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Virology

Neutralization antibody response to booster/priming immunization with new equine influenza vaccine in Japan

Takashi YAMANAKA[1)]*, Manabu NEMOTO[1)], Hiroshi BANNAI[1)], Koji TSUJIMURA[1)], Tomio MATSUMURA[1)], Hiroshi KOKADO[1)], Sarah GILDEA[2)] and Ann CULLINANE[2)]

[1)]Equine Research Institute, the Japan Racing Association, 1400-4 Shiba, Shimotsuke, Tochigi 329-0412, Japan
[2)]Irish Equine Centre, Johnstown, Naas, Co., Kildare, W91 RH93, Ireland

ABSTRACT. Equine influenza (EI) vaccine has been widely used. However, the causative EI virus (H3N8) undergoes continuous antigenic drift, and the vaccine strains must be periodically reviewed and if necessary, updated to maintain vaccine efficacy against circulating viruses. In 2016, the Japanese vaccine was updated by replacing the old viruses with the Florida sub-lineage Clade (Fc) 2 virus, A/equine/Yokohama/aq13/2010 (Y10). We investigated the virus neutralization (VN) antibody response to Fc2 viruses currently circulating in Europe, after booster or primary immunization with the new vaccine. These European viruses have the amino acid substitution A144V or I179V of the hemagglutinin. In horses that had previously received a primary course and bi-annual boosters with the old vaccine booster, immunization with the updated vaccine increased the VN antibody levels against the European Fc2 viruses as well as Y10. There were no significant differences in the VN titers against Y10 and the Fc2 viruses with A144V or I179V substitution in horses that had received a primary course of the updated vaccine. However, a mixed primary course where the first dose was the old vaccine and the second dose was the updated vaccine, reduced VN titers against the European viruses compared to that against Y10. In summary, the new vaccine affords horses protective level of VN titers against the Fc2 viruses carrying A144V or I179V substitution, but our results suggest that the combination of the old and new vaccines for primary immunization would not be optimum.

KEY WORDS: equine influenza vaccine, Florida sub-lineage Clade 2, original antigenic sin, virus neutralization

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Equine influenza (EI) is an important respiratory disease of horses caused by the highly contagious equine influenza virus (EIV), a member of the family *Orthomyxoviridae* of the genus *Influenzavirus A* [2, 4]. The two subtypes of influenza, H7N7 and H3N8, have been isolated from horses [16]. The former is considered to be extinct [15]. In contrast, many large outbreaks of EI caused by H3N8 subtype among horses have been reported worldwide [11, 14, 19] since the first isolation of the latter virus in 1963 in the United States [13]. Vaccinations have been considered an effective measure to prevent or control EI [3, 7, 17]. However, like other influenza viruses, EIV undergoes antigenic drift as its RNA polymerase is highly error-prone. Thus, the strain compositions of EI vaccines has to be periodically reviewed to maintain vaccine efficacy against circulating viruses [3, 5].

EIV (H3N8) diverged into the Eurasian and American lineages in 1980s, and the American lineage subsequently diverged further into the Kentucky, Argentine and Florida sublineages [10]. The Florida sublineage has prevailed, with the emergence of 2 clades; clade 1 (Fc1) viruses predominate in America, while clade 2 (Fc2) viruses predominate in Europe [3, 12, 18]. Since 2010, the World Organisation for Animal Health (OIE) has recommended that EI vaccines contain a virus of each clade [1]. In response to this, the strain selection committee organized by the National Veterinary Assay Laboratory of the Ministry of Agriculture, Forestry and Fishery, Japan, decided to replace A/equine/La Plata/1993 (LP93, Argentine sublineage) and A/equine/Avesta/1993 (AV93, Eurasian lineage) with A/equine/Yokohama/aq13/2010 (Y10, Fc2) in Japanese EI vaccines [6]. The new bivalent vaccines containing Fc1 (A/equine/Ibaraki/1/2007, IBK07) and Y10, complying with OIE's recommendation, have been available in Japan since the fall of 2016. Here, we report a pilot study on the efficacy of this vaccine in the field. We assessed the virus neutralization (VN) antibody responses to the Fc2 viruses currently circulating in Europe with sera collected from Thoroughbred two-year-old racehorses and yearlings vaccinated with the new Japanese vaccine.

*Correspondence to: Yamanaka, T.: yamanaka@equinst.go.jp

MATERIALS AND METHODS

Viruses

All the viruses used in this study, LP93, IBK07, Y10, A/equine/Ayrshire/1/2013 (AY13), A/equine/Carlow/2011 (CL11) and A/equine/Devon/1/2011 (DV11), were propagated in the allantoic sac of ten-day-old embryonated hen's egg. The harvested viruses were aliquoted and stored at −80°C before use.

