# 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 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 (ELISA) and agar gel immunodiffusion (AGID) 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 (RDE) 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 herein 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 – ELISA – 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 States (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 an 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 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 a first analysis it was thereafter found that the main activity of the non-specific inhibitors in horses and guinea pigs was due to serum α2-macroglobulin (α2-M), which binds haemagglutinin through a 4-O-acetyl-*N*-acetyneuraminic acid receptor (19, 20, 21). The α2-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 were 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 (two years old), unvaccinated and not showing any clinical signs of respiratory disease, that were kept on a farm in Sao 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 ($KIO_4$, 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 mammal 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. An HI titre $\geq 20$ 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 herein 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.

Insert 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 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.

Insert Table II

The highest antibody levels against H3N8 and H7N7 subtypes among the 83 sera after RDE and 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).

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 other influenza subtypes 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.

Insert Table III

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/FAO (Food and Agriculture Organization of the United Nations)

network of expertise on animal influenza (OFFLU) algorithm for the detection and characterisation of avian H7N9 (42).

Insert Figure 1

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 has usually 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 low rate of false-positive detection. Thirty-five (out of 83) 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: Kappa (*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

We demonstrated a level of non-agreement among ELISA, AGID and HI tests 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 detection of antibody against EIV owing to the combination of specificity and sensitivity of the tests. Further studies are needed to confirm our preliminary results.

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## References

1. Webster R.G. (1993). – Are equine 1 influenza viruses still present in horses? *Equine Vet. J.*, **25** (6), 537–538. doi:10.1111/j.2042-3306.1993.tb03009.x.

2. Andrews C., Pereira H.G. & Wildy P (eds) (1978). – Viruses of vertebrates. 4th Ed. Ballière & Tindall, London, 42 pp.

3. Cunha R.G. (1970). – Isolamento do vírus de influenza A/equi 2 no Estado da Guanabara. *Rev. Bras. Biol*., **30** (4), 491–498.

4. Cunha R.G., Passos W.S., Pagano M.C.C & Souza D.M. (1986). – Surto de gripe eqüina produzido por vírus de influenza A/Equi 2, no Estado do Rio de Janeiro, Brasil. *Rev. Bras. Med. Vet*., **8** (3), 88–91.

5. Mancini D.A.P., Geraldes E.A., Pinto J.R. & Soares M.A. (1988). – An outbreak of influenza in equines of São Joaquim farm, São Roque, SP, Instituto Butantan. *Rev. Fac. Med. Vet. Zootec*., **25** (1), 93–100. doi:10.11606/issn.2318-3659.v25i1p39-100.

6. Loureiro B.O. (2004). – Estudo de um surto de influenza eqüina ocorrido em 2001 no Estado do Rio de Janeiro mediante a caracterização do vírus e avaliação do esquema vacinal. Dissertation, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

7. Cunha R.G., Passos W.S. & Valle M.C.C. (1978). – Surto de gripe eqüina produzido por vírus influenza A/Eq1 no Estado do Rio de Janeiro, Brasil. *Rev. Bras. Biol*., **38** (3), 549–554.

8. Piegas N.S., Takimoto S., Barbosa H.C., Lacerda J.P., Guarnieri R. & Ishimaru T. (1976). – Isolamento do virus influenza de surto de gripe equina em São Paulo. *In* 15. Congresso Brasileiro de Medicina Veterinária, 25–30 October 1976, Rio de Janeiro, Brazil.

9. World Organisation for Animal Health (OIE) (2009). – Expert surveillance panel on equine influenza vaccines – Conclusions and recommendations. National Institute for Medical Research,

United Kingdom. Available at: www.oie.int/doc/ged/D6325.PDF (accessed on 5 January 2015).

10. World Organisation for Animal Health (OIE) (2011). – Expert surveillance panel on equine influenza vaccines – Conclusions and recommendations. National Institute for Medical Research, United Kingdom. Available at: www.oie.int/doc/ged/D10847.PDF (accessed on 5 January 2015).

