J Vet Diagn Invest 18:264–267 (2006)

BRIEF COMMUNICATIONS

Inability of kaolin treatment to remove nonspecific inhibitors from equine serum for the hemagglutination inhibition test against equine H7N7 influenza virus

Saikat Boliar, Wlodek Stanislawek, Thomas M. Chambers[1]

Abstract. The hemagglutination inhibition test is used by many diagnostic and surveillance laboratories for detection of antibodies to influenza viruses. It is well known that the hemagglutination inhibition test is affected by nonspecific inhibitors present in equine serum. Several serum treatments are in use to remove these inhibitors, including treatment with kaolin. Discrepant results were observed in the authors' laboratories when using kaolin treatment before testing equine sera for antibodies against equine influenza virus (EIV) subtype-1 (H7N7). It is demonstrated here that kaolin treatment leads to false positive results when testing for antibodies against EIV subtype-1, as compared to other standard serum treatments (trypsin-periodate, receptor-destroying enzyme). Against EIV subtype-2 (H3N8), however, false positive results were not evident. Trypsin-periodate and receptor-destroying enzyme (RDE) treatments appear to be superior to kaolin for removal of nonspecific inhibitors from equine serum and should be used for serological diagnosis and surveillance of equine influenza virus.

Key words: Equine influenza virus; HI test; nonspecific inhibitors; serum treatments.

Equine influenza virus (EIV) is a major cause of acute respiratory diseases in horses in most parts of the world.[9] Two subtypes of equine influenza viruses are known. The first subtype, H7N7 or equine-1 (prototype influenza A/equine/Prague/1/56), is believed to be extinct, as there has been no confirmed isolation since 1980.[9] The other subtype, H3N8 or equine-2 (prototype influenza A/equine/Miami/1/63), was first isolated in 1963 and accounts for all recent equine influenza outbreaks. Accordingly the international Expert Surveillance Panel for equine influenza, headed by the Office International des Epizooties (OIE) reference laboratories for this disease, recommended to vaccine manufacturers that the H7N7 strain is no longer required in equine influenza vaccines.[2] In the absence of vaccination, stringent surveillance is necessary to spot any reemergence of equine H7N7 influenza virus.

Although various serological methods are available for the detection of seroconversion against EIV, the World Health Organization, OIE, and many diagnostic laboratories still use the hemagglutination-inhibition assay (HI). This test efficiently detects subtype-specific antibodies against influenza virus hemagglutinin. HI is a rapid and cost-effective test with good sensitivity, and also has a reproducibility of 84% to 96% for myxoviruses.[6] However, one disadvantage with this test is that it is affected by nonspecific inhibitors present in equine serum.

From the Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546, USA (Boliar, Chambers), and the Investigation and Diagnostic Centre-Wallaceville, MAF Biosecurity, Upper Hutt, New Zealand (Stanislawek).

[1] Corresponding Author: Dr. Thomas Chambers, 108 Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099 USA (e-mail: tmcham1@uky.edu).

Francis first described the presence of nonspecific inhibitors in human and animal sera in 1947.[5] Other body fluids such as ovomucin, bovine submaxillary mucin, and pleural fluid are also known to posses nonspecific inhibitors of influenza virus.[12] Three categories of nonspecific inhibitors have been identified in serum. Alpha inhibitors are heat stable but show no virus-neutralizing activity. Beta inhibitors are heat labile and have virus-neutralizing property, while gamma inhibitors are heat stable as well as virus-neutralizing.[12] Beta inhibitors are present in abundance in bovine and mouse sera,[1] whereas equine, guinea pig, and porcine sera contain a class of gamma inhibitors called α–2 macroglobulin,[10,11] a high molecular weight (720 kD) glycoprotein consisting of 4 identical subunits (180 kD) each of which is made of 5 reactive sites.[3] The mode of action of these inhibitors is by competition for the viral receptor binding sites. They bind viral hemagglutinins through their sialic acid residues and thereby prevent erythrocytes from binding the virus particles, leading to false positive or enhanced antibody titers. Equine α–2 macroglobulin is a more potent inhibitor than its human counterpart, which lacks an additional N-O-acetyl group linked to the N-acetylneuraminic acid as it is in horses.[10] Therefore, equine serum has to be processed before the HI test.

