Entomological surveillance for detection RVF virus activity in the risk areas in Egypt.

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Abstract:

In the present study, mosquitoes were collected and tested for the presence of Rift Valley Fever Virus (RVFV) by RT-PCR using specific primers to fragment of the M- segment of RVFV. The mosquitoes were collected through June to November of the year 2009. Mosquitoes collected from previously infected governorates during last outbreak; Sharkia, Dakahlyia, Kafr- El Sheick and Aswan governorates was negative for amplification of RVFV M-segment.; these results may reflect the low levels of circulating RVFV typical in interepizootic periods in the surveyed areas or the virus may be not present in these areas.

Introduction:

Rift Valley fever (RVF) is one of the most serious transboundary animal diseases and is caused by a member of the *Phlebovirus* genus, one of the five genera in the family *Bunyaviridae*. RVF virus (RVFV) is transmitted by mosquitoes, particularly those belonging to the *Culex, Anopheles* and *Aedes* genera [1,2]. Transmission is mostly horizontal, but a vertical mode has been described for some *Aedes* species [3,4] where the RVFV is present in the eggs of *Aedes* mosquitoes which breed in isolated depressions called *dambos* often found in grassland areas. When *dambos* are flooded during periods of extensive and widespread rainfall, these eggs hatch and the subsequent adult mosquitoes are capable of transmitting the virus to domestic animals including sheep, goats, cattle, camels, and buffalos [5]. These depressions are also good breeding habitats for *Culex* and *Anopheles* mosquitoes species. When *Aedes* mosquitoes infect domestic animals with RVFV, viral amplification occurs in these vertebrate hosts resulting in a RVF outbreak. Blood feeding on these infected animals by other competent vectors, including *Culex* and *Anopheles* mosquitoes, can increase the range of the original outbreak due to insect flight or even wind-borne dispersal [6].

Cases of RVF in Human are typically caused by direct contact with the fluids from infected animals after spontaneous abortion or slaughtering of viremic animals. When an epizootic occurs in animals, it is easily transmitted to humans leading to an epidemic [7].

RVF virus was first identified in 1931 in the Rift Valley of Kenya [8]. Egypt has suffered several epidemics of RVF and consequent animal losses and human infection. In 1977, RVF disease was identified for the first time in Egypt and caused abortion in pregnant domestic ruminants and deaths in young lambs [9]. After that epidemic, the next documented RVF outbreak was in early 1981 [10]. After 12 years of absence, the RVF disease was again noted in human patients and domestic ruminants in late May 1993 at Aswan Governorate [11]. RVF outbreaks occurred again in Egypt between April and August 1997 in Aswan and Assiut governorates where infected cattle and sheep exhibited high fever, icterus, bloody diarrhea and abortion [12]. The purpose of the current study is to assess RVFV activity in the risk areas in Egypt during the summer and autumn seasons (2009) by assessing infection rates in wild caught mosquitoes.

Material and methods:

1-Insect collection and identification:

A total of 3840 mosquitoes were captured in the field using CDC - miniature light traps. Mosquitoes surveillance was conducted in the Dakahlia, Sharkia, Kafr-El shick and Aswan governorates and trapping performed at different seasons of the year**.** Trapping was performed as described by the John W. Hock Company (2004). The captured mosquitoes were transported dry to the laboratory and identified according to the species level using morphological keys [13]. After identification, female mosquitoes were grouped into 410 pools (each pool contains 5-10 mosquitoes of one species and sex), according to mosquitoes species, month of collection, location, feeding status. A PCR assay was used to detect RVFV in these pools of mosquitoes.

2-Detection of RVF virus in collected mosquitoes pools using RT-PCR:

2.1- RNA extraction:

Total RNA was extracted from individual insects using RNeasy kit according to manufacturer's instructions (QIAGEN).

