Retrospective serological survey for influenza in horses from Brazil

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Abstract

Equine influenza (EI) virus is one of the most economically important pathogens of respiratory diseases of horses worldwide. Despite availability of vaccines for control of EI, the highly contagious nature and variability properties of the virus mean global outbreaks occur. Thus, continuous surveillance programs, including seroprevalence studies of disease in different countries, may contribute to better control of the disease. In this study, the seroprevalence of equine influenza in 850 horses from Brazil was investigated. The serodiagnosis was based on the single radial hemolysis (SRH) assay using influenza A/equine/Richmond/1/2007 (H3N8) antigen. Antibodies against A/equine/Richmond/1/07 (H3N8) were detected in 44.7% (380/850, 95% CI: 41.4–48.1%) of horses. Seroprevalence was significantly lower ($p=0.001$) in younger animals (< 5 years, 38.6%) than in 'adult' animals (5–14 years, 52.1%). There was also a significant relationship between the year of sampling and seroprevalence ($p<0.0005$). The mean SRH antibody value was 42.0 mm^2 (range 4–238.9 mm^2), with the majority of horses (95.3%) having an SRH value ≤150 mm^2, which is considered an insufficient level for protection of equine hosts against influenza infections and potential virus shedding. These findings indicate the need to reinforce preventive/control measures against equine influenza in Brazil.

Introduction

Alphainfluenzavirus, a renamed genus in the *Orthomyxoviridae* family [1], is comprised of emerging and re-emerging animal and human diseases associated with severe respiratory infections worldwide. Particularly, the equine influenza virus (EIV) is the causative agent of equine influenza (EI). It is one of the most economically important pathogens causing respiratory disorders of horses worldwide because of its rapid spread and highly contagious nature among susceptible hosts. Influenza A viruses of the H3N8 subtype were first isolated from horses in 1963. The equine H3N8 virus is thought to have emerged in South America; phylogenetic analysis demonstrated that genes of the virus isolated from horses shared a most recent common ancestor with avian strains circulating in Argentina, Bolivia, Brazil and Chile a few years before it was first isolated [2]. The equine H3N8 virus was imported to the USA in 1963 when Thoroughbred horses were transported by air from Argentina [3] and further spread to Europe causing an epidemic in 1965. Further major epidemics of equine H3N8 influenza occurred in 1979–1981, 1989 and 2003. The first Brazilian outbreak caused by the H3N8 subtype occurred in 1963 and, subsequently, outbreaks have been reported regularly [4,5].

Initially, the equine H3N8 viruses evolved in a single genetic lineage, but in 1989, divergent evolution led to distinct lineages of viruses circulating in the Americas on the one hand and Europe and Asia on the other [6]. The 'American' lineage further diverged into the 'Florida' and 'Kentucky' sub-lineages [7] and finally, the 'Florida' sub-lineage has been further classified as clade 1 or clade 2, with clade 1 viruses initially predominant in the Americas and clade 2 in Europe [8].

Equine influenza vaccines were introduced shortly after the emergence of the H3N8 virus. The World Organisation for Animal Health (OIE) has overseen a vaccine strain review program for equine influenza since the 1990s; the latest recommendation from the expert surveillance panel (April 2019) is that vaccines should contain both clade 1 and clade 2 viruses of the Florida sub-lineage [9].

In 2018/2019, widespread outbreaks of EI caused by Florida clade 1 viruses have been reported. These were initially identified in South America [10] and subsequently spread to European countries [11]. This recurrent emergence of epidemics of equine influenza from South America emphasizes the need for monitoring the status of equine influenza in South American countries, including Brazil.

