Seroprevalence and Molecular Detection of Rift Valley Fever in Sheep, Gezira state, Sudan (2017-2019)

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

ABSTRACT

Aims: Livestock is the largest subsector of the Sudanese domestic economy and is a growing contributor to exports. Rift Valley fever (RVF) is an economically important arthropod-borne virus disease in Africa, primarily affecting sheep, goats, and cattle. RVF causes paramount loss in sheep industry in Sudan. The objective of this study was to detect RVF in sheep.

Methodology: Five hundred and fifty-six sheep blood Serum from inoculated animals was collected from six study areas in the Gezira (Sudan) was evaluated for the presence of neutralizing antibodies using the diagnostic kit (ID. Vet.) for detecting antibodies directly against the RVFV nucleoprotein (NP) in serum and Reverse transcription (RT)-Polymerase Chain Reaction (PCR) protocol was used as standard test to diagnose and confirm RVFV among studied animals.

Results: The ELISA test indicated that the highest Seroprevalence was found at Shikaira (38%), while samples from Albasabeir, Wad alnaem and Umalqura tested positive for antibodies against
RVFV by 15%, 10% and 9% respectively. The lowest seroprevalence found at Wad anor and Shabona (5% and 2%). The overall seroprevalence was 14.2% in the study sites, there is no significant difference among the six study sites with respect to seroprevalence. The Rt-PCR provided more sensitive and specific detection of RVFV in serum samples (sensitivity= 95% specificity = 80%).

**Conclusion:** The results from this study showed the presence of anti-RVFV antibodies in all study sites, suggesting that RVFV is actively circulating among sheep in Gezira state. The high seroprevalence of RVFV infection in Gezira state indicates its endemicity. Test validation using ROC analysis was able to distinguished between ELISA (actual class) and the PCR (predicted class) and the AUC = 0.79 which indicate that the PCR is gold standard test. Thus Rt-PCR used in the present study is reliable to detect precisely the M segment of RVFV and it is valuable for Wide area survey, among both sheep and Aedes spp. Targeting the prevailing genotypes of RVF-M segment, especially at high-risk areas and as a confirmatory test that should be considered by reference laboratories in the region.

**Keywords:** Rift valley fever; ELISA; Rt-PCR; Sudan.

1. INTRODUCTION

Livestock is the largest subsector of the Sudanese domestic economy and is a growing contributor to exports. In Sudan, the livestock export forms the basis for the foreign exchange earnings. Sudan is ranking 7th in sheep meat production among the top 10 producing countries in the world [1] Sudan exports live sheep and sheep mutton mainly to Saudi Arabia and other Gulf countries. According to FAO [2] the Sudan exported about 1568 tons of mutton, with the value of 8367 thousand USA dollars. Rift Valley fever (RVF) is an economically important arthropod-borne virus disease in Africa, primarily affecting sheep, goats, and cattle [3]. It is caused by Rift Valley Fever virus (RVFV), genus Phlebovirus, which is a member of Bunyaviridae. Sheep are extremely susceptible to RVFV infection with 70–100% mortalities [3]. RVF causes paramount loss in sheep industry in Sudan.

The RVFV was first isolated in 1930 in the Rift Valley Province, Kenya, associated with large epidemic in domestic ruminants and human [4]. It has been widely distributed in sub-Saharan Africa, with epizootic activity affecting animals in Kenya, Tanzania, Zambia, and Uganda [5]. The first report of RVF occurrence in the Sudan was by [6] from human sera derived from Southern Sudan.

The RVFV is associated with mosquitoes. It is transmitted to live stock and human by the bites of infected mosquitoes of the genus Aedes and by exposure to tissues or blood of infected animals [7]. In East and South Africa, the main vector known to transmit RVF is the Aedes mosquito, including Aedescircumluteeolus, and Aedesmicintoshi. More recently, a group of entomologists have identified different vectors that have been responsible for RVF virus transmission in Western Africa, including Aedesvexans, Aedesochraceus [8] Aedesdalzieland Culex mosquitoes [9]. Infected mosquito eggs can survive for years in the soil, once proper environmental conditions exist, such as heavy rainfall, which fills natural depressions in the earth creating stagnant water (dambos) the hardy eggs hatch, and new, infected mosquitoes start a new epizootic cycle [10].

1.1 Virus Isolation

The virus could be isolated from blood, but that should be within the first 8 days of the infection to detect the virus, otherwise the virus will disappear from the blood. Some efforts have been done in this direction to identify fatal human cases of RVF during outbreak periods. Some efforts have been shown by [11] to develop a diagnostic tool to be used in a field lab setting, particularly in the remote rural areas. This trial was used during Kenyan outbreak in 2006-2007 and the results were promising. Virus could also be isolated in hamsters or adult mice intraperitoneally. Moreover, different cell culture types could be used to isolate the virus [12].

