ANTIGENIC SITE OF GLYCOPROTEIN ENCODING GENE IN RABIES VIRUS ISOLATE FROM INDONESIA

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ORIGINAL ARTICLE

ANTIGENIC SITE OF GLYCOPROTEIN ENCODING GENE IN RABIES VIRUS ISOLATE FROM INDONESIA

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ABSTRACT

Rabies is one of the most harmful zoonotic diseases in the world, affecting both animal and human health. Each year, 55,000 people die from rabies: 56% of deaths are in Asia and 54% in Africa. In Indonesia, rabies cases (98%) are usually transmitted by dogs (domestic and wild dogs) (96.79%), cats (1.06%) and apes (0.15%). The aim of this study was to determine the homology score and predict the antigenic sites of the glycoprotein (G-protein) encoding gene from rabies virus in Indonesia using molecular analyses. G-protein gene from isolated samples was amplified using a two-step RT-PCR followed by sequencing. Results show that homology scores of Indonesian rabies virus isolates against reference virus isolates obtained from GenBank records are the following: Indonesia (93-98%), China (89-91%), Thailand (83-86%), India (82-84%), Korea (83-85%) and Pasteur strain (82-83%). Only antigenic site I of isolated sample from Sumatra changed, while antigenic sites II and VI remain unchanged. This suggests that another G-gene strain from a different region might exist in rabies virus isolated from Indonesia.

Key words: amino acid, antigenic site, dog, G-gene, Indonesia, rabies virus

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INTRODUCTION

Rabies in Indonesia was reported in 1889, and it has since spread to 23 provinces, including eight provinces of Sumatra, four of Kalimantan, five of Sulawesi, along with East Nusa Tenggara, Maluku and Bali. According to Susetya *et al.*, (2005), rabies virus (RABV) belongs to the genus *Lyssavirus* from Rhabdoviridae family. Genus *Lyssavirus* differentiated into seven genotypes: rabies virus (genotype 1), Logos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssaviruses type 1 EBL-1 (genotype 5), EBL-2 (genotype 6), and Australian bat lyssaviruses (genotype 7). In Brazil, rabies virus predominantly attacks dogs and cats, and this can be detrimental to a community of dogs if an infected animal enters and transmits the disease. It becomes imperative, therefore, to control possible infection from new rabies strains (Sugiyama and Ito, 2007; Kobayasi *et al.*, 2011).

Rabies virus is a bullet-shaped single-stranded RNA, with size 180 × 75 nm and a 12 kb genome with negative polarity. It contains five gene encoding proteins: N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (polymerase) (Jackson and Wunner, 2002; Faber *et al.*, 2004; Delmas *et al.*, 2008). Glycoproteins (G-gene) are virus-forming proteins, *e.g.*, spikes have around 400 spines, which have an important role in the attachment of viruses to the cell surface, in pathogenicity, and neurovirulence of rabies virus. G-gene consists of nucleotides and 524 amino acids. At position 333, there is

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an amino acid (arginine) that determines its virulence (Nadin-Davis *et al.*, 1994; Plotkin and Mortimer, 1988). It is also reported to have eight antigenic sites, and to date, only five have been successfully mapped: at amino acid position 231 (antigenic site I), 264 (antigenic site VI) and 342-343 (antigenic site a). Specifically, the second antigenic site occurs continuously in the position of amino acids 34-42 or 198-200 (Marissen *et al.*, 200 G protein, a protein that can only be found on the surface of the virus, holds an important role in viral infections. It produces neutralizing antibodies and is used as a diagnostic antigen that detects antibodies to antigens (Hirsh *et al.*, 2004; Li *et al.*, 2010).

Several studies on rabies have used different primers but have failed to determine the expected homology of the elbow point. Currently, measures to properly handle rabies infection in Indonesia are inadequate. As a result, cases have only worsened: areas that used to be rabies-free have now become rabies endemic areas. This study, therefore, was conducted to determine the homology score and predict the antigenic sites of the glycoprotein (G-gene) encoding gene from rabies virus in Indonesia using molecular analyses. The information obtained from this study may serve as reference in producing rabies vaccine in the country.