11. Villalobos E.M.C., Mori E., Lara M.C.C.S.H., Nassar A.F.C., Braga P.R.C., Daniel G.T. & Cunha E.M.S. (2013). – Isolation, sequencing and phylogenetic analysis of equine influenza virus causing the 2012 outbreak in São Paulo, Brazil. *In* 24 Congresso Brasileiro de Virologia, 8 Encontro de Virologia do Mercosul, 01–04 September 2013, Porto Seguro, Bahia, Brazil, 246. Available at: www.sbv.org.br/site/downs/virus_reviews_and_research_volume_18.pdf (accessed on 19 August 2016).

12. Perglione C.O., Gildea S., Rimondi A., Miño S., Vissani A., Carossino M., Cullinane A., Barrandeguy M. (2015). – Epidemiological and virological findings during multiple outbreaks of equine influenza in South America in 2012. *Influenza Other Respir. Viruses*, **10** (1), 37–46. doi:10.1111/irv.1234.

13. Beuttemmüller E.A., Woodward A., Rash A., Ferraz L.E.S., Alfieri A.F. & Alfieri A.A. (2016). – Characterisation of the epidemic strain of H3N8 equine influenza virus responsible for outbreaks in South America in 2012. *Virol. J.*, **13** (45), 1–16. doi:10.1186/s12985-016-0503.

14. World Health Organization (WHO) (2011). – Global influenza surveillance network: Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva, 153 pp. Available at: http://apps.who.int/iris/bitstream/10665/44518/1/9789241548090_eng.pdf (accessed on 11 August 2016).

15. World Organisation for Animal Health (OIE) (2016). – Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 8th Ed., OIE, Paris. Available at: www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.05.07_EQ_INF.pdf (accessed on 11 August 2016).

16. Boliar S., Stanislawek W. & Chambers T.M. (2006). – Inability of kaolin treatment to remove nonspecific inhibitors from equine serum for the hemagglutination inhibition test against equine H7N7 influenza virus. *J. Vet. Diagn. Invest.*, **18** (3), 264–267. doi:10.1177/104063870601800305.

17. Francis T. (1947). – Dissociation of hemagglutination and antibody-measuring capacities of influenza virus. *J. Exp. Med.*, **85** (1), 1–7. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC2135674/pdf/1.pdf (accessed on 19 August 2016). doi:10.1084/jem.85.1.1.

18. Cohen A., Newland S.E. & Biddle F. (1963). – Inhibition of influenza virus haemagglutination: A difference of behavior in sera from a single species. *Virology*, **20** (3), 518–529. Available at: http://ac.els-cdn.com/0042682263901028/1-s2.0-0042682263901028-main.pdf?_tid=c27d67f2-6644-11e6-9ac7-00000aab0f02&acdnat=1471635798_4e29135b49722a99da439f38a64026dd (accessed on 19 August 2016). doi:10.1016/0042-6822(63)90102-8.

19. Hanaoka K., Pritchett T.J., Takasaki S., Kochibe N., Sabesan S., Paulson J.C. & Kobata A. (1989). – 4-O-acetyl-*N*-acetylneuraminic acid in the *N*-linked carbohydrate structures of equine and guinea pig α2-macroglobulins, potent inhibitors of influenza virus infection. *J. Biol. Chem.*, **264** (17), 9842–9849. Available at: www.jbc.org/content/264/17/9842.long (accessed on 19 August 2016).

20. Pritchett T.J. & Paulson J.C. (1989). – Basis for the potent inhibition of influenza virus infection by equine and guinea pig alpha2-macroglobulin. *J. Biol. Chem.*, **264** (17), 9850–9858. Available

at: www.jbc.org/content/264/17/9850.long (accessed on 19 August 2016).

21. Rayan-Poirier K.A. & Kawaoka Y. (1991). – Distinct glycoprotein inhibitors of influenza A virus in different animal sera. *J. Virol*., **65** (1), 389–395. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC240529/pdf/jvirol00044-0411.pdf (accessed on 19 August 2016).

