

Antibody responses after vaccination against equine influenza in the Republic of Korea in 2013

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ABSTRACT. In this study, antibody responses after equine influenza vaccination were investigated among 1,098 horses in Korea using the hemagglutination inhibition (HI) assay. The equine influenza viruses, A/equine/South Africa/4/03 (H3N8) and A/equine/Wildeshausen/1/08 (H3N8), were used as antigens in the HI assay. The mean seropositive rates were 91.7% (geometric mean antibody levels (GMT), 56.8) and 93.6% (GMT, 105.2) for A/equine/South Africa/4/03 and A/equine/Wildeshausen/1/08, respectively. Yearlings and two-year-olds in training exhibited lower positive rates (68.1% (GMT, 14) and 61.7% (GMT, 11.9), respectively, with different antigens) than average. Horses two years old or younger may require more attention in vaccination against equine influenza according to the vaccination regime, because they could be a target of the equine influenza virus.

KEY WORDS: equine influenza, hemagglutination inhibition, seropositivity, vaccination

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Equine influenza is respiratory disease in horses, and it is caused by the equine influenza virus, which belongs to the influenza A virus of Orthomyxoviridae family [21]. Equine influenza outbreaks have been reported all over the world, except for a few countries, such as New Zealand and Iceland [6, 8, 9, 17, 19, 25]. Currently, two subtypes of equine influenza virus are recognized; H7N7 and H3N8. The H7N7 subtype was first isolated in the horse in Czechoslovakia in 1956, and H3N8 was first reported in a race horse in Miami in 1963 [20, 24]. However, the H7N7 subtype has not been isolated since 1979, and it is considered to be extinct [26]. The H3N8 subtype is prevalent among horses worldwide. The H3N8 subtype evolved into two distinct lineages, “American” and “European”, in the late 1980s [4]. The American lineage has further evolved into the South American, Kentucky and Florida lineages, and the Florida lineage has further diverged into two sub-lineages (Florida clade 1 and clade 2), which have been dominantly circulating worldwide [1, 10–12]. Florida clade 1 viruses have caused major outbreaks in Africa, Asia, Australia and Europe [1, 7, 25, 28], and clade 2 viruses have also spread to Europe and Asian countries [6, 18, 23, 29].

The typical clinical signs of equine influenza virus infection in horses are pyrexia, nasal discharge and dry cough [3, 4, 22]. Secondary bacterial infection is observed in rare cases, and

this can cause significant problems [2, 3, 5]. The morbidity rate is high (approximately 100%) in susceptible groups and the mortality rate is low in horses, but a small number of fatalities have been reported in young foals [16, 17]. Equine influenza is considered one of the most important horse respiratory pathogens, because it can spread widely among susceptible horses, affect the performance of the infected animals and eventually cause the cancellation of race meeting or equestrian events. Vaccination is considered a key control measure for equine influenza [3]. Equine influenza vaccine strains are recommended by the World Organization for Animal Health (OIE) according to the antigenic characterization of circulating viruses [15]. Since 2010, vaccines against the clade 1 and clade 2 viruses of the Florida sub-lineage have been recommended for the international market by OIE expert surveillance panels for equine influenza [2].

In Korea, the equine population has increased gradually since the 1980s, and the number of horses raised in Korea was approximately 30,000 as of 2012 (The Statistics Yearbook 2013, Ministry of Agriculture, Food and Rural Affairs, Korea). Vaccination against equine influenza has been practiced with the active involvement of the Korea Racing Authority (KRA) since 1974. The equine influenza vaccine used in Korea since July 2008 is a liquid vaccine containing 2 recombinant canarypox viruses expressing the hemagglutinin (HA) gene from the equine influenza virus strain A/equi-2/Ohio/03 (clade 1) and A/equi-2/Newmarket/2/93 (European representative). Vaccinations against equine influenza are performed twice a year, once from April to May and again from September to November. Horses get vaccinated one or twice a year according to vaccination regimen. In this study, a serological assay (hemagglutination inhibition (HI) assay) was performed to evaluate the antibody levels in vaccinated horses against equine influenza virus recommended by OIE

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Table 1. No. of horse sera collected for this study

Type	KRA horses	Private farms	Total
Racehorse	516	0	516
Riding horse	92	248	340
Yearling and two-year-olds in training	34	60	94
Broodmare	0	136	136
Stallion	12	0	12
Total	654	444	1,098

expert surveillance panel for equine influenza from 2010.

