

Immunological Study on Rift Valley Fever Virus among Human Beings in Taiz Governorate (Yemen)

Al-Aesayi MSA^{1*}, Al-Helali MF¹, Mubarak JS², Al-Galibi SM¹ and Al-Qadasi AM³

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¹Microbiology Division, Department of Biology, Faculty of Science, Sana'a University, Yemen

²Department of Biology, Faculty of Science, Sana'a University, Yemen

³Department of Laboratories, Faculty of Medicine, Hodeida University, Yemen

***Corresponding author:** Mansour Shayif Abdullah Al-Aesayi, Microbiology Division, Department of Biology, Faculty of Science, Sana'a University, Yemen, Tel: 00967-773458276; Email: abushaef@gmail.com

Abstract

Rift Valley Fever Virus (RVFV), which is transmitted to human beings by mosquitoes or direct contact with infected animals, is the etiologic agent of Rift Valley Fever (RVF). Using Enzyme Linked immunosorbent assay (ELISA), this study was performed to prepare RVFV antigen, which is used in detecting anti-RVFV antibodies, from liver of infected lambs. The study also aimed to study immunological prevalence of anti-RVFV IgM and IgG antibodies among human beings, which were conducted in the Research Center of Taiz University and Central Health Laboratory, Taiz Governorate, Southwestern Yemen. RVFV antigen was successfully prepared at a titer dilution of 1: 3200 by Chessboard (CB) ELISA assay. However, out of the 234 samples, 154 samples were positive for ELISA assay, of which 49 samples (31.8%) were positive for anti-RVFV IgM and 105(68.2%) were positive for anti-RVFV IgG antibodies. The antigen was successfully produced, and successfully adhered to ELISA plates. Simple and inexpensive methods gave good results. The result can be used to develop and refine predictive database for RVF transmission based on environmental and remote sensing data.

Keywords: Rift Valley fever; Antigen; ELISA; Immunology; Yemen

Abbreviations: RVFV: Rift Valley Fever Virus; RVF: Rift Valley Fever; ELISA: Enzyme Linked immunosorbent assay; IgM: Immunoglobulin M; IgG: Immunoglobulin G; rpm: Round Per Minute; CB: Chessboard; 3SD: three standard deviation.

Introduction

RVFV is the causative agent of RVF, a zoonotic disease, a member of the family *Bunyaviridae*, genus *Phlebovirus*,

and an enveloped contains tri-segmented genome of single strand negative-sense RNA that affects both ruminants and human beings [1,2]. In ruminants, it induces almost 100% mortality among young animals and a high rate of abortion in pregnant females [3]. In human beings, the severity of infection can vary from mild to very severe clinical symptoms, including fever, blindness, encephalitis, and hemorrhagic fever with a fatal outcome [3,4]. It is transmitted to human beings by mosquitoes

and through direct contact with tissues of infected animals or their products [5].

RVF is an endemic disease in Africa and the Arabian Peninsula. The outbreak appeared for the first time in Saudi Arabia and Yemen in 2000–2001. In Saudi Arabia, 11,882 human cases with 164 deaths were reported [6–8]. In Yemen, 1080 human cases with 141 deaths were reported [7–9], over 20,000 aborted animals with 620 dead [7,10]. Several outbreaks were followed after heavy rainfall in Egypt, Kenya, Somalia, Tanzania, Sudan, Mayotte, and Mauritania from 2003 to 2012 [11].

Several methods used in RVFV diagnosis. Scott, et al. [12] determined that the accuracy of serological methods in detecting anti-RVFV antibodies indicated that Enzyme Linked immunosorbent assay (ELISA) was the most precise serological method. So, this study aimed at preparing RVF antigen from liver of infected lamb to be used in detecting anti-RVFV antibodies by using indirect ELISA technique and studying immunological prevalence of anti-RVFV IgM and IgG antibodies among human beings as well as establishing primary database of RVF prevalence in Yemen.

