

Comparison among three different serological methods for the detection of equine influenza virus infection

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Summary

The equine influenza virus (EIV) H3N8 subtype is responsible for all EIV outbreaks worldwide while the H7N7 subtype is less pathogenic and is considered extinct as it has not been confirmed in outbreaks since 1980. Although EIV is enzootic in Brazil, few reports describe the actual EIV antibody status in the country. The aims of this study were:

- to evaluate the efficiency of different serum treatments described by the World Organisation for Animal Health (OIE) and the World Health Organization (WHO) to remove non-specific haemagglutination inhibitors for the haemagglutination inhibition (HI) assay for EIV
- to evaluate the presence of EIV antibodies by HI, enzyme-linked immunosorbent assay and agar gel immunodiffusion in 83 non-vaccinated equines from São Paulo State
- to evaluate a strategy to better analyse equine sera for EIV antibodies.

Although there was no statistical difference among treatments, receptor-destroying enzyme treatment followed by chicken erythrocyte adsorption showed more consistent results, which corroborate the OIE and WHO recommendation to use this treatment preferentially. The HI results suggest equine H3N8 virus circulation among the animals tested from São Paulo State. The algorithm suggested here could be used to guide antibody detection against equine influenza virus in equines, improving the test specificity by aiming to avoid false positive results.

Keywords

Agar gel immunodiffusion – Antibody – Brazil – Enzyme-linked immunosorbent assay – Equine influenza virus – Haemagglutination inhibition – Influenza A – Non-specific inhibitor of haemagglutination.

Introduction

Equine influenza virus (EIV) (an influenza A virus) subtypes H3N8 and H7N7 cause similar clinical signs of acute respiratory disease in horses (*Equus caballus*), donkeys (*Equus asinus*) and mules (*Equus mulus*). Subtype H3N8 is distributed worldwide and has been responsible

for all recent outbreaks. Currently, the H7N7 subtype is considered to be extinct as it has not been confirmed since 1980 (1).

The first Brazilian outbreak caused by the H3N8 subtype (A/Equi/SP/63) occurred in 1963 (2). Further outbreaks caused by H3N8 in São Paulo State were described in 1969 (3), 1985 (4) and 1988 (5), and in Rio de Janeiro

State in 2001 (6). Isolations of H7N7 EIV from outbreaks in Rio de Janeiro State (A/Equi/RJ/76) (7) and in São Paulo (A/Equi/SP/76) have also been reported (8). According to the World Organisation for Animal Health (OIE) (9, 10), outbreaks occurred in Brazil in 2008 and 2010 involving an H3N8 virus but there was no description of the viral lineage. An outbreak in Brazil also occurred in 2012 and was caused by a H3N8 subtype virus (11, 12, 13).

The serological assays for the detection of antibodies against influenza A listed by the World Health Organization (WHO) (14) and the OIE (15) are the virus microneutralisation assay (14), haemagglutination inhibition (HI) (14, 15) and single radial haemolysis (SRH) (15).

Certain factors present in equine serum may confound results obtained in the HI test, thus allowing the potential for inaccurate detection of a specific antibody against influenza A subtypes. The presence of antibodies against the erythrocytes used in the HI test will cause haemagglutination even if the serum contains antibodies against the virus, leading to a false-negative result. In order to prevent this problem, pre-treatment of serum with an erythrocyte suspension is recommended to remove such antibodies (14). Conversely, other serum factors may be present that inhibit viral haemagglutination, leading to false-positive HI tests (16).

The first description of serum factors (non-specific inhibitors of haemagglutination) active against type B influenza viruses in human, ferret and rabbit sera was published in 1947 by Francis (17). In 1963, the non-specific inhibitors of haemagglutination were divided into three types based on their electrophoretic mobility: α , β and γ (18). Although non-specific inhibitors from horses, pigs and rabbits seemed similar on first analysis, it was thereafter found that the main activity of the non-specific inhibitors in horses and guinea pigs was due to serum α 2-macroglobulin (α 2-M), which binds haemagglutinin through a 4-O-acetyl-N-acetylneuraminic acid receptor (19, 20, 21). The α 2-M is a thermostable protein group present in human and animal plasma and fluids, has a molecular weight of approximately 720 kDa and occurs as monomers, dimers or tetramers of identical subunits containing five binding reactive sites (22).

The OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (15) provides guidelines for serum treatment before the HI test. Heating at 56°C for 30 min is always recommended, with one of the following additional treatments:

- potassium periodate
- receptor-destroying enzyme (RDE)
- kaolin and adsorption of erythrocytes.

According to the *Manual*, treatments with potassium periodate and RDE should be prioritised. In addition, WHO (14, 23) recommends treatment with either RDE or trypsin–potassium periodate to remove antibodies against erythrocytes.

The aims of this study were: *a*) to compare the different treatments of serum recommended by WHO (14) and the OIE (15) for removing non-specific inhibitors of haemagglutination in sera from horses vaccinated against EIV; and *b*) to assess the level of agreement among HI, enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) for the detection of anti-EIV antibodies in horses in Brazil.

Materials and methods

Panel of non-vaccinated equines

A panel of sera from 83 non-vaccinated equines (82 horses and one mule) from four farms in São Paulo State was collected between 2008 and 2012 and stored at –20°C. The animals were between 2 and 20 years old, and there were six of unknown age. Thirty-seven were male and 46 were female.

Serum treatments for removing non-specific inhibitors of haemagglutination

A group of ten horses (2 years old), unvaccinated and not showing any clinical signs of respiratory disease, that were kept on a farm in São Paulo State, Brazil, were vaccinated with a commercial subtype H3N8 EIV vaccine (A/Equi/Kentucky/1/97), and sera were collected 30 days afterwards. In order to remove non-specific inhibitors of haemagglutination these sera were subjected to three different treatments:

- kaolin (Sigma-Aldrich®) followed by chicken erythrocyte adsorption (15)
- trypsin–periodate: trypsin (GIBCO® Trypsin 1:250) followed by metaperiodate potassium (KIO₄, Synth®) and chicken erythrocyte adsorption (14)
- RDE: receptor-destroying enzyme at a concentration of 0.02 U/ml (receptor-destroying enzyme™, SIGMA® cat. 6514) followed by chicken erythrocyte adsorption (14, 15).

Detection of anti-influenza A virus antibodies

Untreated sera from the non-vaccinated equines were tested with a commercial competitive ELISA (BioChek® AI multispecies assay, London, UK), designed to detect antibodies against type A influenza virus nucleoprotein in avian and mammalian species, and AGID used for detecting antibodies against influenza A virus (National Veterinary

Services Laboratories [NVSL]/Animal and Plant Health Inspection Service [APHIS]/United States Department of Agriculture [USDA] antigen part 3001301 A/TY/MN/3689-1551/81-H5N2 and positive control antiserum part 3051301 A/TY/MN/3689-1551/81-H5N2 and Eq1-Bel-H7N1).

The sera were also tested with the HI test according to WHO (14), using 4 haemagglutinating units (HAU)/25 µl. A HI titre ≥ 20 that completely inhibited chicken erythrocyte agglutination was considered positive.

As recommended by WHO (14), subtype-specific HI tests were performed against subtype H3N8 (A/Equi/SP/1.19/2012) and H7N7 (A/Equi/SP/1/56) viruses. The virus was not treated with Tween80/ether because of the consequent decrease in specificity, which may increase the variability of HI titres obtained (14). The sera used for HI were previously treated with RDE (receptor-destroying enzyme™, SIGMA® cat. 6514) at a concentration of 0.02 U/ml and chicken erythrocyte adsorption as recommended by WHO (14) and the OIE (15). All sera were tested by HI, even those with an ELISA–AGID negative result.

Statistical analysis

Statistical differences among the HI results after the three different treatments for the removal of non-specific inhibitors of haemagglutination in the ten vaccinated horse sera were analysed using the Friedman test. The Wilcoxon test was used to investigate whether titres against the H3N8 virus were significantly different from those for the H7N7 virus. Differences in the positivity for H3N8 and H7N7 were tested with the two-proportion test. The agreement between the diagnostic algorithm proposed here and the use of ELISA–HI or AGID–HI tests was tested with Kappa statistics as described by Landis and Koch (24).

Results and discussion

The Friedman test showed no statistical difference ($p > 0.05$) among the treatments (kaolin, trypsin–periodate and RDE). The HI test titres after different serum treatments are shown in Table I.

