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TOPIC – DETECTION OF PHLEBOVIRUS BY USING QUALITATIVE REAL TIME (RT) - PCR AND APPLICATION OF SILVER NANOPARTICLES TO CONTROL IT

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ABSTRACT

The phlebovirus (family- bunyaviridae) is an enveloped negative strand RNA virus with tripartite genome. A method quantifying the small RNA segment by real time detection reverse transcriptase (RT-PCR) using Taqman technology and targeting the targeting the nonstructural protein coding region was developed and used successfully in-vitro from plasmid sample consisting of RVFV infected Vero cell. It was also useful in detecting RVFV in animal sera and also the efficiency of drugs (ribavirin, alpha interferon, and 6-azauridine) for antiviral activity. This was initially evaluated in animals and then on human samples also. The potential antiviral activity of silver nanoparticles formulated as Argovit against RVFV tested on Vero cell culture and type I IF receptor deficient mice by 2 methods was carried out (1) In–vitro: different dilutions of Argovit were administered to animal infected with a lethal dose of virus or to previously infected cells. (2) In-vivo: Viruses were pre-incubated with Argovit in different dilutions before inoculated in cell/mice. The ability of silver nanoparticle to control RVFV was limited but the incubation of virus with argovit before the infection reduced the infectivity titers.

KEYWORDS: RT-PCR, silver nanoparticles, animal are sera, Argovit, RVFV.

INTRODUCTION

The Rift Valley fever (RVF)/rift valley fever virus (RVFV) is a member of (genus - *Phlebovirus*, family-*Bunyaviridae*).[7] It contains a negative-stranded, tripartite RNA genome

comprising of a large, medium and a small (S) segment. Like other phleboviruses, the S segment utilizes an ambisense strategy to code for two proteins, the nucleocapsid protein and the nonstructural protein (NS_s), which are synthesized from subgenomic viral complementary and viral sense mRNA, respectively.[16]

Rift valley fever (RVF) is a kind of mosquito born disease that is caused due to the mosquito bite and was first found in sheeps by Daubney et al. in Kenya in 1931 cattles and other animals and was even spread to human by the body fluids of animals and also by mosquito bite.[3]

Transmission- mosquito bite, body fluids from animals.

