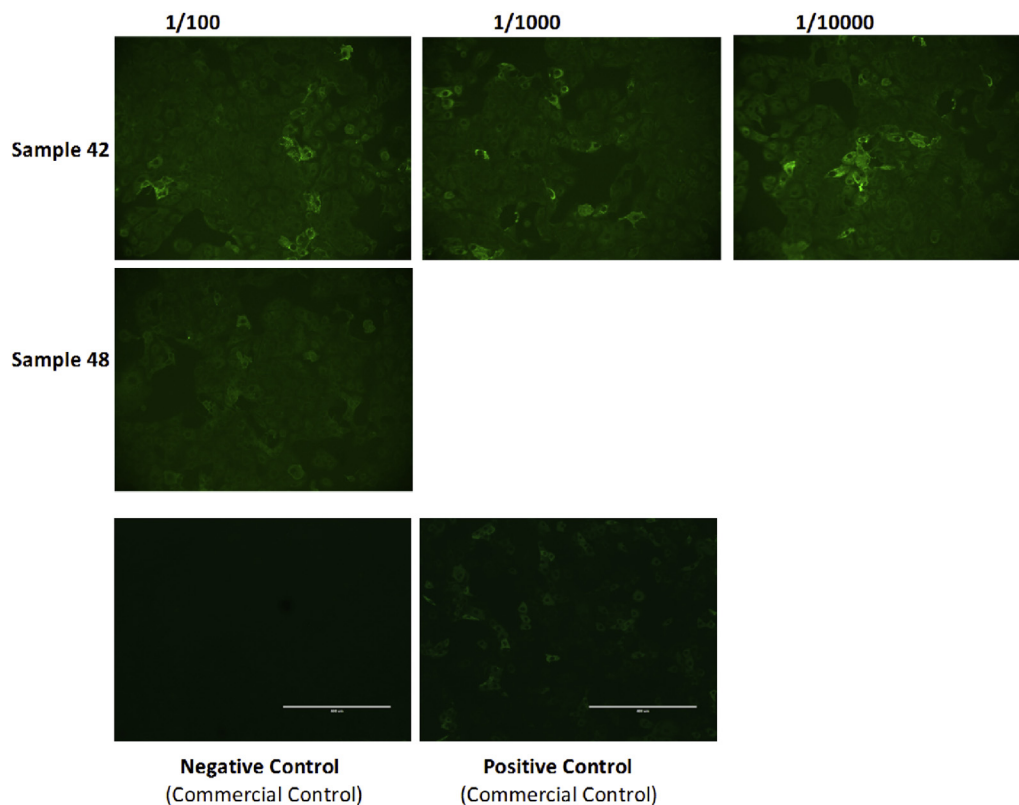
Positive Control
(Commercial Control)

FIG. 3. Immunofluorescence showing level of fluorescence across several titrations up to dilution 1/10 000 for sample 42 and 1/100 for sample 48. Images of positive and negative controls tested are also shown.

[20]. Accumulation of surface water and changing surface to-pology affect bird migratory routes and vector population size; both factors result in a high risk of an increase in prevalence of zoonotic disease [16]. The potential for cross-border transmission through the exchange of economically important animal species from neighbouring countries may also result in the importation of zoonotic pathogens at the wildlife—domesticated livestock interface. Circulation of West Nile virus in the region is recognized, and investigations have suggested an increasing prevalence which may be attributable to anthropogenic environmental changes [21]. These events illustrate the need for improved and comprehensive surveillance in areas where environmental change is recognized to track relative risk to human and animal health to arthropod-borne diseases such as RVFv and West Nile virus.

Rift Valley fever, while primarily being transmissible through the bite of the mosquito vector species, may also be transmitted through direct contact with blood. There are several anecdotal reports of veterinary workers engaged in animal necropsies being exposed to high levels of RVFv during the course of their duties [22], and surveillance activities have detected prevalence of reactive antibody and the virus itself in abattoir and slaughterhouse workers [7,14]. Critical to the control measures imposed during the outbreak in Saudi Arabia in 2000 was the education of agricultural workers to avoid close contact sick animals and consumption of diseased meat in order to reduce risk of spread through direct contact [9]. This risk may be substantially increased during certain religious festivals in Tunisia, where the slaughter of domestic ruminants by individual families disseminates the risk to a much wider population. It is notable that in our study no abattoir or agricultural workers sampled tested positive for IgM, which is indicative of recent exposure; instead, they only showed signs of previous historical exposure. However, this may be an artefact of our results resulting from the small numbers of samples collected from these populations.

Other countries perceived as being at high risk of RVFv outbreaks are neighbouring Algeria and Libya [15]. Travel and trade across the border are common and risk disease importation. The strong IgM response detected in the course of this study is indicative of a recent and strong reaction to exposure to RVFv or a serologically related virus. It is notable that there was a lack of IgG response seen in any sample from febrile patients, suggesting collection soon after exposure before detectable IgG appearing or nonspecific reactivity with a closely related *Phlebovirus*. Additionally, the lack of a detectable virus genome complicates confirmatory testing, something which may be attributed to sampling after the viraemic stage of disease has occurred [23]. Detailed medical records were collected for all patients and high-risk participants in this study. Of the 18 seroreactive cases, records indicated that none had travelled abroad in the 2 months preceding sampling. Additionally there was no identifiable link between seropositive abattoir workers and the importation of livestock from abroad for slaughter, as the peak of importation occurred after the sampling period. Even so, the possibility remains of a case introduced through travel or movement of livestock in a country with optimal conditions for an outbreak.

This study provides evidence to support more detailed and thorough surveillance activities for RVFv in order to conclusively demonstrate whether these cases are autochthonous findings and to survey its prevalence in the region's indigenous mosquito populations.

Conclusion

Because of the potential for RVFv to create a devastating agricultural burden, its control becomes critically important when it is discovered to be circulating in a region. A campaign of active surveillance, prevention strategies, increased research and vaccination of livestock all play a role in the control of RVFv and serve not only to reduce the potential economic burden but also, by interrupting the virus transmission amongst livestock, to interrupt transmission across the human—livestock interface, thus significantly reducing the risk to public health. Active collaboration between public health and animal health institutions of Tunisia is critical, as is increasing cohesive planning with the institutes of neighbouring countries. Though our study is limited in scope, we hope that it provides a foundation for further surveillance and investigation of RVFv in Tunisia.

Acknowledgements

Conflict of Interest

None declared.

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