22. Borth W. (1992). – Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J*., **6** (15), 3345–3353. Available at: www.ncbi.nlm.nih.gov/pubmed/1281457 (accessed on 19 August 2016).

23. World Health Organization (WHO) (2002). – WHO Manual on animal influenza diagnosis and surveillance. Geneva. Available at: http://apps.who.int/iris/bitstream/10665/68026/1/WHO_CDS_CSR_NCS_2002.5.pdf (accessed on 19 August 2016).

24. Landis J.R. & Koch G.G. (1977). – The measurement of observer agreement for categorical data. *Biometrics*, **33** (1), 159–174. doi:10.2307/2529310.

25. Tauraso N.M.T., Pedreira F.A., Spector S.L. & Bernier G.M. (1971). – Effect of various methods of removing non-specific inhibitors to virus hemagglutination upon serum proteins and immunoglobulins. *Arch. Ges. Virusforsch*., **34** (3), 214–222. doi:10.1007/BF01242995.

26. Pagano M.C., Passos W.S. & Cunha R.G. (1985). – Anticorpos inibidores de hemoaglutinação para os vírus de influenza eqüina em soros de eqüídeos de várias regiões do Brasil. *Rev. Bras. Med. Vet*., São Paulo, **7** (7), 194–198.

27. Loureiro B.O., Oliveira Jr J.G., Schivago P.A., Oliveira G.S., Portz C., Brito S.N., Bittercourt A.J. & Andrade C.M. (2002). – Soroepidemiologia da Influenza eqüina, subtipo A/Eqüino-2 (H3N8) no Estado do Rio de Janeiro. *Rev. Bras. Med. Vet*., **24** (1), 11–13.

28. Oliveira G.S., Schiavo P.A., Mazur C. & Andrade C.M. (2005). – Prevalência de anticorpos para o vírus da influenza equina, subtipo H3N8, em equídeos apreendidos no Estado do Rio de Janeiro. *Rev. Ciência Rural*, **35** (5), 1213–1215. doi:10.1590/S0103-84782005000500038.

29. Diel D.G., Almeida S.R., Weiblen R., Frandoloso R., Anziliero D., Kreutz L.C., Groff H.S. & Flores E.F. (2006). – Prevalência de anticorpos contra os vírus da influenza, da arterite viral e herpesvírus em eqüinos do Estado do Rio Grande do Sul, Brasil. *Ciên. Rural*, **36** (5), 1467–1673. doi:10.1590/S0103-84782006000500019.

30. Pena L.J., Pena D.A., Barrios P.R., Dale R., Lamêgo M.R.A. & Moraes M.P. (2006). – Levantamento soro-epidemiológico da infecção pelo vírus da anemia infecciosa eqüina, da influenza eqüina-2 e do herpesvírus eqüino-1 em rebanhos do sul do Estado do Pará, Brasil. *Braz. J. Vet. Res. Anim. Sci.*, **43** (4), 537–542. doi:10.1590/S1413-95962006000400014.

31. Aguiar D.M., Cavalcante G.T., Lara M.C.C.S.H., Villalobos E.M.C., Cunha E.M.S., Okuda L.H., Stéfano E., Nassar A.F.C., Souza G.O., Vasconcellos S.A., Labruna M.B., Camargo L.M.A. & Gennari S.M. (2008). – Prevalência de anticorpos contra agentes virais e bacterianos em eqüídeos do Município de Monte Negro, Rondônia, Amazônia Ocidental Brasileira. *Braz. J. Vet. Res. Anim. Sci.*, **45** (4), 269–276. doi:10.1590/S1413-95962008000400003.

