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Genotype-specific neutralizing antibody titers against Japanese encephalitis virus genotypes 1 and 3 in horses immunized with a genotype 3 vaccine

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Purpose: Japanese encephalitis is one of the most important mosquito-borne and zoonotic diseases in Asia and the Pacific region. Although the dominant Japanese encephalitis virus (JEV) genotype has shifted from G3 to G1 in Korea since 1990, a G3 strain (Anyang 300) has been used in vaccines for horses for almost 40 years. This study aimed to investigate the seroconversion rates and geometric mean titers (GMTs) of virus-neutralizing antibodies (VNAs) against JEV G1 and G3 in horses immunized with the G3 vaccine.

Materials and Methods: Serum samples of 1,231 horses immunized with the Anyang 300 vaccine were collected in 2018. VNA titers against JEV KV1899 (G1) and Anyang 300 (G3) were measured in all serum samples using the virus neutralization test. Titers were analyzed according to blood sampling time (prior to and following annual revaccination), age, and region.

Results: Rates of VNA titer >10 were 45.1% and 77.8% for G1, and 49.1% and 82.9% for G3 in samples taken before and after revaccination, respectively. GMTs of genotype-specific VNAs against JEV G1 and G3 were 8.3 and 11.6 before revaccination and rose to 27.2 and 65.4 following revaccination. Overall sero-positivity did not significantly differ between genotypes, but GMTs significantly differed among genotypes and sampling times. No significant difference was found in GMTs among age groups or regions.

Conclusion: Genotype-specific neutralizing antibody titers against JEV G1 and G3 differed significantly in horses immunized with the G3 vaccine. Antigenic differences between genotypes could reduce the vaccine's efficacy, requiring the development of a new vaccine.

Keywords: Encephalitis, Japanese, Horses, Genotype, Antibodies

Introduction

Japanese encephalitis (JE), caused by Japanese encephalitis virus (JEV), is one of the most important mosquito-borne zoonotic diseases occurring in Asia and the Pacific region [1,2]. JEV affects approximately 3 billion people in its endemic area and causes 30,000–50,000 cases of disease annually [1,3]. The geographic distribution of JEV is in East, South, and Southeast Asia, extending to the Kathmandu Valley of Nepal, Papua New Guinea, and the Torres Strait of Northern Australia [4-6].

JEV is one of the most medically significant arboviruses and belongs to the *Flavivirus* genus within the family *Flaviviridae* [7,8]. JEV is comprised of single-stranded positive-sense RNA with two untranslated regions and a single open reading frame (ORF)

[5,8]. The ORF encodes seven nonstructural proteins (NS1, NS2A and B, NS3, NS4A and B, and NS5) and three structural proteins (capsid protein, precursor to the membrane protein, and envelope protein) [5].

JEV is classified into five genotypes (G1–G5) based on phylogenetic analysis of the envelope protein gene [5]. JEV G3 was the predominant genotype in Korea with Japan and Taiwan. However, G1 JEVs were introduced in 1990 and have subsequently replaced G3 JEVs as the dominant genotype in Asian countries including Korea, Japan, China, Vietnam, and Thailand [9-14]. In Korea, two antigenically distinct JEVs were reported (G1 and G3) around 1994, and primarily G1 viruses have been isolated since then [14,15].

Natural transmission of JEV occurs mainly via mosquitoes of the genus *Culex*, particularly *Culex tritaeniorhynchus* [2,8]. Wading ardeid water birds such as herons and egrets are virus reservoirs, and pigs with high viremia serve as amplification hosts for epizootics and epidemics of JEV [1,2]. JEV spills over into horses and humans, causing fatal encephalitis or subclinical infection. However, the low viremia titers of JEV in humans and horses indicate that they are dead-end hosts [1,2].

In Korea, epidemics of JE generally occur during the summer season with northern countries including Japan, China, Nepal, and Taiwan [5]. JE has not been officially reported in swine since 2008, and no clinical cases of JEV infection in horses have been reported to date in Korea [16,17]. In humans, approximately 1,000 cases of JEV infection occurred annually in the 1960s, but the number fell to 20 cases per year in the 2000s due to vaccination. However, cases of JEV infection in adults have increased since 2010 [18,19].