1.2 Viral Genetic Components Detection

Recently scientists have been able to detect the RVFV genetic material. Reverse Transcriptase PCR was used for this purpose and to analyze the virus phylogentic which is very useful to detect the viral RNA sequencing, to study the strain type of RVFV and could identify the source
or region where the virus came from [13]. The virus is well known as an enveloped tripartite single stranded RNA genome. According to [14] and [15] this tripartite genome named (due to its size) as large (L), Medium (M) and Small (S) segment. The L encodes the L protein and the RNA polymerase. The M encodes the virion glycoproteins GI and G2 which are involved in attachment to the host cell through unidentified receptors and elicit neutralizing antibodies. While the S encodes the nucleocapsid (N) protein, which encapsulates each of the genome RNA segments to form ribonucleoprotein complex (RNP). The RNP is the template for the viral RNA polymerase [16]. RVF virus may withstand environmental temperatures of 25 – 30 °C for approximately 80 minutes. Exposure to contaminated body fluids of infected animals stills the major mode of transmission. This is a particular hazard to those exposed to contaminated blood and abortion products in the abattoir, and in areas of food preparation in endemic regions [17]. Compared with other viral haemorrhagic diseases, such as Ebola and Marburg disease, the RVF has a low mortality.

RVFV infects many animals; including sheep, goats, cattle, camels, and Asian water Buffaloes. The symptoms of the disease, in the different animal are similar, and include fever, hepatitis, and abortion. Additionally, sheep and cattle, once infected, become highly viraemic, thus allowing both early and highly effective infection of the vector and hence efficient disease transmission. In less severe cases, infected animals may present with injected conjunctiva, nasal discharge, weakness and decreased milk production. In humans, the virus is hepatotropic, and shows intrahepatic viral replication leads to massive and patchy hepatic necrosis [18].

1.3 Detection of the RVFV RNA in Mosquitoes

Investigation of RVFV in mosquitoes as the main carrier for the disease during the Madagascar epizootic outbreak in 1991 and Mauritanian in 1998 outbreak was unable to find the virus [19]. But this changed when [20] could detect RVFV RNA using RT-PCR method in Egypt in two different areas. Similarly in Sudan the virus was isolated from the mosquitoes during 2007 outbreak [21]. These results were promising and could help to identify the circulation of the virus during interepidemic or cryptic cycle as early as possible. This could aid in the allocation of the resources to the high-risk regions at the right time. The discrepancy in finding the virus in mosquitoes might be attributable to the diagnostic techniques that were used. RVFV can be detected by classical virological methods including histopathology, virus isolation [22], detection of antigen and antibodies [23,24,25] and molecular assays [26,27]. The RVFV infection can be further confirmed by immune staining using specific antibodies [28,29]. However, technical expertise is needed for this approach. Currently, a combination of serological and molecular methods is preferred in laboratory diagnosis. Enzyme-linked immune sorbent assay (ELISA) is commonly used to confirm RVFV infection. Several assays are available for the detection of both RVFV antigens and anti-RVFV antibodies [30,31]. Molecular methods are rapid to detect the viral RNA. Therefore, a number of highly sensitive nucleic acid based molecular tests have been developed and proven useful during RVF outbreaks [32]. In Areas of endemic disease winds will disseminate infected mosquitoes, as well as infected eggs, which go on to remain dormant in previously virgin soil for several years [33].

1.4 Rift Valley Fever Virus in Sudan

Before 1977 RVF was geographically limited to sub-Saharan Africa causing sporadic epizootics in animals and accidental contact infection in humans, which was rarely fatal. However, in 1977 and again in 1978 extensive epizootics of RVF with unprecedented human disease and fatalities occurred in Egypt [34-36]. It was following its dramatic appearance in Egypt that RVF came into prominence as a potential international disease problem.