Considering that the horses live in an area enzootic for EIV and had been previously immunised with a commercial vaccine, it was expected that they would have antibody levels in the HI test against an H3N8 equine strain. If treated serum gives a titre lower than 20, this could be due either to removal of a specific antibody or to a high level of removal of non-specific inhibitors of haemagglutination, or both. Although there was no statistical difference, trypsin–periodate treatment seemed to have removed antibodies and non-specific inhibitors in sera 1, 2, 3 and 4 from

Table I
Antibody levels from haemagglutination inhibition test after different serum treatments, against equine influenza virus H3N8 (A/Equi/SP/1.19/2012), 4 HAU/25 µL, in vaccinated horse serum

Equine	Trypsin–periodate	Kaolin	RDE
1	<20	<20	40
2	<20	20	40
3	<20	40	80
4	<20	80	80
5	80	40	80
6	160	80	80
7	160	160	80
8	160	160	160
9	160	160	160
10	160	320	640

HAU: haemagglutination unit
RDE: receptor-destroying enzyme

the vaccinated horses. This did not occur with the kaolin treatment, which only had a titre lower than 20 in serum 1, or with RDE, which did not remove antibodies from any of the sera from the vaccinated horses. The absence of antibodies in equines 1, 2, 3 and 4 could have been due to deterioration caused by trypsin–periodate.

The RDE treatment showed more consistent results, given that it was expected that the horses should have titres against H3N8 in the HI test; this corroborates the OIE and WHO recommendations to use this treatment preferentially. Previous studies have demonstrated that RDE inactivates only non-specific inhibitors, whereas kaolin with erythrocyte adsorption can remove the gamma globulin fraction from sera (25), which agrees with the present study.

Subtype-specific HI tests in the non-vaccinated equines from São Paulo State against H3N8 A/Equi/SP/1.19/2012 and H7N7 A/Equi/1/56 are shown in Table II.

The highest antibody levels against H3N8 and H7N7 subtypes among the 83 sera after RDE and chicken erythrocyte (CE) treatment were 640 and 160, with mean values of 173.49 and 29.39, respectively.

The Wilcoxon test demonstrated that the titres of the equine sera against H3N8 virus were always higher than or equal to those for H7N7 virus ($p < 0.05$), and the two-proportion test showed higher frequencies of positives against H3N8 virus (100%; 83/83) than against H7N7 virus (91.56%; 76/83).

Table II
Antibody levels from haemagglutination inhibition (H3N8 and H7N7) test and results from enzyme-linked immunosorbent assay and agar gel immunodiffusion test for 83 equine sera from São Paulo State, Brazil

Equine	Animal	H3N8	H7N7	ELISA	AGID	Equine	Animal	H3N8	H7N7	ELISA	AGID
1	H	80	20	Positive	Positive	46	H	80	20	Positive	Negative
2	H	160	20	Negative	Positive	47	H	40	20	Positive	Negative
3	H	160	20	Positive	Positive	48	H	40	20	Positive	Negative
4	H	320	40	Positive	Positive	49	H	160	40	Positive	Positive
5	H	640	20	Positive	Positive	50	H	80	40	Positive	Positive
6	H	160	40	Negative	Negative	51	H	160	20	Negative	Negative
7	H	160	20	Positive	Positive	52	H	160	40	Negative	Negative
8	H	320	40	Positive	Positive	53	H	80	40	Positive	Positive
9	H	640	20	Negative	Negative	54	H	80	20	Positive	Negative
10	H	160	40	Positive	Positive	55	H	80	40	Positive	Negative
11	H	640	40	Positive	Positive	56	H	80	40	Positive	Positive
12	H	160	20	Positive	Positive	57	H	160	20	Positive	Negative
13	H	640	40	Positive	Positive	58	H	320	20	Positive	Negative
14	H	160	40	Positive	Positive	59	H	160	20	Negative	Negative
15	H	640	20	Positive	Positive	60	H	160	20	Negative	Negative
16	H	80	20	Positive	Positive	61	H	80	20	Positive	Negative
17	H	160	80	Positive	Positive	62	H	80	20	Positive	Negative
18	M	80	40	Positive	Positive	63	H	320	20	Negative	Negative
19	H	160	160	Positive	Positive	64	H	80	40	Negative	Negative
20	H	80	40	Positive	Positive	65	H	80	20	Positive	Negative
21	H	80	20	Positive	Positive	66	H	160	20	Positive	Negative
22	H	160	80	Positive	Negative	67	H	160	20	Negative	Negative
23	H	160	40	Positive	Positive	68	H	160	20	Positive	Negative
24	H	160	40	Positive	Negative	69	H	80	20	Positive	Negative
25	H	80	20	Positive	Negative	70	H	80	20	Positive	Negative
26	H	160	40	Negative	Negative	71	H	160	20	Positive	Negative
27	H	80	20	Positive	Negative	72	H	160	40	Positive	Negative
28	H	320	40	Negative	Negative	73	H	80	40	Positive	Positive
29	H	320	80	Positive	Negative	74	H	80	40	Positive	Positive
30	H	160	40	Positive	Positive	75	H	40	20	Positive	Positive
31	H	160	20	Positive	Negative	76	H	160	20	Positive	Negative
32	H	160	40	Positive	Negative	77	H	160	20	Positive	Negative
33	H	160	20	Positive	Negative	78	H	80	20	Positive	Negative
34	H	640	20	Negative	Negative	79	H	160	20	Positive	Negative
35	H	80	<20	Negative	Negative	80	H	80	20	Positive	Negative
36	H	320	20	Positive	Negative	81	H	80	40	Positive	Negative
37	H	80	<20	Positive	Positive	82	H	80	40	Positive	Positive
38	H	160	<20	Positive	Positive	83	H	80	40	Positive	Positive
39	H	160	<20	Positive	Positive						
40	H	160	<20	Positive	Negative						
41	H	160	<20	Positive	Negative						
42	H	160	40	Positive	Negative						
43	H	80	20	Negative	Negative						
44	H	80	40	Negative	Negative						
45	H	40	<20	Positive	Negative						