A total of 1,098 horse sera were obtained from the Korea Racing Authority (KRA, Gwacheon, Korea) in 2013 (Table 1). Of the 1,098 sera, 654 sera were from horses raised by the KRA. Most of these horses (78.9%) are racehorses. Four hundred forty-four sera were collected from horses raised in privately owned farms. Half of these horses are riding horses (55.9%). One thousand and forty one horses (94.8%) were vaccinated against equine influenza less than 12 months before blood sample collection. Fifty-seven horses (5.2%) out of 1,098 were vaccinated against equine influenza more than 12 months before blood sample collection.

The equine influenza viruses, A/equine/South Africa/4/03 (H3N8) (American lineage, Florida sublineage clade 1) and A/equine/Wildeshausen/1/08 (H3N8), were used as antigens in the HI assay. A/equine/Wildeshausen/1/08 is classified into Florida sublineage clade 2 according to the phylogenetic tree analysis reported by Woodward *et al* [27]. The virus A/equine/South Africa/4/03 was kindly provided by Dr. Debra Elton from the World Organisation for Animal Health (OIE) reference laboratory of equine influenza, Center for Preventive Medicine, Animal Health Trust, U.K., and the A/equine/Wildeshausen/1/08 virus was kindly provided by Dr. Armando Damiani, OIE reference laboratory of equine influenza, Institute of Virology, Veterinary Medicine, Freie Universitat Berlin in Germany. These viruses were propagated in 10-day-old embryonated specific pathogen free (SPF) eggs (VALO, MD, U.S.A) and incubated at 37°C for 3 days. The allantoic fluid were harvested after chilling at 4°C and stored at -70°C before use.

Virus titers were measured by HA assay as previously described [14]. Briefly, 25 μ l of allantoic fluid were serially diluted 2 fold with 25 μ l of phosphate buffer saline (PBS). Fifty μ l of 0.5% chicken red blood cells (RBCs) was added to each well. The virus and RBC mixture was incubated at room temperature until a distinct RBC button formed (30–60 min) in the control well.

Antibody titers were measured by the HI assay as previously described [14]. Briefly, 50 μ l of horse serum was treated with 100 μ l of 0.016 M potassium periodate at room temperature (RT) for 15 min, and 50 μ l of 3% glycerol in PBS was added. The mixtures were placed at RT for 15 min and then incubated at 56°C for 30 min. Twenty-five μ l of treated serum samples were serially diluted with PBS two fold, and antigens (4 HA/25 μ l) were added. After a one-hr incubation at RT, 50 μ l of 0.5% chicken RBCs was added to

each well. The titers were recorded after 1-hr incubation at RT. Seropositivity was defined as a HI titer greater than or equal to 8 in this study.

Chicken RBC were collected from 8 chickens of 12-week-old specific pathogen-free (SPF) provided from Namdeok SPF Co. (Suwon, Korea). These animals were taken care of in biosafely level 2 animal facilities at the Animal and Plant Quarantine Agency, Korea, according to the protocol of the Institutional Animal Care and Use Committee of the Republic of Korea.

For statistical analyses, population proportion testing was performed using the Minitab® 16 program (Minitab®, State College, PA, U.S.A.). If the absolute value of the Z value was smaller than 1.645 (95% confidence interval (CI)), the null hypothesis was accepted. The geometric mean titers (GMT) were calculated using Microsoft Excel™.