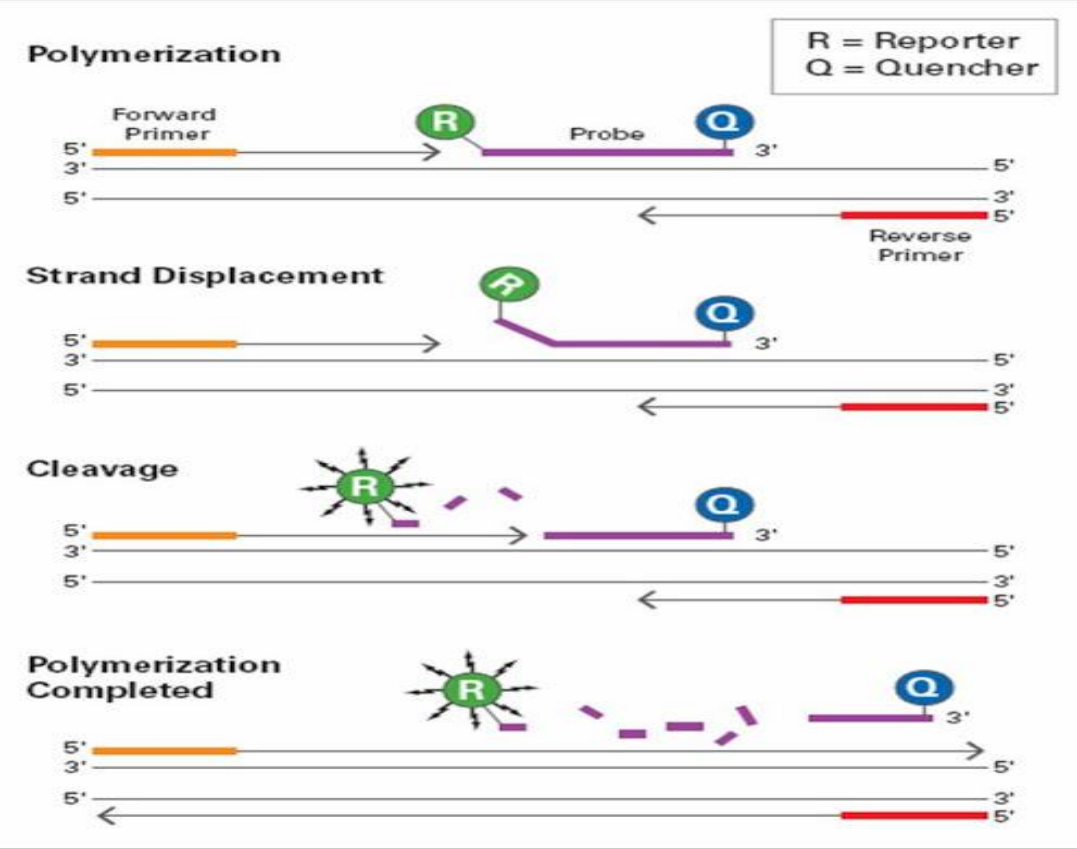
Incubation period: 2-6 days

Symptoms-asymptomatic for a very long time with acute febrile illness or sometimes may cause hemorrhagic fever, flu like fever, muscle pain, joint pain, encephalitis, rhinitis etc.

The symptoms of the rift valley fever last only for 4-7 days after which the immune responses become detectable and the appearance of antibiotics in the blood occurs with the disappearance of virus from blood.[14]

Mortality rate- 10-15% of the patients

A method quantifying the small RNA segment by real time detection reverse transcriptase (RT-PCR) using Taqman technology and targeting the targeting the nonstructural protein coding region was developed and used successfully$_8$ in-vitro from plasmid sample consisting of RVFV infected Vero cell. It was also useful in detecting RVFV in animal sera and also the efficiency of drugs (ribavirin, alpha interferon, and 6-azauridine) for antiviral activity.



RT-PCR test

As this was initially evaluated in animals and then in human samples

The potential antiviral activity of silver nanoparticles formulated as$_{17}$ Argovit against RVFV was tested on Vero cell culture and type I IF(interferon) receptor deficient mice by 2 methods was carried out

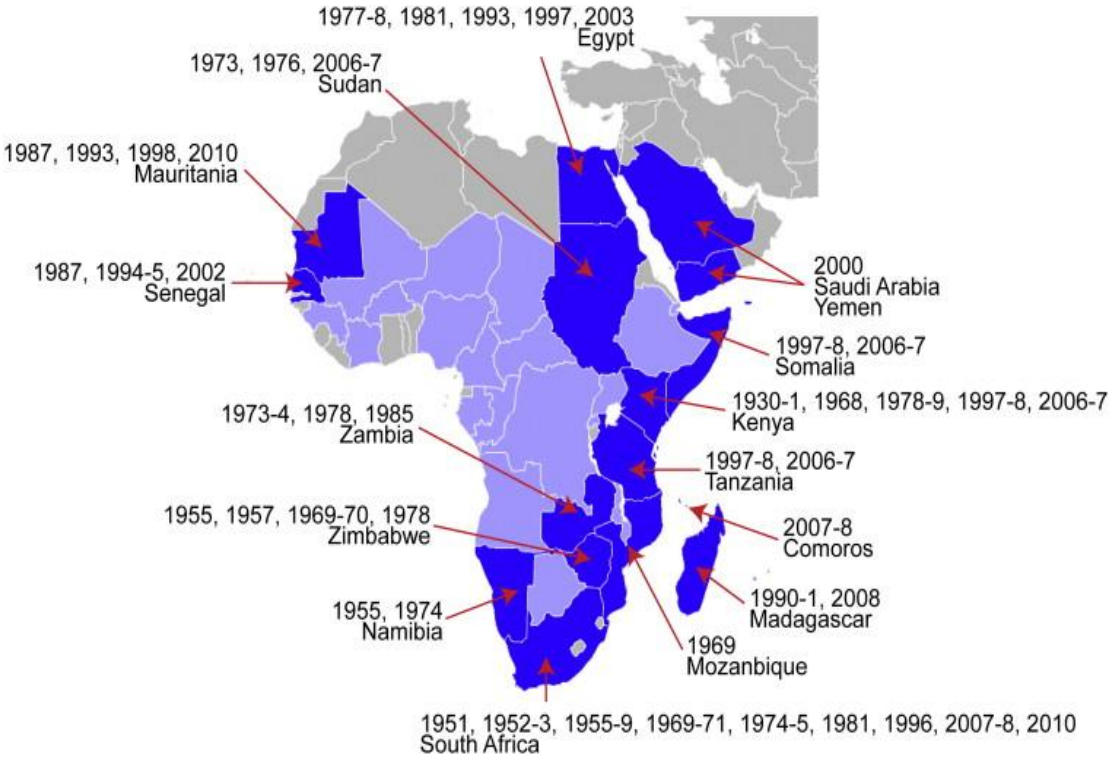
1. In–vitro: different dilutions of Argovit were administered to animal infected with a lethal dose of virus or to previously infected cells.
2. In-vivo: Viruses were pre-incubated with Argovit in different dilutions before inoculated in cell/mice.