AGID: agar gel immunodiffusion
 ELISA: enzyme-linked immunosorbent assay
 H: horse
 M: mule

A higher titre and frequency of H3N8 can be explained by the fact that this subtype is enzootic in several Brazilian regions (26, 27, 28, 29, 30, 31, 32, 33) and that it was isolated from Brazilian outbreaks in 2008 and 2010 (9, 10).

It should be noted that H7N7, less pathogenic than H3N8, has not been detected anywhere in the world since 1980 (1). Though H7N7 has not been directly identified in sick horses, antibodies have been detected in non-vaccinated animals in other countries (1, 34, 35, 36). In Brazil, antibodies against H7N7 have also been reported (26, 31, 32, 33, 37), with frequencies between 2.7% and 77.75%.

The lack of cross-reaction between H3N8 and H7N7 was demonstrated by Tumová and Pereira (38), Cunha *et al.* (4) and Lee *et al.* (39). Although there is no cross-reaction between these EIV subtypes in the HI test, this may not be true for other influenza subtypes (38, 39). Therefore, considering that avian influenza virus is capable of crossing species barriers (40), and of infecting equines (41), it may be speculated that antibodies against H7N7 detected in an HI test might be a result of an equine infection with influenza subtypes other than equine H7N7 (35). Alternatively, they may be due to the incomplete removal of non-specific inhibitors of haemagglutination prior to HI assay, or may even indicate that H7N7 virus is circulating among horses and causing subclinical disease.

The results of the HI, ELISA and AGID tests are shown in Table III. The surveillance of antibodies against influenza virus in populations of horses is routinely performed on the basis of the results of the HI test (16) as it has high sensitivity and allows differentiation among subtypes. This serodiagnosis is performed by targeting the identification of horses that have had previous contact with the virus and also detecting virus circulation in unvaccinated equines.

Table III
Comparison of positive results with enzyme-linked immunosorbent assay (influenza A), agar gel immunodiffusion (influenza A) and haemagglutination inhibition (H3N8) tests for 83 equine sera from São Paulo State

ELISA	AGID	HI	n
+	+	+	32
+	-	+	35
-	-	+	15
-	+	+	1
Total			83

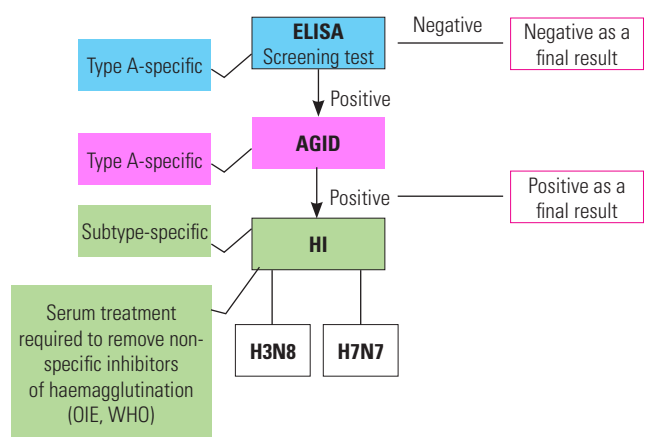
AGID: agar gel immunodiffusion
 ELISA: enzyme-linked immunosorbent assay
 HI: haemagglutination inhibition
 n: number with these results