MATERIALS AND METHODS

Sample collection

Twelve samples were collected from the brains of infected dogs in Sumatra, Kalimantan, Sulawesi and Bali islands from 2005 to 2010. All infected samples were processed in 10% formalin suspension. These were marked as 6, 7, 8, C4, C9, 285, 382, 391, 436, and 533. One uninfected brain sample served as negative control and one rabies-infected brain served as positive control.

RNA extraction and RT-PCR

The study used TRIzol® LS RNA (Invitrogen, CA, USA), following the manufacturer's protocols for RNA isolation and the methods employed by Rantam (2007). Synthesis and amplification of cDNA were conducted according to the manufacturer's protocols on RT-PCR beads (GE Healthcare Bio-Sciences Corp, NY, USA). As shown in Table 1, specific primers were used to amplify the glycoprotein encoding gene of rabies virus (Yang *et al.*, 2011). Samples were then subjected to annealing temperature of 50°C for 35 cycles. Gel electrophoresis was done using 1% agarose gel (Prati 2001; Fatchiyah, 2006). RT-PCR product was visualized under UV light with wavelength of 302 nm. The final product was prepared for sequencing.

Sequencing

RT-PCR product was purified following the instructions from the gen clean kit NucleoSpin®Extract II column. After this, the product was sequenced according to manufacturer's protocols using BigDye® XTerminator™ Solution Kit T and SAM™ Solution Kit (Applied Biosystems, CA, USA). Sequencing was done using an automated

Table 1. Primers used for DNA amplification of Indonesian rabies virus isolates from Indonesia.

Primer	Nucleotide sequence (5'-3')	Position	Sense	Primer position
RVN1F	ATGGATGCCGACAAGATTGTATTC	71-94	+	1-24
RVN1R	GAATTCCTCTCCCAGATAGCC	1097-1118	–	1027-1047
RVG1F	ATGGTTCTTCAGGCTCTCCTGTTTGT	3317-3342	+	28-53
RVG1R	GACTGACTTGTAGTGAGCATCGGC	4346-4369	–	1057-1080

sequencer (3130 Genetic Analyzer, Applied Biosystems, CA, USA). Molecular data were obtained and homology nucleotide scores were measured using NCBI Blast Mega 5.05.

RESULTS AND DISCUSSION

Results from RT-PCR showed that G-gene band had a length of 1053 bp. This indicates that the specific primers used were capable of amplifying the targeted gene of rabies virus. Figs. 1-4 show the result of amplified fragment of G-gene from rabies virus collected from different regions (Sumatra, Sulawesi, Kalimantan, and Bali). G-gene RT-PCR products in each region can be seen, except for codes 7 and 8 from Kalimantan and code C3 from Sulawesi, which did not indicate the presence of DNA. G-gene shows the amplicon with a length of 1053 bp.

Table 2 shows the homology scores of Indonesian rabies virus isolates against reference virus isolates from Indonesia (93-98%), China (89-91%), Thailand (83-86%), India (82-84%), Korea (83-85%), and Pasteur strain (82-83%) obtained from GenBank records. In all samples, homology score from Kalimantan samples with codes 6, 7 and 8 cannot be detected

Table 3 shows that the G-gene was only found in antigenic site I (amino acid position 231) from samples obtained in Sumatra. Changes in amino acid sequence in cysteine-arginine were found in codes 391 and 533, and changes in cysteine-leucine were found in code 436. In antigenic site VI, no change in amino acid sequence of tryptophan-tryptophan was seen. Meanwhile, in antigenic site II, amino acid position 198-200, no change was seen in the sequence aspartate-tyrosine-threonine. In this study, antigenic site III of the G-gene was not detected.

Amplification of G-gene fragments using primers in the nucleotide position 4346-4369 produced an amplicon with length of 1053 bp. Primers used were suitable for samples of natural strains in Indonesia. RT-PCR was followed by sequencing to obtain the nucleotide sequences of G-gene fragments, then a homology analysis was performed to determine the level of relationship between Indonesian rabies virus samples and existing strains from GenBank (Indonesia, China, Thailand, India, Korea, and Pasteur strain).