Vaccination and serum sample collection

Twenty-five two-year-old Thoroughbred racehorses (19 males and 6 females) were vaccinated with the new vaccine containing ≥100 chicken cell agglutinin (CCA)/dose of IBK07 and Y10 each (Nisseiken, Tokyo, Japan) in November 2016. These horses had received bi-annual booster vaccinations following a primary course at the age of one, with the old vaccines containing ≥150CCA/dose of IBK07, ≥100CCA/dose of AV93 and LP93 each (Nisseiken). The last booster vaccinations with the old vaccines were administered in May 2016. The serum samples were collected from the horses on the day they first received the new vaccine (Pre, November 2016) and one month later (Post, December 2016).

Twenty-six Thoroughbred yearlings with no history of EI vaccination were divided into two groups NN and ON of 13 horses each. The NN group (7 males and 6 females) were vaccinated twice with the new vaccine one month apart (January and February 2017). The ON group (5 males and 8 females) were vaccinated with the new vaccine (February 2017) one month after the administration of the old vaccine (January 2017). Serum samples were collected from all the yearlings on the day of first vaccination (January 2017) and one month after the second dose (March 2017). The sera collected on the day of first vaccination for VN antibodies (see below) against IBK07 to confirm the naivety of the yearlings. Additionally, we tested the serum samples from our previous study [21], which were collected from nine yearlings one month after the completion of primary vaccination with two doses of the old vaccines administered one month apart (OO group, 5 males and 4 females).

These investigations were approved by The Promotion and Ethics Committee for Research of the Equine Research Institute. Owners gave consent for their horses to be included in this study.

Serum treatment and VN test

All the sera were pretreated by trypsin-heat-potassium metaperiodate and the VN tests were conducted as previously described [20, 21]. Briefly, two-fold serial dilutions of the treated horse serum were prepared. Equal volume of the virus ($10^{3.6}$–$10^{4.4}$ 50% egg infectious dose/200 μl was added to each dilution of serum and incubated for 60 min at 34°C. Each dilution of the serum with virus was inoculated into the allantoic sacs of five ten-day-old embryonated hens' eggs (200 μl/egg). The allantoic fluid was harvested after three days of incubation at 34°C and examined for hemagglutination using 0.5% chicken red blood cells. The VN titre was determined as the reciprocal of the highest dilution of the antiserum at which ≥3 eggs showed <2 HA unit/50 μl. Due to the limited volumes of the yearlings' sera, VN titers of the OO group were determined only against Y10 in this study.

Data analysis

Regarding the data obtained from the two-year-old horses, the geometric mean (GM) titer against IBK07 at initial sampling was designated the base-line in this study as this virus was contained in both the original and the updated vaccine. The multiple comparisons of GM VN titers of the two-year-old horses were performed by Friedman test with Dunn's post hoc test to identify differences between the base-line and the VN titres at other sampling points. The frequencies of the two-year-old horses showing ≥64 of the VN titers against each Fc2 virus between Pre and Post were compared by McNemar's test. Regarding the data obtained from the yearlings the multiple comparisons of GM VN titers of each group one month after the second vaccination were performed by Friedman test with Dunn's post hoc test for identifying differences between the antibody response to Y10 and the other Fc2 viruses. These statistical calculations were done using Prism 6 for Windows (GraphPad Software, San Diego, CA, U.S.A.). VN titers at <8 were provisionally considered 4 for statistical analysis in this study. P value at <0.05 were deemed to be significant in this study.

RESULTS

Two-year-old horses

The GM VN titers obtained from the two-year-old racehorses are presented in Table 1. The GM VN titer against Y10 (199.5) on the day of vaccination was similar to the base-line (205.1). The Pre GM titer against AY13 was approximately 1.8-fold lower than the base-line but this was not statistically significant (P=0.08). In contrast, the Pre GM VN titers against the viruses carrying A144V substitution, CL11 (49.9) and DV11 (57.3), were about four (4.1 or 3.6, respectively)-fold lower than the base-line which was significant (P<0.01). The Post GM titers against CL11 (114.6) and DV11 (108.4) increased approximately two (2.3 or 1.9, respectively)-fold higher than each Pre GM VN titer. While the Post GM titer against DV11 remained significantly lower than the base-line titre (P=0.04), no significant differences were observed between the Post VN titers against AY13 or CL11 and the base-line (P>0.99 or P=0.12, respectively). The increase in the percentage of horses with VN titers of ≥64 against CL11, DV11 or AY13 post vaccination was significant (P=0.02, 0.04 or 0.02, respectively) whilst the increase in the percentage of horses with VN titers of ≥64 against Y10 was not significant (P=0.48) (Table 2).