2.2. One-step RT-PCR on RNA assay for RVFV on RNA samples:

A total PCR reaction volume of 20 µl contained 12 µl of reaction mix provided with the kit (Qiagen kits) and 8 µl of RNA. The PCR primers for this assay (RVS:AAAGGAACAATGGACTCTGGTCA,RVAs:CACTTCTTACTACCATGTCCTCC AAT, RVP: AAAGCTTTGATATCTCTCAGTGCCCCAA) anneal to G_2 glycoprotein region within the M segment of the tripartite negative-sense single stranded RNA genome of RVFV [14]. RT-PCR of RVFV with 5' nuclease probe detection involved reverse transcription at 50 °C for 30 min, initial denaturation at 95°C for 15 min, and 45 cycles with 95°C for 5 sec and 57 °C for 35 sec. fluorescence was read at the combined annealing extension step at 57 °C. PCR was performed using an ABI 7500. The ≥ 95% detection limits of the RT-PCR were determined by probit using the Statgraph Plus 5.0 software package (statistical Graph Inc) According to [14].

2.3. Reverse transcriptase reaction for detecting RNA quality by testing mosquito RNA by insecticide resistance primer:

Because all field-caught mosquitoes were negative for RVFV, it was important to demonstrate that RNA extracts contained amplifiable targets; otherwise, negative results could be due to poor quality RNA. To verify the quality of RNA in extracts, transcripts of two mosquitoes genes involved in insecticide resistance (*ace*-1 and *para*) were amplified from a subset of the mosquitoes pools. Total RNA was extracted from five mosquitoes samples using the guanidinium thiocyanate phenol-chloroform method. RNA was reversibly transcribed into single-stranded cDNA using the Superscript II reverse transcriptase (Life Technologies, France). The reaction was primed with a mixture of 45 µl of Master mix and 5 µl of samples of RNA mosquitoes. The PCR conditions were 30 min at 50°C and 15 min at 95°C then 1min at 94°C, 2min at 48°C and 2min at 72°C for 40 cycles followed by 10 min at 72°C. DNA fragments were separated by electrophoresis on 1.5% agarose gels and were visualised by ethidium bromide staining under UV light. The procedures were carried out according to [15,16].

Results:

1. Entomological results:

A total of 3840 mosquitoes, belonging to 4 genera and 7 species, were collected from Dakahlia, Sharkia, Kafr-El shick and Aswan governorates at different seasons of the year (Table 1).

The predominant species at Sharkia was Culex pipiens (85.71%), followed by *Culex antennatus* (10.31%), *Anopheles pharoensis* (2.87%) and lastly *Culex theileria*, *Aedes dentatus* and *Culiceda* (of each 0.48%). At Dakahlyia, the most abundant species was *Culex antennatus* (96.92%), followed by *Cx. Pipiens* and *Anopheles pharoensis* (1.53%). At Kafr- El Shick, the *Culex antennatus* was the most abundant species (59.09%), followed by *Cx. Perexiguus* (18.18%), *Cx. Pipiens* (13.63%) and lastly Anopheles *pharoensis* (9.09%). The most frequent species at Aswan was *Cx. Pipiens* (50%), followed by *Cx. Antenattus* and *Anopheles pharoensis* (of each 25%). These results indicate that the *Culex* genus was more abundant than *Anophelus* and *Aedes* genera. A total of 410 monospecific pools were constituted regarding to species, and site of collection. Individual mosquitoes were then submitted to RT-PCR assay for RVFV detection.

Table (1): Indicates number and species abundance of mosquitoes during the period from July to November 2009 at different location of Sharkia, Dakahlyia, Kafr-El Sheick and Aswan governorates.

Governorate	Species	Total Number	Species Abundance*
Sharkia	**CX. Pipiens**	216	85.6%
	Cx. antennatus	26	10.31%
	Cx. perxigus	2	0.79%
	Cx. theileria	1	0.48%
	Anoph. Pharoensis	5	2.87%
	Aedes. Det	1	0.48%
	Culiceda	1	0.48%
	Total	**252**	**100%**
Dakahlyia	**Cx. pipiens**	2	1.53%
	Cx. antennatus	126	96.92%
	Anoph. pharoensis	2	1.53%
	Total	**130**	**100%**
Kafr-El Shick	**Cx. pipiens**	3	13.63%
	Cx. antennatus	13	59.09%
	Cx. perxigus	4	18.18%
	Anoph. pharoensis	2	9.09%
	Total	**22**	**100%**
Aswan	**Cx. pipiens**	2	50%
	Cx. antennatus	1	25%
	Anoph. pharoensis	1	25%
	Total	**4**	**100%**

* **Abundance** = number of individuals of one species/ total number of mosquitoes collected (approximated to one decimal).