The viruses were preincubated with argovit in different dilutions before inoculating them into the healthy cell and mice.

The ability of silver nanoparticle to control RVFV was limited but the incubation of virus with argovit before the infection reduced the infectivity titers.

Epidermology

Year of Isolation	Source	Origin	No of People Effected	Death
1931	**sheep's**	**Kenya**	None	**-**
1944		**Uganda**		
1955	**Mosquito**	**Uganda**	**10%**	**1-2%**
1977	**Mosquito**	**Kenya**	**12%**	**4-5%**
1977	**Infected livestock trade and cattle**	**Egypt**	**Only cattles**	**_**
1978	**Bovine**	**Zimbabwie**	**10%**	**6%**
1983	**Human and also mosquito**	**Burkina faso**	**20%**	**10-12%**
1983	**Mosquito,sheep**	**Senegal**	**5%**	**1-2%**
1997	**Infected livestock-Nile irrigation system**	**Kenya[18]**	**143**	**21**
2000	**Cattle and sheep**	**Saudi Arabia and yeman**	**516,1087**	**87,121**
2003	**Livestock and camel**	**Egypt**	**148**	**27**
2006	**Mosquito (aedes-genus)**	**Kenya, Somalia and Tanzania**	**684,114, 264**	**234,51, 109**
2007	**Mosquito**	**Sudan**	**738**	**230**
2008	**Ruminant trade and cattles**	**Madagascar**	**476**	**19**
2008-2009	**Ruminant trade**	**Madagascar**	**236**	**7**
2010	**Animals**	**South Africa**	**237**	**26**
2012	**Mosquito**	**Mauritania**	**36**	**18**
2016	**Human**	**Niger**	**105**	**28**



MATERIAL AND METHODOLOGY

The different types of materials and methods were used for the determination of the virus in both vevo and vivo culture.

Cell, virus and mouse sera

In vero cells

Cells were grown in 5% co_2 at 37^o in 199 culture medium (M199) which was supplemeted with 10% inactivated fetal calf serum. RVFV strains like MP12, ZH548, ZH501, 74HB59,clone 13 and also the closely related various other phleboviruses like Toscana, Icoaraci, and Belterra were grown in vero culture(ATCC CLL-81) by using serial passage.

Virus titres were determined by 2 methods

1) By counting PFU under agarose layer.[17,4,7]
2) By 50% tissue culture infective dose ($TCID_{50}$) method.

In vivo culture

Mice were inoculated intraperitoneally with 10^4 PFU of RVFV strain ZH548 and blood was collected at different times postinoculation by veinal puncture at the retro-orbital sinus.

In vitro cells (RNA in vitro transcription)

The RNA fragment (750-bp) containing the complete NS_s open reading frame of RVF virus MP12 strain in the genomic sense orientation was transcribed in vitro from the pGem3-NSs plasmid by using the T7 RNA polymerase (Promega) under the conditions recommended by the supplier. The DNA template was eliminated by extensive treatment with RNase-free DNase. Proteins were eliminated by phenol-chloroform extraction, and RNA was concentrated after isopropanol precipitation.[19] The amount of RNA was estimated by spectrophotometry, and known amounts were used to determine the standard curve for real-time RNA quantification.

Vero cells (RNA extraction)

In this the vero cells were infected with the MP12 virus at a rate of 0.01 PFU per cell, and virus present in the maintenance $medium_{12}$ was collected after 72 h when the cytopathic effect was evident. Cell debris was eliminated by low-speed centrifugation. RNA was extracted according to the manufacturer's instructions either from.

a) 200 µl of virus stock by using the RNA Instapur kit (Eurogentec, Seraing, Belgium)

b) 140 µl of mouse sera by using Trizol method (Invitrogen Life Technologies SARL, Cergy Pontoise, France),
c) 140 µl of human sera by using the QI Aamp Viral RNA kit (Qiagen, Courtaboeuf, France).