In the Sudan serological evidence of the existence of RVF was available in 1936, when a limited survey including parts of East and Central Africa revealed neutralizing antibodies in 6-7 % of 164 human sera derived from Southern Sudan [37]. The disease was of little or no concern until 1973, when RVF virus was identified for the first time as the cause of an extensive epizootic involving sheep, cattle and less severely goats in Kosti District on the White Nile, some 200 km south of Khartoum [37]. The disease emerged again in 1976 after rainy summer in a farm in north of Khartoum where new cattle from White Nile state added to the existed herd suggested that the virus was carried to Khartoum from the central of Sudan where the virus was enzootic [37]. In addition, after the two epizootic outbreaks of RVF in Sudan 1973 and 1976, the human
illness that associated with outbreaks promoted some researchers to conduct serological study among Sudanese human population to show to what extent the RVFV circulated in the country. In 1980, 846 human sera collected from Hospital of Khartoum and military were tested to the antibodies towards RVFV. The result showed that 3.2% of them had antibody of RVF but unfortunately the geographic and age cluster were not available for those samples but in general the sera of 138 male and females belong to patients from Khartoum and Gazira states and the rest of the samples were from military recruits who belong to many regions in Sudan [38]. During an unusual heavy rainfall season, in October 2007, a serological diagnosis was conducted using Rift Valley Fever (RVF) IgM Enzyme Linked- Immuno Sorbent Assay (ELISA) for detection IgM immunoglobulin in suspected outbreak of hemorrhagic fever in The White Nile, Gezira, Sinnar and Blue Nile States of the Sudan. A total of 323 blood samples were collected from cattle, sheep and goats and analysed. The overall percentages of IgM antibodies in the three species of animals in the study areas were; 53.6% for caprine, 48.3% for ovine and 21.3% for bovine. Gazira State showed highest morbidity (50%) followed by The White Nile (24.6%) and then Sinnar and Blue Nile states, (21%). Bovine showed low infection whereas caprine morbidity was high followed by the ovine. The overall positive percentage of all animal examined (cattle, sheep and goats) from the study areas was 35.6% [39]. RVF becomes a central issue in a new global economy of world trade [40]. It has hard impact on the African countries whose economies are based on animal resources. It will be difficult for them to compete. Sudan is a good example for this severe economic impact as it is a big producer of animal resources as well as one of the main exporters of live animals to many of the Middle East Countries and mainly Saudi Arabia [41]. In 2007 [42] reported that there is a new outbreak of RVF in animals and humans lasted approximately five months from September 2007 to January 2008. It seems that the virus has circulated endemicity in Sudan since the first outbreaks before 30 years as reported in 1973 [43], this gave evidence that the virus could persist for many decades without sign of outbreak; however, it could emerge when the conditions are appropriate [44]. The main objectives of this study are to diagnose and characterize the Rift Valley Fever Virus in sheep in high-risk area and to estimate prevalence of RVFV in sera of sheep by using ELISA and to confirm RVFV by Rt-PCR methods.

2. MATERIALS AND METHODS

2.1 Study Design

A prospective descriptive study was conducted in six different sites in Gezira State, in the period 2017-2019.

2.2 Study Area

This study was conducted in Gezira State (Fig. 1) which is located in the central region of Sudan and has an area of 23,737 km² and an estimated population of approximately 3.2 million people according to the 2008 national census (CBS, 2008). The State is dissected by the Blue Nile River with Wad Madani town as its capital. The average daily temperature is 32°C during summer (April, May, June to middle of July) and 22°C during winter (November to the end of January). The rainy season starts in July and ends by October, with an estimated annual rainfall of 140 – 225 mm. The relative humidity is 38% in autumn (end of July to the middle of October) and 30% during winter.

2.3 Study Population

Sudan’s vast multitudes of domestic animals represent a large proportion of all African Livestock. The country ranks first to third among all African countries in the number of cattle, sheep, goats and camels, third in the number of poultry and fifth in the number of donkeys [45]. In 2009 Sudan official sources estimated cattle numbers at 41.653 million, sheep 51.555 million, goats 43.270 million and camels 4.521 million. In addition to these there were also 7.515 million donkeys and 784 thousand horses [46, 47].

2.4 Blood and Serum Samples

Five hundred and fifty-six sheep blood samples were collected from six sites across the study area; these sites represented the south, east, west and north of Gezira state. Each blood sample was obtained with a separate needle and vacuum tube containing anticoagulant. One set of samples was immediately mixed with AVL (Qiagen, Valencia, CA), a lysis buffer for nucleic acid purification from the Qiagen QiaAmp viral RNA extraction kit (Qiagen), so as to inactivate RNA viruses in samples [48] and resolving the issue of infectious samples. All samples were
carried out in ice bags in the field and then stored at -80°C.

2.5 Serological Test by ELISA

Serum from sheep blood samples was evaluated for the presence of neutralizing antibodies using the diagnostic kit (ID. Vet.) for detecting antibodies directly against the RVFV nucleoprotein (NP) in serum.

2.6 Sample Preparation

A 96-plate containing the test and control samples was prepared before transferring them into an ELISA microplate using a multi-channel pipette in order to avoid differences in incubation times between samples.