Materials and Methods

A total of 234 people who suffered from fever, aged from 4 years to over than 50 years old, admitted in some hospitals of Taiz City, Southwestern Yemen, during the period from January 2013 until August 2016. About 5 ml of blood were collected by venipuncture into sterile dry glass tube, labeled with a date, time of collection, the patient's name and number of sample, transferred to laboratory as soon as possible (within one hour) after the blood has been collected, allowed to clot for 30–45 min at room temperature, centrifuged at 2000 rpm for 10 min for separation of serum, transferred into new sterile dry tube, capped tightly, labeled, inactivated in water bath at 56°C for 30 min for inactivation of complement, and stored in refrigerator at -20 °C until used by ELISA. Accepted samples were clear, non-hemolyzed and non-lipemic, but rejected samples were excessive hemolysis, presence of large clots, leakage, microbial growth and repeated freeze-thaw cycles [12–14].

Preparation of RVFV Antigen

According to the information mentioned by Smithburn, et al. [15] the concentration of RVFV was highest in the liver of infected animal. Based on this data, RVFV antigen was prepared from a liver of lamb by using sucrose acetone extraction method according to the methods

described by Clarke and Casals [16]. Lamb inoculation: under strict control measures, two lambs, aged 2 and 4 months, were inoculated intraperitoneally with 0.5 ml of infected human serum by RVFV containing 10^4 LD₅₀, which was kindly supplied by the Department of Virology, Central Researches laboratories, Taiz Governorate. Sufficient virus was present in serum of lambs after 4 days of inoculated, and the optimal titer dilution of virus in the serum was 1/1600 when the lambs seemed unsteady gait. No one of the lambs died during the virus incubation period.

Procedure for preparing RVFV antigen: 10 g of the liver of the infected lamb was put in sterile mortar and thoroughly minced, four volumes of 8.5% aqueous solution of sucrose, after sterilized by filtration, were added to the liver tissue and mixed well until homogenized, and one volume of homogenate was added to 20 ml of chilled acetone after vigorous shaking. Then, the tightly stoppered bottles were centrifuged at 1800 rpm for 5 min at 4°C. The supernatant fluid was aspirated and chilled acetone equal to the aspirated amount was added to the sediment. The bottles were placed in an ice bath for at least one hour to dehydrate the gummy sediment. The centrifugation process was repeated twice, and the supernatant was aspirated and the sediment was completely dried by attaching a vacuum pump through a filter flask to the bottle containing the sediment. Normal saline was added to the dried sediment in a volume equal to 0.4 volume of the original volume of the homogenate. The sediment was dissolved within 2 hours and the solution was left overnight in a refrigerator. After a complete dissolving, the solution was centrifuged in a refrigerated centrifuge at 20,000 rpm for 30 min. The supernatant containing the viral antigen was inactivated with binary, 20% sodium thiosulphate solution sterilized by autoclaving, used to neutralize the 2-Bromoethylamine hydrobromide action, and then was kept in a bottle, frozen at -70°C until used.

Serological Examination

Chessboard (CB) titration ELISA procedures for the prepared antigen was done according to Rose, et al. [17]. The prepared antigen was subjected to evaluation of the binding activity against specific antibody molecules IgM, IgG, and subjected to serial 2-fold dilution in coating buffer, carbonate-bicarbonate buffer pH 9.6 composed of NaHCO₃ 2.93g; Na₂CO₂ 1.59g; distilled H₂O 1L, to obtain dilutions of 1:50; 1:100; 1:200; 1:400; 1:800; 1:1600 ...etc. Row H was left empty as blank. 50µl of each dilution were dispensed into horizontal rows A–G wells in polystyrene microtiter plates (Maxisorp; Nunk, Copenhagen, Denmark,

