Gene S characterization of Hantavirus species Seoul virus isolated from *Rattus norvegicus* on an Indonesian island

Dian Perwitasari,[1] Ima Nurisa Ibrahim,[1] Andi Yasmon[2]

[1] National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia
[2] Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Corresponding author: Dian Perwitasari
E-mail: dian@litbang.depkes.go.id or perwita_d_s@yahoo.com

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Abstrak

Latar belakang: *Hantavirus hidup dan berkembang biak di tubuh hewan pengerat, salah satunya Rattus norvegicus yang banyak ditemukan di daerah kepulauan di Indonesia. Hantavirus spesies Seoul virus (SEOV) adalah virus RNA negatif rantai tunggal yang termasuk dalam keluarga Bunyaviridae, mempunyai beberapa gen spesifik terutama gen S yang dapat dikembangkan untuk uji diagnostik. Tujuan penelitian ini ialah untuk mengetahui karakter dari gen S dari Hantavirus spesies Seoulvirus.*

Metode: *Pada penelitian ini dilakukan sekuensing gen S yang berasal dari jaringan paru-paru rodensia. Fragmen DNA yang disekuensing menggunakan primer DNA* SEOS-28F *dan* SEOS -360R,VNS-1501F *dan* VNS-CSR. *Hasil sekuensing dianalisis menggunakan program* seqscape *dan dianalisis menggunakan program Bioedit dan Mega5. Analisis filogenetik untuk homologi nukleotida dan asam amino dari ketiga strain Kepulauan Seribu tersebut dibandingkan dengan spesies hantavirus lainnya yang diambil dari genebank.*

Hasil: *Analisis Homologi nukleotida dan asam amino antara strain Kepulauan Seribu dengan SEOV menunjukkan homologi nukleotida tertinggi pada strain KS74 (88,4%) dan terendah pada KS90 (87,2%), sedangkan homologi asam amino tertinggi adalah strain KS74 (91.3%) dan terendah pada strain KS90 (89,5%).*

Kesimpulan: *Karakter gen S virus yang ditemukan di Kepulauan Seribu sebanding dengan virus SEOV yang ditemukan di Singapura dan Korea.* ***(Health Science Indones 2014;1:1-6)***

Kata kunci: *Seoul virus, gen S, Kepulauan Seribu, Indonesia*

Abstract

Background: Hantavirus lives and reproduces in the body of rodents. *Rattus norvegicus* was one found in the Kepulauan Seribu islands of Indonesia. Hantavirus species Seoul virus (SEOV) is a negative single chain RNA viruses included in the family Bunyaviridae. It has a few specific genes, especially genes S that can be developed for a diagnostic test. The aim of this study was to ascertain the character of gene S of hantavirus species Seoul virus.

Methods: Gene sequencing of S Seoul virus from lung tissue of rodents was conducted. DNA fragment sequencing used primer pairs of SEOS-28F and SEOS -360R, VNS-1501F and VNS-CSR. The results of sequencing were analyze by *seqscape* program to obtain a sequence of nucleotides, and analyzed by Mega5 programs. Phylogenetic analysis was done for homology nucleotides and amino acids which were compared to other hanta virus species from the gene bank.

Results: The comparison analysis showed, the highest homology from strain KS74 was 88.4% and the lowest from strain KS90 was 87.2%. The highest homology of amino acids sequence compared with Seoul virus came from strain KS74 was 91.3% and the lowest came from strain KS90 was 89.5%.

Conclusion: Gene S of viruses was found in Kepulauan Seribu in Indonesia and it was comparable to that found in Singapore and Korea. ***(Health Science Indones 2014;1:1- 6)***

Key words: Seoul virus, S segment, Kepulauan Seribu

Hantavirus was first recognized in 1950 in the Korean War. The first overall number of Hemorrhagic Fever cases with Renal Syndrome (HFRS) ever reported worldwide was approximately 60000-150000.[1] More than 90% of these cases occurred in Asia countries,[1] while Hantavirus Pulmonary Syndrome (HPS) cases were reported to have occurred in Europe and America.[2] Hantavirus species causing HFRS have a mortality (Case Fatality Rate/CFR) ranging between 5% -15% (WHO, 1982).