The seropositive rate and mean antibody titers against equine influenza: A total of 1,007 horses (91.7%) out of 1,098 were antibody positive for the equine influenza virus, A/equine/South Africa/4/03 (Florida lineage, clade 1 sub-lineage) (Table 2). Riding horses and broodmares showed a higher antibody positive rate (98.8% and 97.1%) than the average rate of the horses tested in this study. Young horses under two years old in training showed a lower positive rate (68.1%) than the average. Overall, except for yearlings and 2-year-olds in training, all groups showed a fairly high positive rate (all approximately 90%). The highest antibody levels were detected in riding horses and broodmares (GMT 129.1 and 143.9, respectively). The rest of the groups showed low levels of antibody titers (GMT 14–34).

In the HI assay using the antigen A/equine/Wildeshausen/1/08 (Florida lineage clade 2 sub-lineage), 1,028 horses (93.6%) out of 1,098 horses were antibody positive (Table 2). Broodmares, riding horses and racehorses showed a higher antibody-positive rate (96.3–97.7%) than average. Again, young horses under 2 years old in training showed a lower positive rate (61.7%) than the average. Racehorses showed the highest antibody level (GMT 138.7). Except for the yearlings and two-year-olds, which showed a low antibody level (GMT 11.9), all groups showed similar antibody levels (GMT 122.1 in riding horses, 115.6 in broodmares and 90.5 in stallions).

The seropositive rate and mean antibody titers by age of horse: The age of the horses tested varied (from 0 to 26 years old), and the results were analyzed after the horses were divided into 5 groups (0–1, 2, 3–5, 6–10 and over 10 years old). For antigen A/equine/South Africa/4/03, younger hors-

Table 2. Antibody responses in the HI test by horse type

Type	No. tested	A/Eq/South Africa/4/03 antigen					A/Eq/Wildeshausen/1/08 antigen				
		No. positive	Positive rate	Z value	z (0.05) 95% CI	GMT*	No. positive	Positive rate	Z value	z (0.05) 95% CI	GMT
Racehorse	516	464	89.9%	-1.475	-1.645	34	497	96.3%	-9.727	-1.645	138.7
Riding horse	340	336	98.8%	4.756	1.645	129.1	328	96.5%	-3.113	-1.645	122.1
Yearling and two-year-olds in training	94	64	68.1%	-8.309	-1.645	14	58	61.7%	0.359	1.645	11.9
Broodmare	136	132	97.1%	2.262	1.645	143.9	133	97.8%	4.062	1.645	115.6
Stallion	12	11	91.7%	-0.006	-1.645	24	12	100.0%	2.589	1.645	90.5
Total	1,098	1,007	91.7%			56.8	1,028	93.6%			105.2

* Geometric mean titer

Table 3. Antibody responses in the HI test in different age groups

Years	No. tested	A/Eq/South Africa/4/03 antigen					A/Eq/Wildeshausen/1/08 antigen				
		No. positive	Positive rate	Z value	z (0.05) 95% CI	GMT	No. positive	Positive rate	Z value	z (0.05) 95% CI	GMT
0-1	46	24	52.2%	2.504	1.645	7.9	16	34.8%	-16.203	-1.645	3.3
2	159	135	84.9%	2.148	1.645	27.1	146	91.8%	-0.877	-1.645	88.4
3-5	460	424	92.2%	-12.668	-1.645	46.3	445	96.7%	2.795	1.645	135.5
6-10	206	205	99.5%	1.99	1.645	165.3	202	98.1%	2.641	1.645	160.4
Over 10	226	218	96.0%	0.904	1.645	81.8	218	96.0%	1.789	1.645	97.7

es (0-1 and 2 years old) showed lower seropositivity rates (52.2% and 84.9%, respectively) than the average, although the 2-year-olds showed much higher seropositivity than the 0-1-year-olds (Table 3). Horses over 5 years old showed a higher seropositivity rate (96.0-99.5%) than average. Horses 3-5 years old showed similar seropositivity rates (92.2%) to the average. Similarly, horses between 0-2 years old showed the lowest antibody titers (GMT 7.9-27.1), and horses older than 2 years showed high titers (GMT 46.3-165.3).