In order to decrease the likelihood of non-specific inhibitors or test sensitivity/specificity levels interfering in the classification of a horse either as negative or positive for anti-EIV antibodies, an algorithm for the detection of anti-EIV antibodies is proposed. The anti-EIV antibody detection starts with screening using ELISA and AGID. Sera that are positive in at least one test are treated with RDE (14, 15), HI tested and the result reported (Fig. 1). The algorithm was based on the OIE/Food and Agriculture Organization of the United Nations (FAO) network of expertise on animal influenza (OFFLU) algorithm for the detection and characterisation of avian H7N9 (42).

The analytical sensitivity (ASen) is the capacity to detect a minimal amount of antibody in a sample. The analytical specificity (ASpe) is the ability of the test to specifically detect the reaction of the antibody with the antigen, without the interference of non-specific test reactions (43).

Diagnostic specificity (DSpe) and sensitivity (DSen) are parameters for diagnostic test validation. The DSpe corresponds to the percentage of true negative samples that are classified as negative by the test, and the DSen is the proportion of true positives that are classified as positive by the test (43, 44). Low DSen and DSpe have implications in yielding a negative result for a positive animal and a positive result for a negative animal, respectively.

The ELISA and HI in general have higher ASen than AGID, while AGID usually has higher DSpe than ELISA or HI. The one serum sample with an ELISA-/AGID+ result may represent a false-negative on the ELISA because AGID has a low rate of false-positive detection. Thirty-five (out of 83)



AGID: agar gel immunodiffusion
 ELISA: enzyme-linked immunosorbent assay
 HI: haemagglutination inhibition
 OIE: World Organisation for Animal Health
 WHO: World Health Organization

Fig. 1
An algorithm for the indirect serodiagnosis of equine influenza virus in equine serum

sera were ELISA+/AGID–, which could represent some true and some false positives. Most probably, the true positives were outside the analytical sensitivity limit of AGID and the false positives were outside the 99% DSpe of the ELISA. The ELISA specificity and sensitivity discussed are from the manufacturer's literature of the BioChek® AI multispecies assay.

If AGID is positive, HI should be positive for at least one subtype of influenza A, as happened with three sera (out of 83) which were negative for H7N7 and positive for H3N8.

An AGID–/HI+ result could indicate a true positive but with a lower antibody concentration than AGID is able to detect, because the HI ASen is higher than the AGID ASen. In this case, it is worthwhile testing with the ELISA to confirm this result, since the ELISA has high DSpe and would identify the false positives and true negatives. If the ELISA has a negative result, as happened with 15/50 (against H3N8) and 14/46 (against H7N7) sera, the HI positive may have been due to residual non-specific inhibitor, even after RDE serum treatment.

Using the algorithm, the HI-positive results decrease from 100% to 81.93% (68/83) against H3N8 and from 91.56% to 74.7% (62/83) against H7N7. The algorithm showed almost perfect agreement (H3N8 and H7N7: $K = 0.96$, 95% confidence interval [CI] 1.0–0.745; $p = 0.001$) when compared to the use of the ELISA and HI tests and fair agreement (H3N8: $K = 0.254$, 95%CI 0.397–0.111,

$p = 0.001$; H7N7: $K = 0.322$, $p < 0.001$, 95%CI 0.48–0.164) when compared to the use of AGID and HI tests (24). The combination of ELISA and AGID (being positive in at least one test) and HI could be an alternative strategy for antibody detection against EIV, giving an improvement in test specificity and therefore minimising false-positive results.

Conclusions

The authors demonstrated a level of non-agreement among ELISA, AGID and HI test results. Incomplete removal of non-specific inhibitors of haemagglutination from the equine sera even after RDE and CE treatment may have been the cause. The algorithm presented may be a good strategy for the detection of antibodies against EIV due to the combination of specificity and sensitivity of the tests. Further studies are needed to confirm these preliminary results.