In 2002, studies in Indonesia have reported that the prevalence of hantavirus infection in the population living on the harbor at Batam island and Makassar was 1.6%.[2] From 1052 serum of rodents from Batam island, Serang, Jakarta, Subang, Semarang, and Makassar that were examined serologically by ELISA, hantavirus was found as much as 2.3%, 9.0%, 28.6%, 4.2%, 12.4% and 5.1%, respectively.[3,4] Hantavirus infections were also reported in several species of rodents (*Rattus norvegicus* and *Rattus tanezumi*) and animal insectivores (*Suncus murinus*) in the port of Jakarta and the Kepulauan Seribu.

Transmission of hantavirus infection through air containing the virus can be derived from the urine and saliva of infected rodents.[5] The environment passed by rodents was the place that had the potential to spread hantavirus.[1,3,6] Infection can occur in humans if the human is in the environment and accidentally inhaled contaminated hantavirus aerosol.[3,6] On the other hand, infection can occur in rodents through direct contact with other animals (rodents) previously infected with hantavirus.

Hantavirus is a negative single stranded RNA virus, which belonged to the Bunyaviridae family. Hantavirus genome consists of three genes: S (small) segment, M (medium) segment, and L (large) segment. RNA S segment encodes the nucleocapsid protein (N, 48 to 58 kDa) and M RNA encodes the two glycoproteins, Gn external polyprotein (68-76 kDa) and Gc (52-58 kDa), while transcriptase L segment (246-247 kDa) encodes the L protein.[1] Of the third gene, the S gene has immunogenic properties and contain many conserve region.[7]

Therefore, in this initial study Hantavirus, the S gene isolated from the Kepulauan Seribu will be characterized. The result of this study is expected to be the initial data for the complete genome sequence of cloning studies on Hantavirus strain Indonesia. Further results of this study can be used for characterization of the hantavirus genes M and L, which is expected to contribute in the development of vaccines, antivirals, and immunodiagnostic tests.

METHODS

Sample

This study was a continuation of a previous study (2009) of 83 serum samples from the animal reservoir (rodents) and examined by ELISA.[8] The positive results obtained in 3 samples of KS (Kepulauan Seribu)74, KS80 and KS90 hantavirus were examined. Then RNA extraction and isolation were performed. Once that was done, the reverse reaction of RNA into cDNA was performed and the results stored at-20^{o}C.[9]

PCR (Polymerase chain reaction)

Amplification of cDNA from the RT reaction was performed by PCR using Taq Gold DNA polymerase (AB applied) and a pair of specific primers. For the PCR I reaction mixture (25 ml) to the PCR micro tube was added 2.5 ml of 10X PCR buffer [200 mm Tris-HCl (pH 8.4), 500mm KCl], 0.75 ml of 50 mM MgSO4, 0.5 ml of 10μM dNTP mix, 0.5 ml of 10 μM forward and reverse primer 10 μM, 0.2 ml Taq DNA polymerase Gold, 1-3 ml cDNA products, and aquabidest or (Diethylpyrocarbonate) DEPC until the volume of the mixture reached 25 ml. Then PCR was performed using the sequencer machine (Biorad). The reaction begins with heating at a temperature of 94^{o}C for 2 min. Then, denaturation phase at a temperature of 94^{o}C, elongation at a temperature of 66 – 40°C with 4 touchdown cycles, and extensions at a temperature of 68^{o}C. The next stage was carried out at a temperature of 94^{o}C (30 seconds), 50^{o}C (30 seconds), and 68^{o}C (2 minutes), this stage was carried out for 35 cycles. The last stage at 68^{o}C for 10 minutes, then stored at 4°C to be used for electrophoresis. The heating stage can vary depending on the length of the sequence bands. To simplify the process, the Seoul virus cDNA PCR was amplified into two sections.[10] The primers pairs used were SEOS28F (TAGTAGTAGACTCCCTAAAGAGCTACTA) and SEOS-360 (TGTCCTGT AGGTTCATCA-ATGTCAAG), VNS1501F (AGCA CAAT-CACTGCCATGTA), and VNS-CSR (TAGTA GTAGGCTCCCTAAAAAGACAA).

