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A Novel Approach for Detection of Sindbis Viral RNA – with QPCR

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Abstract

SINV was approved as a causative agent of pogosta disease. The seroprevalence of SINV antibodies for the Finnish population is around 2%, considering the prevalence varies between different regions of Finland. While the seroprevalence of SINV antibodies in Sweden highest in central parts of the country. The annual incidence rate in endemic regions of affected countries ranges from 2.7/100,000 in Finland and 2.9/100,000 in Sweden to 18/100,000 in Northern Karelia. This is the most widely distributed of all known arboviruses, affecting all age groups. This study describes the design and evaluation of a rapid and robust quantitative PCR assay able to detect a wide range of different SINV. Primers with the potential to detect all SINV were designed from conserved regions of all different strains of sindbis virus sequences, as identified from multiple alignments. By using SYBR-green-based quantitative real-time PCR (QPCR) protocols, this QPCR assay is able to detect 50-100 target molecules of synthetic DNA and less than 100 copies of viral RNA of different SINV. SINV RNA was also detected in clinical samples of patients with SINV has been linked to Pogosta disease in Finland. Ockelbo is a disease in Sweden and Karelian fever in Russia. The real-time RT-PCR assay is specific and sensitive for detection of SINV and can used for screening SINV in wildlife. This current assay provides a powerful tool for research and diagnostic laboratories where different strains of SINV are circulating worldwide and may be useful in surveys with the purpose of finding new SINV in man and other species.

Keywords: Pogosta disease; SINV; QPCR; mosquito species; bats; ticks; humans; viral RNA.

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1. Introduction

1.1. Togaviridae

The Togaviridae are a family of viruses, including the following genera: Genus *Alphavirus*; type species: *Sindbis virus*, Eastern equine encephalitis virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, Ross River virus, O'nyong'nyong virus, Chikungunya, Semliki Forest virus [1]

1.2. Alphavirus

An **alphavirus** belongs to the group IV Togaviridae family of viruses. There are 30 alphaviruses able to infect various vertebrates such as humans, rodents, fish, birds, and larger mammals such as horses as well as invertebrates. Transmission between species and individuals occurs mainly via mosquitoes making the alphaviruses a contributor to the collection of Arboviruses – or Arthropod Borne Viruses. Alphaviruses particles are enveloped, have a 70 nm diameter, tend to be spherical (although slightly pleomorphic), and have a 40 nm isometric nucleocapsid [2, 3]. The alphaviruses are small, spherical, enveloped viruses with a genome of a single positive sense strand RNA. The total genome length ranges between 11,000 and 12,000 nucleotides, and has a 5’ cap, and 3’ poly-A tail. The four non-structural protein genes are encoded in the 5′ two-thirds of the genome, while the three structural proteins are translated from a subgenomic mRNA colinear with the 3′ one-third of the genome.

There are two open reading frames (ORF’s) in the genome, non-structural and structural. The first is nonstructural and encodes proteins (nsP1–nsP4) necessary for transcription and replication of viral RNA. The second encodes three structural proteins: the core nucleocapsid protein C, and the envelope proteins P62 and E1 that associate as a heterodimer. The viral membrane-anchored surface glycoproteins are responsible for receptor recognition and entry into target cells through membrane fusion [4, 5].

SINV First time was discovered and isolated from pool of *Cx.univittatus* and *Culex pipiens* mosquitoes in the Nile River (Egypt) in 1952 [1]. SINV, a member of the western equine encephalomyelitis complex of the genus *Alphavirus* in the family *Togaviridae*, is an enveloped virus with a genome of single-stranded, positive-polarity, 11.7-kb RNA, that is contains of two domains. The 3’ one third codes for the structural proteins –the membrane glycoprotien and the capsid proteins. The nonstructural protein are coded by the 5’ two thirds of the genome[6]. SINV is present throughout the Old World but has never been found in the New World (the Americas). SINV seropositivity in humans has been reported in various areas, and antibodies to SINV have also been found from various bird and mammal species (1-3).

