

ZH501-VSVRI: Is it Still the Best Choice for Vaccination Against Rift Valley Fever in Egypt?

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Abstract

ZH501 strain of Rift valley fever virus (RVFV) was originally isolated from a human patient during the outbreak of 1977 in Egypt. This virus strain was used since 1980 for preparation of an inactivated RVF vaccine at Veterinary Serum and Vaccine Research Institute (VSVRI), Egypt. Two subpopulations of ZH501 with a single nucleotide polymorphism (A/G) at nucleotide position 847 of Gn gene of M segment have been described. This nucleotide substitution affected significantly RVFV virulence in the mouse model. In this report, the genetic makeup of the Gn gene of ZH501-VRVSI vaccine virus stock was analyzed for verification of its safety and stability. Plaque assay of the vaccine stock virus revealed the presence of two populations that produced different plaque forms. The viruses that developed large plaques resembling those produced by the virulent virus strains were isolated for genetic analysis. Comparison of the Gn gene nucleotide sequence of the isolated viruses with those of the wild-type ZH501 and other reference strains identified five nucleotide substitutions, of which three are capable to induce amino acid changes in the mature protein. Protean analysis suggested a potential change in the three dimensional structure of the Gn protein in relation to the parent strain. The results of this study throw light on the changes occurred in the master seed virus used for preparation of RVFV vaccine in Egypt. Further studies focusing on the other gene segments of ZH501-VSVRI are required to conclude is it still the best choice for vaccination against RVF in Egypt.

Keywords: RVFV; ZH501-VRVSI strain; Vaccine; Egypt

Introduction

Rift valley fever virus (RVFV) is a member of the genus *phlebovirus* that belongs to the family *Bunyaviridae*. The virus is primarily transmitted by mosquitoes (*Aedes* species) and causes a potentially severe disease in man and animals [1-3]. RVFV was first identified in 1930, during an outbreak of sudden deaths and abortions among sheep along the shores of Lake Naivasha in the greater Rift valley of Kenya [4,5]. Nowadays, the geographical distribution of RVF includes most countries of the African continent and Madagascar [6]. An epidemic of RVF infection was recorded for the first time in Egypt at 1977 [7,8] and appeared again at 1993 [9].

RVFV is an enveloped single-stranded tripartite RNA virus. The viral genome is typically split into three segments; large (L), medium (M) and small (S), of negative- or ambi-sense [10,11]. The anti-viral-sense M segment encodes for two envelope glycoproteins; G1 (Gc) and G2 (Gn), beside two accessory proteins: 14 kDa (NSm) and 78 kDa. The open reading frame (ORF) of M gene contains five in-frame translation-initiation codons within the pre-Gn region that is located upstream of the Gn and Gc genes [12-14].

As a result of its significant socio-economic impact, vaccines were required for the control of RVFV outbreaks, at least in the veterinary field. The first RVF vaccine was developed by serial intracerebral inoculation of mice with Entebbe strain [15]. This attenuated virus, designated as Smithburn strain, had partially lost its original virulence but it had the ability to induce abortions and teratogenesis in pregnant ewes, goats and cows [3,16,17]. To overcome these drawbacks, an inactivated vaccine was produced, however it was not as potent as the attenuated vaccine and it usually requires booster inoculations [18].

During the first Egyptian RVF outbreak at 1977, a viral isolate designated as ZH501 strain, was isolated from a human case in Zagazig, Sharqia province, Egypt. This virus strain was used for preparation of a safe and potent inactivated RVF vaccine at Veterinary Serum and

Vaccine Research Institute (VSVRI), Cairo, Egypt [19]. Sequence analysis of the Gn gene of wild-type ZH501 strain revealed that the virus is composed of two major viral subpopulations, with single nucleotide heterogeneity at the nucleotide position 847 of M gene segment (M847). In one subpopulation, M847 contained a G residue encoding for glycine, while the other carried A residue encoding for glutamic acid. Intra-peritoneal inoculation of ZH501(M847-A) and ZH501(M847-G) in mice demonstrated that ZH501(M847-A) is as virulent as the wild-type ZH501, whereas ZH501(M847-G) had a reduced mouse virulence [20].

In this report, an extensive genetic analysis of Gn gene of the ZH501 RVFV stock, used for vaccine preparation at VSVRI, was described. These data are invulnerable for identifying the inter-relationship between this virus stock and the different RVFV isolates worldwide including the wild-type ZH501 strain, a mean to provide basis for the development of safer and more effective vaccine(s).