Gel electrophoresis

The agar rose gel was made by putting 0.5 g of 3 tablets in 100 ml of TAE buffer and heated until it was melted. Ethidium bromide was then added (2.5 ml) before poured into chamber. The sample was introduced into the gel wells and another marker

added was in the wells. The sample was run in the gel for 15 – 25 minutes.[11]

Purification

The purification procedure can use two types of products, MinElute or QIAquick from QIAGEN. This study used MinElute QIAGEN products for purification. For the short steps, 5 volumes of Buffer PBI was added to 1 volume of PCR products using a column to bind DNA and centrifuged for 1 min at 13,000 rpm. It was then washed using 750 ul of PE buffer that served to remove residual ethanol and then centrifuged for 1 min. To dissolve the DNA, 10 ml EB was added in the middle column, centrifuge for 1 minute and was ready for sequencing.[9]

Sequencing

The sequencing procedures were done with BigDye (ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit) and 5 sequence buffers were used according to the manufacturer's instruction. The volume to be added in the reaction was 0.5 ml of 10 µM primers and predicted cDNA product 1 – 50 ng (estimated 100 – >2000 bp) of PCR products. There were several stage of reaction temperature for PCR; the first phase of 96°C for 1 min, second stage 96°C for 10 sec, 50°C for 5 seconds and 60°C for 4 min (this step is repeated as many as 25 cycles), and the fourth was 4°C. Before sequence analysis, excess dye was removed with terminator removal kit (Dye XTM. 2.0 spin kit 250) Avg. 63 206) or with BigDye Ex which was a mixture of 10 ml solution with 45 ml SAM Buffer solution.[9] Some samples can be analyzed using a 3130 DNA sequence system (Applied Biosystems) or (fmol DNA sequencing system; Promega) or by sequence fluorescent techniques (Terminator Cycle Sequencing kit dRodhamine; Applied Biosystem) using ABI 377 automatic sequencer.

The results of S gene sequence were edited and analyzed using the seqscape program (AB). Having obtained the complete sequence of the S gene, the gene was then compared with other strains to determine phylogenetic tree. The comparison of sequence was read using Bioedit program. To output data was using Mega 5 program and ClustalW program (European Bioinformatics Institute). The result of a complete nucleotide sequence of the gene S of strain KS74, KS80 and KS90 was compared with the complete sequences of Hantavirus species taken from the Gene Bank.

RESULTS

The identification of S segment of Hantavirus was done by RT-PCR with two pairs of primers, SEOS-28F and SEOS -360R and VNS-1510F and VNS-CS8R. The specific bands of the S segment from the specimens can be seen between 250 to 500 bp (Fig.1 and 2). The length of the specific bands were the same as the positive control. This indicated that the DNA SEOV of Kepulauan Seribu was conserved.

The Figure 1 showed the results of PCR products using primer pairs SEOS – 28F and SEOS – 360R which were analyzed on 1.5% agar rose gel. Sequentially from the left lane: Marker 1 kb. Lanes 1, 2, 3: the sample with the predictions of approximately 332 bp DNA fragment. The marker for lane 4 was Marker 100 bp.

Figure 2 showed the results of PCR products using primer pairs VNS-1501F and VNS-CSR which were analyzed on 1.5% agar rose gel. Sequentially from the left lane; Marker 100 bp. Lane 1: negative control. Lanes 1, 2 and 3: the sample with predictions of approximately 250 bp DNA fragment.

The RT-PCR product was sequenced and the analysis of the sequence was closely related with the Seoul virus. Table 1 showed accession number of the sequencing from another country, this was used for comparison in the study.

