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ASSESSMENT OF GENE AND PROTEIN SIMILARITIES OF BEIJING-1 VACCINE PRODUCING STRAIN WITH JAPANESE ENCEPHALITIS VIRUS STRAINS CIRCULATING IN VIETNAM

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SUMMARY

Since 2006, the inactivated Japanese encephalitis vaccine has been studied and produced by the VABIOTECH company from the Beịing-1 strain on Vero cells. Vaccines produced from the materseed and working seed virus have been evaluated at laboratory and clinical scale in humans. The results showed that the vaccine was safe and created 100% protective antibodies after the booster dose. To officially put this vaccine into production and mass use, the master seed virus BV-MSV-0210 and working seed virus BV-WSV-0310 with the reference standard strain JEV Beijing-Kanonji has been tested for genetic stability. By the method of Sanger sequencing and genetic analysis software, we have evaluated the similarity of nucleotide and proteinsequencesof the E antigen-encoding gene. The results showed that the seed virus similarity of amino acids and nucleotides is 100% compared with the reference strain. Thus, it can be concluded, the seed virus has antigen stability. Nucleotide and amino acid gene sequences of E genomic regions of the two seed lots were compared with virus strains isolated from human, pig and mosquito in Vietnam. The results showed that the nucleotide similarity of seed virus compared with the JEV strains isolated from humans ranged from 86.67 to 97.54%; from pigs is 87.47 to 88.33%, and from mosquitoes is 86.05 to 99%. Meanwhile, the amino acid similarity of the seed virus with the JEV strains isolated from humans ranged from 96.73 to 99.02%; from pigs is 98.00 to98.40% and from mosquitoes 94.55 to 98.40%. The sequence of amino acids in the epitope producing neutralizing antibodies of the seed virus did not differ from that of the JEV strain circulating in humans isolated in 2014.

Keywords: *Beijing-1 strain, Master Seed Virus strain, Working Seed Virus strain, Envelope protein gene, nucleotide homology, amino acid similarity, neutralizing antibody-produce epitopes.*

INTRODUCTION

Japanese Encephalitis virus has many strains (about 290 strains have been isolated in Asia), belonging to 5 genotypes. However, not all strains can be used to produce human vaccines. In order to be selected for the production of vaccines for human use, the virus strain needs to meet many criteria as prescribed by the World Health Organization. Currently, most strains used to produce vaccines belong to genotype 3 such as: Nakayama strain; Beijing -1; Beijing-3; SA-14-14-2 (Sharma *et al.*, 2014; Huynh Phuong Lien *et al*., 2011).

According to statistics, Japan is the first research country to produce the first inactivated JE vaccine with strains of Nakayama and/or

Beijing-1. However, the actual clinical research results show that the Beijing-1 strain has superior immunity compared to the Nakayama strain. Therefore, since 1989, the production of the JE vaccine was officially switched to use the strain Beijing-1. After Japan, China also researched and produced vaccines using strains Beijing-3 and SA-14-14-2. Subsequently, Korea started using the Nakayama strain; India and Austria used the strain SA-14-14-2.

In Vietnam, in 1989 Vabiotech company researchedand used the Nakayama strain to produce inactivated JE vaccine on mouse brains. The vaccine has been put into mass use since the early 1990s up to now with very good protection results. From 2006 up to now, Vabiotech company has researched and developed a technological process to produce inactivated JE vaccines on Vero cells from Beijing-1 strain to gradually replace vaccines produced in the brain of mice today. This new vaccine has gone through a number of stages such as the establishment of a Master Seed Bank with code BV-MSV-0210 and a Working Seed Bank with code BV-WSV-0310.The assessment and monitoring of the quality of the strains produced by the time of preservation and over the quality of 10 batches of finished vaccines at the laboratory scale and on the clinical scale in humans have been carried out from 2013-2018. The results showed that the vaccine was safe and created 100% protective antibodies after the booster injection. To officially put this vaccine into production and mass use, the evaluation of the E gene region stability (specific protective antibody-forming region) of these strains compared with the original strain and the similarity of nucleotides and amino acids with the JEV strains circulating in Vietnam is essential to confirm whether the vaccine using this batch of strains is really effective in preventing the disease from the JEV strains circulating in Vietnam. For that reason, we performed E gene sequencing and analysis of nucleotide and amino acid similarity of master seed virus (MSV) batch BV-MSV-0210 and working seed virus (WSV) batch BV-WSV-0310 with the original standard strain (Reference JEV Beijing-Kanonji) provided by Kanonji, Japan to assess genetic stability and compare with existing JEV strains in Vietnam to evaluate antigen similarity.