The result of homology score of glycoprotein gene from Indonesian rabies virus against Indonesian isolates from GenBank ranged from 93-98%, depending on the region. In 2003, code C4 from Sulawesi showed 93% homology to reference Indonesian isolate. In contrast, code C9 from Sulawesi showed 96%, while for Sumatra and Bali, it ranged from 95% to 98%. Meanwhile, compared to Thai isolates, Indonesian isolates showed a homology score of 83-86%. Against Indian isolates, homology score ranged between 82-84%. Further, compared with Chinese isolates, Indonesian isolates attained homology score of 89-91%, wherein homology score from Sulawesi was 89%. Against Korean isolates, Indonesian isolates showed homology level of 83-85%. Finally, compared to G-gene of Pasteur strain, the degree of homology was 82-83%.

It is clear that homology scores against reference virus isolates largely vary. Low homology scores may translate to emergence of new rabies virus strains. Notably, however, there is a high degree of similarity, *i.e.*, 93-98%, between existing Indonesian isolates and Indonesian references isolates from GenBank, suggesting that genetics of rabies is more tied geographically and related to isolation years rather than species-specific (Yang *et al.*, 2011; Lang *et al.*, 2012).

The results in Table 2 show that antigenic areas are exposed to antigenic sites I, II and VI. The amino acid change in antigenic site I lies in the position of amino acid 231 (nucleotide 693-695), which is apparent in Sumatran samples with code 391 and 533 (cysteine-arginine) TGC-CGT and code 436 (cysteine-leucine) TGC-CTC. In contrast, in Kalimantan, Sulawesi and Bali regions, no changes in amino acids were seen; they remained similar to amino acid virus Pasteur–cysteine. In antigenic site VI, at the position of amino acid 264 (nt 792-794) TGG–TGG, Indonesian samples did not exhibit change in amino acid sequence in tryptophan - tryptophan. Similarly, in antigenic site II, at position 198-200, no change in amino acid sequence was seen in all regional samples, conserving the sequence aspartate-tyrosine-threonine.

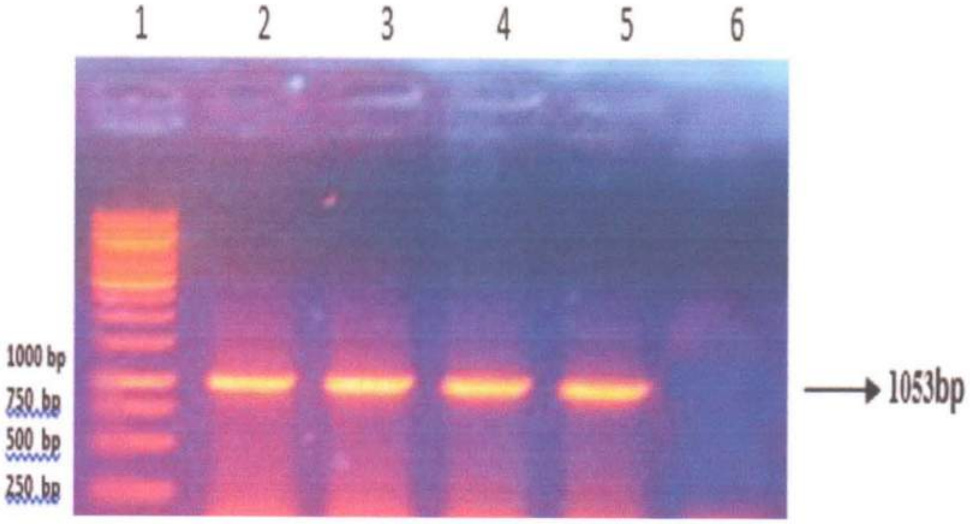

Fig. 1. Results of G-gene amplification of rabies virus from Sumatra via PCR. Columns (1) DNA marker, (2) Sample code 533, (3) Sample code 391, (4) Sample code 438, (5) Positive control and (6) Negative control.