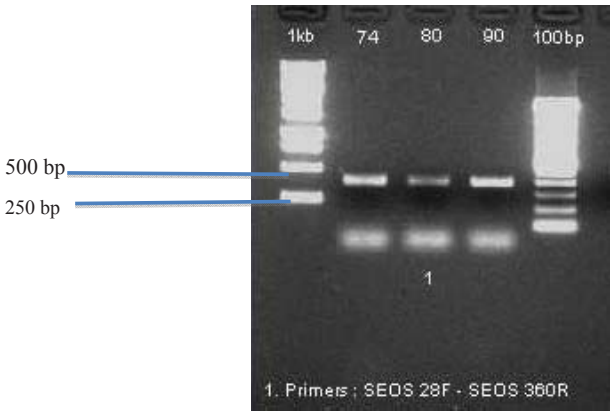

Figure 1. Primer SEOS 28F – SEOS 360R

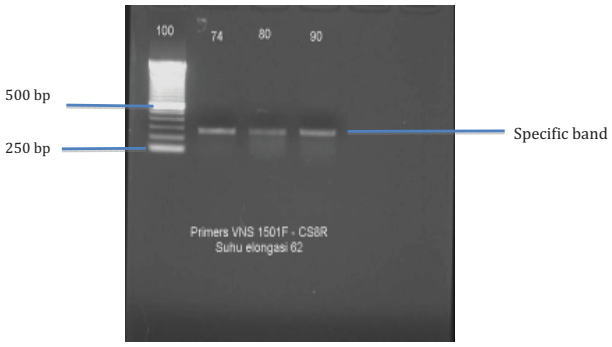

Figure 2. Primer VNS-1501F and VNS-CSR

Phylogenetic tree analysis can be used to determine the relation of kinship, epidemiology, and geographical distribution found in the strains.[12,13,14] From the analysis of the phylogenetic tree, all three strains were used in this study adjacent to the strain from Seoul virus (Korea) and Singapore strain. The first branching indicated the possibility of strain KS74 and KS80 were derived from strain KS90 and from Singapore. Branching is still on a branch with a strain originating from Seoul virus/ Korea, so that the strain found in the Kepulauan Seribu were closely related to the Seoul virus derived from Singapore and Korea (figure 3).

Table 1. Comparisons to the Seoul Virus Strain

Strain	Place/Country	Years	Sources/accession number
KS74	Kepulauan Seribu/Indonesia	2009	Used in this study
KS80	Kepulauan Seribu/Indonesia	2009	Used in this study
KS90	Kepulauan Seribu/Indonesia	2009	Used in this study
Singapore/06(RN46)	Singapore	2009	GQ274945
Strain 80-39	Korea	2003	NC__005239
Thailand 741	Thailand	2004	AB186420
Serangvirus	Serang/Indonesia	2004	AM998808.1
Dobravavirus	Rusia	2011	JF920152
Tulavirus	Europe	2011	NC__0055227.2
Andesvirus	Argentina	2002	NC__003466.1
Araucariavirus	Brazil	2004	AY740633.1
Cao Bang strain 3	Vietnam	2007	EF54324.1
Gen S	China	2000	AY006465.1.

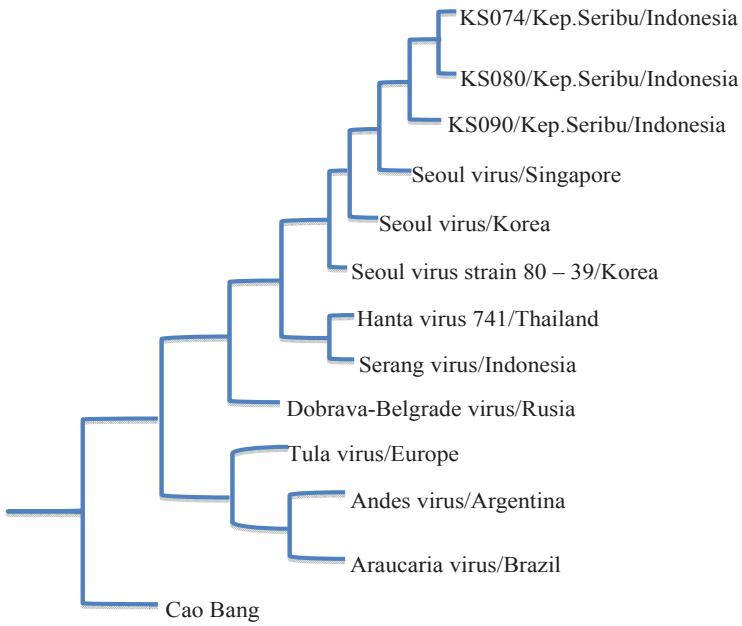

Figure 3. Phylogenetic tree of Indonesia viruses found in Kepulauan Seribu Phylogenetic tree was generated by Mega 5 software; Cladogram UPGMA analysis of S gene nucleotide Seoul virus traces of three strains of virus found in the Kepulauan Seribu in 2009 by Hantavirus species S gene from other countries.