In this study, we present some results on the E gene region similarity of 2 batches of Beijing-1 strains being used in JE vaccine production with standard virus strains and JEV strains circulating in Viet Nam.

MATERIALS AND METHODS

The object of study is the seed bank of batch BV-MSV-0210 and the seed bank for production batch BV-WSV-0310. Reference JEV Beijing-Kanonji provided by Kanonji, Japan, is the strain used to produce 2 batches of seed strains used in research. Total RNA was extracted and purified from each sample using the QIAamp Viral RNA Mini Kit (Qiagen). E gene region was amplified by RT-PCR using OneStep PCR Kit (Qiagen) with primer pair: JEV-E-p1: 5'-TTCAACTGTCTGGAATGG-3'; JEV-E-p2: 5'-AGCATGCACATTGGTAGCT- A-3 'with the program: 50°C for30 minutes, 95°C for15 minutes; 30 cycles (94°C for 1 minute, 50°C for 1 minute, 72° C for 1 minute), 72°C for10minutes, hold at 4^{o}C. PCR products were tested on the 1% agarose gel. The band containing the PCR product is eluted and purified using the QIAquick PCR Purification Kit (Qiagen) for sequencing. Gene sequencing was performed with Applied Biosystems™ Sanger Sequencing Kit (Thermo Fisher Scientific) and ABI 3500 gene sequencing machine of the Institute of Biotechnology, Vietnam Academy of Science and Technology. E gene sequences were analyzed using available software on the internet such as Blast, BioEdit, MEGA6.0 to evaluate the similarity and build phylogenetic trees based on regional sequence E gene of 2 strains Beijing-1 with standard strain and JEV strains isolated from humans, pigs, and mosquitoes in Vietnam from 1964 to 2014 (Table 1). Analysis of E region antigen epitopes was conducted according to the method of Luca *et al*. (2012).

RESULTS

Results of E gene amplification of master seed, working seed, and reference strains

The gene encoding the E antigen of the master seed strain BV-MSV-0210, working seed strain BV-WSV-0310 and the reference standard strain of JEV Beijing-Kanonji were amplified by RT-PCR. The results showed that the PCR product is very specific, without byproducts, the size is equivalent to the length of E gene according to theoretical calculations (about1500 bp). Results are shown in Figure 1.

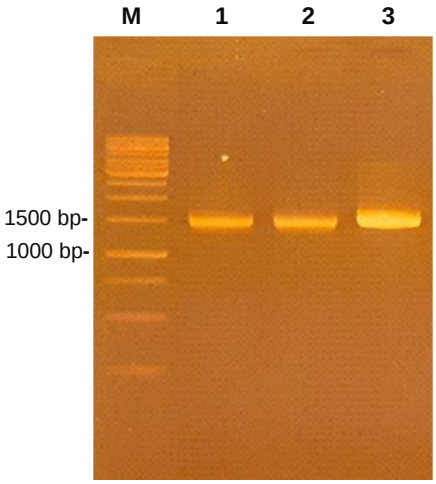

Figure 1. Agarose gel 1% electrophoresis for testing RT-PCR products amplified gene encoding E antigens of strains: original strain BV-MSV-0210 (lane No. 1); production strain BV-WSV-0310 (lane No. 2); Standard strain reference original JEV Beijing-Kanonji (lane No. 3). M: Farmentas 1 kb DNA ladder.