32. Heinemann M.C., Cortez A., Lara M.C.C.S.H., Cunha E.M.S., Nassar A.F.C., Villalobos E.M.C., Neto J.S.F. & Ferreira F. (2009). – Soroprevalência do vírus da influenza equina no Município de Uruará, PA, Brasil, Amazônia oriental. *Arq. Inst. Biol. (S. Paulo)*, **76** (4), 697–699. Available at: www.biologico.sp.gov.br/docs/arq/v76_4/heinemann.pdf (accessed on 19 August 2016).

33. Cunha E.M.S., Villalobos E.M.C., Nassar A.F.C., Lara M.C.C.S.H., Peres N.F., Palazzo J.P.C., Silva A., Stefano E. & Pinto

F.A. (2009). – Prevalência de anticorpos contra agentes virais em equídeos no sul do Estado de São Paulo. *Arq. Inst. Biol. (S. Paulo)*, **76** (2), 165–171. Available at: www.biologico.sp.gov.br/docs/arq/v76_2/cunha2.pdf (accessed on 21 August 2016).

34. Madic J., Martinovic S., Naglic T., Hajsig D. & Cvetnic S. (1996). – Serological evidence for the presence of A/equine-1 influenza virus in unvaccinated horses in Croatia. *Vet. Rec*, **138** (3), 68. doi:10.1136/vr.138.3.68.

35. Olusa T.A.O., Adegunwa A.K., Aderonmu A.A. & Adeyefa C.A.O. (2010). – Serologic evidence of equine H7 influenza virus in polo horses in Nigeria. *Sci. World J.*, **5** (2), 17–19. doi:10.4314/swj.v5i2.61509.

36. Diaz-Mendez A., Viel L., Hewson J., Doig P., Carman S., Chambers T., Tiwari A. & Dewey C. (2010). – Surveillance of equine respiratory viruses in Ontario. *Can. J. Vet. Res.*, **74** (4) 271–278. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC2949340/pdf/cjvr_04_271.pdf (accessed on 21 August 2016).

37. Mancini D.A.P., Mendonça R.M.Z., Pinto J.R., Mori E. & Fernandes W.R. (2004). – Anti-human protector antibody detected in horses as a zoonotic viruses. *Braz. J. Vet. Res. Anim. Sci.*, **41** (6), 379–383. doi:10.1590/S1413-95962004000600004.

38. Tumová B. & Pereira H.G. (1968). – Antigenic relationship between influenza A viruses of human and animal origin. *Bull. WHO*, **38** (3), 415–420. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC2554487/pdf/bullwho00236-0087.pdf (accessed on 21 August 2016).

39. Lee C.W., Senne D.A. & Suarez D.L. (2006). – Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of

chickens. *Clin. Vaccine Immunol*., **13** (3), 395–402. doi:10.1128/CVI.13.3.395-402.2006.

40. Horimoto T. & Kawaoka Y. (2001). – Pandemic threat posed by avian influenza A viruses. *Clin. Microbiol. Rev*., **14** (1), 129–149. doi:10.1128/CMR.14.1.129-149.2001.

41. Guo Y., Wang M., Kawaoka Y., Gorman O., Ito T., Saito T. & Webster R.G. (1992). – Characterization of a new avian-like influenza A virus from horses in China. *Virology*, **188** (1), 245–255. doi:10.1016/0042-6822(92)90754-D.

42. Food and Agriculture Organization of the United Nations (FAO) (2013). – Laboratory protocols and algorithms. Addressing avian influenza A (H7N9). Rome. Available at: www.fao.org/docrep/019/i3596e/i3596e.pdf (accessed on 21 August 2016).

43. Saah A.J. & Hoover D.R. (1997). – 'Sensitivity' and 'specificity' reconsidered: The meaning of these terms in analytical and diagnostic settings. *Ann. Internal Med*., **126** (1), 91–94. doi:10.7326/0003-4819-126-1-199701010-00026.

44. Guimarães M.C.S. (1985). – Exames de laboratório: Sensibilidade e especificidade, valor preditivo. *Rev. Soc. Bras. Med. Trop*., **18** (2), 117–120. doi:10.1590/S0037-86821985000200009.