The results of the HI assay using the antigen A/equine/Wildeshausen/1/08 were quite similar to those using the antigen A/equine/South Africa/4/03. Horses 3 years old or older showed a higher seropositivity rate (96.0-98.1%) than the average (Table 3). Horses 0-1 year old showed a lower seropositivity rate (34.8%) than average, while 2-year-old horses showed a similar seropositivity rate (91.8%) to the average. Horses 0-1 year old showed the lowest antibody levels (GMT 3.3), while horses 6-10 years old showed the highest levels (GMT 160.4).

The seropositivity rate against A/equine/South Africa/4/03 was 91.7% among 1,098 vaccinated horses. Interestingly, the seropositivity rate against A/equine/Wildeshausen/1/08 was 93.6%. The A/equine/Wildeshausen/1/08 virus belongs to the clade 2 sub-lineage, and this lineage was not contained in the vaccine used in Korea in 2012 and 2013. Regardless, comparatively high seropositivity and antibody titers (93.6% and GMT 105.2) against the clade 2 virus, which were even higher than the seropositivity against clade 1 (91.7%, GMT 56.8), were observed in this study. There could be several explanations for this phenomenon. First, some of the horses tested in this study were not from Korea. There is a possibility that they were exposed to clade 2 sub-lineage equine

influenza viruses and developed antibodies against those viruses before they were imported to Korea. Second, the antibodies against clade 2 sub-lineage could be from clade 2 sub-lineage infection in Korea. Third, antibodies against clade 1 sub-lineage may show cross reactivity to clade 2 sub-lineage viruses.

The first and second possibilities are unlikely. Only 175 horses (15.9%) tested in this study were introduced from foreign countries, so, over 93% positivity could not be due to the first possibility. Infection inside Korea has little chance too, because clade 2 virus isolation or clade 2-related outbreaks have not been reported in Korea to date. Only clade 1 sub-lineage virus isolation was reported in Korea from horses with typical respiratory symptoms in 2011 [13]. Therefore, cross reactivity between clades is the most likely explanation of high antibody levels against the clade 2 sublineage. Several reference sera obtained from various sources (in-house reference sera in the QIA, reference sera from National Veterinary Services Laboratory, U.S.A., and reference sera from the European Directorate for the Quality of Medicines (EDQM) Council of Europe) against equine influenza were tested for various equine influenza virus strains (Eurasian, Florida clade 1 or clade 2 sub-lineage). Similar or 1 or 2 log lower Hi titers than HI titers against the same lineage were observed in the HI assay with virus from different lineage (data not included). This type of cross reaction has been reported previously [27]. So, there is a high chance that antibodies against the clade 1 sub-lineage virus could cross react with virus from the clade 2 sub-lineage.

Horses 0-1 year old showed lower antibody levels than the other groups except for 2-year-olds in the HI test with clade 2 virus (52.2% and GMT 7.9 against clade 1 and 34.8%

and GMT 3.3 against clade 2 sublineage virus). This finding may be observed due to the comparatively lower number of vaccinations after their birth. These 46 horses between 0 and 1 year old were vaccinated only once by the time, blood samples were collected for this study, and less than 5 months had passed since vaccination. However, 2-year-old horses showed rapid increases in antibody levels (84.9%, GMT 27.1 in clade 1 antigen and 91.8%, GMT 88.4 in clade 2 antigen). This was thought to be from multiple vaccinations, because out of 159 horses, 63 horses (39.6%) were vaccinated twice before blood sample collection.

This study was performed to monitor the status of antibody levels against equine influenza, because we believe that equine influenza vaccination has been practiced systematically on a regular basis in Korea with the active involvement of the KRA, though the vaccines applied in this study did not contain vaccine strains recommended by OIE expert surveillance panel from 2010. Overall, riding horses, racehorses, broodmares and stallions, which are generally over 3 years old, showed high seropositivity rates and high antibody titers against equine influenza virus Florida clade 1 or clade 2. Horses 2 years old or younger may need more attention in vaccination against equine influenza during the vaccination regime, because they could be a target of equine influenza virus.

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