Results of sequencing and analysis of genes encoding E antigens of master seed and working seed strains

PCR products were collected by gel eluted method, purified by QIAquick PCR Purification Kit, and sequenced by ABI 3500 gene sequencing machine. After sequencing, we used specialized software programs such as Blast, BioEdit, MEGA6.0 ... to analyze, evaluate the similarity and build phylogenetic trees based on the E region sequence of the master seed BV-MSV-0210and working seed BV- WSV-0310strains. The results showed that the gene encoding E antigen has a length of 1500 bp, a G/C ratio of 52%, which encodes a protein with a length of 500 amino acids.

The length and nucleotide and protein sequences of the gene coding for E-antigen the MSV, WSV and reference strain are completely identical. These strains have 100% similarity for both nucleotides and amino acids. However, when comparing the nucleotode and amino acid sequences of the seed strains with the gene sequences encoding E antigens of JEV strains isolated in Vietnam, the similarity percentage is very different (Table 1).

Based on the results of comparing the nucleotide and protein sequences of the gene coding for E antigen of MSV and WSV strains compared with the reference strain (Beijing-Kanonji) and the JEV strains isolated in Vietnam from 1964 to 2014, we created a phylogenetic tree to evaluate the nucleotide and protein correlation between strains studied (Figures 2 and 3).

The results determined the epitope positions of protein E of the WSV and MSV strains

Comparing nucleotide sequence and E gene protein of the WSV strain and MSV strain with JEV strains isolated in Vietnam, especially strains isolated from human, the results show that this rate ranged from 86.67 to 97.53% for nucleotides and 96.73 to 99.00% for proteins. We have examined whether the epitopes crucial to the immune response for neutralized antibodies change or not. Using the method of Luca *et al*. (2012), we determined the positions of the epitopes and the positions of amino acids that determine the epitope for antigenic properties. The results are shown in Table 2.

As results, (Table 2) the amino acids of the epitopes of the gene encoding the E antigen in the WSV strain, the reference strain, the SA14-14-2 vaccine strain, and the JEV strain isolated from humans in Vietnam in 2014 did not change.

Table 1. Nucleotide and protein sequence similarity ratio of E antigen-encoding gene of master seed strain (BV-MSV-0210) and working seed strain (BV-WSV-0310) compared to E-gene sequence of JEV strainsisolated in Vietnam over different years and from different hosts.

No.	Accession in GenBank	Years of isolation	Hosts	Nucleotide and protein sequence similarity ratio (%)	
				Nucleotide	Protein
1	LC000631	1964	Human	97.45	98.31
2	AY376461	1986	Human	96.40	99.00
3	AY376463	1989	Human	96.73	99.20
4	HQ009263	2004	Human	97.53	97.20
5	LC000634	2007	Human	87.45	96.73
6	KP876007	2014	Human	86.67	97.04
7	AY376464	2001	Pig	88.33	98.40
8	AY376465	2002	Pig	88.27	98.40
9	HQ009265	2005	Pig	87.47	98.00
10	JEU70420	1979	Mosquito	99.00	98.06
11	AB933311	1994	Mosquito	88.05	98.22
12	AY376468	2002	Mosquito	88.33	98.40
13	JN574431	2005	Mosquito	87.80	98.04
14	LC000635	2010	Mosquito	87.54	96.77
15	LC000637	2011	Mosquito	86.05	94.55