In previous serological surveys of equine influenza in Brazil, the hemagglutination inhibition (HI) test has been used to determine seropositivity [12]. However, the HI test is not readily standardized. In experimental infections of ponies, only animals with antibody levels greater than 154 mm^2 measured by single radial hemolysis (SRH) assay were protected against infection with an antigenically-related equine influenza strain, although animals with SRH levels above 85 mm^2 did not show clinical signs of disease and therefore could 'silently' shed virus [13]. Further analysis of data from vaccination and challenge studies and natural outbreaks of EI among vaccinated horses allowed a threshold of 150 mm^2 to be defined above which horses were protected against infection with virus related to strains included in vaccines [14]. Thus, serological surveillance using the SRH assay can give a more precise evaluation of the numbers of animals likely to have protective immunity.

In this study, the seroprevalence of equine influenza in 850 horses in Brazil based on single radial hemolysis (SRH) was investigated.

Materials and Methods

This study was approved by the Ethics Committee on Animal Use (CEUA) guidelines of the School of Veterinary Medicine and Animal Sciences, São Paulo State University, UNESP, Botucatu, SP, Brazil (protocol number 155/2018).

Sample collection and serological analyses

A convenience serum sample was collected once from 850 horses for equine infectious anemia testing, which is mandatory in Brazil for movement of equids, between 2005 and 2017. Blood (10 mL) was aseptically collected from the jugular by venipuncture. The horses belonged to farms from the southwest region of Brazil, where equine rearing is common. The horses were used for farm work, sport, and/or leisure activities and, at sample collection, none presented with pulmonary signs compatible with EI.

Data including age, sex (male/female) and breed, and location (state) and year of sampling were collected. There was no consistent data regarding the status of influenza vaccination among horses sampled, and this information was not used to further analysis.

Antibody levels against influenza A/equine/Richmond/1/2007 (H3N8), a representative of Florida clade 1, were measured using the single radial hemolysis (SRH) assay performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [9]. The area of zones of hemolysis (mm^2) were calculated from the diameter measured using digital calipers. All SRH areas below the lower limit of detection (4 mm^2; [15]) were set to 4 for analysis.

Statistical analysis

Overall seroprevalence was estimated using the Wilson method to determine confidence limits [16]. Pearson's chi-square test was used to find associations between serological results and explanatory variables in SPSS 25.0 (Statistical Package for Social Sciences, Inc., Chicago, IL, USA). For statistical analysis, animals were divided into three age categories (<5 years = 'young', 5–14 years = 'adult', and ≥15 years = 'old'). Due to the occasional use of the same horse in two activities, this parameter was not considered in further statistical analysis.

Results

Antibodies against influenza A/equine/Richmond/1/2007 (H3N8) were detected in 380 of the 850 horses tested giving an overall seroprevalence of 44.7% (95% CI: 41.4–48.1%).

There was no significant difference in seroprevalence between male and female or mixed and pure-breed horses or location of sampling, however the majority of samples (82%) were obtained from São Paulo state (Table 1). There was a significant difference in seroprevalence according to age (χ^2=3.30, p=0.001), with 52.1% of 'adult' and 50.7% of 'old' horses *versus* 38.6% of 'young' horses seropositive. The age range of animals sampled was from 4 days to 33 years. There was also a significant relationship between year of sampling and seroprevalence,

χ^2 =41.11, p<0.0005 with the highest seroprevalence in 2005 (64.3%) and lowest seroprevalence (around 31%) in 2009–2011.

The mean SRH antibody value was 52.7 mm^2 (range 4–238.9 mm^2), a low antibody level likely to be insufficient clinical protection as it is below the threshold of 85 mm^2. Furthermore, only 4.7% of horses had SRH antibody levels above 150 mm^2, associated with protection against infection, and only 19.8% had levels between 85 and 150 mm^2. When individual SRH antibody values were plotted for different age groups (Figure 1), it was apparent that the highest mean SRH values were seen in horses aged 1 month or less (75.63 mm^2) but with a clear division with half of the foals having SRH values 109.8–193.9 mm^2 and half (6/12) having values of 4–34.8 mm^2. Mean SRH values were similar in all other age groups (35.9–58.3 mm^2). The highest median SRH values were also seen in horses 1-month-old or less (72.3 mm^2). However, the median SRH value was lower in 29 horses classified as 6 months old (33.6%) and the median value in horses aged 1–4 years was below the lower limit of detection (4 mm^2). In 5–9-year-old horses, the median SRH value was 51.0 mm^2, but was again 4 mm^2 in horses aged 10 years or older.