plates), incubated overnight at 4°C, and washed 4 times by washing buffer (0.5ml tween 20 in 1L Phosphate buffer saline BPS which composed according to Voller, et al. [18] of NaCl 8.00g; KCl 0.20g; KH₂PO₄ 0.20g; Na₂HPO₄.12H₂O 2.90g; distilled H₂O 1L; pH 7.4). 50 µl/well of blocking buffer, 10g bovine serum albumin in 1L BPS, were dispensed to all wells, incubated for 1h at 37°C and washed 4 times. Two reference sera (strong positive and known negative) were diluted 2-fold 1:10; 1:20; 1:40 and so on in diluting buffer (5g of bovine serum albumin; 50µl tween 20; in 1L BPS). 50µl of each dilution were added to vertical columns (1-10) while the columns (11-12) received 50 µl of the known negative serum sample, incubated at 37°C for 1 h in titer IgG or 2 h in IgM and washed 4 times. 50µl of diluted horseradish peroxidase conjugated goat anti-human IgG or swine anti- human IgM (Sigma Chemical Co.) were added to all wells, incubated (1 h in 37°C), and washed 4 times. The substrate buffer, Citric acid (2.1g in 100ml distilled H₂O) 24.3ml; Na₂HPO₄ (7.1g in 100ml distilled H₂O) 25.7ml; distilled H₂O 50ml; Orthophenylenediamine 40mg; H₂O₂ 20µl, was added to all test wells (50 µl / well), incubated at 37°C in dark place for 20 min. 50µl of stopping buffer, 2.5M sulfuric acid (H₂SO₄); pH 3.3, were added to all wells. The optical density was measured at 492 nm using plate readers. The highest dilution of antigen gave a value of 1.1 optical density (OD) after 20 min of substrate incubation with the strong positive serum antibodies IgG while gave a value of 1.2 OD with the strong positive serum antibodies IgM, and under 0.1 OD with negative serum was considered the optimal dilution of the antigen.

ELISA procedures for detection of anti-RVSV IgM and IgG antibodies were done according to Voller, et al. [18]. The serum samples were analyzed for detecting anti-RVSV IgM and IgG antibodies by using indirect ELISA assay. Ninety-six well polystyrene microtiter plates (Cooke M 29 AR; Dynatech plates) were coated with 100µl/well (Row H wells were left empty as blank) of RVSV antigen diluted in coating buffer, covered, incubated at 4°C overnight, and washed 3 times using washing buffer. 100µl/well of blocking buffer were dispensed to all wells, incubated for 1h in moist chamber at 37 °C for 30 min, and washed 3 times. Serum samples were diluted (1:10 for test IgG or 1:40 for IgM) in diluting buffer and 100µl of each serum sample were added in duplicate. Each plate included a positive control serum (wells A1-A6) and a negative control serum (wells A7-A12). Plates were incubated at 37 °C for 1 h in test IgG or 2 h in IgM. After being washed 3 times, 100µl of horseradish peroxidase conjugate labeled goat anti-human IgG or swine anti-human IgM (whole molecules of IgG or IgM diluted according to the manufacturer's recommendation, Sigma

Chemical Co.) were added into each well, and incubated for 1h at 37 °C. The plates were washed 3 times and blotted on paper towels. 100 µl/well of Orthophenylenediamine hydrochloride substrate were added and plates were left for 20 min at 37 °C in dark. The reaction was stopped within 10 min after adding 50µl /well of stopping buffer. The plates were read by ELISA using dynastic plate reader at 492 nm. The samples with optical density value more than 3SD above the mean of the negative control serum samples (Cutt-off) were considered positive.

$$\text{Cutt-off} = \bar{X} + 3SD$$

\bar{X} = mean of negative control serum samples.

SD = standard deviation of negative control serum samples.

Statistical Analysis

Statistical analyses of the data were performed using statistical software package SPSS version 16. The categorical variables were done using Chi-square test at a 99% confidence level, and a significance level of 0.05 was used to determine the relationships between the data collection and immunological prevalence rates. Asymptotic Significance ≤ 0.05 was considered to be significant.

Ethical Approval

Ethical approval for this study was granted by the Microbiology Division, Biology Department, Faculty of Science, Sana'a University Ethical Committee. Permission to conduct the study was given by Sana'a to Taiz Governorate authorities. Oral and written consents were obtained from all participants.