Vaccination is the most effective preventive measure against JEV infection in its endemic area [20]. A live attenuated JEV G3 vaccine, containing the Anyang 300 strain, was developed for animals and has been used in pigs and horses in Korea since 1980 [21]. In particular, horses are vaccinated annually with the live attenuated vaccine in May [21]. Serological surveys of JEV have been conducted for horses in Korea [16,21,22]. However, few studies have been conducted on cross-neutralizing responses across genotypes in horses receiving the G3 vaccine in Korea [16]. Therefore, this study aims to investigate and analyze the seroconversion rates and geometric mean titers (GMTs) of virus-neutralizing antibodies (VNAs) against two JEV genotypes, G1 and G3, in serum samples from 1,231 horses immunized with the G3 vaccine according to blood sampling time (prior to and following annual revaccination), age, and region.

Materials and Methods

Serum samples

To investigate VNA titers of JEV genotypes 1 and 3 in horses, a total of 1,231 serum samples were collected from the Korea Racing Authority and private horse farms in the Republic of Korea in 2018. These horses were revaccinated annually with the JEV G3 vaccine containing the Anyang 300 strain. Serum samples from 481 horses were collected in 13 provinces and cities (Gangwon, Gyeonggi, Gyeongnam, Gyeongbuk, Jeonnam, Jeonbuk, Gwangju, Daegu, Busan, Seoul, Ulsan, Incheon, and Jeju) from March to June 2018 prior to annual revaccination. The remaining (750) serum samples were obtained in 16 provinces and cities (Gangwon, Gyeonggi, Gyeongnam, Gyeongbuk, Jeonnam, Jeonbuk, Chungnam, Chungbuk, Gwangju, Daegu, Daejeon, Busan, Seoul, Ulsan, Incheon, and Jeju) from May to June 2018 after annual revaccination. The ages of the 481 horses sampled before annual revaccination ranged from 2 to 26 years, and those of the 750 horses after annual revaccination ranged from 1 to 25 years. Serum samples were inactivated at 56°C for 30 minutes and stored at -20°C until use.

Virus and cells

KV1899 (G1) isolated from swine blood in 1999 and the vaccine strain Anyang 300 (G3) isolated from the spleen of a piglet in 1969 were used for the virus neutralization (VN) test. JEVs were maintained and passaged in Vero cells, which were grown in Dulbecco's Modified Eagle's Medium (Gibco-BRL/Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS; Gibco-BRL/Invitrogen), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Gibco-BRL/Invitrogen).

Virus neutralization test

Neutralizing antibodies against JEV KV1899 (G1) and Anyang 300 (G3) were measured in all serum samples using the VN test, as described previously [16]. In brief, 50 μL of each serum sample was added to the first well containing 50 μL medium and serially diluted in the 96-well plate. The diluted serum samples were mixed with each virus at 200 $TCID_{50}$ (50% tissue culture infectious doses)/100 μL. After incubation for 1 hour at 37°C, 100 μL (2×10^5 cells/mL) of Vero cells in medium with 10% FBS were added to each well. The mixture of serum, virus, and cells was incubated at 37°C under 5% CO_2. The neutralizing antibody titers of the serum samples are ex-

pressed as the reciprocal of the highest serum dilution that completely inhibited cytopathic effects.

Statistical analysis

An arbitrary value of 1 was used for seronegative samples of <2 VNA titer in GMT calculations. Statistically significant differences between VN titers of G1 and G3 were assessed using the two-tailed paired t-test. Statistical significances among groups were analyzed according to genotype and sampling time using one-way analysis of variance (ANOVA). Statistical significance was assessed based on p-values <0.05. All statistical comparisons and calculations were conducted using GraphPad Prism software ver. 8.3.0 (GraphPad Software, San Diego, CA, USA).

Results

VNA titers were investigated against JEV G1 (KV1899) and homologous G3 (Anyang 300) in serum samples of 1,231 horses immunized with G3 vaccine containing Anyang 300; 481 samples were collected prior to annual revaccination and 750 samples were collected following annual revaccination. VNA titers were calculated from the reciprocal of the highest serum dilution that completely inhibited cytopathic effects using the VN test in Vero cells. The highest seroconversion rates against JEV G1 and G3 were 45.1% and 47.2%, respectively, before vaccination, and 76.3% and 71.1% after vaccination, with a VNA titer range of 16–256 (Table 1). In the VNA titer range of 2–8, seroconversion rates between the two genotypes (G1 versus G3) were also similar before (32.0% versus 34.3%) and after (15.6% versus 15.9%) vaccination (Table 1). Antibody negative (<2 VNA titer) rates decreased from 22.9% to 6.7% for G1, and from 16.6% to 1.2% for G3 from before to after vaccination (Table 1). On the other hand, high VNA titers above 512 were only 1.5% for G1 after revaccination, but were 1.9% and 11.8% for G3 before and after revaccination, respectively (Table 1). Total seroconversion rates (VNA titer ≥2) before and after revaccination were 77.1% and 93.4% for G1 and 83.4% and 98.8% for G3. The rates with VNA titer >10 before and after revaccination were 45.1% and 77.8% for G1