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Comparaison de trois méthodes sérologiques de détection de l'infection par le virus de la grippe équine

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Résumé

Tous les foyers de grippe équine dans le monde sont dus au sous-type H3N8 du virus. Le sous-type H7N7, moins pathogène, est considéré comme éteint, sa présence n'ayant été confirmée dans aucun des foyers enregistrés depuis 1980. Au Brésil, la grippe équine est enzootique mais la prévalence d'anticorps dans le pays est peu documentée. La présente étude avait trois objectifs :

- évaluer l'efficacité de plusieurs traitements de sérums décrits par l'Organisation mondiale de la santé animale (OIE) et l'Organisation mondiale de la santé (OMS) sur la suppression des inhibiteurs d'hémagglutination non spécifiques, afin de pouvoir utiliser l'épreuve d'inhibition de l'hémagglutination pour la détection de la grippe équine,
- évaluer la présence d'anticorps dirigés contre la grippe équine chez 83 chevaux non vaccinés de l'état de São Paulo en utilisant l'inhibition de l'hémagglutination, l'épreuve immuno-enzymatique (ELISA) et l'épreuve d'immunodiffusion en gélose (IDG) ;

– évaluer une stratégie visant à améliorer les techniques sérologiques de détection des anticorps dirigés contre la grippe équine.

S'il n'y a pas eu de différence statistique significative entre les traitements, celui faisant appel à l'enzyme de destruction du récepteur suivi d'une adsorption sur érythrocytes de poule a permis d'obtenir les résultats les plus cohérents, ce qui corrobore les recommandations de l'OIE et de l'OMS en faveur de ce traitement. Les résultats obtenus au moyen de l'inhibition de l'hémagglutination indiquent que le virus H3N8 est présent parmi les animaux testés de l'état de São Paulo. L'algorithme présenté par les auteurs pourrait servir de modèle pour détecter la présence d'anticorps dirigés contre le virus de la grippe équine chez les chevaux : en effet, il permet d'éviter les résultats faussement positifs, ce qui améliore la spécificité du test utilisé.

Mots-clés

Anticorps – Brésil – ELISA – Épreuve immuno-enzymatique – Épreuve d'immunodiffusion en gélose – Inhibiteur non spécifique de l'hémagglutination – Inhibition de l'hémagglutination – Virus de la grippe équine – Virus influenza de type A.



Comparación de tres métodos serológicos distintos para detectar la infección por el virus de la gripe equina

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Resumen

El subtipo H3N8 del virus de la gripe equina (VGE) es el agente etiológico de todos los brotes que se producen en el mundo, mientras que el subtipo H7N7, menos patógeno, se da por extinto, en la medida en que desde 1980 no se ha confirmado su intervención en brote alguno. Aunque en el Brasil el VGE es enzoótico, existen pocos trabajos que den cuenta de la situación real del país en cuanto a la presencia de anticuerpos contra el virus. Los autores describen un estudio que perseguía los siguientes objetivos:

- evaluar la eficacia de distintos tratamientos séricos descritos por la Organización Mundial de Sanidad Animal (OIE) y la Organización Mundial de la Salud (OMS) para eliminar los inhibidores inespecíficos de la hemaglutinación con objeto de aplicar la técnica de inhibición de la hemaglutinación a la detección del VGE;
- evaluar la presencia de anticuerpos contra el VGE por inhibición de la hemaglutinación, ensayo inmunoenzimático (ELISA) e inmunodifusión en gel de agar en 83 ejemplares equinos no vacunados del estado de São Paulo;
- evaluar una estrategia encaminada a analizar más eficazmente sueros equinos para detectar en ellos anticuerpos anti-VGE.

Aunque no se observaron diferencias estadísticamente significativas entre los tratamientos, el uso de enzimas destructores de receptores seguido de la técnica de adsorción de eritrocitos de pollo arrojó resultados más coherentes, cosa que avala la recomendación de la OIE y la OMS de privilegiar este tratamiento. Los resultados obtenidos por inhibición de la hemaglutinación parecen indicar que el virus H3N8 equino circula entre los animales analizados del estado de São Paulo. El algoritmo aquí propuesto podría servir de guía para detectar

en equinos la presencia de anticuerpos contra el VGE. Puesto que apunta a evitar falsos positivos, su aplicación mejoraría la especificidad de la prueba.

Palabras clave

Anticuerpo – Brasil – ELISA – Ensayo inmunoenzimático – Gripe A – Inhibición de la hemaglutinación – Inhibidor inespecífico de la hemaglutinación – Inmudifusión en gel de agar – Virus de la gripe equina.



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