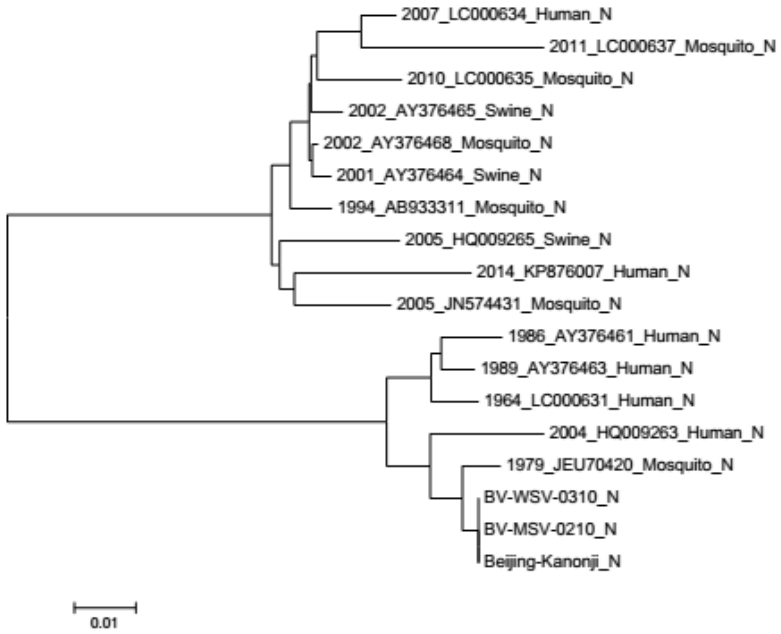

Figure 2. Phylogenetic tree based on E gene nucleotide sequence of the WSV strain (BV-WSV-0310), MSV (BV-MSV-0210), original reference strain (Beijing-Kanoji), and the JEV strains isolated in Vietnam from 1964 to 2014.

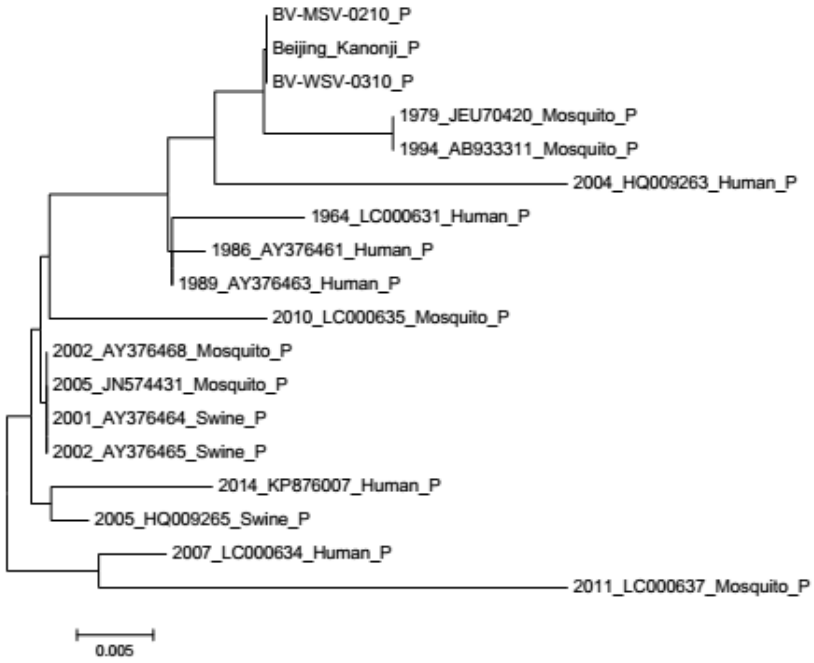

Figure 3. Phylogenetic tree based on E gene protein sequence of the WSV strain (BV-WSV-0310), MSV (BV-MSV-0210), original reference strain (Beijing-Kanoji), and the JEV strains isolated in Vietnam from 1964 to 2014.

Table 2. Position of epitopes and positions of amino acids determining antigens of epitope on protein E of the WSV strain (BV-WSV-3010), reference strain (Beijing-Kanonji), JEV strain isolated in humans 2014 and vaccine strain SA14-14-2.