Several techniques are in use to eliminate these nonspecific inhibitors from horse serum, including 1) heat treatment, 2) kaolin and erythrocyte absorption, 3) trypsin-periodate (TP), and 4) receptor-destroying enzyme (RDE) from *Vibrio cholerae*. Sometimes inhibitor-resistant influenza viruses are also used, but these have not proved very efficient in detecting rises in antibody titer.[13] Since nonspecific inhibitors present in equine serum belong to the heat-stable gamma class, heat treatment alone is not an efficient method to eliminate them.

In 2004, one of the authors (WS) observed suspicious HI results on a set of equine sera that were tested following kaolin and heat treatment. Several of the set tested positive against equine H7N7 influenza virus. New Zealand is one of the few countries that does not practice vaccination, because its horse population has historically been free from equine influenza. The finding of apparent seroconversion to equine H7N7 influenza, in the absence of vaccination or a recognizable outbreak, was confirmed by an OIE reference laboratory (TC), using kaolin treatment, but the same sera were negative when TP treatment was used. This led the authors to compare the effectiveness of kaolin treatment versus TP and RDE treatment of equine serum samples for the HI test.

Two different sets of horse sera were studied. One was the set from New Zealand horses. Initially 1 serum sample was collected from a horse showing peripheral edema and respiratory signs from a herd of about 70 horses on an isolated farm in New Zealand. Further investigation determined that a mild respiratory disease had affected many horses over a few weeks, and consequently blood samples from 15 horses were collected for bacterial and viral examinations. A second set of 24 sera was obtained from University of Kentucky ponies ($n = 6$), 3 of which were influenza-naïve yearlings. The remaining 3 were 3-year-olds that had been vaccinated as yearlings with equine influenza vaccine[a] containing both equine-1 and equine-2 strains, and later challenged with influenza A/equine/KY/1999 (H3N8). All 6 were experimentally infected with equine H3N8 influenza virus (influenza A/equine/Ohio/1/2003), and sera were obtained once before and on 3 different occasions following infection.

As antigens for the HI testing, influenza A/equine/Prague/1/56 (H7N7), influenza A/equine/Miami/1/63 (H3N8), and influenza A/equine/Ohio/1/2003 (H3N8) viruses were grown in the allantoic fluid of 10-day old embryonated chicken eggs. After 48 hours of incubation at 37°C, viruses were harvested in sterile tubes and their titers were measured by the hemagglutination test. Viruses were not treated with ether for the HI test, since such procedure increases sensitivity but decreases specificity.[8]

Kaolin, TP, and RDE treatments were done according to published methods[7] with some modifications. Kaolin treatment of serum was carried out by mixing 2 volumes of serum with 10 volumes of 10% kaolin[b] solution (v/v in PBS) for 20 minutes followed by centrifugation at 1,200 × g for 20 minutes. Then 1 volume of 5% chicken RBC suspension and 7 volumes of PBS were added and centrifuged again at 400 × g for 20 minutes. After the centrifugation, the supernatant (a 1 : 10 dilution of kaolin treated serum) was collected.

For TP treatment, 1 volume of serum was treated with 1 volume of 0.4% trypsin[c] (v/v in PBS, pH 7.2) at 56°C for 30 minutes. After cooling to 25°C, 3 volumes of 0.01M sodium periodate[d] ($NaIO_4$) was added and incubated for 15 minutes. Then $NaIO_4$ was inactivated by adding 3 volumes of 1% glycerol[e] (v/v in normal saline) for 15 minutes at 25°C. Finally, 2 volumes of normal saline were added to achieve a 1 : 10 dilution of treated serum.