After precipitation with isopropanol, RNA was resuspended in 50 µl of RNase-free water and stored at −80°C.[8]

Design and synthesis of primers and fluorogenic probe

The available NS_s sequences published by Sall et al. (34) were aligned with DNAsis (version 2.6; Hitachi Software Engineering, Olivet, France) and Primer Premier Software was used to design the primers and probe,[16] (version 4.04; Premier Biosoft International, Palo Alto, Calif.).

The primers S432 (5′-ATG ATG ACA TTA GAA GG GA3′) hybridized at positions 432 to 450 and NS3m (5′ATG CTG GGA AGT GAT GAG3′) hybridized at positions 712 to 729 which was modified from the NS3 primer,[14] in genomic sense RNA, generating a 298-nucleotide DNA fragment. The primers were synthesized by Invitrogen Life Technologies. The Taq Man probe CRSSAr (5′ATT GAC CTG TGC CTG TTGCC3′) was synthesized by Oligo Genset (Paris, France). It contains a fluorescent $reporter_8$ dye (6-carboxyfluorescein) at the 5′ end and a fluorescent quencher dye (6-carboxy-tetramethyl-rhodamine) at the 3′ end.

The reaction was carried out at 37°C for 60 min with 10 µl of RNA in a final volume of 30 µl with 200 IU of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and 1 µM forward primer S432 under the conditions recommended by the supplier.[11]

Finally, the reaction mixture was heated at 95°C for 10 min to denature the enzyme.

Detection of RVFV RNA genome in serum sample.

RT-PCR Rounds	Primer	Nucleotide Sequence
First round	NS_{NG} NS_{CA}	5′-TATCATGGATTACTTTCC-3′ 5′-CCTTAACCTCTAATCAAC-3′
Second round	NS_{2G} NS_{3A}	5′-GATTTGCAGAGTGGTCGTC-3′ 5′-ATGCTGGGAAGTGATGAGCG-3′

During the outbreak investigation, samples were obtained from humans and animals suspected of having RVF.[12] Nucleic acids were extracted from 100-µl serum samples by disruption in guanidium thiocyanate, adsorption on silica particles, and elution in sterile distilled water, followed by nested RT-PCR as previously described.[12,4] As a precaution against contamination, aerosol barrier tips and separate rooms were used for template preparation, reaction assembly, and processing and analysis of the PCR products, on the one hand, and positive and negative controls were included among samples for the extraction and RT-PCR steps, on the other hand.

Extraction

a) Three negative controls WERE PRESENT

1. 100 µl of previously tested negative serum
2. Sterile PCR-grade water
3. Leibovitz 15 maintenance medium

b) Positive controls included serum with 10^4 PFU of RVFV strain MP12.

For the two steps of the nested RT-PCR, appropriate RT-PCR mixes like PCR-grade water to the negative one and cDNA prepared from MP12 RNA strain to the positive control tubes were added.[8] Each serum sample was independently processed twice to ensure confirmation of the results.

Identification and immunofluorescent tests using pools of hyperimmune ascitic fluid were performed 3 days postinfection by different methods,[16] In addition, samples were screened for specific RVFV immunoglobulin M (IgM) antibodies using the ELISA method previously described by Digoutte et al. Comparison of the different methods was performed by using the Fisher exact test or chi-square values calculated using Mc-Nemar's test.[20]

Among the different samples analysed the sensitivity range was 65-100% and specificity was 90-100% depending on the comparison. It had overall sensitivity of 93%, positive prediction value 97.5% and specificity upto 96%.

Real-time PCR

The amplified reaction mixture contained 2 µl of cDNA in a final volume of 20 µl, and the reaction was carried out with the LightCycler fast-start DNA Master hybridization probes kit (Roche Diagnostics, Meylan, France),[12] $MgCl_2$ at a 3.5 mM final concentration, the primers

NS3m and S432 at 0.5 μM final concentrations, and the fluorogenic probe CRSSAr at a 0.5 μM final concentration.[18] PCR was carried out in the,[8] Light Cycler (Roche) for 45 cycles at 95°C for 15 s and 60°C for 1 min.