Table 1. Genotype-specific seroconversion rate in horses raised in Korea before and after annual revaccination

VNA titer	KV1899 (G1)		Anyang 300 (G3)	
	Before annual revaccination	After annual revaccination	Before annual revaccination	After annual revaccination
<2	110 (22.9)	50 (6.7)	80 (16.6)	9 (1.2)
2–8	154 (32.0)	117 (15.6)	165 (34.3)	119 (15.9)
16–256	217 (45.1)	572 (76.3)	227 (47.2)	533 (71.1)
512–1,024	0	11 (1.5)	7 (1.5)	85 (11.3)
>1,024	0	0	2 (0.4)	4 (0.5)
Total samples	481	750	481	750

Values are presented as number of samples (%).
VNA, virus-neutralizing antibody.

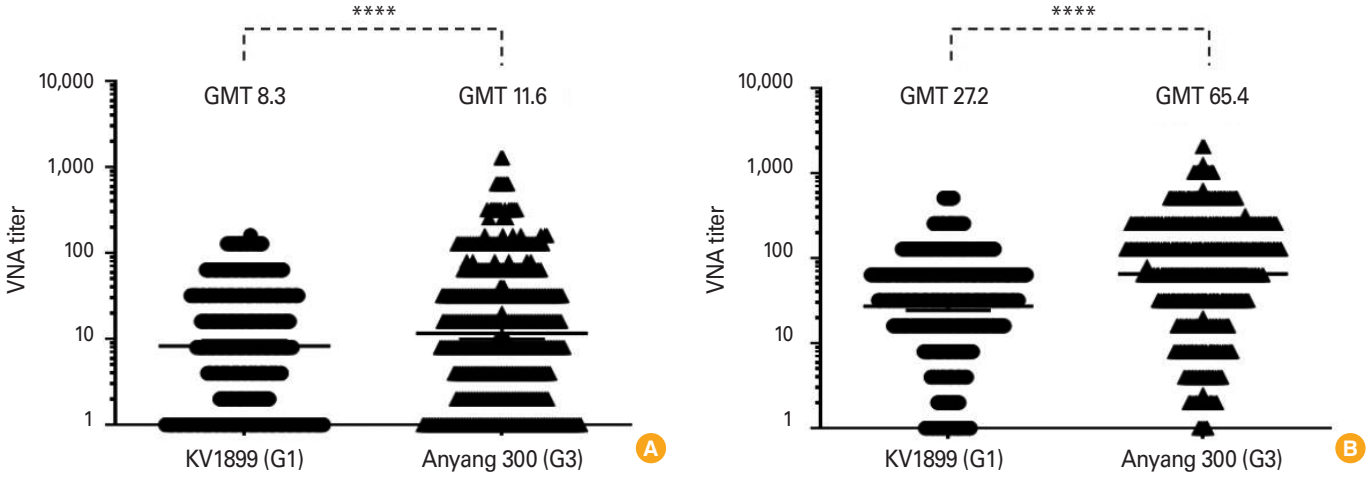

Fig. 1. GMTs of genotype-specific VNAs against JEV genotype 1 (G1) KV1899 and genotype 3 (G3) Anyang 300 in serum samples collected from horses immunized with a G3 vaccine. VNA titers of horse serum samples collected before (A) and after annual revaccination (B) were calculated from the reciprocal of the highest serum dilution that completely inhibited cytopathic effects in the virus neutralization test in Vero cells. The line indicates GMTs calculated from VNA titers against JEV G1 and G3. GMTs, geometric mean titers; VNA, virus-neutralizing antibody; JEV, Japanese encephalitis virus. ****p<0.0001; significant differences in VNA titers were found between JEV genotype G1 and G3 using the two-tailed paired t-test.

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and 49.1% and 82.9% for G3, respectively. Differences of 4%–6% were observed between the seroconversion rates of the two genotypes.

The GMTs of genotype-specific VNAs against JEV G1 (KV1899) and G3 (Anyang 300) were 8.3 and 11.6 in serum samples collected before annual revaccination, respectively (Fig. 1A). Following revaccination, the GMTs of JEV G1 and G3 were 27.2 and 65.4, respectively, and the difference in GMTs between the two genotypes was greater than that before revaccination (Fig. 1B). The VNA titers showed significant differences between JEV genotypes G1 and G3 based on two-tailed paired t-test ($p<0.0001$).