Results

Using specific IgM and IgG antibodies through CB ELISA titration assay, the antigen was successfully produced and titrated from liver of lamb. However, the highest titer dilution of antigen was investigated 1: 3200 by using CB titration ELISA assay for both IgM and IgG antibodies.

Out of the total 234 samples, 154(65.8%) of the cases were positive for ELISA assay, and 80(34.2%) were negative. However, the overall positive results rate was 49 (31.8%) and 105(68.2%) for antibodies IgM and IgG respectively as shown in Table 1. Regarding table 1, the immunological prevalence rate of anti-RVSV IgM and IgG among human beings. Results revealed that the highest prevalence rate 56(36.4%) in age group 31-40 years was

20(13.0%) and 36(23.4%) of anti- RVFV IgM and IgG respectively. However, the overall prevalence rate of RVF cases 77 (50.0 %) was equally recorded in urban and rural patients. Noteworthy, the urban patients were equivalent in infection to the rural. The relationship among age groups, localities, and immunity response to IgM and IgG are not significant >0.05 . In general, rate of males was higher than females. So, 97(63.0%) and

57(37.0%) of patients were males and were more infected than females respectively. However, the highest immunological prevalence rate connected with patients' occupations was 65(42.2%) in shepherds, who were more subjected to contact with animals. The relationship amongst sex, occupations, RVFV infection, and immunity response of the patients shows a statistical significant ≤ 0.05 .

Variables	ELISA assay of prepared antigen					
	Anti-IgM	Anti-IgG	Total	P-Value	df	Asymp. Sig.
	No.(%)	No.(%)	No.(%)			
Age Groups						
<10	0(0.00)	2(1.3)	2(1.3)	0.396	1	0.529
Oct-20	2(1.3)	13(8.4)	15(9.7)			
21-30	12(7.8)	19(12.3)	31(20.1)			
31-40	20(13.0)	36(23.4)	56(36.4)			
41-50	8(5.2)	20(13.0)	28(18.2)			
>50	7(4.5)	15(9.7%)	22(14.3)			
Total	49(31.8)	105(68.2)	154(100)			
Localities						
Urban	25(16.2)	52(33.8)	77(50.0)	0.03	1	0.863
Rural	24(15.6)	53(34.4)	77(50.0)			
Sex						
Males	25(16.2)	72(46.8)	97(63.0)	4.386	1	0.036
Females	24(15.6)	33(21.4)	57(37.0)			
Occupation						
Shepherds	13(8.4)	52(33.8)	65(42.2)	12.78	1	0
Employee	19(12.3)	41(26.6)	60(39.0)			
Farmers	17(11.0)	12(7.8)	29(18.8)			

Table 1: Immunological prevalence rate of anti-RVFV IgM and IgG among human beings in relation to socio-demographic characteristics.

No: Number of cases; Symp. Sig.: Asymptotic Significance; df: Degree of Freedom; P-Value: Possibility Value.

Actually, immunological prevalence rate of anti-RVFV IgM and IgG among human beings in relation to clinical symptoms and season is shown in Table 2. Table 2 shows that headache had the highest distribution rate 105 (68.2%) among IgM and IgG patients, decreased to 19(12.3%) Vomiting and 16(10.4%) Hemorrhagic fever among anti-IgM patients while Ocular diseases and Encephalitis had lowest distribution rate 8(5.2%) and 6 (3.9%) among anti-IgG patients respectively. The relationship between clinical symptoms and immunity

response is significant ≤ 0.05 . However, the results revealed that the highest immunological distribution rate demonstrably appeared 64(41.6%) in Summer and, decreased to 56(36.4%) in Autumn. Therefore, the prevalence rate decreasingly started at end of Autumn and dramatically increased at end of Spring. The relationship among immunity response for the patients, RVFV infection, and seasons of year doesn't have a statistically significant > 0.05 .