Assay of antiviral activity of compounds in cell culture

Glycyrrhizin, ribavirin, and 6-azauridine were purchased from Sigma-Aldrich (St. Quentin-Alpha 2b interferon (IFN-α2b)[7] was purchased from Schering-Plough (Herouville St-Clair, France).

Treated cells

Confluent layers of Vero cells in 24-well tissue culture plates were infected with 0.1 ml of diluted viral suspension (0.01 $TCID_{50}$ per cell), and 2 ml of maintenance medium containing the test compound at an appropriate concentration was added (4 wells per concentration).[8] Five concentrations of the antiviral substances were tested in decreasing order from the maximally tolerated dose, which was the highest dose that did not cause microscopically detectable cytotoxic effects.

24 Well Tissue Culture Plate

Untreated cells (controls)

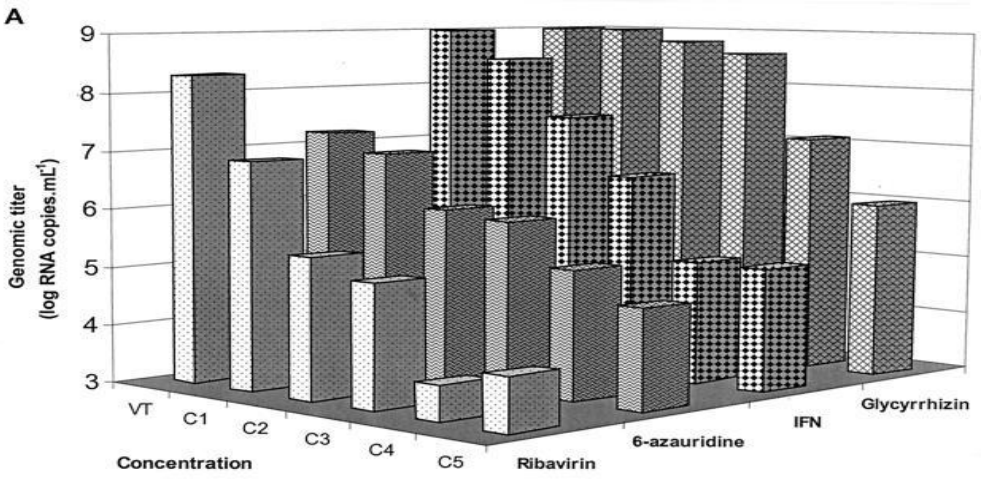
Four wells were not treated with the drugs and served as controls. After 40 h of incubation, when a maximal infectious RVFV titer was reached in the untreated cells, intra- and extracellular RVFV were obtained by three cycles of freezing and thawing. The viral titer was determined in cell culture ($TCID_{50}$ per milliliter), [7] and RNA was extracted and used to quantify the RVFV genome by real-time RT-PCR detection (RTD-PCR).

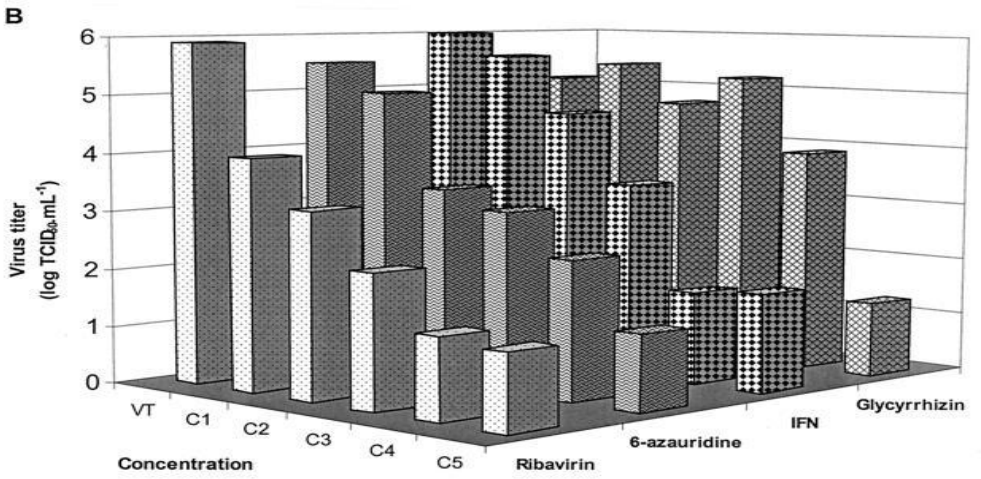
4 Well Tissue Culture Plate

Statistical analysis

Statistical analysis of the data was carried out using one-way analysis of variance and Spearman's test.[11]