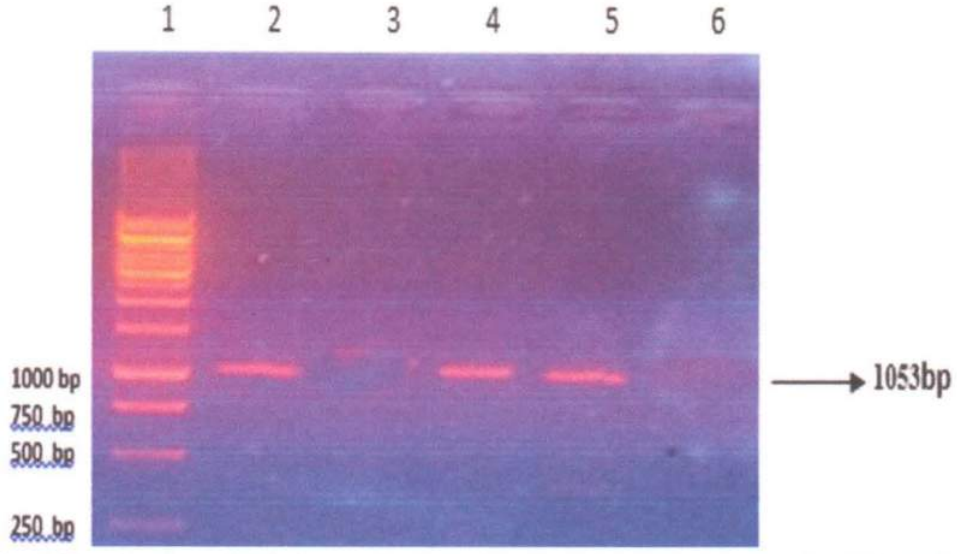

Fig. 2. Results of amplified G-gene of rabies virus from Sulawesi. Columns (1): DNA marker, (2) Sample code C9, (3) Sample code C3, (4) Sample code C4, (5) Positive control and (6) Negative control.

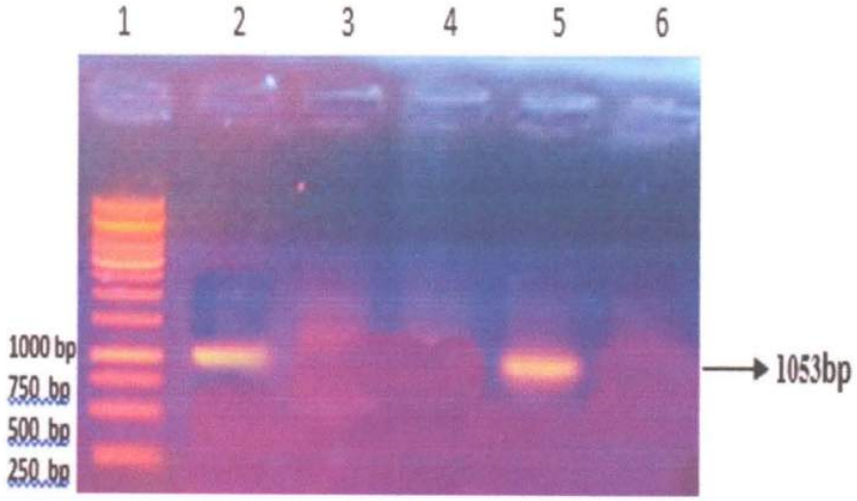

Fig. 3. Results of amplified G-gene of rabies virus from Kalimantan. Columns (1) DNA Marker, (2) Sample code 6, (3) Sample code 7, (4) Sample code 8, (5) Positive control and (6) Negative control.

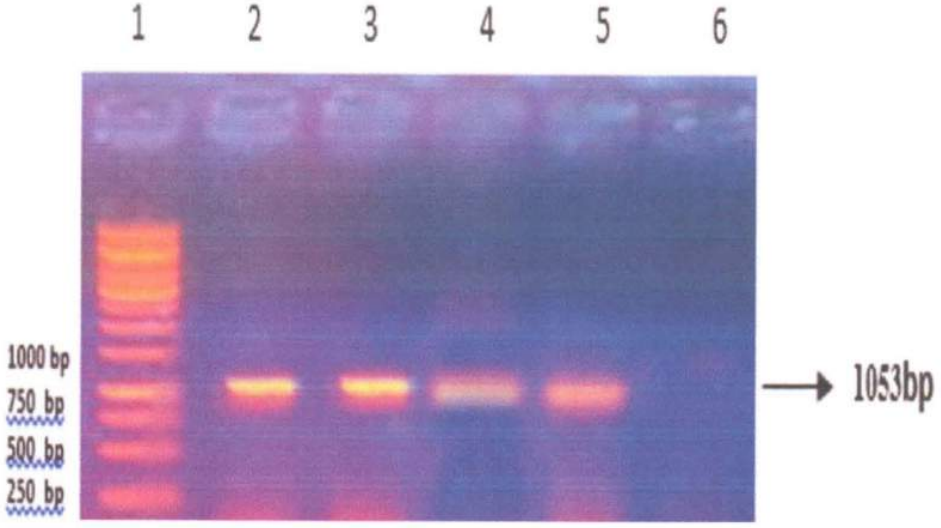

Fig. 4. Results of amplified G-gene of rabies virus from Bali. Columns (1) DNA Marker, (2) Sample code 148, (3) Sample code 285, (4) Sample code 382, (5) Positive control and (6) Negative control.