	Domain II-fusion loopprotein E			**Domain I-IIhinge**				**Side chains of Domain I**	**Side chains of Domain III**			
Position ofneutralizing epitopes	104	106	107	52	126	136	275	179	337	360	302	387
AA of the strain SA14-14-2 (Luca et al.)	G	G	L	Q	I	K	S	K	I	F	G	R
AA of the strain BV-WSV-3010	G	G	L	Q	I	K	S	K	I	F	G	R
AA of the strain 2014_KP876007_Human_ProE	G	G	L	Q	I	K	S	K	I	F	G	R
AA of the strain Kanonji	G	G	L	Q	I	K	S	K	I	F	G	R

DISCUSSION

Japanese Encephalitis virus (JEV) is the leading cause of viral encephalitis globally. The envelope protein of the JEV (protein E) facilitates virus binding on the cell surface and membrane fusion, which is the primary target of antibodies that neutralize the virus (Luca *et al*., 2012). Therefore, the efficacy of JE vaccine depends on the ability to produce antibodies to neutralize E protein. Since 2006, inactivated Japanese encephalitis vaccine has been

researched and produced by Vabiotech company from Beịing-1 on Vero cell. The results of clinical trials on humans showed that the vaccine was safe and created 100% protective antibodies after the booster injection. To officially put this vaccine into production and mass use, the evaluation of genetic stability and E-antigens similarity of the WSV strain and MSV strain compared with the JEV strains circulating in Vietnam are essential. The gene encoding the E antigen of the seed lots includes the Master Seed Bank BV-MSV-0210 and the Working Seed Bank BV-WSV-0310 with the reference standard strain JEV Beijing-Kanonji were sequenced and analyzed for similarity. The results showed that 2 batches of WSV have the similarity of amino acids and nucleotides of 100% compared with the reference strains. Thus, it can be concluded that the MSV strain and the WSV strain have genetic stability. However, the question that needs to be answered is, do the strains used in production have similar antigens to neutralizing antibodies against JEV strains circulating in Vietnam? To answer this question, the E gene region nucleotide sequences of the two strains were compared with the E gene region sequences of JEV strains isolated from humans, pigs and mosquitoes in Vietnam and submitted in GenBank from 1964 to 2014 (Table 1). The results showed that, the similarity of the nucleotide of the strain compared with the strains VNNB isolated from humans ranged from 86.67 to 97.54%; from pigs is 87.47 to 88.33% and from mosquitoes is 86.05 to 99%.Meanwhile, the amino acid similarity of the strain compared to the strains of JEV isolated from human ranged from 96.73 to 99.02%; from pigs is 98.00–98.40% and from mosquitoes 94.55 to 98.40%. Looking closely at the variation of the nucleotide sequence as well as the protein sequence of the JEV strains in Table 1, we can see that the similarity level decreases over time. For example, the nucleotide similarity of JEV isolated from humans in 1989 was 96.73% and protein was 99.20, by 2014 this similarity level was only 86.67% for nucleotides and 97.04. % for protein. So, is it because the pressure when using JE vaccine since 1990 has created mutant strains to gradually evade the protective ability of the vaccine? According to Roy *el al*. (2020), when the structure of the neutralizing antibody epitopes changes, the JEV can evade the protective ability of the neutralizing antibody produced by vaccines.To be able to elucidate this problem, we have used the method of Luca *et al*. (2012) to compare the amino acid sequence in the neutralizing antibody epitope region of Domain I, II and III of MSV strain, WSV strain and reference strain with JEV strains circulating in human isolates in 2014. Results showed that, no change of amino acids determining the configuration of antigens belonging to these epitopesfound (Table 2). That partly explains that vaccines produced by the WSV strains derived from the MSV strains still create 100% protective antibodies. According toDo Tuan Dat*et al*. (2017), the JECEVAX vaccine produced from the MSL BV-MSV-0210 and the WSL BV-WSV-0310 was safe and hadeffective protection100%after the third injection. The titer of antibodies to neutralize GMT using the PRNT method increased after 2 doses of 2.09 logs and 3.04 logs after 3 doses.This result is equivalent to the vaccine of the same type of Japan (CC-JEV) and the Republic of Austria (IXIARO).The protective effect of this vaccine was even better than that of the control group using the JEVAX vaccine (the vaccine being used in the expanded vaccination program in Vietnam, produced on mouse brain with Nakayama strain) only reached 99%. Some other vaccines currently circulating in Vietnam are Imojev (live chimeric vaccine, strain SA14-14-2, produced by France) or JEEV vaccine (made in India) also has a protective effect of 95–98%.