Graphs[8]





Diagnosis

RT-PCR is the fastest and the easiest method of detecting RVFV in both animals and human but other methods that can even be employed for this are:

Differential diagnoses includes brucellosis, Bluetongue, Wesselsbron disease, enterotoxemia, Bovine ephemeral fever, vibriosis, trichomonosis, Nairobi sheep disease, heartwater, ovine enzootic abortion, toxic plant ingestion,17 bacterial septicaemias,8 peste des petits ruminants, anthrax and Schmallenberg disease. Laboratory diagnosis would ideally rely upon a combination of serological and molecular approaches.

Treatment

Target	Drug name	p-value	Viability	Refrence
Growth factor receptor inhibitors	Sorafenib (Nexavar) Masitinib Pazopanib HCl Paclitaxel (Taxol)	0.0001 0.0002 0.0007 0.0003	103 89 86 97	Adnane et al.,2006 Humbert et al.,2009 Zerbe et al.,2008 Welsh and Fife,2015
Microtubule assembly and disassembly modulators	Vincristine Docetaxel (Taxotere) Toremifene Citrate (Fareston, Acapodene)	0.0009 0.0027 0.0004	87 108 119	McGrail et al.,2015 LaPointe et al.,2013 Mizuuchi et al.,2015
Synthetic estrogen receptor modulators	Tamoxifen Citrate Fulvestrant (Faslodex)	0.0005 0.0035	102 84	Vogel,2015 Lai et al.,2015
Anti-parasitic[8]	Fenbendazole (Panacur) Ivermectin	0.0001 0.0015	100 95	Samaee,2015 Arndts et al.,2015
DNA synthesis inhibitor	Gemcitabine HCl (Gemzar)	0.0002	81	Sai et al.,2015,;
Antifungal agent	Itraconazole (Sporanox) Clotrimazole (Canesten)	0.0003 0.0079	88 94	Feldstein et al.,2015 Chung et al.,2015
Histamine H1 antagonist	Clemastine Fumarate	0.0008	119	Apolloni et al.,2014
Calcium antagonist	Manidipine dihydrochloride (CV-4093)	0.008	140	Rizos and Elisaf,2014
Nucleoside analog	Floxuridine	0.0042	108	Vivian and Polli,2014
Noradrenaline reuptake inhibitor	Maprotiline hydrochloride	0.0006	84	Chew and Ong,2014
Serotonin receptor agonist	Quetiapine fumarate (Seroquel)	0.001	81	Pisu et al, 2010

Use of Silver Nanoparticles for the Control of Rvfv

Silver has been known from a very long for its potent anti microbial activity and also various different formulations of silver nanoparticles are been known which contain anti viral activity in them.[12] However, the mechanism underlying this activity has not yet understood. Comparted to other anti viral agents the use of silver nano particles for the treatment is the most appropriate one as it contains many advantages like Non emergence of resistance variance, contains anti-inflammatory effect, antimodulatory effect, Safer use, Low price. Argovit is a formulation of silver nanoparticles that is being used commercially which has shown broad spectrum of antimicrobial activity and also,[8] anti-inflammatory effect.[18]

In this experiment we have tested the potency of silver nanoparticles for its antiviral activity against RVFV infection, both in cell culture as well as infected mice. The result that came out from this experiment was that silver nano particles were useful for the control of RVFV.

Methods

- Silver nanoparticle preparation
- Viruse cells and normal cell preparation
- Addition of silver nanoparticle to the cells
- Cell viability test (between treated cells and untreated cells)
- In-vitro infection experiment
- RVFV infection of mice
- Treating some mices with argovit
- Testing the treated and untreated cells.

RESULT

The assay sensitivity range was 65-100% and specificity was 90-100% depending on the comparison. It had overall sensitivity of 93%, positive prediction value 97.5% and specificity upto 96%. It reveals the useful application of silver nanoparticle in the control of important zoonotic pathogen.