Table 1. Antibody response of vaccinated horses boosted with the updated EI vaccine

Indicator viruses	Virus profiles	GM VN	
		Pre V	Post V
A/equine/La Plata/1993	Removed from the updated vaccine	143.0	188.7
A/equine/Ibaraki/1/2007	Contained in the original and updated vaccine, Florida sub-lineage Clade (Fc) 1	205.1	337.8
A/equine/Yokohama/aq13/2010	Newly added to the vaccine, Fc 2	199.5	410.1
A/equine/Ayrshire/1/2013	Fc2 with I179V substitution	111.4	173.6
A/equine/Carlow/2011	Fc2 with A144V substitution	49.9[a)]	114.6
A/equine/Devon/1/2011	Fc2 with A144V substitution	57.3[a)]	108.4[a)]

GM VN, geometric mean virus neutralization titre (n=25); Pre V, on day of vaccination; Post V, one month post vaccination. a) Statistical significance between the Pre GM VN titer against A/equine/Ibaraki/1/2007 (base-line, 205.1) and the Pre and Post GM VN titers against the four Fc2 viruses ($P<0.05$, Friedman's test with Dunn's multiple comparison tests).

Table 2. The percentage of horses (n=25) with VN titers of ≥64 prior to and post booster vaccination

Indicator viruses	Pre V VN ≥64 (%)	Post V VN ≥64 (%)
A/equine/Yokohama/aq13/2010	92.0 (74.0–99.0)[a)]	100.0 (86.3–100.0)
A/equine/Ayrshire/1/2013	68.0 (46.5–85.1)	96.0 (79.7–99.9)[b)]
A/equine/Carlow/2011	60.0 (38.7–78.9)	88.0 (68.8–97.5)[b)]
A/equine/Devon/1/2011	60.0 (38.7–78.9)	84.0 (63.9–95.5)[b)]

VN, virus neutralization; Pre V, on day of vaccination; Post V, one month post vaccination with the updated vaccine. a) 95% confidence interval in brackets. b) Statistical significance between the ratios between Pre and Post in each row ($P<0.05$, McNemar's test).

Table 3. Geometric mean virus neutralization (VN) titers of the sera collected from the yearlings one month after the second dose of the primary vaccination course

Indicator viruses	Groups[a)]		
	NN (n=13)	ON (n=13)	OO (n=9)
A/equine/Yokohama/aq13/2010	27.3	35.6	34.6
A/equine/Ayrshire/1/2013	41.8	39.6	ND[b)]
A/equine/Carlow/2011	16.9	5.0[d)]	6.3[c,d)]
A/equine/Devon/1/2011	19.8	9.4[d)]	8.0[c,d)]

a) NN or OO indicates the group of yearlings was vaccinated twice one month apart with the new updated vaccine or the old vaccine, respectively. ON indicates the group of yearlings was vaccinated twice one month a part with the old vaccine and new updated vaccine sequentially. b) Not determined. c) These data were cited from our previous study [21]. d) Statistical significance between the VN titer against A/equine/Yokohama/aq13/2010 and the other two (OO) or three (NN and ON) Fc2 viruses in each column (Friedman's test with Dunn's multiple comparison tests, $P<0.05$).

Yearlings

All the yearlings were seronegative (VN titer: <8 against IBK07) on the day of first vaccination confirming that they were naïve at the beginning of this study. The GM VN titers one month after the second dose of vaccine are presented in Table 3. There were no significant differences in the GM VN titers against Y10 and the other 3 Fc2 viruses in the horses that received two doses of the updated vaccine (NN group). On the other hand, the GM VN titers against CL11 (6.3) and DV11 (8.0) were significantly lower than that against Y10 (34.6) ($P<0.01$ and $P=0.01$, respectively), in the group vaccinated with old outdated vaccine, OO group, and this was also the case when the first dose was the old vaccine and only the second was updated, ON group. The GM VN titers of ON group against CL11 (5.0) and DV11 (9.4) were significantly lower than that against Y10 (35.6) ($P<0.01$ and $P=0.02$, respectively).