CONCLUSION

With the results obtained above, it can be confirmed that 2 lots of Beijing-1strains (BV-MSV-0210 and BV-WSV-0310) produced by Vabiotech company have high genetic stability with standard strains. The nucleotide sequence similarity of the gene encoding E antigen of the WSV strain compared to the JEV strains circulating in Vietnam from 1964 to 2014 is from

86.67 to 97.54% in humans; 87.47–88.33% in pigs and 86.05–99% in mosquitoes. Meanwhile, the amino acid similarity of the WSV strain compared to the strains of JEV isolated from human ranged from 96.73 to 99.02%; from pigs is 98.00-98.40% and from mosquitoes 94.55 to 98.40%. When comparing the amino acid sequence in the neutralizing antibodyepitopes of the WSV strain and the MSV strains with the JEV strain circulating in humans in 2014, there was no change of amino acids that determine the conformation of the antigen.

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ĐÁNH GIÁ SỰ TƯƠNG ĐỒNG VỀ GEN VÀ PROTEIN CỦA CHỦNG SẢN XUẤT VACCINE BEIJING-1 SO VỚI CÁC CHỦNG VIRUS VIÊM NÃO NHẬT BẢN ĐANG LƯU HÀNH TẠI VIỆT NAM

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TÓM TẮT

Từ năm 2006, vaccine viêm não Nhật Bản bất hoạt đã được nghiên cứu sản xuất bởi công ty VABIOTECH từ chủng Beịing-1 trên tế bào Vero. Vaccine sản xuất từ các lô chủng giống gốc và giống sản xuất đã được đánh giá ở quy mô phòng thí nghiệm và quy mô lâm sàng trên người. Kết quả cho thấy, vaccineđạt an toàn và tạo kháng thể bảo vệ 100% sau mũi tăng cường. Để chính thức đưa vắc xin này vào sản xuất và sử dụng đại trà, các lô chủng giống gồm chủng giống gốc BV-MSV-0210 và chủng giống sản xuất BV-WSV-0310 cùng chủng chuẩn tham chiếu JEV Beijing-Kanonji do Nhật Bản cung cấp đã được kiểm tra về tính ổn định di truyền. Bằng phương pháp giải trình tự Sanger và phân tích gen với các chương trình phần mềm khác nhau, chúng tôi đã đánh giá sự tương đồng về trình tự nucleotde và protein của gen mã hóa kháng nguyên E. Kết quả cho thấy, 2 lô chủng giống có sự tương đồng về amino acid và nucleotide là 100% so với chủng tham chiếu. Như vậy, có thể kết luận, chủng giống gốc và chủng giống sản xuất có tính ổn định về kháng nguyên. Trình tự nucleotide và amino acid vùng gen E của 2 lô chủng giống đã được so sánh với các chủng virus phân lập từ

người, lợn và muỗi ở Việt Nam. Kết quả cho thấy, sự tương đồng về nucleotide của chủng giống so với các chủng virus VNNB phân lập từ người dao động từ 86,67 đến 97,54%; từ lợn là 87,47 đến 88,33% và từ muỗi là 86,05 đến 99%. Trong khi đó, sự tương đồng về amino acid của chủng giống so với các chủng virus VNNB phân lập từ người dao động từ 96,73 đến 99,02%; từ lợn là 98,00-98,40% và từ muỗi là 94,55 đến 98,40%. Trình tự amino acid trong vùng epitope sinh kháng thể trung hòa của chủng giống không có sự khác biệt so với chủng virus VNNB lưu hành trên người phân lập năm 2014.

Từ khóa: *Chủng Beijing-1, Chủng giống gốc, chủng giống sản xuất, gen mã hóa kháng nguyên E, sự tương đồng nucleotide, sự tương đồng amino acid, epitope sinh kháng thể trung hòa.*