2.6.1 Wash solution preparation

The wash concentrate (20x) was brought to room temperature (21±5°C) and was mixed thoroughly to ensure that the Wash concentrate is completely solubilized. Then the Wash solution (1x) was diluted by the Wash concentrate (20x) in distilled/deionized water.

2.7 Description and Principles

The Well was coated with a recombinant Rift Valley Fever nucleoprotein. Samples were tested and the controls were added to the micro wells. Anti-nucleoprotein antibodies, if present, form an antibody- antigen complex which masks the nucleoprotein epitopes, then an antinucleoprotein peroxidase (HRP) conjugate was added to the micro wells to fixe the remaining nucleoprotein epitopes, forming an antigen-conjugate- HRP complex. After one hr. of incubation after washing in order to eliminate the excess conjugate, the substrate solution (TMB) is added. The resulting coloration depends on the quantity of specific antibodies presenting the sample to the test:

- In the absence of antibodies, a blue solution appears which becomes yellow after addition of the stop solution.
- In the presence of antibodies, no coloration appears.

Fig. 1. Map of Sudan showing Gezira states and study sites
2.8 Testing Procedure

The following reagents were added to the plate; 50 µl of Dilution Buffer-19 to each well, 50 µl of the Positive control to wells A1 and B1, 50 µl of the Negative control to well C1 and D1, 50 µl of each sample to be tested to the remaining wells. Then the plate was incubated for 1 hour ± 4 min at 37(±2 0C) and the plate was washed 3 times with approximately 300 µl of the washing solution. Drying of the wells was avoided between washings.

The Anti-RVF-NP Conjugate 1x was prepared by diluting the Anti-RVF- NP Po Conjugate 10x to 1/10 in Dilution Buffer 19. 100 µl of the Conjugate 1X was added to each well using the micropipette. Then the plate was incubated for 30 min ± 3 min at 21 0C (± 5 0C). Each well was washed 3 times with approximately 300 µl of the wash solution. Drying of the wells was avoided between washings, then 100 µl of the Substrate Solution was added to each well, then the plate was incubated for 15 min ± 2 min at 21 0C (± 5 0C) in the dark. 100 µl of the stop solution was added to each well in order to stop the reaction. The result read using the reader and optical density was recorded at 450nm.

2.9 Virus Isolation and RNA Extraction

2.9.1 RNA extraction

Viral RNAs were extracted from the serum samples using QIAamp viral RNA kit. About 140 µl of serum was added to 560 µl AVL buffer containing carrier RNA into 1.5 ml microcentrifuge tube and was mixed by pulse-vortexing for 15 seconds and then followed by incubation at room temperature for 10 minutes. About 560 µl of About 630 µl of the mixture was transferred to QIAamp spin column mounted on 2 ml collection tube and was centrifuged at 6000 x (8000rpm) for 1 minute. The column was transferred to another collection tube and the 630µl of the mixture was passed through it. The column was washed twice with 500 µl of washing buffers WB1, WB2, respectively. RNA was eluted by 60 µl buffer AVE equilibrated to room temperature. Viral RNAs and total nucleic acids extracted from serum samples were quantified using a spectrophotometer at 260 nm wavelength.

2.9.2 Reverse transcription (RT)-Polymerase Chain Reaction (PCR) protocol

Two primers specific for amplification of RVFV M segment were designed and they were produced a 342 bp Rt-PCR product. Below are the primers used:

| Primer | Sequence |
|--------|----------|
| RVF2   | 5’-CTG TCT GGC ACA GCA TTG AT-3’ |
| RVF    | 5’-CAC ATT GAA ACA CCC ACA CC-3’ |

A single tube RT-PCR amplification was carried out using One step Access RT-PCR system (QIAGEN, USA), stander of 50 µl reaction mixture contained in a final concentration 1x enzyme mix reaction buffer, 0.5 µl of 10Mm dNTPs mix 5.0 µl of 25 Mm MgCL, 1.0 µl of 5.0 Units enzyme mix, 2.0 µl of 20 picomole of each primer, 5.0 µl of target RNA was used. The total volume was completed to 50.0 µl using RNase free water.

The cycling program was consisted of a reverse transcription step at 50 0C for 30 min, a pre denaturation step at 95 0C for 15 min, this was followed by 40 cycles of denaturation at 94 0C for 1 min, annealing temperature at 56 0C for 30 seconds each, extension at 72 0C for 45 seconds. The reaction mixture in each Rt-PCR tube was then subjected to a final extension step at 72 0C for 10 minutes. All Rt-PCR amplifications were carried out at 50 ul volume per tube.