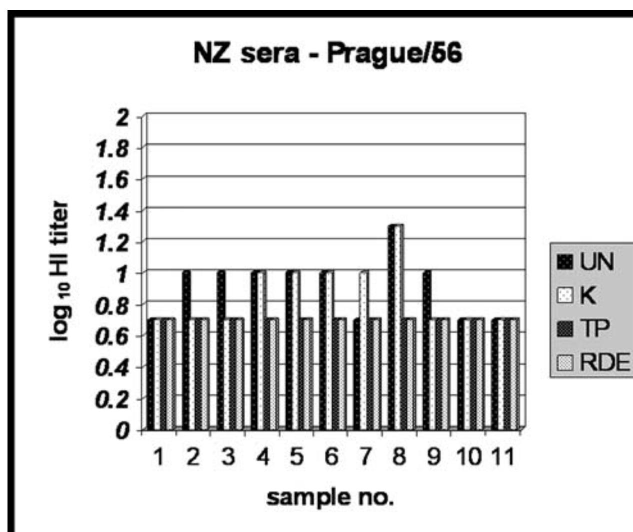

Figure 1. HI titers of representative New Zealand (NZ) sera against influenza A/equine/Prague/1/56 virus, showing differential reactions of several sera depending on treatment. UN = untreated sera, K = kaolin-treated, TP = trypsin-periodate treated, RDE = receptor destroying enzyme treated sera. Serum samples that tested negative (< 10, the lower limit of detection) were arbitrarily set to a value of 5 for calculation, thus 0.70 is the minimum log value. Positive samples (titer ≥ 10) have log values ≥ 1.0 according to their degree of reactivity.

Lyophilized RDE[f] (50 units) was reconstituted in 5 ml of sterile distilled water. One volume of serum was treated with 4 volumes of RDE working solution (1 : 10 dilution in calcium saline solution). This was incubated overnight (12–18 hours) in a 37°C water bath. Then RDE was inactivated by addition of 3 volumes of a 2.5% sodium citrate solution for 30 minutes at 56°C. Lastly, 2 volumes of PBS were added to obtain a 1 : 10 dilution of RDE treated serum.

HI tests were performed as described,[7] with some modifications, in U-bottom 96-well microtiter plates. Serum samples were serially diluted across the columns up to the 12th well using a 2-fold dilution series. The lowest dilution used was 1 : 10. Four HA units of virus and 0.5% chicken RBC suspension (v/v in PBS) were used. Each test included a negative control without virus. The HI titer was read as the reciprocal of the highest serum dilution that completely inhibited erythrocyte agglutination. Titers ≥10 were considered positive, while negative samples were given a score of 5 for calculation.

Both sets of serum samples were tested against influenza A/equine/Prague/1/56 (equine-1) and 1 EIV subtype-2 virus, namely, influenza A/equine/Miami/1/63 for New Zealand serum samples, while influenza A/equine/Ohio/1/2003 was used for Kentucky serum samples. HI tests were also carried out with untreated sera to compare the effects of different treatments. Untreated sera were diluted with PBS to make the starting 1 : 10 dilution for HI testing.

For New Zealand sera, 7 untreated and 5 kaolin-treated sera out of 15 samples tested were positive against H7N7 virus subtype, while none of the TP and RDE treated sera was positive (Fig. 1). Regardless of treatment, all of these

Table 1. HI titers of serum samples.

Serum sample	Antigen (virus) used for HI test	HI titer* (mean ± SE)			
		UN $_a$	K $_b$	TP $_c$	RDE $_d$
New Zealand sera set (n=15)	Prague/56 (H7N7)	7.3 ± 1.1	6.6 ± 1.1	5.0 ± 0.0	5.0 ± 0.0
	Miami/63 (H3N8)	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
Kentucky sera set (n=24)	Prague/56 (H7N7)	19 ± 1.2	13 ± 1.1	5.2 ± 1.0	5.7 ± 1.1
	Ohio/1/2003 (H3N8)	91 ± 14	82 ± 14	59 ± 14	82 ± 14