Nucleotide sequences of S gene from the Kepulauan Seribu viruses were compared to the complete S gene derived from the Gene Bank with ascension number AY006465.1 with length of the S gene was 582 bp. Although there were differences in DNA sequence compared to other Hantanvirus species, comparison with the Seoul virus showed high sequence homology among species (figure 3).

The homology of amino acid and nucleotide of gene S can be seen in Table 2. The results of nucleotide homology to Seoul virus between species showed the highest homology to the KS74 strain (88.4%), while the lowest homology was shown in strain KS90 (87.2%). The results of homology between strains of the Kepulauan Seribu showed the highest homology between strains KS74 to KS80 was equal to 98.5%. Comparison of nucleotide homology between the Kepulauan Seribu strains, showed the lowest was between strains KS74 and KS90 which was equal to 96.8%.

Table 2. Homology of amino acid and nucleocapcid protein (gen S)

Nucleotide / Amino acid	Seoul virus/ Korea (%)	Seoul virus strain 80-39/ Korea (%)	Seoul virus/ Singapore (%)	KS074/ Kepulauan Seribu/ Indonesia (%)	KS080/ Kepulauan Seribu/ Indonesia (%)	KS090/ Kepulauan Seribu/ Indonesia (%)
Seoul virus/Korea	-	96.5	96.9	88.4	87.5	87.2
Seoul virus strain 80-39/Korea	99.0	-	96.9	88.3	87.5	87.0
Seoul virus/Singapore	98.8	99.7	-	89.3	88.6	88.2
KS074/Kepulauan Seribu/Indonesia	90.4	91.3	91.1	-	98.5	96.8
KS080/Kepulauan Seribu/Indonesia	89.9	90.9	90.6	98.9	-	97.9
KS090/Kepulauan Seribu/Indonesia	89.5	90.4	90.2	98.4	99.4	-

DISCUSION

Primer pair SEOS-28F and SEOS -360R was used for amplification of DNA fragment, which have length of 332 base pairs. The amplifications result was analyzed on 1.5% gel agar rose. The DNA migration band was lower than DNA marker 400 bp, the band was between marker 250 – 500 bp. The length of the DNA band was expected. The second fragment used primers pair VNS-1510F and VNS-VNS-CSR, where the length was around 250 bp. This suggested that the gene was a piece of S specific genes from Seoul virus, because the primers used were specific primers for Seoul virus. The primers were used to view characterization from many samples from other country, such as from the Nakamura study in Thailand.[15] In another study by Plyusnin and Ibrahim same primer were used.[16] Specific primers were used for amplifications of S Seoul virus genes. The estimation fragment length using this primer was approximately 1039 bp. The result showed migrations of DNA fragment were above the DNA marker of 1000 bp.[17-19]

The phylogenetic tree showed a close relationship between viruses found in the Kepulauan Seribu to other viruses found in South East Asian countries. In recent years, in the Asian region of the Russia Federation, around 100 to 100 HFRS cases were reported each year.[20, 21]

The highest amino acid homology comparison between strains of the Kepulauan Seribu, Korea and Singapore was indicated by the strain KS74 which was equal to 91.3%, while the lowest homology was shown by the strain KS90, which was 90.2%. Comparison between the Kepulauan Seribu strains, showed the highest homology was between strain KS80 and KS90 which was equal to 99.4%, whereas the lowest homology was between the strains KS74 and KS90 which was equal to 98.4%. Comparison of nucleotide sequence homology and amino acid homology to the S gene obtained from the Kepulauan Seribu virus strains showed three different strains of other viruses. These strains can be regarded as 3 new strains derived from the Kepulauan Seribu, strain KS74 from Tidung Island, while KS80 and KS90 was derived from the same island but from the island grill. Nucleotide changes that occur in several places provide information that these changes were due to substitution mutation and no mutations, deletions or insertions were found.[22, 23] This meant the pattern of mutations that occurred did not cause changes in reading frame (frame shift) in the S gene

open reading frame which caused the protein to be truncated or changes in the composition of amino acids.[21, 23] The analysis showed that the amino acid sequences between Seoul virus strain showed high homology. Accordingly, the N protein from strains of the Kepulauan Seribu can be used as an antigen basis for the development of a diagnostic test to detect Hantanvirus Seoul virus in the future.

In conclusion, gene S of viruses was found in Kepulauan Seribu in Indonesia and it was comparable to that found in Singapore and Korea.

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