2.9.3 Preparation of agarose gel

The agarose gel was prepared by adding 1 gram of agarose powder to 100ul of Tris acetate buffer mixed together and dissolved by microwave, then ethidium bromide was added to the gel for staining, after cooling down the gel was poured in rack.

Following amplification, 12 ul from each Rt-PCR tube containing amplified product was loaded onto gels of 1.5% agarose and electrophoresed. The Rt-PCR products were visualized under UV light.

2.10 Data Analysis

Seroprevalence, with 95% confidence intervals, was calculated for each area and overall, taking into account the different sampling weights in each area. Seroprevalence was compared between areas, using the ANOVA test, also it was used to estimate the association of age, sex and locality with the outcome (seropositive to RVFV) while controlling for possible confounding.
For this purpose, age was categorized into four groups, >1, 1< 3, 3-5, >5.

2.10.1 Screening test

Screening test will be used to identify animals more likely to have a RVFV infection. There are two measures that are commonly used to evaluate the performance of screening tests: the sensitivity and specificity of the test. Sensitivity tests the ability to correctly identify animal carrying the virus (true positive rate), whereas specificity tests the ability of the test to correctly identify the animals without having the virus (true negative rate). Sensitivity and specificity test was calculated by the following equations:

Sensitivity% = No. of true positives/No. of true positive+ No. of false negative
Specificity%= No. of true negative/No. of true negatives+No. of false negatives
Likelihood ratio = No. of true negative/No. of true negative + No. of false negative

2.10.2 Receiver Operating Characteristic (ROC) curve analysis

To evaluate performance and accuracy of ELISA and RT-PCR to discriminate infected cases from normal cases ROC curve analysis [49] was used.

3. RESULTS AND DISCUSSION

3.1 Characteristics of Study Animals

Serum samples were randomly collected in a cross-sectional survey in 2017 from six sites namely; Shikaira, Wad anor, Wad alnaem, Shabona, Albasabeir and Umalquora which represent a number of sites reported as area infected by virus in 2007 and 2010 (Fig. 1). As shown in Table 1, total number of 556 animals were sampled and the animals were distributed according to gender, females represent 479 (86.2%) and males 77 (13.8%). The age was grouped in four categories about 62% of animal aged between 1 to 3 years, while 22.1% in age 3 to 5 years (Table 2). These animals represent four ecotypes of sheep that are normally raised by nomads in Savannah and semi-arid zones in Sudan; they are of Dobasi, Ashgar, Whatsich and Baladi ecotypes.

3.2 Detection of RVFV Using ELISA

ELISA is reported as the most sensitive test for the detection of border line levels of antibody to RVF, total of 556 serum samples from non-vaccinated sheep were subjected for detection of the antibodies against the virus infection using IgG ELISA-kit. The ELISA test indicated that the highest Seroprevalence was found at Shikaira (38%), while samples from Albasabeir, Wad alnaem and Umalquora tested positive for antibodies against RVFV by 15%, 10% and 9% respectively (Fig. 2). The lowest Seroprevalence found at Wad anor and Shabona (5% and 2%). The overall seroprevalence was 14.2% in the study sites. However, there is no significant difference among the six study sites with respect to seroprevalence. A similar result obtained by [50] who reported no significant difference among five districts in Mozambique with respect to RVFV seroprevalence.

As shown in Table 3, the females showed increased numbers of animals with respect to anti-RVFV IgG positivity than the males (Table 4). This trend of female susceptibility was supported by [50] and [51] in both goat and sheep.

| Area           | No. of samples | Gender       |
|----------------|----------------|--------------|
|                |                | Female       | Male        |
| Shikaira       | 37             | 26 (4.7%)    | 11 (1.97%)  |
| Wad anor       | 100            | 83 (14.9%)   | 17 (3%)     |
| Wad alnaem     | 101            | 85 (15.3%)   | 16 (2.9%)   |
| Shabona        | 99             | 86 (15.5%)   | 13 (2.3%)   |
| Albasabeir     | 119            | 111 (20%)    | 8 (1.4%)    |
| Umalquora      | 100            | 88 (15.8%)   | 12 (2.2%)   |
| Total          | 556            | 479          | 77          |
| Percentage     | 86.2           | 13.8         |             |
Table 2. The distribution of animals according to age