Overall, GMTs appeared to increase as horses aged, and GMTs in horses over 16 years tended to maintain a constant level or decrease in a comparison of GMTs among age range classes of 1 to 5, 6 to 10, 11 to 15, 16 to 20, and 21 to 25 years (Fig. 2). However, no significant difference in GMTs was found between age groups ($p=0.0623$). Prior to annual vaccination, both genotypes showed similar GMT levels, whereas after revaccination, GMTs against G3 increased more strongly than those against G1 (Fig. 2). Comparison among groups via one-way ANOVA according to genotype and sampling time revealed a significant difference ($p=0.0071$).

In a comparison of GMTs by region, GMTs against G3 increased more strongly than those against G1 after revaccination in all regions except Gyeongnam (Fig. 3). No significant difference was observed in GMTs among regions, but a significant difference was found in GMTs among groups according to genotype and sampling time based on one-way ANOVA ($p<0.0001$).

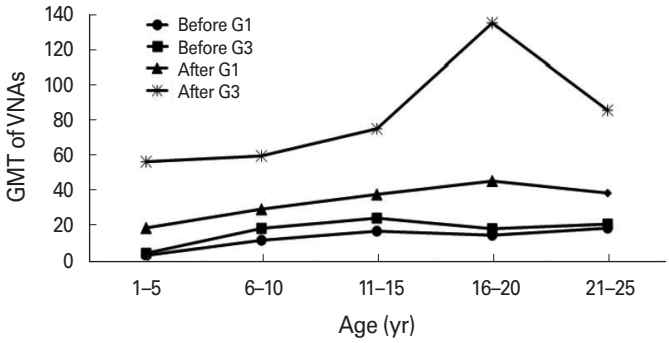

Fig. 2. GMTs of VNAs against JEV G1 (KV1899) and G3 (Anyang 300) in serum samples collected before annual revaccination (before) and after annual revaccination (after) according to the following age ranges (yr): 1–5, 6–10, 11–15, 16–20, and 21–25. A significant difference was found in the comparison among groups according to genotype and sampling time based on one-way analysis of variance ($p=0.0071$). GMTs, geometric mean titers; VNA, virus-neutralizing antibody; JEV, Japanese encephalitis virus.

Discussion

Most horses raised in Korea have been vaccinated annually with a live attenuated JE G3 vaccine, containing the strain Anyang 300, since 1980 [21]. Although the dominant JEV

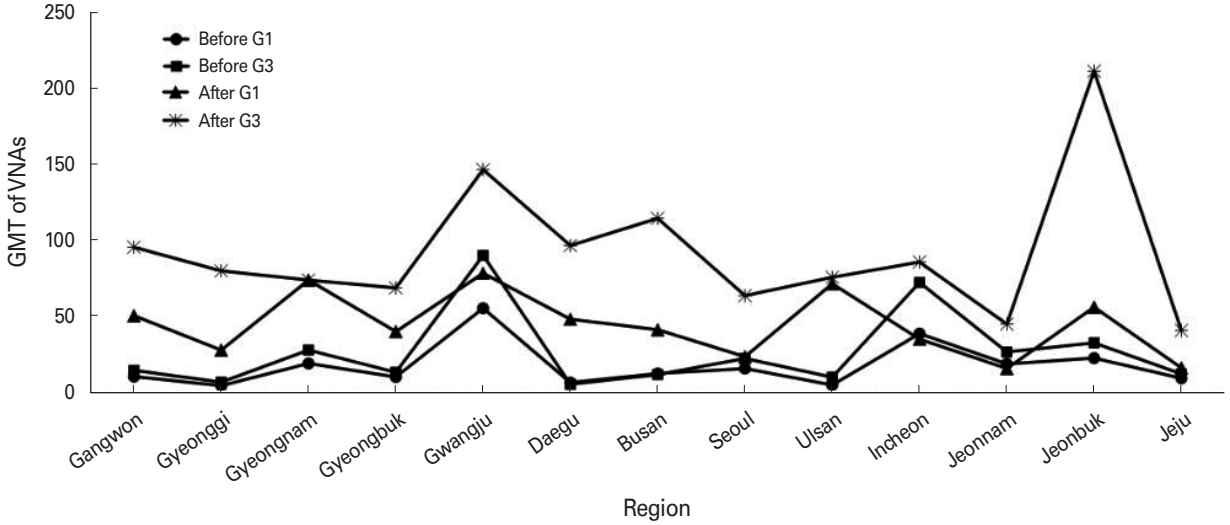

Fig. 3. GMTs of VNAs against JEV G1 (KV1899) and G3 (Anyang 300) in serum samples collected before annual revaccination (before) and after annual revaccination (after) according to region of residence. A significant difference was found in GMTs among groups according to genotype and sampling time based on one-way analysis of variance ($p<0.0001$). GMTs, geometric mean titers; VNA, virus-neutralizing antibody; JEV, Japanese encephalitis virus.