The virus has been isolated from several mosquito species such as bats [7], ticks and humans [8]. SINV is a member of the Western equine encephalomyelitis virus complex. SINV is classifying under *Alphavirus* genus related to the *Togaviridae* family. SINV is an arthropod –borne-virus infects several kinds of mosquitoes and transmission in nature depending upon cycle between invertebrate and vertebrate hosts [9]. The structure of SINV has been studied using X-ray scattering and electron cryomicroscopy, providing important data about SINV structure (figure 1).

The external diameter of SINV is 690Å and the molecular mass of SINV is 40 $*10^6$ daltons. SINV RNA genomic is around (4.2 $*10^6$ daltons) surrounded by an icosahedral nucleocapsid of T = 4 symmetry with a diameter of 410A° (14). SINV have two nested icosahedral protiens capsid surrounded by lipids which spiked by E1 and E2 glycoproteins [10]. The nuclocapsid is penetrated by 80 ‘‘spikes’’glycoprotein (Figure 2). Each one of these spikes includes the recognition site for the receptor on the host cell.

The life cycle of SINV which is follow in general the life span of alphavirus is drawn in figure (3).

1.3. The life cycle for SINV

SINV enter target cells by endocytosis. A few receptors such as integrins, heparin, laminin and sulphate are involved in this process. After endocytosis, conformational changes happen in viral envelope because of the acidic environmental as a result of endosome triggers, that expose E1 peptide which mediate virus –host cell fusion. This release the viral genome and permits cytoplasmic delivery of the core. (nsPs) are precursor of non-structural proteins are translated from SINV mRNA and the cleavage of this precursor gives nsP1–nsP4. nSP1 has RNA capping properties and play an important role in synthesis of negative strand of viral RNA. nsP2 displays RNA triphosphatase, proteinase activities and RNA helicase, nsP3 is unit of replicase enzyme and nsP4 is the viral RNA polymerase. The RNA drives the expression of the C–pE2–6K–E1 polyproteins, which is done by autoproteolytic serine protease. The E1 and pE2 glycoproteins are generated by cleaved into E2 and E3, The capsid C is released [4]. Viral assemply is confirmed by binding of the viral RNA to the viral nucleocapsid. The assembled SINV particle with an icosahedral core, budding from the cell membrane.

The main way for SINV transmission is via these mosquitoes which are count as a prime vector, with Migratory birds and specially grouse which has been suggested to be the most commonly infected animal and serving as the prime reservoir host. The typical symptoms of SINV (pogosta disease, Ockelbo disease, and Karelian fever) includes of itching rach, arthritis, fever, headache, fatigue, and muscle pain [11].

SINV is found in Africa, Eurasia, and Oceania but so far the clinical infection cases are reported in northern Europe especially in Finland (9, 11, and 12). After isolating the virus from several patients in 2002, SINV was approved as a causative agent of pogosta disease. The seroprevalence of SINV antibodies for the Finnish population is around 2%, considering the prevalence varies between different regions of Finland [12]. While the seroprevalence of SINV antibodies in Sweden highest in central parts of the country [13, 14].

The most cell cultures used in virology derive from primates, including monkeys and humans; rodents, including hamsters, mice, and rats and birds, most notably chickens. After that the cells were cultivated by incubate them in a nutrient medium in the presence of blood serum, which contains hormones and factors that is required for cells growth. The bovine serum is most commonly used as a blood serum.

1.4. Serology test

The diagnostic of SINV is depending on serology. Just around 40% of patients show IgM antibodies during the first week of sickness.

That gives an indication that is serology diagnostic is not precise enough to give a full picture of SINV infection. In addition, the time factor also important especially when large amount of samples need to process within short time. Because of that we looking for new method that could be serve to diagnose the SINV in easy way and short time. SINV RNA has been detected from skin lesions by using the polymerase chain reaction (PCR) method in Sweden, but no genetic sequence is available [15].

AIM

The overall aim of this work is to detect SINV by using RT-PCR and measuring the RT-PCR performance with mosquitoes samples.