Real-time RT detection-PCR of RVFV

In the Past year RT-PCR were already done for the detection of RVFV in different animals and also was proved to be useful for the diagnosis of RVFV in human. A high level of sensitivity was gained in done RT-PCR test, which detected 0.4 PFU of MP12 viruses and was approx. 100-fold more sensitive than the simple one.[17] To avoid the complications,

contaminations and also to speed up the reaction, we employed novel Light Cycler instrument (Rays Diagnostics), which contained ultrarapid thermal cycling along with TaqMan technology. In this new primers and probes were employed in the NS_S region (highly conserved) which was previously amplified by the RT-PCR.[8]

For all the **experiments** that were performed, the threshold level set was above the noise band and also the threshold cycle (c_t) was also determined. The conditions that were required for the optimization of salt and primer were obtained by RVFV MP12 RNA; those yielding the lowest C_T values were selected.[18] In the **standard conditions**, the concentrations of the forward and reverse primers and the probe were 450 nM, and the concentration of $MgCl_2$ was 2.5 mM.

Evaluation of sensitivity and specificity of the genomes was done by using serial dilutions of a known amount of RNA transcribed in vitro from plasmid pGem-NSs. The detection and quantification were linear over the range of concentrations examined, from at least 10^6 to 100 copies per run (data not shown). To test the reproducibility of the results, four aliquots of the same samples were independently amplified during the same cycles the intra-assay coefficient of variation (CV) calculated by using the C_T values (table 1) were found to vary between 0.2 and 1.8%, depending on the quantity of RNA.

Sensitivity and intraexperimental variability of RVFV strain MP12 synthetic RNA[a]

No. of synthesis RNA[b] copies	Detection rate (%)	C_T(mean ± SD)	CV (%)
5×10^6	100	18.79±0.03	1.2
5×10^5	100	24.22±0.33	1.3
5×10^4	100	29.98±0.49	1.7
5×10^3	100	31.81± 0.09	0.9
5×10^2	100	35.70 ±0.38	1.4
10^2	100	33.14 ±0.24	0.2
5×10^1	26	36.25[C]	N.D

To assay RNAs from RVFV stock, serial dilutions of samples in MP12-infected cell culture supernatants containing from 10^6 to 10 $TCID_{50}\cdot ml^{-1}$ were reverse transcribed and amplified. We observed a linear response over 10^6 $TCID_{50}\cdot ml^{-1}$ and the method could detect less than 10 $TCID_{50}$ ml of $RVFV^{-1}$ Moreover; variation with time were also tested by assaying the same samples in three different experiments carried out with a 1-day interval. The interassay CV calculated by using the C_T values was found to vary between 0.8 and 3.2%, depending on the quantity of RNA.[8] As real-time RT-PCR is of higher use for the diagnosis, we evaluated

sera from mice experimentally infected with the virulent strain ZH548 and collected it at different times after inoculation of the virulent$_{12}$. Viremia was determined by plaque assay, and after RNA extraction and amplification, the concentrations of genome equivalents were calculated (Table-2). The values obtained were in good correlation with the infectious titers. In addition to this seven human serum samples artificially contaminated with the MP12 strain were successfully collected and amplified.[15]

Real-time quantification of RVFV RNA extracted from MP12-infected from Vero cell supernatant and from sera of infected mice collected at different times post infection.

Source, infection status, and time (h)post infection	Virus titer[a]	Quantification(log RNA copies.ml^{-1})
Vero cell supernatant	6.2	9.3
infection with MP^{12}	5.0	7.0
	3.5	6.7
	2.8	4.0
	3.0	5.0
	2.7	8.0
	1.9	6.9
Not infected	Not detected	Not detected
Mouse sera	-	-
Infection with 2H548	Not found	-
24	4.3	7.5
35	3.0	8.0
50	8.8	9.5
Not infected	Not detected	Not detected

Mouse serum samples- PFU per milliliter.