genotype has shifted from G3 to G1 in Korea since 1990, the G3 veterinary vaccine has been used against JEV for almost 40 years [16,17]. The majority of JE vaccines licensed to date include the G3 virus [23,24]. Antigenic differences that affect the vaccine's efficacy could exist among strains and genotypes [24,25]. Therefore, the positive rate of neutralizing antibodies was investigated using viruses of two genotypes (G1 and G3) with the same serum samples from 216 horses in Korea [16]. By contrast, in the present study, seroconversion rates and GMTs of VNAs against the JEV genotypes G1 and G3 were analyzed in serum samples of 1,231 horses immunized with the G3 vaccine according to blood sampling time (prior to and following annual revaccination).

In serosurveillance studies, sera of 492 horses (49.7%) were seropositive against JEV KV1899 (G1) based on the hemagglutination inhibition (HI) test using 989 blood samples collected between October 2005 and March 2007 in Korea [21]. Horses with an HI antibody titer ≥160 accounted for 3.9% of animals tested [21]. Antibody positive rates of 57.8% (737/1,274) and 58.9% (837/1,421) against JEV Anyang 300 (G3) were obtained using an *in vitro* neutralization assay in 2012 and 2013, respectively [22]. In this study, the total seroconversion rates (VNA titer ≥2) before and after revaccination were 77.1% and 93.4% for G1, and 83.4% and 98.8% for G3. The rates of VNA titer >10 were 45.1% and 77.8% for G1, and 49.1% and 82.9% for G3 in samples taken before and after revaccination, respectively. Total seroconversion rates (VNA titer ≥2) and rates of VNA titer >10 showed 4%–6% differences between the two genotypes in this study (Table 1). A difference of approximately 10% in seroconversion rates of the two genotypes was found in samples with VNA titers above 512 after revaccination. In a previous study, seropositive rates showed a large difference, with 4.2% against JEV KV1899 (G1) and 89.4% against Anyang 300 (G3) in a VN test using 216 horse serum samples collected in Korea [16]. Compared to previous findings, the overall sero-positive rate did not significantly differ between genotypes, although GMTs differed significantly with genotype in this study ($p<0.0001$) (Fig. 1). In accordance with our results, examination of VNA titers against G1–4 viruses after inoculation with two G3 inactivated vaccines in humans showed no significant difference in seroconversion rates between genotypes, but did show a difference in GMTs [26]. In particular, both G3 inactivated vaccines induced lower GMTs against G1 viruses compared to other genotypes [26]. In another study, antibody positive rates differed significantly with the age of horses [21]. However, in this study, GMTs did not differ significantly among age groups and regions, although there was a significant difference in GMTs with genotype and between samples collected before and after revaccination (Figs. 2, 3). These results suggest that reduced VNA titers against the G1 virus can be induced in horses vaccinated with the G3 vaccine, as observed in pigs [27]. Reductions in the protective efficacy and cross-neutralizing responses of G3 vaccines to heterologous JEV genotypes have been reported in mice and humans [24]. In addition, the antibody titer prior to vaccination was significantly lower than that after vaccination, suggesting that annual revaccination against JEV is required for horses.

The horse population has not been officially designated as experiencing a clinical outbreak in Korea [16]. Previous research suggested that antibody titers ≥640 based on the HI test indicate field infections in the vaccinated group [28]. Although the test method used differs from those of previous studies, field infections were suspected in domestic horses due to the presence of high antibody titers (≥1,024) in the VN test results in this study. JE occurrence in humans has been steady, with 17 cases in 2018 and 34 cases in 2019 (Korea Centers for Disease Control and Prevention, Infectious Disease Portal), suggesting continuous circulation of JEV in Korea.

In conclusion, genotype-specific neutralizing antibody titers against JEV G1 and G3 differed significantly in horses immunized with the G3 vaccine. Antigenic differences between genotypes could reduce the vaccine's efficacy, requiring the development of a new vaccine that includes the JEV G1 strain. Because the Anyang 300 strain used in this study is homologous with the vaccine strain, future research evaluating heterologous JEV G3 strains and serum samples from other animals such as pigs is necessary.

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