**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

**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**

| Equine | Animal | H3N8 | H7N7 | ELISA | AGID |
|---|---|---|---|---|---|
| 1 | H | 80 | 20 | Positive | Positive |
| 2 | H | 160 | 20 | Negative | Positive |
| 3 | H | 160 | 20 | Positive | Positive |
| 4 | H | 320 | 40 | Positive | Positive |
| 5 | H | 640 | 20 | Positive | Positive |
| 6 | H | 160 | 40 | Negative | Negative |
| 7 | H | 160 | 20 | Positive | Positive |
| 8 | H | 320 | 40 | Positive | Positive |
| 9 | H | 640 | 20 | Negative | Negative |
| 10 | H | 160 | 40 | Positive | Positive |
| 11 | H | 640 | 40 | Positive | Positive |
| 12 | H | 160 | 20 | Positive | Positive |
| 13 | H | 640 | 40 | Positive | Positive |
| 14 | H | 160 | 40 | Positive | Positive |
| 15 | H | 640 | 20 | Positive | Positive |
| 16 | H | 80 | 20 | Positive | Positive |
| 17 | H | 160 | 80 | Positive | Positive |
| 18 | M | 80 | 40 | Positive | Positive |
| 19 | H | 160 | 160 | Positive | Positive |
| 20 | H | 80 | 40 | Positive | Positive |
| 21 | H | 80 | 20 | Positive | Positive |
| 22 | H | 160 | 80 | Positive | Negative |
| 23 | H | 160 | 40 | Positive | Positive |
| 24 | H | 160 | 40 | Positive | Negative |
| 25 | H | 80 | 20 | Positive | Negative |
| 26 | H | 160 | 40 | Negative | Negative |
| 27 | H | 80 | 20 | Positive | Negative |
| 28 | H | 320 | 40 | Negative | Negative |
| 29 | H | 320 | 80 | Positive | Negative |
| 30 | H | 160 | 40 | Positive | Positive |

| | | | | | |
|---|---|---|---|---|---|
| 31 | H | 160 | 20 | Positive | Negative |
| 32 | H | 160 | 40 | Positive | Negative |
| 33 | H | 160 | 20 | Positive | Negative |
| 34 | H | 640 | 20 | Negative | Negative |
| 35 | H | 80 | <20 | Negative | Negative |
| 36 | H | 320 | 20 | Positive | Negative |
| 37 | H | 80 | <20 | Positive | Positive |
| 38 | H | 160 | <20 | 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 |
| 46 | H | 80 | 20 | Positive | Negative |
| 47 | H | 40 | 20 | Positive | Negative |
| 48 | H | 40 | 20 | Positive | Negative |
| 49 | H | 160 | 40 | Positive | Positive |
| 50 | H | 80 | 40 | Positive | Positive |
| 51 | H | 160 | 20 | Negative | Negative |
| 52 | H | 160 | 40 | Negative | Negative |
| 53 | H | 80 | 40 | Positive | Positive |
| 54 | H | 80 | 20 | Positive | Negative |
| 55 | H | 80 | 40 | Positive | Negative |
| 56 | H | 80 | 40 | Positive | Positive |
| 57 | H | 160 | 20 | Positive | Negative |
| 58 | H | 320 | 20 | Positive | Negative |
| 59 | H | 160 | 20 | Negative | Negative |
| 60 | H | 160 | 20 | Negative | Negative |
| 61 | H | 80 | 20 | Positive | Negative |
| 62 | H | 80 | 20 | Positive | Negative |
| 63 | H | 320 | 20 | Negative | Negative |
| 64 | H | 80 | 40 | Negative | Negative |
| 65 | H | 80 | 20 | Positive | Negative |
| 66 | H | 160 | 20 | Positive | Negative |
| 67 | H | 160 | 20 | Negative | Negative |