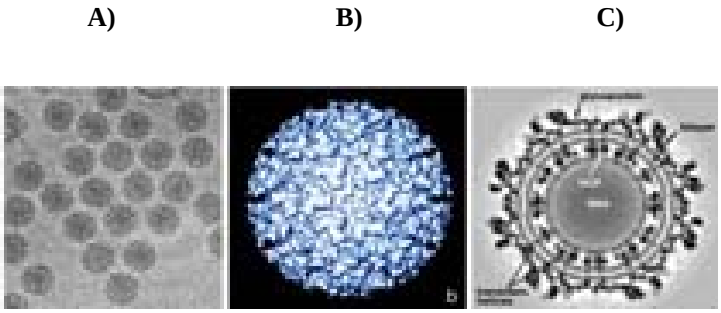


Figure 1: A) Cryo-electron microscope picture of purified SINV particles. B) Structure of SINV at 20 Å resolution. C) Cross section of SINV at 11 Å resolution included capsid protein, lipid bilayers and glycoprotiens.

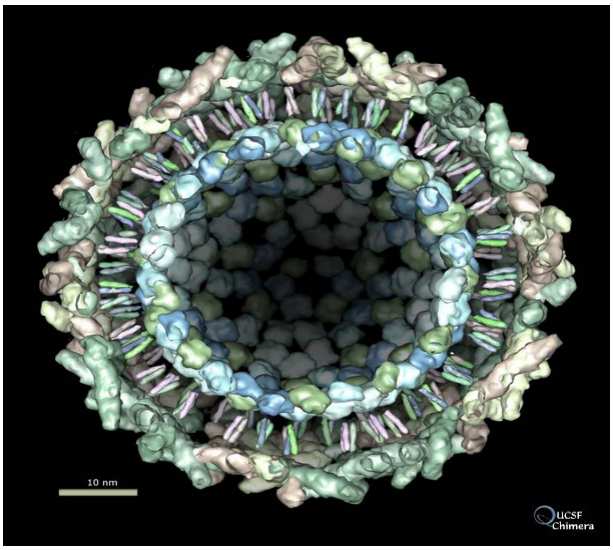


Figure 2: SINV have two nested icosahedral proteins capsid surrounded by lipids which spiked by E1 and E2 glycoproteins. Each one of these spikes includes the recognition site for the receptor on the host cell The glycoproteins have a coiled coil domain extending through the lipid bilayer [2].

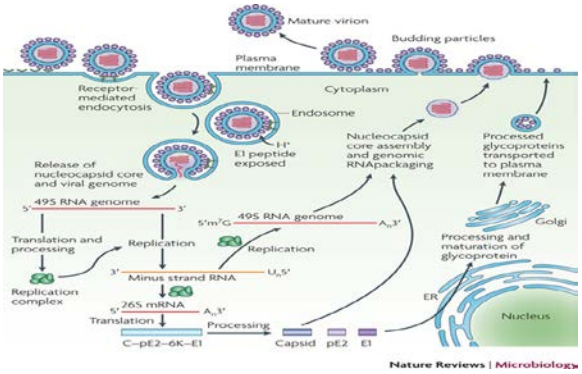


Figure 3: The life cycle for alphavirus genus[16]

2. Material and methods

2.1. SINV Samples

Two Green Monkey kidney cell lines (GMK) were used to cultivate SINV from two patient serum samples (1997,2003) and infected cell lines with adenovirus were used as a positive control to isolate SINV. Infected A549 cell line with adenovirus and uninfected cells (A549 and GMK) were used as a negative control

2.2. SINV Viral RNA Extraction

RNA was extracted and purified by using RNA Mini Kit (QIAGEN). 140µl of Viral culture was adding to 560 µl of buffer AVL including RNA carrier into 1.5ml tube, incubate for 10 min at room temperature, centrifuge the sample to remove the drops from inside the led , add 560 µl of 96% ethanol to the sample, vortex for 15 s, centrifuge the sample to remove the drops from inside the led, apply 630 µl of sample solution to the QIAamp Mini column, centrifuge for 1 min at 8000 rpm, discard the tube containing the filtrate and place QIAamp Mini column into a new 2 ml collection tube, repeat the step until all the solution loaded onto QIAamp Mini column, add 500 µl of AW1 washing buffer, centrifuge for 1 min at 8000 rpm, discard the tube containing the filtrate and place QIAamp Mini column into a new 2 ml collection tube, add 500 µl of AW2, centrifuge for 3 min at full speed, discard the tube containing the filtrate and place QIAamp Mini column into a 1.5 ml tube, add 40 µl of AVE elution buffer, centrifuge for 1 min at 8000 rpm.