| Area          | No. of samples | Age Group | <1 | 1 < 3 | 3 - 5 | >5 |
|---------------|----------------|-----------|----|-------|-------|----|
| Shikaira      | 37             |           | 6(1.07%) | 13(2.33%) | 12(2.16%) | 6(1.07%) |
| Wad anor      | 100            |           | 12(2.15%) | 62(11.15%) | 24(4.31%) | 2(0.36%) |
| Wad alnaem    | 101            |           | 14(2.51%) | 64(11.51%) | 21(3.77%) | 2(0.36%) |
| Shabona       | 99             |           | 10(1.79%) | 63(11.33%) | 23(4.13%) | 3(0.54%) |
| Albasabeir    | 119            |           | 21(3.77%) | 79(14.21%) | 17(3.05%) | 2(0.36%) |
| Umalquora     | 100            |           | 10(1.79%) | 61(10.97%) | 26(4.67%) | 3(0.54%) |
| **Total**     | **556**        |           | 73 | 342   | 123   | 18 |
| **Percentage**|                |           | 13.1 | 61.5 | 22.1 | 3.2 |

The high infection rate at Shikaira site reported from this study, was due to many factors which include sand dunes in the area coupled with permanent water sources (stagnant water) and these create suitable conditions for vector (Aedes mosquitoes) for breeding, survival and development. These findings are strongly supported by other works done elsewhere [52,53].

High percentage of infection (33.5%) in old age (> 12 month) was reported [50] this result is in agreement with findings of this study (61.5%), usually the old females that constitute the bulk of our sample size are kept for breeding purposes thus could serve as reservoir for the virus and their movement increased the wide spread of the disease. Generally, the seroprevalence increased with age in this study similar to the result reported by [51] in sheep while [54] reported the same trends of age effect in camels. It is worth mentioning that in Sudan the husbandry of camel is usually accompanied by sheep breeding, for purpose of maximizing the profit from camel investment, a situation that may assist maintaining the transmission of RVFV between these highly susceptible two animal bread.

In our study the non-vaccinated samples recorded 14.2% this result in accordance with the findings of [51] who reported 14.9% however they reported 19.2% in vaccinated samples. There is no significant difference in prevalence between male and female sheep ($\chi^2= 0.699, P = 0.4031$), this result in accordance with that of [54] (who reported no significant difference between gender in sheep and camel as well as the study of [55] in cattle.

Table 3. Anti-RVF IgG seroprevalence among female sheep according to ages

| area          | <1 | 1 < 3 | 3 - 5 | >5  | %+ve by area |
|---------------|----|-------|-------|-----|--------------|
| Shikaira      | 0  | 5(19.23%) | 5(19.23%) | 1(3.84%) | 11(42%) |
| Wad anor      | 0  | 0 | 5(7.58%) | 0 | 5(6%) |
| Wad alnaem    | 1(2.78%) | 7(19.44%) | 0 | 0 | 8(9.4%) |
| Shabona       | 0 | 2(2.53%) | 0 | 0 | 2(2.3%) |
| Albasabeir    | 5(5.10%) | 11(11.22%) | 2(2.04%) | 0 | 18(16.21%) |
| Umalquora     | 2(2.29%) | 5(5.74%) | 2(2.29%) | 0 | 9(10.22%) |
| **Overall**   | 11.01% |

Table 4. Anti-RVF IgG seroprevalence among male sheep according to ages

| Area          | >1 | 1 - 3 | 3 - 5 | <5  | %+ve |
|---------------|----|-------|-------|-----|------|
| Shikaira      | 3(27.27%) | 1(9.09%) | 1(9.09%) | 0 | 5(45.45%) |
| Wad anor      | 0 | 0 | 0 | 0 | 0 |
| Wad alnaem    | 1(5.88%) | 1(5.88%) | 0 | 0 | 2(12.5%) |
| Shabona       | 0 | 0 | 0 | 0 | 0 |
| Albasabeir    | 0 | 0 | 0 | 0 | 0 |
| Umalquora     | 0 | 0 | 0 | 0 | 0 |
| **Overall**   | 9.01 |
Table 5. *Anti-RVF IgG seroprevalence* according to sheep gender

|        | +ve | -ve | $\chi^2$ | P. value |
|--------|-----|-----|---------|----------|
| Male   | 7   | 63  | 0.699   | 0.4031   |
| Female | 51  | 341 |         |          |

**Fig. 2. Prevalence of RVF among sheep in the study area using ELISA**

Table 6. Prevalence of RVFV infection by Rt-PCR test according to sex

| Area       | female (+VE) | % of infection |
|------------|---------------|----------------|
|            | <1            | 1 - 3          | 3 - 5         | >5            |
| Shikaira   | 0             | 1 (3.84%)      | 3 (11.53%)    | 1 (3.84%)     |
| Wad anor   | 0             | 0              | 0             | 0             |
| Wad alnaem | 0             | 0              | 0             | 0             |
| Shabona    | 0             | 7 (50%)        | 2 (14.3%)     | 0             |
| UmAlqura   | 0             | 0              | 1 (5.3%)      | 0             |
| Overall    |               |                | 22.2%         |