2- RT-PCR amplification using RVFV specific primer set:

A reverse transcriptase polymerase chain reaction (RT-PCR) was applied to detect Rift Valley Fever Virus (RVFV) in *Culex pipiens* mosquitoes pools collected during the period from July to November 2009 from Sharkia, Dakahlyia, Kafr- El

Shick and Aswan governorates of Egypt. The RT-PCR approach is a fast, efficient alternative to the more time consuming and cumbersome conventional virus isolation laboratory procedure [17].

Figure (1&2), shows the amplification plot and report of RT-PCR analysis, which indicated that all collected mosquitoes pools were negative for RT-PCR for RVF virus. The blue and brown lines indicate the experimental positive control (RVFV RNA) which was used at different dilution (1:10 and1:100).

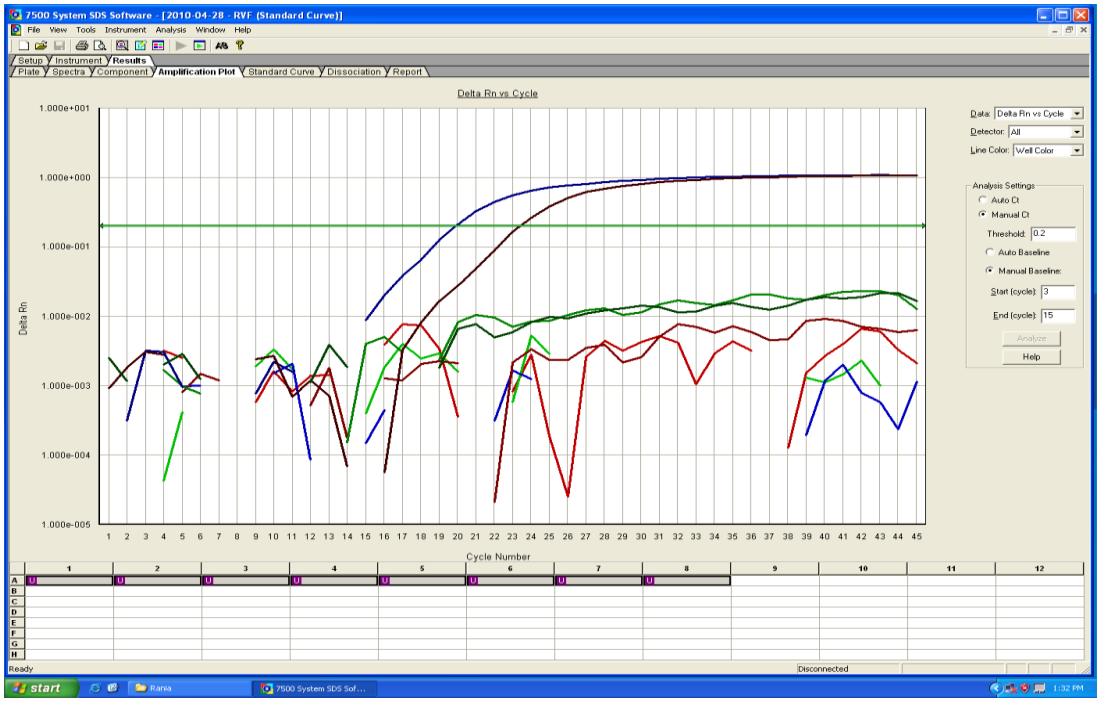

Figure (1): The real-time detection of the specific PCR products by fluorescence (*y* axis) (F1 detection wavelength or F1/F2 ratio) dependent on the PCR cycle number (*x* axis).