2.3. Measurement of the concentration of purified viral RNA

Nanodrop (saveen Werner) was used to measure the concentration of the purified RNA samples.

2.4. Generation cDNA from SINV Viral RNA

Construction of cDNA strands from SINV viral RNA by using Gscript Rverse Transcriptase Kit.Reverse transcription reaction Mix was prepared by adding 1.0µl dNTP (final concentration 0.5mM each dNTP), GoScript™ 5X Reaction Buffer 4.0µl, Recombinant RNasin® Ribonuclease Inhibitor 1.0µl, GoScript™ Reverse Transcriptase 1.0µl, MgCl2 (final Concentration (1.5–5.0mM) 4.0µl, Nuclease-Free Water (to a final volume of 15µl) 4µl.

The other mixture contain 5µl from purified viral RNA was added to 1µl of Random Primer (0.5µg/reaction), heat block for 5 min in 70°C. Immediately chill in ice for at least 5 minutes. Combine 15 µl from reverse transcription mix to 5 µl viral RNA and random primer mix. PCR program was set to 25^0C (5 min); 42^0C (1h); 70^0C (15min) and 4^0C (14h) (Bior thermal cycler).

2.5. PCR Screening

2.6. Extract RNA of SINV from mosquito

Mosquitos samples were collected during late summer (Aug-Sep) 2012 from north Sweden and separated according their species and sex using the morphological character. *Aedes.cinereus, A. Communis, A. cantans* and *A.diantens are four* Mosquitoes species were used in this study .Mosquitoes were frozen at -70°C, transported to the laboratory. Then five mosquitoes in 500 µl of cell culture medium (high-glucose Dulbecco's modified Eagle's medium [DMEM; Sigma-Aldrich, St Louis, MO] with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 _g/ml streptomycin (23). Add the solution mixture to 2ml Lysing MatrixD tube that contains 1.4mm ceramic spheres, Homogenized the samples in the morta FastPrep-24 machine for 2 min at 50 Oscillation/s. The suspensions were clarified by centrifugation (5,000×*g*)**.** For 1 min), and the supernatant was used for RNA extraction with a viral RNA mini kit according to the manufacturer's protocol (Qiagen).

3. Real –time RT-PCR

Specific primers were designed to amplify 75 bp from sindbis genome which is binding in nsP1 regions (5´to 3´ direction). This region represent the target and conserved region of different strains of sindbis virus, forward primer 5´-GGTTCCTACCACAGCGACGAT-3´; nt postion 227 to 247, and reverse primer 5´-TGATACTGGTGCTCGGAAAACA-3 nt postion 279 to 301; (24). PCR reaction was prepared by adding 25µl KappaTaq 2x Ready mix with Mg^{+2}(kappa Biosystem), 2µl of forward primer (final concentration 0.4 µM), 2µl of reverse primer (final concentration 0.4 µM), 1µl of SINV cDNA template, up to 50µl of MQ water. The SybrGreen (Applied biosystems , USA) was labeled at the 5´-end . The assay was carried out using ABI prism 7700 sequence detection system (Applied biosystems , USA) the reaction was set according to this program ; 95^0C (15min);35 cycle of 95^0C (15s), and 60^0C (30 s) (figure 5)

4. Results

4.1. Evaluation of SINV infected cells by using PCR

Firstly we want to evaluate SINV by using SINV infected cell lines (1997) and uninfected cells were used as negative control. After purify RNA from infected cell lines and measuring the concentration of Viral RNA, we construct cDNA strands using SINV RNA as template Then by using specific designed primers we amplify 75 bp from sindbis genome which is represent the target and conserved region of different strains of sindbis virus. The result shown that the PCR fragments successfully generated and the result of gel electrophoresis showed the PCR fragment bands presented as the same and expected sizes (figure 3). Tenfold serial dilution (10^7-10^1) of viral RNA of Sindbis virus that is purified from infected cell line from patient serum in 1997 for investigating the numbers of copies and determine the concentration of SINV.