3.3 Detection of RVFV Using Reverse Transcriptase-Polymerase Chain Reaction

In the current study, due to the fact that none of our reference virological diagnostic laboratories satisfy the minimum requirements for virus isolation (level 3). So we adopted Rt-PCR as standard test to diagnose and confirm RVFV among studied animals. The Rt-PCR provided more sensitive and specific detection of RVFV in serum samples (sensitivity 95%, specificity = 80%). The medium genome of RVFV is preferable and had been tested [26,50,51]. The pair of primers designed for detecting the M segment was produced 342 bp primary Rt-PCR product (Fig. 3). The Rt-PCR amplification products were visualized onto an ethidium bromide stained agarose gel. From Table 6 the Rt-PCR test indicated that positive result was found among animals at Shikaira and Shabona and Umalqura. The Rt-PCR test revealed that the highest prevalence was reported at Shikaira 19%, while in Shabona was 11.10% and Umalqura tested positive for antibodies against RVFV 0.80% (Fig. 4). The lowest prevalence found at Wad anor and Wad alnaem 0%. The overall prevalence was 15.23% in the study sites. This study did not reveal significant difference between male and female with respect to Rt-PCR ($\chi^2$= 2.34, $P= 0.1261$), similar to IgG result.
Fig. 3. The 1.5% ethidium bromide stained agarose gel for detecting 342 bp: Lane 1 DNA ladder, Lane 2 negative control, Lane 4 positive sample 342bp

Table 7. Prevalence of RVFV infection by Rt-PCR test according to age

| Area        | Male (+ve) | % of infection |
|-------------|------------|----------------|
|             | Age group  |               |
|             | >1         | <1 - 3        | 3 - 5 | <5 |
| Shikaira    | 1 (10%)    | 1 (10%)       | 0     | 0  |
| Wad anor    | 0          | 0             | 0     | 0  |
| Wad alnaem  | 0          | 0             | 0     | 0  |
| Shabona     | 1 (4.8%)   | 1 (4.8%)      | 0     | 0  |
| UmAlqura    | 0          | 0             | 0     | 0  |
| Total       |            |               |       | 7.7% |

Fig. 4. Prevalence of RVF in the study area by using Rt-PCR
Table 8. Prevalence of RVFV according to sheep gender using Rt-PCR

|          | +ve | -ve | $\chi^2$ | P. value |
|----------|-----|-----|----------|----------|
| Male     | 4   | 52  | 2.34     | 0.1261   |
| Female   | 15  | 81  |          |          |

![Graph showing prevalence of RVFV](image)

Fig. 5. Prevalence of RVF in the study area by using Rt-PCR and ELISA

3.4 Validation of Rt-PCR and ELISA in detecting RVFV

Fig. 4 showed that the percentages of RVFV infections were high when using the ELISA for the five areas expect Shabona site. It was stated that the Rt-PCR is suitable for detection of recent infection of the virus within first three days, while the ELISA may detect the antibodies after infection during period ranging from one week to months by identifying the Immunoglobulin M (IgM) and Immunoglobulin G (IgG) [56]. There is high percentage of infection in Shabona site, when using Rt-PCR, a result likely due to circulating of the virus during data collection. These findings taken together highlight the complementary nature of molecular detection assays and serologic tests and the importance of using a combination of assays for reliable diagnosis of virus infection.

Analysis of variance was performed using the Statistix 8 software. The analysis was carried out for the pooled data from the study areas and for each area separately for both ELISA and Rt-PCR tests. As shown in Table 4 and Table 5 the analysis of variance showed that there is no significant difference between the age groups and sex (male and female) with respect to both tests. This result indicates that the infection with this virus doesn’t depend on age or sex of the sheep. All age groups and both sexes are vulnerable to virus infection.

3.5 Sensitivity and Specificity of ELISA and Rt-PCR

The sensitivity test is defined as the ability of a screening test to detect a true positive, being based on the true positive rate, reflecting a test ability to correctly identify all infected animals. Table 6 showed that there is a high-sensitivity and low specificity test. In other words, they are good for catching actual cases of the virus. It was reported [57] that the rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of RVFV infection during an outbreak of the disease among humans and susceptible animals. The RT-PCR is often used as a supportive diagnostic assay to the time consuming and cumbersome conventional virus isolation laboratory procedure.