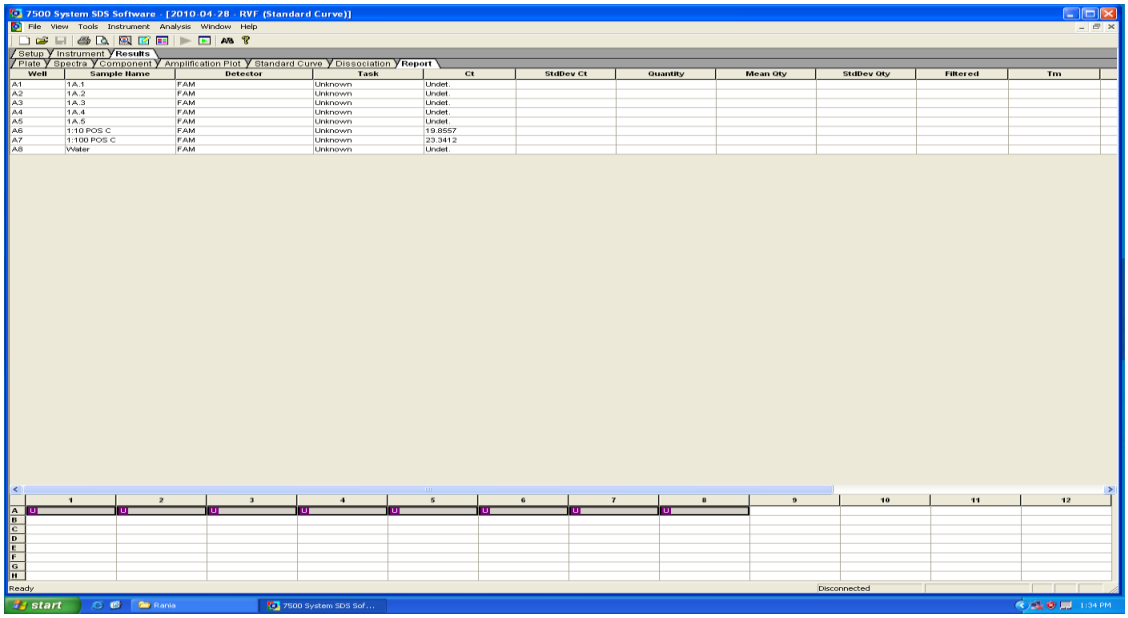

Figure (2): RT-PCR result for detection of RVF virus in mosquitoes pools were collected during the period from July 2009 to November 2009 from Sharkia, Dakahlyia, Kafr- El Shick and Aswan governorates.

Reverse transcriptase reactions were done on extracted RNA of mosquitoes samples by using mosquitoes insecticide resistance primers for check on the quality of RNA extracted step. Figure (3) clearly shows that field caught mosquitoes contain the expected 194 bp amplification product for *ace*-1. Figure (4) shows the same RNA pools but with a second target amplified. Again, all field caught mosquitoes produce a band of expected size (481-510 bp) for *para*. In both experiments, the bands generated by the field mosquitoes is the same size as that produced by Laboratory *Cx.pipiens* DNA and Laboratory *Cx.pipiens* RNA which used as controls positive.

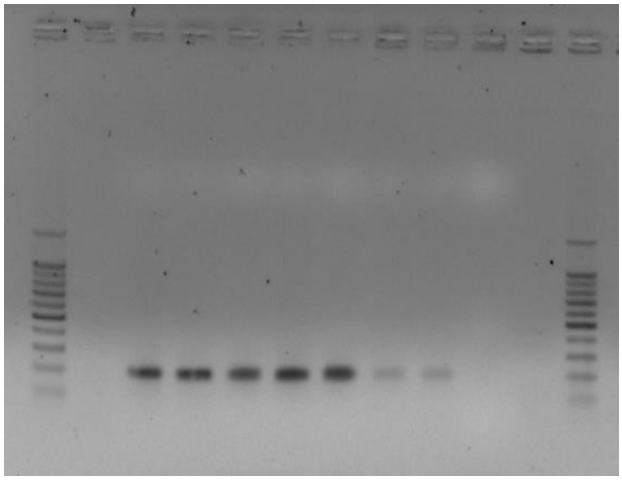

Figure (3): Gel image of reverse transcriptase reaction done on extracted RNA of mosquitoes samples by using mosquitoes organophosphrus resistance primer (Ace 1) for showing the quality of RNA extracted.