* HI Titer = highest serum dilution completely inhibiting chicken erythrocyte agglutination. Serum samples tested negative (< 10, the lower limit of detection) were arbitrarily set to a value of 5 for calculation of means. Positive samples have titer ≥ 10 according to their degree of reactivity. a. UN= untreated sera; b. K= kaolin treated sera; c. TP= trypsin-periodate treated sera; d. RDE= receptor destroying enzyme treated sera.

serum samples tested negative against equine-2 (H3N8) influenza virus (Table 1). For the Kentucky experimental sera, all but 1 untreated serum sample (B27-Day 14) were positive against H7N7 virus subtype. For kaolin-treated sera, only 2 samples, including B27-Day 14, came out as negative, and the rest (22/24) were positive against H7N7 virus. For TP-treated sera only 1/24 and for RDE-treated sera only 2/24 serum samples were positive when tested against equine-1 (H7N7) virus. Representative test results have been shown in Fig. 2. These results did not distinguish between the naïve and previously exposed ponies, except for a slight trend of the naïve ponies to show higher titers postinfection.

These results demonstrate that HI titers of untreated horse sera are higher in comparison to treated sera when tested against EIV subtype-1. But when HI tests of those serum samples were carried out using EIV subtype-2, there was little difference among untreated and serum samples treated by 3 different methods (Table 1). These results indicate that EIV subtype-1 is sensitive to the nonspecific inhibitors present in horse serum, whereas EIV subtype-2 is less or not at all sensitive to them. These findings corroborate the findings of Connor et al. showing that equine H3 influenza viruses are resistant to hemagglutination inhibition by horse serum.[4]

New Zealand is considered to be free from all equine influenza viruses, and Kentucky is believed to be free of equine H7N7 virus. While equine H3N8 influenza is enzootic in Kentucky, the ponies used in the challenge experiment were raised and maintained in a carefully regulated herd and had been monitored since birth for evidence of natural influenza infection using HI of TP-treated sera. HI test results from both New Zealand and Kentucky samples showed that kaolin-treated serum samples tested positive against equine-1 influenza virus, as did untreated serum samples. The same samples treated with TP or RDE gave largely negative results when tested against equine-1 influenza virus. TP treatment does not eliminate specific antibodies from equine serum, since known equine-1 influenza seropositive sera from vaccinated dams tested positive in HI tests following TP treatment (Holland & Chambers, unpublished data). Moreover, HI titers of kaolin-treated sera were close to those of untreated serum samples, showing that kaolin treatment was not an efficient method for removing nonspecific inhibitors from horse sera. Therefore, kaolin treatment is not adequate to eliminate false-positive results for equine H7N7 influenza virus antibodies. Periodate acts by oxidizing cis-hydroxyl groups on carbohydrates, and RDE cleaves N-acetylneuraminic acid residues from sialyloligosaccharides of α–2 macroglobulin.[12] These should be effective in inactivating the nonspecific inhibitors, since they bind viruses through their sialic acid residues. Kaolin adsorbs nonspecific inhibitors[7] but may not completely inactivate them. It can be concluded that kaolin is not an adequate treatment for horse sera used in the HI test, especially for detection of antibodies against EIV subtype-1 (H7N7). RDE and TP treatments appear to effectively remove nonspecific in-