3.6 Receiver Operating Characteristic (ROC) Curve Analysis

ROC analysis was used to create cumulative values for failure and success then the values of the False Positive Rate (FPR) and the True Positive Rate (TPR) for each row was calculated. An excellent model of ROC analysis has AUC near to the 1 which means model has good
To distinguish between the ELISA (actual class) and the Rt-PCR (predicted class) we used the class predictions produced by the model. Table 10 and Fig. 6 illustrated that the AUC is 0.79 which indicate that the Rt-PCR is gold standard test.

Table 9 (a,b,c,d,e,f). Sensitivity and specificity values for the ELISA and RT-PCR assays for RVFV for study sites

(a)

| Area      | Test    | +VE | -VE | Positive predictive value | Negative predictive value |
|-----------|---------|-----|-----|---------------------------|---------------------------|
| Um alqura | Rt-PCR  | 1   | 15  | 0.9% (95% CI)             | 99% (95% CI)              |
|           | ELISA   | 9   | 89  |                           |                           |
|           |         |     |     | Sensitivity= 90%          | Specificity= 52%          |

(b)

| Area      | Test    | +VE | -VE | Positive predictive value | Negative predictive value |
|-----------|---------|-----|-----|---------------------------|---------------------------|
| Wad alneem| Rt-PCR  | 0   | 24  | 0.1000 (95% CI)           | 1.000 (95% CI)            |
|           | ELISA   | 10  | 53  |                           |                           |
|           |         |     |     | Sensitivity= 100%         | Specificity= 52.63%       |

(c)

| Area      | Test    | +VE | -VE | Positive predictive value | Negative predictive value |
|-----------|---------|-----|-----|---------------------------|---------------------------|
| Shabona   | Rt-PCR  | 11  | 24  | 0% (95% CI)               | 99% (95% CI)              |
|           | ELISA   | 1   | 91  |                           |                           |
|           |         |     |     | Sensitivity= 0%           | Specificity= 47%          |

(d)

| Area      | Test    | +VE | -VE | Positive predictive value | Negative predictive value |
|-----------|---------|-----|-----|---------------------------|---------------------------|
| Albasabeir| Rt-PCR  | 0   | 118 | 14.4% (95% CI)            | 100% (95% CI)             |
|           | ELISA   | 17  | 88  |                           |                           |
|           |         |     |     | Sensitivity= 100%         | Specificity= 53.88%       |

(e)

| Area      | Test    | +VE | -VE | Positive predictive value | Negative predictive value |
|-----------|---------|-----|-----|---------------------------|---------------------------|
| Wadanor   | Rt-PCR  | 9   | 20  | 0.0500 (95% CI)           | 99% (95% CI)              |
|           | ELISA   | 5   | 72  |                           |                           |
|           |         |     |     | Sensitivity= 100%         | Specificity= 51.3%        |
Table 10. Receiver operating characteristic curve for ELISA and PCR

| Age  | +ve ELISA | +ve RT-PCR | FPR | TPR | AUC  |
|------|-----------|------------|-----|-----|------|
| <1   | 12        | 2          | 0.79| 0.89| 0.52 |
| >1-3 | 34        | 10         | 0.21| 0.33| 0.057|
| 3-5  | 10        | 5          | 0.034| 0.06| 0.0019|
| >5   | 2         | 1          | 0.034| 0.06| 0.0019|

Fig. 6. Receiver Operating Characteristic curve

4. CONCLUSIONS

The results from this study showed the presence of anti-RVFV antibodies in all study sites, suggesting that RVFV is actively circulating among sheep in Gezira state.

The high seroprevalence of RVFV infection in Gezira state indicates its endemicity.

The present study highlights the importance of tracking the spread of the virus and its implication for analyzing changes in RVFV endemicity in specified areas over time.

Our results revealed that the seroprevalence for RVF virus was (14.2%) in Gezira state.

The Rt-PCR used in the present study is reliable to detect precisely the M segment of RVFV. It is valuable as a confirmatory test and should be considered by reference laboratories in the region.

ETHICAL APPROVAL

The field work and laboratories investigations has been conducted at Gezira State and Blue Nile National Institute for Communicable
Diseases. the author received an ethical permission from the general directorate of animal health, federal ministry of agriculture and animal resources; the author also received an ethical clearance from veterinary ethics committee (vec). animals sampled were housed and cared in accordance with national international legislation and local animal regulation requirements. the blood collection procedure from sampled sheep was performed by qualified veterinarian following proper physical restraint of animal to ensure both personnel and animal safety.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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