| | | | | | |
|---|---|---|---|---|---|
| 68 | H | 160 | 20 | Positive | Negative |
| 69 | H | 80 | 20 | Positive | Negative |
| 70 | H | 80 | 20 | Positive | Negative |
| 71 | H | 160 | 20 | Positive | Negative |
| 72 | H | 160 | 40 | Positive | Negative |
| 73 | H | 80 | 40 | Positive | Positive |
| 74 | H | 80 | 40 | Positive | Positive |
| 75 | H | 40 | 20 | Positive | Positive |
| 76 | H | 160 | 20 | Positive | Negative |
| 77 | H | 160 | 20 | Positive | Negative |
| 78 | H | 80 | 20 | Positive | Negative |
| 79 | H | 160 | 20 | Positive | Negative |
| 80 | H | 80 | 20 | Positive | Negative |
| 81 | H | 80 | 40 | Positive | Negative |
| 82 | H | 80 | 40 | Positive | Positive |
| 83 | H | 80 | 40 | Positive | Positive |

AGID: agar gel immunodiffusion

ELISA: enzyme-linked immunosorbent assay

H: horse

M: mule

**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

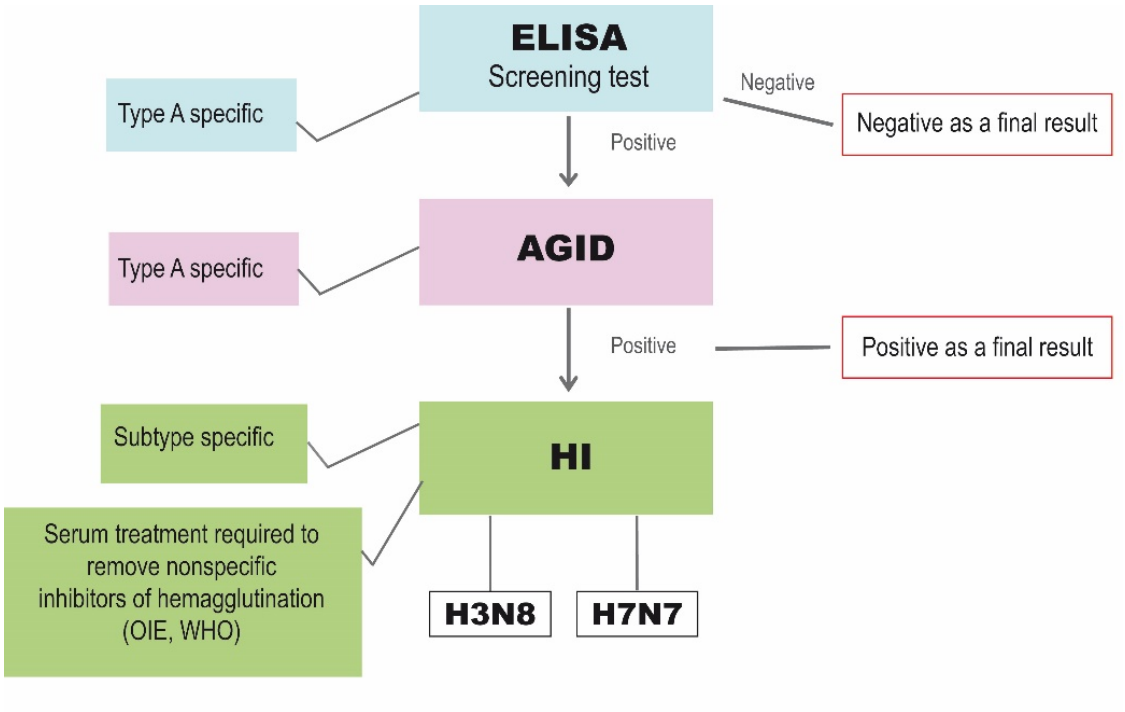


**Fig. 1**

**An algorithm for the indirect serodiagnosis of equine influenza virus in equine serum**

AGID: agar gel immunodiffusion

ELISA: enzyme-linked immunosorbent assay

HI: haemagglutination inhibition