Construction of cDNA strands using purified SINV RNA as template then by using specific designed primers we amplify 75 bp from Sindbis genome which is represent the target and conserved region of different strains of Sindbis virus. The result shown that we got the same expected bands size in all dilution. We can observe the brightness of the bands decrease Proportionally from left to right regarding to samples dilution (figure 4). From these results we can conclude that we successfully evaluate the SINV in infected cells.

SINV was successfully evaluated in the infected cell lines. SINV RNA will be prepared from eight mosquitos samples from Sweden and just three mosquitos samples(A1,A2 and A5) were used as a positive control by spiked them with RNA SINV from (1997) sample to test how RT-PCR assay performance on mosquitos samples Figure (6).The results shown that qPCR assay detected viral RNA of all spiked mosquitoes samples.

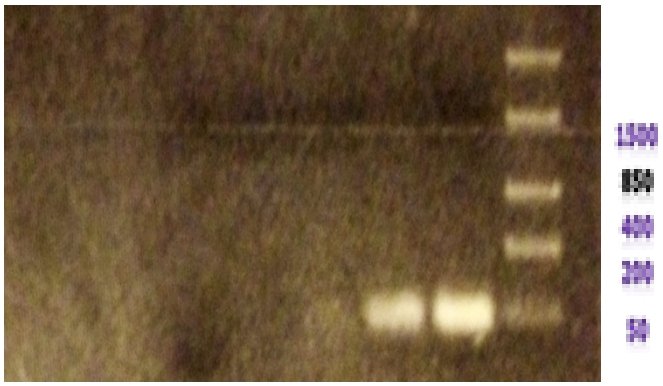


Figure 3: Agarose gel electrophoresis of amplified PCR fragments of conserved region from Sindbis virus. The two lanes directly right of the ladder shows PCR construct for the conserved region of SINV which is represents 75bp. The lanes number 3 and 4 as negative control for the uninfected cells

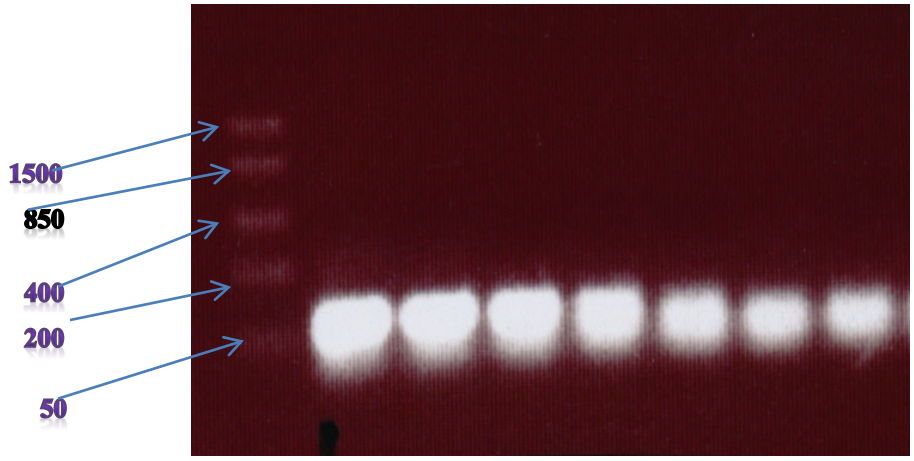


Figure 4: Agarose gel electrophoresis of different diluted amplified internal of viral RNA of sindbis virus (1997). The first lane right the ladder represent low diluted ($^{10\text{-}1}$) SINV PCR fragments, the lanes from (2-7) were gradually descending until SINV PCR fragments become high diluted ($^{10\text{-}7}$).