Materials and Methods

Virus and cells

The ZH501 strain of RVFV was kindly supplied by VSVRI, Cairo,

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Egypt. The virus was originally isolated from a human case at Zagazig, Sharqia province, Egypt during the outbreak of 1977 by intracerebral inoculation of suckling mice for two successive passages. After initial passaging in suckling mice, the ZH501 virus stock was generated and titrated on BHK-21 cell line. The virus used in this study had an infectivity titer of 10^8 TCID₅₀/ml.

Virus plaque assay

Selection of the different RVFV phenotypes was carried out using plaque assay as described previously [21]. Ten-fold serial dilutions of ZH501 virus were prepared in MEM (Sigma, St. Louis, Mo, USA) containing 2% fetal bovine serum; FBS (Gibco BRL, Grand Island, NY, USA). Fifty microliters of each dilution were adsorbed on duplicate BHK-21 cell monolayers in a 24 well-plate for 1 hour at 37°C. After adsorption, the non-adsorbed virus was aspirated and the monolayer cells were washed twice with MEM. One milliliter of a mixture containing 1x MEM, 1% agarose 2% FBS, 50U of penicillin/ml and 50 µg streptomycin was used to overlay the cell monolayers. After incubation for 3 days at 37°C, each monolayer sheet of cells was stained by adding a second agarose overlay containing 4% neutral red. After incubation for 24 hours, the different developed plaques particularly those large in size, were selected, propagated in BHK-21 cells and designated RVFV ZH-501-VSVRI.

RNA isolation

Viral RNA was isolated from the supernatant aliquots collected from BHK-21 cells infected with the selected large plaques using Trizol reagent (Invitrogen, San Diego, CA), according to the manufacturer's recommendations. Briefly, 400 µl of the harvested culture supernatant were added to 1 ml Trizol reagent and incubated at RT for 10 min. Two hundred microliters of chloroform were then added and the tubes were vigorously shaken several times before incubation at RT was continued for 15 min. The tubes were centrifuged at 14,000 rpm for 15 min at 4°C and the upper aqueous phase containing the RNA was collected and transferred to another tubes containing 500 µl isopropanol. Following incubation at RT for 10 min, the mixture was centrifuged at 14,000 rpm for 10 min. The precipitated RNA was finally washed by 75% ethanol, air dried for 10 min and resuspended in 30 µl nuclease free water.

RT-PCR and DNA sequencing

The viral RNA (5 µl) was reverse transcriptase (RT)-PCR amplified using Access quick one-step RT-PCR kit (Promega, Madison, USA). The optimized cycling conditions were: 1 cycle at 45°C for 45 min; 1 cycle at 95°C for 2 min; 45 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 min, and finally 1 cycle at 72°C for 5 min. The Gn gene of M segment was amplified utilizing the following primers: RVFV: 5'- GTC TTG CTT GAA AAG GGA AAA -3' and RVFVR: 5'- CCT GAC CCA TTA GCA TG -3' [22]. The RT-PCR products were analyzed in 1.25% agarose gel containing 0.5 µg/ml ethidium bromide. Specific bands were excised from the gel and purified using Montage DNA gel extraction kit (Millipore, Concord Road Billerica, MA, USA). The purified DNA fragments were sequenced by primer walking of both strands using ABI Big-Dye 3.1 dye chemistry and ABI 3110 Prism automated DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence and phylogenetic analysis

The obtained sequence data was analyzed using BLAST web interface (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Nucleotide sequence editing and prediction of amino acid sequences were accomplished using Lasergene software, version 3.18 (DNASTAR, Madison, WI,

USA). For sequence alignment, previously published nucleotide sequences of 22 Egyptian and references RVFV strains were obtained from the GenBank (Table 1). Phylogenetic tree on the partial sequence of Gn gene (570 bp) was constructed using MegAlign program of the Lasergene software.

Results

Sequence analysis of RVFV ZH-501-VSVRI Gn gene

Sequencing of RT-PCR product of Gn gene was conducted in both direction, and a consensus sequence of about 570 bp was used for nucleotide and deduced amino acid analysis. The sequence data was submitted to the GenBank and has obtained the accession number GU953292. Sequence analysis of ZH-501-VSVRI, in comparison to the reference strains (Table 1), revealed a high degree of sequence homology with strains H501Egy sh77, BEgyAs78, HEgyGh78, MEgySh78, OEgyGh79 (91.1%), H548EgySh77 and BZimBea74 (98.9%). A lower identity was observed with the most recent RVFV human and animal isolates (95.3% and 96.1%). ZH-501-VSVRI sequence had only 96.3% identity with Smithburn strain.