Discussion

The seroprevalence of equine H3N8 influenza estimated using SRH in this study was 44.7% (95% CI: 41.4–48.1%). This value is in good agreement with the mean (52%) and mode (42%) seroprevalence estimated from HI data in 14 studies in different states and regions from Brazil published between 1985 and 2014 [12]. Prevention and control of EI is based mainly on biosecurity procedures and vaccination. Mathematical modelling has been used to demonstrate that vaccination of around 40% of an equine population with an effective EI vaccine will reduce virus transmission sufficiently to provide herd immunity [17]. However, modelling also demonstrated that where there is an antigenic mismatch between a vaccine strain and challenge strain, even very high levels of SRH antibody may not afford protection and more animals must be vaccinated to achieve herd immunity.

In the current study, seroprevalence differed significantly between different age groups of horses, with the highest seropositivity (52.1%) in adult horses (aged 5–14 years) when compared with horses under 5 years of age (38.6%). Similarly, Jurado-Tarifa et al. [18] found the main risk factor associated with equine H3N8 influenza seroprevalence in Spain was age; significantly higher seropositivity was observed in old or geriatric (odds ratio, OR = 6.1, P = 0.008, 95% CI = 1.6–23.1) and adult (OR = 4.8, P < 0.001, 95% CI = 2.5–9.0) horses compared to young animals. The higher seroprevalence of adult horses could be attributed to repeated exposure of these animals to equine influenza virus or vaccination. In Brazil, movement of horses between states or entry

to equestrian events requires a certificate confirming that the horse has been vaccinated against EI (maximum 360 days prior to transit) or a certificate confirming that no clinical cases or outbreaks of infectious diseases (including equine influenza) occurred at the farm of origin 30 days before issuing an animal movement permit [19]. Nevertheless, no consistent data are available regarding vaccination/revaccination status of the horses sampled, which may be considered a limitation of the current study.

In addition to horse age, the year of sampling was identified as a risk factor for seropositivity in this study with the highest seroprevalence value in 2005. In the period 2005–2017, the most outbreaks of EI reported to the OIE in any one year was 144 outbreaks in 2006 (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home). During 2009–2011, when seroprevalence values were lowest in this study, a total of 86 outbreaks were reported over the 3 years. In 2012, there was an extensive outbreak of EI in South America that was first reported in Chile then spread to Brazil (129 outbreaks were notified to the OIE), Uruguay and Argentina, where both vaccinated and unvaccinated animals were affected by a Florida clade 1 strain [20]. Although no outbreaks were reported to the OIE in 2015–2017, an outbreak was identified in vaccinated and unvaccinated horses in a veterinary school hospital in São Paulo in 2015, again caused by a Florida Clade 1 strain [4].

SRH is a reliable *in vitro* assay that is more reproducible than hemagglutination (HI) for measuring antibodies against equine influenza [21]. Evidence supports that levels above 150 mm^2 of antibodies measured by SRH are required for protection of the host against challenge with a similar influenza virus strain [14]. Recently, a large-scale serological study with 2,645 analyzed serum samples of horses in France found that 12.4% (n=328) had antibody levels below 85 mm^2 (risk of infection), 27.3% between ≥85 mm^2 and <154 mm^2 (horses clinically protected), and 60.3% ≥154 mm^2 [22]. In contrast, in the present study, the mean SRH antibody level was 42.0 mm^2 (range 4–238.9 mm^2), with the majority of horses (95.3%) having an antibody values below the protective level of 150 mm^2 [14]. This finding indicates the need to emphasize prevention and control measures targeting EI in Brazil, mainly biosecurity and vaccination procedures, as this country possesses one of the largest population of horses globally, estimated 6 million horses [23] and, with the addition of mules and donkeys, close to 10 million equids.