Variables	ELISA assay of prepared antigen					
	Anti-IgM	Anti-IgG	Total	P-Value	Df	Asymp. Sig.
	No.(%)	No.(%)	No.(%)			
Clinical symptoms						
Headache	19(12.3)	86(55.8)	105(68.2)	3.819	1	0.051
Vomiting	16(10.4)	3(1.9)	19(12.3)			
Hemorrhagic fever	14(9.1)	2(1.3)	16(10.4)			

Ocular diseases	0(00.0)	8(5.2)	8(5.2)			
Encephalitis	0(00.0)	6(3.9)	6(3.9)			
Season						
Summer	23(14.9)	41(26.6)	64(41.6)	1.973	1	0.16
Autumn	19(12.3)	37(24.0)	56(36.4)			
Winter	0(00.0)	4(2.6)	4(2.6)			
Spring	7(4.5)	23(14.9)	30(19.5)			

Table 2: Immunological prevalence rate of anti-RVFPV IgM and IgG among human beings in relation to clinical symptoms and season.

No.: Number of cases; Symp. Sig.: Asymptotic Significance; df: Degree of Freedom; P-Value: Possibility Value.

Discussion

This study was performed to prepare RVFPV antigen from lamb liver to be used in detecting anti-RVFPV antibodies in human beings or animals' serum samples by using modern ELISA technique and to study immunological prevalence of anti-RVFPV IgM and IgG among human beings.

The prepared antigen from lamb liver was successfully prepared using sucrose acetone extraction technique and also assessed successfully by IgM and IgG antibodies. This antigen was successfully adhered to CB ELISA plates directly at a high titer dilution of 1: 3200. This result is in consistency with those recorded by Said, et al. [19], who produced same antigen from the liver of sheep at a dilution of 1:200. Similar antigen prepared by Paweska, et al. [20] from infected mouse liver at a dilution of 1:400. Moreover, Maysa, et al. [21] prepared RVFPV antigen from the liver of hamster at a dilution of 1:3200. From the mentioned above, the difference in titration results may be related to variance in methods of test, accuracy and sensitivity of test.

The present study showed that 154 out of 234 cases were positive for ELISA assay. Their ages ranged from 4 years to over than 50 years. Nearly similar to those obtained in Egypt, the Kingdom of Saudi Arabia, Nigeria, Kenya, Somalia, and Mauritania by Olaleye, et al. [22], Nabeth, et al. [23], Seleem, et al. [8], Woods, et al. [24], Tariq, et al. [4], Youssef [25].

The overall immunological prevalence rate of anti-RVFPV IgM was 31.8%. This is similar to those obtained by MMWR [26] recorded 33.4% in Egypt, and is nearly similar to those reported by Sarthou, et al. [27] and Olaleye, et al. [22] during their survey in Mauritania and Nigeria whose findings were 24.3% and 23.2% respectively. The highest immunological prevalence rate 13.0% was in the age group ranging from 31-40 years. This is in agreement with MMWR [26], Olaleye, et al. [22],

Tariq, et al. [4], Memish, et al. [28] whose results were similar or with slightly lower percentages, and may be referred to individuals with ruminant exposure, or patients with specific age groups, variance in sample size, specificity of test and low concentration of the antibodies in the serum samples.

Moreover, the overall immunological prevalence rate of anti-RVFPV IgG was 68.2%. In contrary to this, lower findings were reported by MMWR [26], Thonnon, et al. [29] Nabeth, et al. [23], Seleem, et al. [8], Woods, et al. [24], Youssef, et al. [25], Maysa, et al. [21], Memish, et al. [28], Tigoi, et al. [30] whose records 13%, 15.3%, 24.4%, 10.6%, 15%, 10.3%, 10.53%, 11.1% and 19.5% respectively. The difference in results may be resulted because we collected our samples from patients with acute RVF infection. The results also revealed that the highest prevalence rate 23.4% was in the age group ranging from 31-40 years. This finding is in a disagreement with previous studies in Egypt by Youssef, et al. [25], Maysa, et al. [21] who recorded lower rate of 13.7% in the age group 31-40 years. Therefore, there was a significant difference between age groups and immunity response of human beings > 0.05.