Five unique nucleotide substitutions were observed along the nucleotide sequence of ZH-501-VSVRI. The sites of these characteristic substitutions are located at positions 1033 that changed from C to T, 1206 from A to C, 1252 from A to G, 1257 from T to A and 1258 from G to C.

Analysis of the deduced amino acid sequence of ZH-501-VSVRI in relation to the reference strains identified three unique amino acid substitutions: T287I, S336R and K351R that correspond to the nucleotide substitutions at positions 1033, 1206 and 1252, respectively. Sequence identity remained high (98.4%) when the deduced aa sequence of ZH-501-VSVRI compared to animal, arthropod and human RVFV sequences, regardless the year of isolation (except for bat strains, BZimSin74 and Mug44). However, the deduced aa similarity with Smithburn strain did not exceed 95.2%.

Phylogenetic analysis of RVFV ZH-501-VSVRI Gn gene

Using nucleotide sequences for building Phylogenetic tree, the ZH-501-VSVRI grouped with the parent ZH501EgySh77 and the viruses that were isolated from the seventies in the region. However ZH-501-VSVRI was located on a separate branch (Figure 1). There were obviously many clusters of RVFV that were sequenced from Zimbabwe. Only one virus from Zimbabwe clustered with the Egyptian group. Bat, mosquito and human viruses isolated in later years clustered away from the ZH-501-VSVRI. Phylogenetic analysis of the deduced aa sequences support the finding that numerically, no major changes in the ZH-501-VSVRI were present, compared to the parent ZH501 (Figure 2). It also supports the suggestion that ZH-501-VSVRI is more suited for vaccination against the circulating strains in the region.

Protean analysis of RVFV ZH-501-VSVRI Gn protein

Protean analysis showed that the aa substitutions may have changed the three dimensional structure of the Gn protein in the vaccinal strain comparing with the parent ZH501EgySh77 strain. The aa substitution at position 287 lead to the abolishment of a calculated coil at position 286 (as calculated by the Garnier-Robson method, Lasergene), as well as reducing flexibility around this region (as calculated by the Karplus-Schulz method, Lasergene) and reducing the antigenicity index (as calculated by the Jameson-Wolf method, Lasergene) (Figure 3). The aa substitution at 336 produced a predictably less flexible region in the

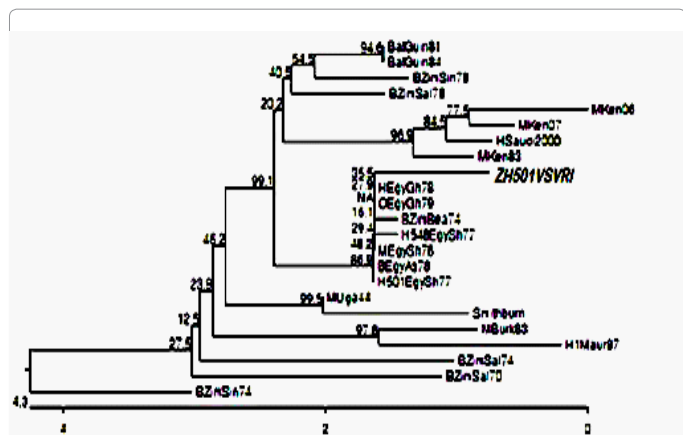


Figure 1: Phylogram of RVFV Gn gene sequence. The nucleotide sequence of Gn gene of ZH-501-VSVRI was aligned with the corresponding sequences of 22 RVFV reference strains using Clustal W program. The phylogenetic tree was constructed using MegAlign software (Bootstraps trails = 1000 and seed = 111).

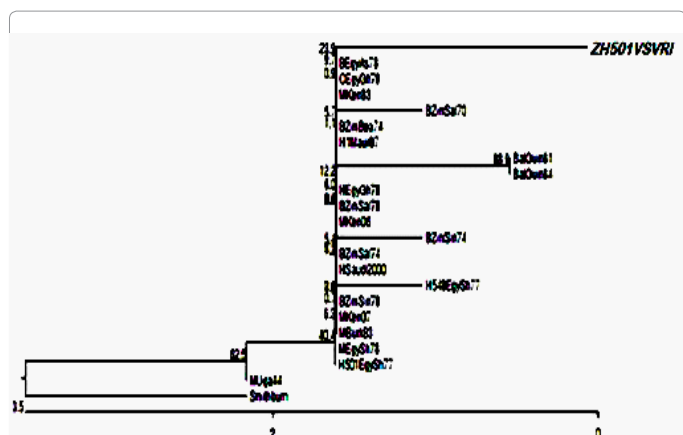


Figure 2: Phylogram of RVFV Gn protein sequence. The amino acid sequence of Gn protein of ZH-501-VSVRI was aligned with the corresponding sequences of 22 RVFV reference strains using Clustal W program. The tree was constructed using MegAlign software (Bootstraps trails = 1000 and seed = 111).