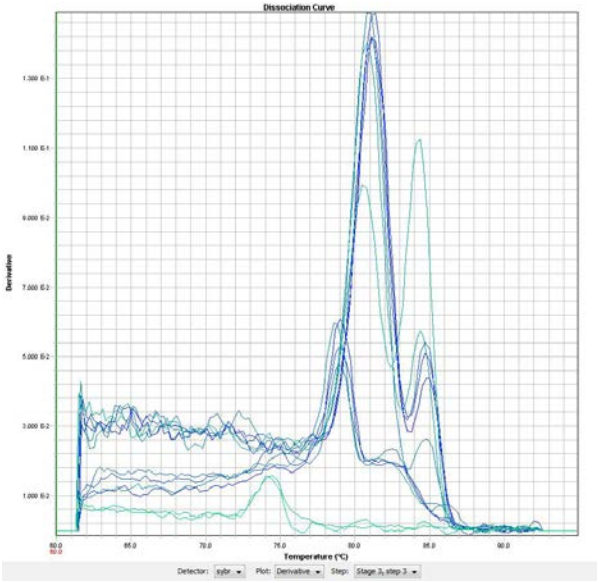
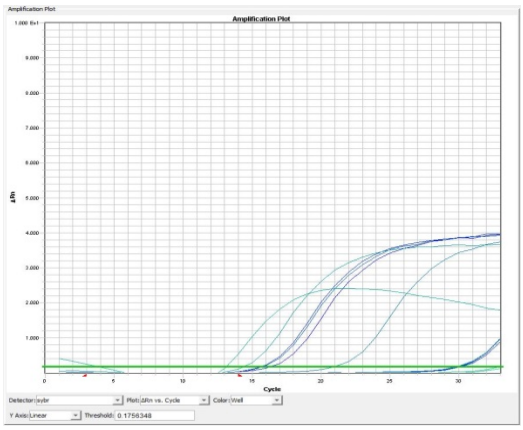


Figure 5: the dissociation curve of SINV from mosquitos spiked with RNA SINV from sample 1997



Posi	ID	Dye	Type	Ct	Tm
A1	Sind-M1-S*	sybr	Unknown	16,5	81,1
A2	Sind-M2	sybr	Unknown	0	79,0
A3	Sind-M3-S*	sybr	Unknown	15,7	81,4
A4	Sind-M4	sybr	Unknown	0	78,9
A5	Sind-M5-S*	sybr	Unknown	15,8	81,1
A6	Sind-M6	sybr	Unknown	29,9	78,9
A7	Sind-M7	sybr	Unknown	21,1	80,8
A8	Sind-M8	sybr	Unknown	0	78,6
A9	Virus 97	sybr	+Ve	14,5	80,8
A10	Virus 2003	sybr	Unknown	13,0	84,4
A11	GMK	sybr	-ve	0	74,5
A12	H2O	sybr	Unknown	0	74,2

Figure 6: real-time RT-PCR analysis of SINV from mosquitos spiked with RNA SINV from sample 1997.

5. Discussion

Clinical infections have been reported for Sindbis virus in North Europe, Africa and Australia. The SINV diagnosis is based on serology tests in previous studies [15, 17, 18]. Described a double –sandwich SINV which is catch IgM enzyme immunoassay (EIA) coated with anti-human chain antibody are first managed and evaluated by test serum and after that with purified SINV, rabbit Anti-SINV immunoglobulin, and with labeled anti rabbit swine IgG. The qPCR assays perform a great sensitivity: with the sensitivity little than 1 ng of total RNA [19-21]. In addition, the Specificity of this assay which is transported by PCR master mixes and the combination of SYBR Green primers insures a single product of the predicted size from every reaction without secondary products [20]. The high reproducibility of the qPCR system, with replicate correlation coefficients > 0.99, gives us indication that experimental samples can be reliably compared across plates and runs. A real-time RT-PCR assay was developed to detect SINV and serves as a diagnostic tool for SINV human infection.

Our results indicate that SINV was successfully detected with conventional PCR & qPCR in the infected cell lines. Also the results shown that qPCR assay successfully detected viral RNA of all spiked mosquitoes samples (A1,A3 and A5) (figure 6) . We got a weakly positive results in samples (A6 and A7) (figure 6), and that's could be contamination, because of that we sequenced them to confirm if we have SINV in these samples or not. Another advantage that we observed when we used qPCR with Mosquitoes samples, the viral RNA of the SINV are not affecting or inhibiting with Mosquitoes DNA, RNA and the other molecular components .That's show us how much qPCR is sensitive and specific [21].

Finally we can conclude that the real-time RT-PCR assay is specific and sensitive for detection of SINV and can used for screening SINV in wildlife.

Acknowledgment

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