DISCUSSION

Recently, sequence analysis of haemagglutinin gene has revealed that Fc2 viruses have diverged into two sub-groups, the A144V group and the I179V group [1, 12, 18]. The former has the amino acid substitution from alanine to valine, at position 144 of the hemagglutinin protein and the latter has the amino acid substitution at position 179 from isoleucine to valine. The majority

of isolates in the United Kingdom and Ireland belongs to A144V group, in contrast, the majority of the circulating viruses in mainland Europe belongs to I179V group [1, 12, 18]. We previously reported that the VN titers of the horse antiserum raised to LP93 against the viruses carrying A144V substitution were ≥8-fold lower than those against the homologous virus and the Fc2 viruses lacking the A144V substitution [21]. Furthermore, we demonstrated that horses vaccinated with the inactivated Y10 lacking A144V and I179V substitutions showed milder pyrexia and less virus shedding following challenge with the Fc2 virus carrying A144Vsubstitution, CL11, than horses vaccinated with the inactivated LP93 vaccine. We hypothesized that this was due to the higher VN titers against CL11 of the horses which were administered the inactivated Y10 vaccine compared to those administered the inactivated LP93 vaccine [22]. Therefore, it is important to survey the VN antibody responses of horses vaccinated with the newly updated vaccine not just against the Fc2 virus included in the vaccine, Y10, but also the Fc2 viruses carrying the A144V substitution.

The comparatively high Pre GM VN titers against Y10 (199.5) of the two-year-old horses despite the absence of Fc2 in the old vaccine which they have received, corresponds to our previous reports of cross-neutralization with horse antiserum raised to LP93 [21, 22]. Also, in comparison with the Pre GM VN titer against Y10, the low Pre GM VN titers against the Fc2 viruses with A144V substitution, CL11 and DV11 (49.9 and 57.3, respectively), is consistent with our previous findings [21, 22].

The VN titer in horse serum against EIV that correlates with protection has yet to be unequivocally established. In our previous report of a ten yearlings which were experimentally challenged with CL11 ($10^{9.4}$ EID_{50}/horse), four horses with VN titer at ≥64 against CL11 did not suffer pyrexia following the challenge while five horses out of the remaining six with VN titers at <64 were pyretic [22]. From these results, the VN titer at ≥64 was considered to correlate with protection against pyrexia caused by EIV infection (Fisher's exact test, P=0.048, data not shown). The frequency of the two-year-old horses with VN titers at ≥64 against the Fc2 viruses carrying A144V or I179V substitution significantly increased one month after the booster immunization with the new vaccine. This suggests that the new inclusion of Y10 into the EI vaccine is beneficial to the protection against Fc2 viruses currently circulating in Europe.

Because of the longer shelf life of EI vaccines (2 or 3 years) than that of human seasonal influenza vaccines (1 year) [9], there is often an overlap of vaccines on the market and in the stores in veterinary hospitals when an updated vaccine is released. Thus, we considered it important to investigate the effect of a mixed primary course and created the ON group as well as the NN group for the yearlings. The GM VN titers of the ON group against the viruses carrying A144Vsubstitution, namely CL11 and DV11, one month after their primary course were about two or three times lower than those of the NN group, and were similar to those of the OO group [21]. Moreover, they were significantly lower than the GM VN titer against Fc2 vaccine strain, Y10. These suggest that the benefit of using the updated vaccine for primary vaccination is lost by the sequential combination of the old and updated vaccine. It was demonstrated in mice that the sequential exposure to two antigenically different *influenza A* viruses leads to reduced immunity to the second virus, a phenomenon known as original antigenic sin [8]. Unfortunately, due to the limited volumes of sera, the VN titers of the yearlings against the old viruses removed from the vaccine, e.g. LP93, were not measured in this study. However, the low GM VN titer against the A144V viruses of the ON group may be due to original antigenic sin. Further studies will be needed to investigate this. Regarding I179V viruses which primarily circulate in mainland Europe [1, 12, 18], not only the NN but also the ON group had similar or higher GM VN titer against these viruses than that against Y10, suggesting that the new vaccine induces a good VN antibody response to I179V viruses.

In summary, our data suggest that the use of the new vaccine which includes the Fc2 virus, Y10, as a booster affords horses protective levels of VN titers against the Fc2 viruses carrying A144V or I179V substitution. However, primary vaccination mingling the old and new vaccines afforded the horses insufficient VN antibody response against Fc2 viruses with A144V substitution which circulate mainly in the United Kingdom and Ireland. This may be due to the original antigenic sin and suggests that the new vaccines should be used for both vaccinations when administering the primary course.

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