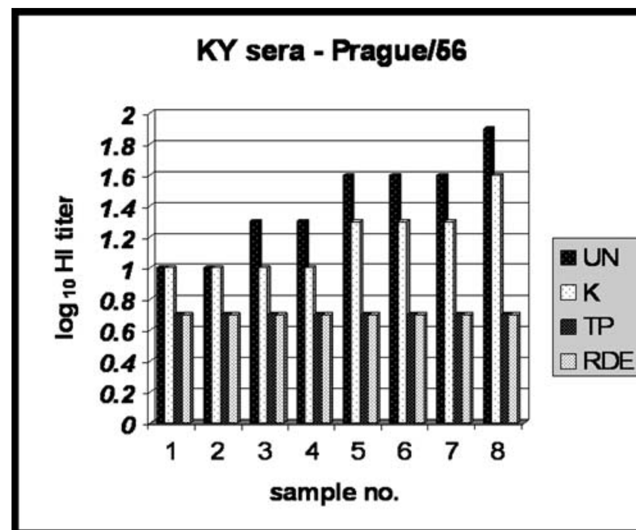

Figure 2. HI titers of representative Kentucky (KY) sera against influenza A/equine/Prague/1/56 virus, demonstrating differential reactivity depending on treatment. UN = untreated sera, K = kaolin-treated, TP = trypsin-periodate treated, RDE = receptor destroying enzyme treated sera. KY sera sample no. : 1, 2, and 3 are preinfection samples from ponies D33, D35, and D37 respectively; 4, 5, 6, 7, and 8 are postinfection (Day 8) samples from ponies B23, B27, B33, D33, and D35 respectively. Serum samples tested negative (< 10, the lower limit of detection) were arbitrarily set to a value of 5 for calculation, thus 0.70 is the minimum log value. Positive samples (titer ≥ 10) have log values ≥ 1.0 according to their degree of reactivity.

hibitors from equine sera and should be the preferred treatments for HI-based serodiagnosis of EIV subtype-1.

Acknowledgements. We thank Ms. Lynn Tudor for expert technical assistance. This work was supported in part by USDA/CSREES Project No. KY014014. This work is in connection with a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director (publication no. 05-14-107).

Sources and manufacturers

a. Encevac TC-4, Intervet Inc., Millsboro, DE
b. Kaolin, Fisher Scientific, Pittsburgh, PA.
c. Trypsin, Worthington Biochemical Company, Freehold, NJ.
d. Sodium meta-periodate, Matheson Coleman & Bell, Norwood, OH.
e. Glycerol, Sigma Chemical Company, St. Louis, MO.
f. RDE, Denka Seiken Company LTD., Chuo-ku, Tokyo, Japan.

References

1. Anders EM, Hartley CA, Jackson DC: 1990, Bovine and mouse serum beta inhibitors of influenza A viruses are mannose-binding lectins. Proc Natl Acad Sci USA 87: 4485–4489.
2. Anonymous: 2005, Conclusions and recommendations from the 2005 meeting of the Expert Surveillance Panel on Equine Influenza Vaccines. OIE bulletin: 26–27.
3. Borth W: 1992, Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J 6: 3345–3353.
4. Connor RJ, Kawaoka Y, Webster RG, Paulson JC: 1994, Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205:17–23.
5. Francis T, Jr: 1947, Dissociation of hemagglutinating and antibody-measuring capacities of influenza virus. J Exp Med 85:1–7.
6. Hierholzer JC, Suggs MT, Hall EC: 1969, Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. Appl Microbiol 18: 824–833.
7. Hsiung GD: 1973, Diagnostic virology: an illustrated handbook, pp. 26–34,172. Yale University Press, New Haven, CT.
8. Kendal AP, Cate TR: 1983, Increased sensitivity and reduced specificity of hemagglutination inhibition tests with ether-treated influenza B/Singapore/222/79. J Clin Microbiol 18: 930–934.
9. Mumford JA, Chambers TM: 1999, Textbook of influenza, pp. 146–162. Blackwell Science, Oxford, England.
10. Pritchett TJ, Paulson JC: 1989, Basis for the potent inhibition of influenza virus infection by equine and guinea pig alpha 2-macroglobulin. J Biol Chem 264:9850–9858.
11. Ryan-Poirier KA, Kawaoka Y: 1993, Alpha 2-macroglobulin is the major neutralizing inhibitor of influenza A virus in pig serum. Virology 193:974–976.
12. Ryan-Poirier KA, Kawaoka Y: 1991, Distinct glycoprotein inhibitors of influenza A virus in different animal sera. J Virol 65:389–395.
13. Subbarao EK, Kawaoka Y, Ryan-Poirier K, et al.: 1992, Comparison of different approaches to measuring influenza A virus-specific hemagglutination inhibition antibodies in the presence of serum inhibitors. J Clin Microbiol 30:996–999.