Regarding Localities, the overall distribution rate of RVF cases 77(50.0%) in urban areas was similar to those in rural areas 77(50.0%). On the other hand, lower percentages of 13.6%, 11.8% in rural areas and 5.3%, 8.7% in urban one was previously recorded by Seleem, et al. [8], Maysa, et al. [21] respectively. From the mentioned above, we find that the distribution rate 25(16.2%) and 24(15.6%) of anti-RVFPV IgM is similar to 52(33.8%) and 53(34.4%) of anti-RVFPV IgG in rural and urban areas respectively. This may be attributed to many predisposing factors such as presence of animal reservoir, agricultural activities, irrigation, and people sleeping on the roofs of buildings in Summer. There was also a significant difference between Localities and immunity response of human beings > 0.05, but it showed important with P-value 0.030.

Obviously, the overall immunological prevalence rate in males and females was 97(63.0) and 57(37.0%) respectively. The higher percentages in males than females may be attributed to that males are repeatedly exposed to mosquito more than the females during sleep outdoors. The obtained results are in agreement with Woods, et al. [24]. The prevalence rate of anti-IgM cases was similar 25(16.2) and 24(15.6) in males and females respectively while the prevalence rate of anti-IgG was higher 72(46.8%) in the males than the females 33(21.4%). This result may be attributed to the fact that males are more involved than females in occupations that require direct contact with susceptible animals and the infectious agents Turell, et al. [31]. This result is in agreement with Woods, et al. [24], Seleem, et al. [8], Youssef [25], Maysa, et al. [21], Byomi, et al. [32]. There was a statistical significant amongst gender, RVFV infection, and immunity response of human beings < 0.05 .

The highest prevalence rate 65(42.2%) was in shepherds while it decreased to 29(18.8%) in Farmers. This result may be attributed to that shepherds contacted with susceptible animals many times, while the farmers only contacted with breeding habitats of mosquitoes. These results are in consistence with those reported in Mauritania by Boushab, et al. [11] who recorded lower prevalence rate 26% in shepherds and 3% in farmers. There was also a statistical significant between occupations and immunity response of human beings < 0.05 .

The most common symptoms were 12.3%, 10.4% and 9.1% of anti-IgM accompanied with headache, vomiting and Hemorrhagic fever while the lowest prevalence was 5.2% and 3.9% of anti-IgG accompanied with Ocular diseases and encephalitis respectively. This is in agreement with Tariq, et al. [4], Arthur, et al. [33], Woods, et al. [24]. who recorded similar prevalence rate? There was a statistical significant among symptoms of disease, RVFV infection, and immunity response of human beings < 0.05 .

The highest immunological prevalence rate recorded 14.9% and 26.6% in Summer and followed by 12.3% and 24.0% in Autumn of anti-RVFFV IgM and IgG respectively. These findings are in agreement with Olaleye, et al. [22] who mentioned that the infection rate was significantly higher during the wet season than during the dry season of the same year. This is in consistence with Maysa, et al. [21], who recorded lower prevalence rate of anti-IgG was 20% in Summer, 7.14% in Autumn. The difference in results may be attributed to the environmental factors, vegetation, rainfall, slope degree, abundance of vectors,

and selected specimens. There was a significant difference between seasons of year and immunity response of human beings > 0.05 .

Conclusion

The prepared antigen was successfully produced and adhered to ELISA plates at a highest titer dilution of 1: 3200. Simple and inexpensive methods gave good results. The overall positive results rate 154(65.8%) was positive for antibodies IgM and IgG. Immunological prevalence of RVF among human beings would be the highest in Autumn and in Summer, especially during rainfall and Greater Feast. People aged between 31-40 years are the most infected, and males are more infected than females. The result can be used to develop and refine predictive database for RVF transmission based on environmental and remote sensing data.

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Conflicts of Interest

There are no personal or professional conflicts of interest.

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