Understanding the age-related SRH antibody profile may help inform targeted vaccination strategies. The two distinct clusters of higher or lower SRH antibody levels in foals aged 1 month or less in this study suggests that the dams of at least half were vaccinated during pregnancy to confer maternally-derived antibodies (MDA). The median level of MDA in foals classified as 6-months old was around half of that in foals 1 month or less and the median level had declined to below the limit of detection of the SRH assay in young horses between 1 and 4

years of age. The median SRH value of 51.0 mm^2 in horses aged 5–9 years most likely reflects greater likelihood of exposure to virus through mixing with other horses and / or vaccination. However, further studies are needed to better understand vaccine uptake in this equine population.

Overall, this study revealed influenza seroprevalence of 44.7% in horses in Brazil. However, the generally low SRH antibody levels revealed in sampled horses reinforces the need for control measures against the disease, particularly adequate vaccination approaches.

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Conflicts of interest

The authors declare no conflict of interest.

Table 1 Univariate analysis of risk factors associated with equine influenza virus seropositivity determined by single radial hemolysis (SRH) assay among 850 Brazilian horses

Factor	**Level**	**SRH**			***P* value**
		Positive	**Total**	**% prevalence (95% CI)**	
Sex	Male	180	417	43.17 (38.50–47.96)	0.653
	Female	190	425	44.71 (40.05–49.46)	
Age	Young (<5 years)	182	471	38.64 (34.35–43.11)	**0.001**
	Adult (5–14 years)	134	257	52.14 (46.05–58.17)	
	Old (≥15 years)	36	71	50.70 (39.34–61.99)	
Breed	Mixed	100	255	39.22 (33.43–45.33)	0.065
	'Pure'[1]	271	388	46.09 (42.10–50.13)	
State[2]	São Paulo	311	697	44.62 (40.97–48.33)	0.389
	Paraná	15	34	44.12 (28.88–60.55)	
	Santa Catarina	10	25	40.00 (23.40–59.26)	
	Mato Grosso	11	21	52.38 (32.37–71.66)	
	Espírito Santo	10	20	50.00 (29.93–70.07)	
	Rio Grande do Sul	5	15	33.33 (15.18–58.29)	
	Goiás	6	15	40.00 (19.82–64.25)	
	Mato Grosso do Sul	1	11	9.09 (1.62–37.74)	
Year	2005	54	84	64.29 (53.62–73.70)	**0.0005**
	2006	72	130	55.38 (46.81–63.65)	
	2007	87	182	47.80 (40.67–55.03)	
	2008	40	119	33.61 (25.76–42.50)	
	2009	30	96	31.25 (22.85–41.09)	
	2010	20	65	30.77 (20.89–42.80)	
	2011	6	19	31.58 (15.36–53.99)	
	2012	19	50	38.00 (25.86–51.85)	
	2013	16	31	51.61 (34.84–68.03)	
	2014	15	33	45.45 (29.84–62.01)	
	2015 & 2016[3]	7	14	50.00 (26.80–73.20)	
	2017	10	27	37.04 (21.53–55.77)	

[1]Pure-breed horses included: Appaloosa (n=44), Arabian (n=55), Brazilian pony (n=31), Campolina (n=59), Mangalarga (n=138), Paint horse (n=41), Quarter horse (n=145), Thoroughbred (n=43), Other (n=32, Crioulo, Andaluz, Brazilian sport horse, Breton, Lusitana, American trotter, Minihorse, Friesian, Westfalen, Haflinger).

[2]As the numbers sampled in Minas Gerais (*N*=10) and Rondônia (*N*=2) were too low to meet the assumption that no cells have an expected count of <5 in the Pearson's chi-squared test, they were not included in analysis.

[3]In order to meet the assumption that no cells have an expected count of <5, samples obtained in 2015 and 2016 were merged for the Pearson's chi-squared test.

Fig. 1 Single radial hemolysis (SRH) values of equine influenza. Dotted lines represent the thresholds for clinical and virologic protection at 150 mm^2 and clinical protection only at 85 mm^2.

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