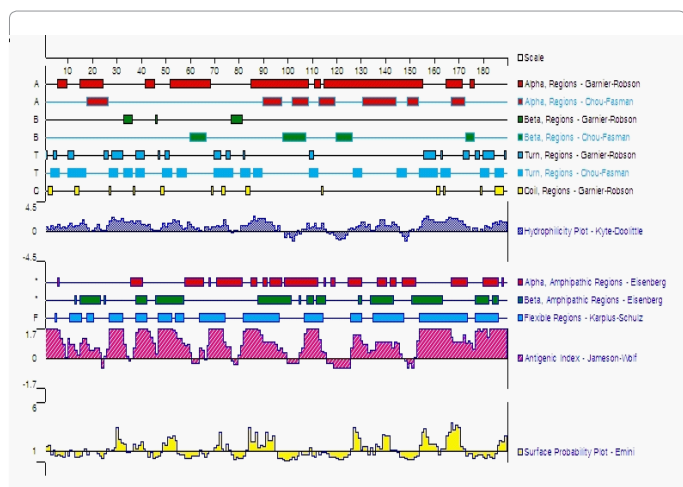


Figure 3: Protean analysis of RVFV ZH501-VSVRI Gn protein in comparison to the parent strain ZH501EgySh77.

vaccine sequences with an increased hydrophilicity index (as calculated by Kyle-Doolittle method, Lasergene) and, an additional predictable alpha region. This lead to an increased surface probability index (as calculated by Emini method, Lasergene) and, antigenicity index downstream of that region. The aa substitution at position 351 produced an additional turn region in the vaccine thus reducing the expected antigenicity index at positions 346-348.

Discussion

Rift valley fever (RVF) is an acute, sub-acute or mild arthropod-born viral disease that causes serious and fatal disease in many animal species and man. The disease is characterized by high mortality rates among lambs and calves and abortion in pregnant ewes and cows [23]. An outbreak of RVF disease was recorded for the first time in Egypt at 1977, causing high mortality and abortion rates among domestic animals with extensive human involvement [24].

The ideal RVFV vaccines for medical and veterinary use should satisfy the following criteria: a) they should prove safety in target recipients; b) they should elicit rapid humoral immune responses that neutralize the prevalent RVFV strains; and c) they should induce long-term protective immunity [25]. Historically, a live-virus vaccine prepared from Smithburn strain was used for the control of RVF in non-pregnant cattle and sheep in endemic areas and during outbreaks. The inactivated vaccines were the only choice for use in pregnant animals and in RVF-free countries [16]. Inactivated virus vaccines are usually prepared from highly immunogenic strains of RVFV propagated in cell culture. The process of vaccine production involves inactivation of the virus strain with formaldehyde and emulsification in suitable adjuvants for an enhanced immunogenicity. The principle quality control measures used for evaluation of the inactivated RVFV vaccines are the safety testing that ensures complete loss of the residual pathogenicity and the identity verification that confirm the presence of just the vaccine seed virus in the vaccine preparation [26].

In a country like Egypt, where recurrent outbreaks of RVF are consecutively occurring in man and animals since 1977, it is pivotal to fully characterize the national RVFV vaccine strain. Two main reasons justify this need: first, to ensure that the antigenic makeup of the vaccine strain is adequate to provide the required level of protective immunity in vaccinated animals, and second, to be able to provide the proper level of in-process and final quality control. Moreover, rumor have risen during the last RVF outbreak in Egypt at 2003 to be a result of improperly inactivated vaccine strain that was produced at VSVRI, however no evidence was provided to support this claim. We therefore set this study to provide the genetic reference information of VSVRI master seed RVFV strain.

Our first task was to provide evidence that vaccine strain is a homogenous mix of viruses capable of producing uniform size plaques. However, plaque assay showed only that the vaccine strain contained RVFV that produced at least two plaque sizes. The larger and more abundant of the plaques (resembled those produced by the wild-type ZH501) were picked. The smaller minute size plaques were not selected. Changes in plaque size (phenotype) have been reported for RVFV [27]. Multiple plaque purification of the virus stock does not prevent the development of small size plaques [28]. The capacity of the virus to form plaques was associated with determinants on the S gene segment [28].