Table 2. Homology scores of glycoprotein gene of Indonesian rabies virus isolates against Indonesian, Chinese, Thai, Indian, Korean and Pasteur strain isolates from GenBank.

Sample code	Origin	Glycoprotein gene (%)					
		Indonesia	China	Thailand	India	Korea	Pasteur strain
6	Kalimantan	-	-	-	-	-	-
7	Kalimantan	-	-	-	-	-	-
8	Kalimantan	-	-	-	-	-	-
C4	Sulawesi	93%	89%	83%	82%	83%	82%
C9	Sulawesi	96%	91%	85%	84%	84%	83%
148	Bali	96%	91%	85%	84%	85%	83%
285	Bali	95%	90%	85%	83%	84%	83%
382	Bali	98%	89%	84%	82%	83%	82%
391	Sumatra	97%	91%	86%	84%	85%	83%
436	Sumatra	96%	90%	85%	84%	83%	83%
533	Sumatra	98%	91%	86%	84%	85%	83%

Most antibody neutralization of rabies virus is targeted at antigenic site II, linear epitope glycoprotein and all rabies variants have non-silent mutations in the epitope (Marissen *et al.*, 2005).

Genetic material of viruses is susceptible to mutations, primarily due to errors in replication. In particular, a change in the arrangement of RNA molecules in a virus causes the emergence of new viruses, genetically different from the previous strains. Rabies is an RNA virus and high mutation rate in its RNA structure appears to equip new viral strains to be adaptive. Mutations are changes that occur in the genetic material (DNA and RNA). In this study, mutations tend to be more common in G-gene fragments from Sumatra and Sulawesi. Genetic diversity seems to provide adaptive potential that varies according to natural history (Nagarajan *et al.*, 2006).

This study illustrates that G-gene fragments of rabies virus were amplified using specific primers, producing an amplicon with a length of 1053 bp. Meanwhile, the amino acid sequence of G- gene is composed of amino acids 63 to 309. Homology scores of Indonesian isolates, in contrast to reference isolates, largely varied, the degree of homology was lowest against Pasteur strain (82-83%), highest against Chinese isolates (89-91%), and ranged from 82-86% when compared to Thai, Indian, and Korean isolates. G-gene on antigenic site I from Sumatra sample showed changes in amino acid sequence. However, no changes were seen in antigenic sites II and VI.

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Table 3. Amino acid changes on glycoprotein genes of Indonesian rabies isolate virus against Pasteur strains.

No.	Rabies virus isolate	Antigenic site I		Antigenic site VI		Antigenic site II	
		Amino acid position	Amino acid change	Amino acid position	Amino acid change	Amino acid position	Amino acid change
1	Pasteur	231	C – C	264	W – W	198-200	D – Y – T
2	C4 Sul (2009)	231	C – C	264	W – W	198-200	D – Y – T
3	C9 Sul (2009)	231	C – C	264	W – W	198-200	D – Y – T
4	148 Bali (2008)	231	C – C	264	W – W	198-200	D – Y – T
5	285 Bali (2008)	231	C – C	264	W – W	198-200	D – Y – T
6	382 Bali (2008)	231	C – C	264	W – W	198-200	D – Y – T
7	391 Sum(2005)	231	C – R (cysteine-arginine)	264	W – W	198-200	D – Y – T
8	436 Sum(2005)	231	C – L (cysteine-leucine)	264	W – W	198-200	D – Y – T
9	533 Sum(2005)	231	C – R (cysteine-arginine)	264	W – W	198-200	D – Y – T
10	6 Kal(2010)	231	C – C	264	W – W	198-200	D – Y – T
11	7 Kal(2010)	231	C – C	264	W – W	198-200	D – Y – T
12	8 Kal (2010)	231	C – C	264	W – W	198-200	D – Y – T

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