Vero cell samples- TCID$_{50}$ per milliliter.[7]

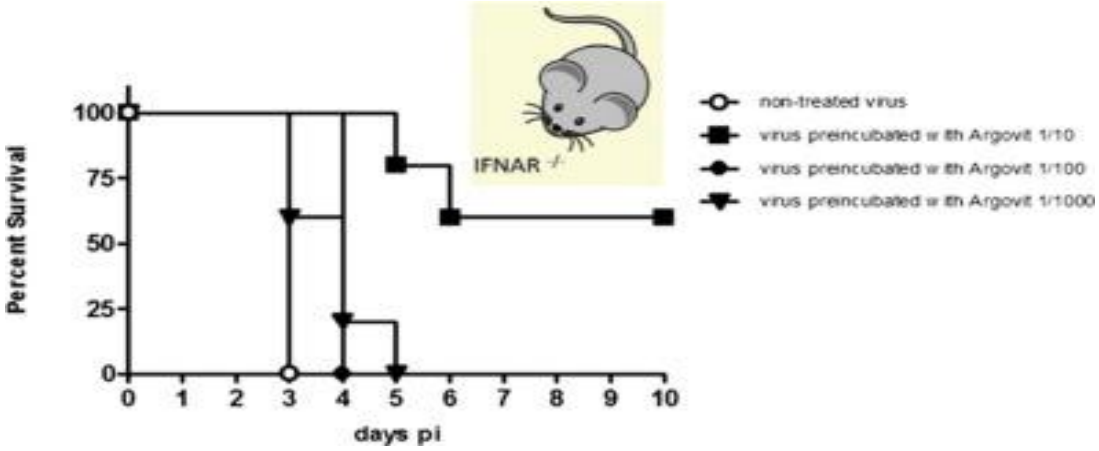
Analysis of the antiviral activity of Argovit against RVFV on cultured cells

Effect of Argovit

We found that daily administration of 120 mg/kg of Argovit silver by oral route beginning one day after infection was able to slightly reduce the viral load in lethally infected animals. However this reduction was not enough to prevent their final death.[7] This result resembled our previous finding in cell culture assays,[12] where Argovit silver nanoparticles displayed only a partial control on an ongoing RVFV infection, which could lead to a minor delay in the appearance of signs of disease and death. Since no signs of toxicity due to Argovit administration by oral route were recorded, some room is left for higher dosage treatments. Other delivery patterns, with earlier, higher and/or more frequent doses should be tested in order to impair viral production more drastically with significant delay or prevention of

disease signs and death, so that the use$_2$ of Argovit might be considered as a therapeutically post-exposure antiviral treatment.[7] In contrast, preincubation of RVFV with Argovit was much more effective in reducing viral infectivity, both in vitro and in vivo. Incubation with concentrations near the cytotoxicity threshold (12µg/ml) abolished almost completely viral propagation, leading to a 98% of infectivity reduction. In a similar way, mice inoculated with a lethal dose of virus previously incubated with 1.2 mg/ml of Argovit silver showed a delayed-onset clinical disease and mortality, with a survival rate of 60%.These results show that interaction of silver nanoparticles with RVFV severely affects its infectivity, maybe by interfering with virus-cell attachment and viral entry as suggested for other viruses such as HIV and vaccinia virus respectively, or by morphological changes in the virion that render it un-infectious, as described for influenza.[10] The latter mechanism may explain the fact that mice surviving in this experiment did not become protectively immunized as a result of the inoculation. Although still to be defined, the physical interaction of RVFV with a certain amount of silver nanoparticles seems to destroy the virus in a way that it is no longer able to productively infect animals to cause disease or death, but at the same time its antigenic structure or immunogenicity is strongly affected so that the virus is no longer recognized properly by the immune system. Thus, regardless the ability of silver nanoparticles to reduce RVFV infectivity, their use for RVFV inactivation would not be recommended.

In summary, our results open the possibility of using silver nanoparticles formulated as Argovit to control the infectivity of RVFV, which represents an important zoonotic pathogen and potential biological weapon. In order to support the use of Argovit as an antiviral agent for post-exposure treatment, further studies must be done to optimize in vivo delivery protocols.[12]



1. Effect of argovit on mice.

WHO response

For the 2016, Niger outbreak, WHO sent a multisectoral national rapid response team, including members from the Ministry of Health, veterinary services and Centre de Recherche Médicale ET Sanitaire (CERMES). The unit was deployed for field investigation on 31 August 2016.[2]

In Niger, the WHO Country Office provides technical and financial support for surveillance, outbreak investigation, technical guidelines regarding case definition, case management, shipment of samples, and risk communication.

The Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE), and WHO are coordinating on animal and human health and providing additional support to Niger for the outbreak response.

WHO is working with partners in the Global Outbreak Alert and Response Network (GOARN) to coordinate international support for the response.[2] The International Federation of Red Cross and Red Crescent Societies (IFRC) and UNICEFare supporting outbreak response.

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