The PCR fragment length of 746 bp was amplified as expected [28]. Sequence analysis of the obtained PCR fragment of ZH-501-VSVRI in comparison to ZH548 and ZH1776 showed that the PCR product lengths

Gen Bank Accession Number	Strain	Symbol	Source	Origin	Year of isolation
DQ380200	Zh-501	H501 Egy Sh 77	human	Egypt-Sharqiya	1977
DQ380206	ZH-548	H548 Egy Sh 77	human	Egypt-Sharqiya	1977
DQ380207	Zc-3349	B Egy As 78	bovine	Egypt-Asyut	1978
DQ380204	Zm-657	M Egy Sh 78	mosquito	Egypt-Sharqiya	1978
DQ380205	ZS-6365	O Egy Gh 79	ovine	Egypt- Gharbiya	1979
DQ380203	ZH-1776	H Egy Gh 78	human	Egypt- Gharbiya	1978
DQ380209	2250/74	B Zim Bea 74	bovine	Zimbabwe-Beatrice	1974
DQ380188	763/70	B Zim Sal 70	bovine	Zimbabwe- Salisbury	1970
DQ380220	1853/78	B Zim Sin 78	Bovine	Zimbabwe- Sinoia	1978
DQ380222	2269/74	B Zim Sin 74	bovine	Zimbabwe- Sinoia	1974
DQ380194	2373/74	B Zim Sal 74	bovine	Zimbabwe- Salisbury	1974
DQ380214	1260/78	B Zim Sal 78	bovine	Zimbabwe- Salisbury	1978
DQ380215	ANK-3837	Bat Guin 81	bat	Guinea	1981
DQ380216	ANK-6087	Bat Guin 84	bat	Guinea	1984
DQ380187	ARD-38388	M Burk 83	mosquito	Burkina Faso	1983
DQ380191	Entebbe	M Uga 44	mosquito	Uganda	1944
DQ380198	Kenya 83 (21445)	M Ken 83	mosquito	Kenya- Ruiru	1983
EF467178	KEN06-ELH131B08	M Ken 06	mosquito	Kenya	2006
EF467177	KEN07-KLF112	M Ken 07	mosquito	Kenya	2007
DQ380186	OS-1	H1 Maur 87	human	Mauritania	1987
DQ380197	Saudi 2000-10911	H Saudi 2000	human	Saudi Arabia	2000
DQ380193	Smithburn	Smithburn	Vaccinal strain	Uganda	1949

Table1: RVFV strains used for nucleotide and amino acid sequence comparison in the study.

can be expected with different viruses isolated within the same temporal period. Nucleotide sequence-based phylogenetic analysis revealed that BZimBea74 is clustered with the Egyptian viruses reported in the first Egyptian RVF outbreak of 1977 (Figure 1). This is a strong indication that BZimBea74 could be the virus responsible for the outbreak. However, we believe that it is very difficult to support this claim in the absence of the original samples collected during the outbreak. Retrospective serosurveys have indicated that RVFV was not present in Egypt before the 1977-78 epidemics [29,30]. ZH-501-VSVRI is therefore confirmed to belong to the same group of viruses that was isolated during that first outbreak or shortly thereafter.

Bat, mosquito and human viruses isolated in later years (Table 1), including the HSaudi2000, were found cluster away from the ZH-501-VSVRI. In the absence of sequence information pertaining to the viruses involved in the recent outbreaks of the virus in Egypt and the surrounding region, we can conclude that none of the recent outbreaks were caused by the parent virus from which the vaccine virus was isolated or ZH-501-VSVRI itself. This means that the national Egyptian vaccine is exonerated from the most recent outbreaks in Egypt and the region. We can go back to reexamine this conclusion when sequence data is made available about the recent RVFV outbreaks in Egypt.

Amino acid sequence homology and protease analysis suggest that ZH-501-VSVRI has retained the antigenic structures characteristic for the Egyptian lineage of RVFV (Figure 3). Compared to Smithburn, ZH-501-VSVRI is more suited for vaccination against the Egyptian RVFV lineage viruses, which are placed much further on the phylogenetic tree (Figure 2). ZH-501-VSVRI carries several unique regions that can be used for the development of real-time RT-PCR assays for rapid in process quality control during vaccine manufacture. It will also be a very valuable tool for the identification of whether outbreaks were caused by improper inactivation of the virus during vaccine manufacture. It is pivotal that we continue to fingerprint the remaining segments of the ZH-501-VSVRI together with the circulating virus strains to confirm it remains the ideal vaccinal strain for current circulating RVFV strains or not.

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