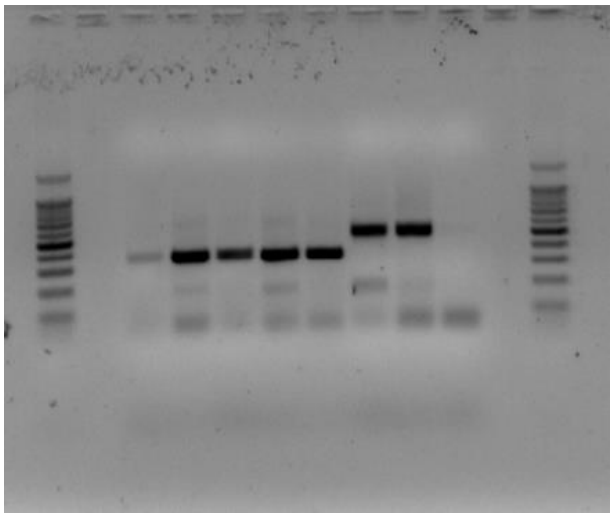

Figure (4): Gel image of reverse transcriptase reaction done on extracted RNA of mosquitoes samples by using mosquitoes pyrethroid resistance primer (Kdr mutations) for showing the quality of RNA extracted.

Discussion:

RVF is a common zoonotic disease transmitted between animal and man through mosquitoes. Mosquitoes perpetuate transmission either by vertical (transovarial) or by horizontal transmission to vertebrates which then infect other blood-feeding mosquitoes. The relative importance of vertical versus horizontal transmission in

maintaining virus transmission or in RVF outbreaks remains speculative. Because of this, entomological surveillance is necessary to assess of RVF virus activity in wild mosquitoes. Mosquitoes surveillance was conducted in Nile Delta and Aswan during the summer and autumn seasons 2009, and focused on areas where RVF outbreaks were previously detected by using different epidemiological and biotechnological tools.

From the entomological surveillance during the period of study and areas under investigations, the *Cx.pipiens* was more abundant in Sharkia and Aswan governorates but *Cx. Antennatus* was more abundant in Dakahlyia and Kafr- El Shick. The difference of the mosquitoes species collected in this survey depend on the difference on biology and ecology behavior of each mosquitoes species. During flooding, *Culex* mosquitoes have the highest abundance and breed in dambos.

The mosquitoes density was recorded in July, August and peaked during September, followed by decline in October at Nile Delta (Sharkia, Dakahlyia and Kafr- El Shick governorates). The summer season is predicted to pose the highest risk of large mosquitoes populations in Sharkia, Dakahlyia and Kafr-El Shick. This risk is associated with the rice growing season. Conversely, the highest mosquitoes density in Aswan was recorded in November, perhaps due to increased humidity and warm temperature. High humidity or flooded rice fields, coupled with warm temperatures, act as risk factors for RVF virus transmission and maintenance in these governorates. The high humidity with warm temperature or flooded water rice field with warming temperature affected on the biology of vectors, by increasing feeding frequency and egg production, and by decreasing the length of the development cycle and the extrinsic incubation period lead to increase vector density and increase vector capacity to transmit the virus [18].

RT-PCR is a rapid, sensitive and specific tool for detecting arthropod-borne viruses [19]. Although RVFV was not detected in wild caught mosquitoes, these data do not necessarily prove that the study areas are free of infection. Egypt is currently experiencing an inter-epizootic period; consequently, circulating virus levels may be very low in the mosquitoes population. This scenario is consistent with [20], where in an outbreak situation, RVFV was isolated from sixteen-aborted foeti and one dead calf in Madagascar, but analysis of 11,371 mosquitoes (61% *Culex antennatus*) collected at the same localities failed to isolate the virus. Thus, even in areas with high levels of RVFV transmission, entomologic surveys may sometimes fail to detect the viral load in insects.

Taken together, it is recommended that seroprevalence studies of animal and mosquitoes are necessary to obtain epidemiological data about RVFV in Egypt. Also, existing surveillance systems must be alert for outbreaks in neighboring countries and the possibility of sporadic or flareup infections during inter-epizootic period.

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