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Seroepidemiological Survey of Equine Influenza a H3N8 in Horses from the Eastern Region of the United States-Mexico Border

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Abstract: Seroprevalence studies of equine influenza virus in Mexico are very limited. For this reason, the main objective of this study is to start taking a series of samples looking for specific antibodies for equine influenza virus AH3N8 and the pandemic AH1N1 influenza virus. The higher seroprevalence of influenza A H3N8 Equine/Kentucky/CA09 was mainly associated with the dry season (MH-OR = 1.91; p<0.0001), completely farmed horses (MH-OR = 1.554; p<0.0001) with the permanent presence of dogs in the stables (MH-OR = 1.327; p<0.0001), males and sgeldings (MH-OR = 1.239 and 2.136, respectively p<0.0001) of undefined breed and quarter horse cross breed (MH-OR = 1.367 and 1.565, respectively p<0.0001) which were used for ridding and jumping (MH-OR = 1.274 and 1.244, respectively p<0.0001).

Key words: Equine influenza, AH3N8, AH1N1, seroprevalence, Eastern United States, Mexico border region

INTRODUCTION

The influenza A virus of the Orthomyxoviridae family affects a wide range of birds and mammals including human beings (Lorono-Pino *et al*., 2010). This etiological agent is classified into different subtypes based of its Haemagglutinin (HA) and Neuraminidase (NA) proteins, recognizing at the present day 16 HA subtypes (H1-H16) and nine NA subtypes (N1-N9) (Daly *et al*., 2008). Equine Influenza (EI) is caused by H7H7 or H3N8 subtypes. The H7N7 subtype was first identified in Czechoslovakia in 1956 (A/equine1/Prague/1/56) (Lorono-Pino *et al*., 2010) and at present time, it is considered as extinct among equines (Bogdan *et al*., 1993; Webster, 1993; Yamanaka *et al*., 2010).

The H3N8 subtype was first isolated from a horse in the USA in 1963 (A/equine2/Miami/1/63) and has been identified worldwide in horses (Wadell *et al*., 1963; Bogdan *et al*., 1993; Webster, 1993; Yamanaka *et al*., 2010) and is cause of several outbreaks (Daly *et al*., 2011). In addition, it has been recently demonstrated that horses could be a reservoir of H1N1 (Mancini *et al*., 2006). EI is cause of important economic losses due to its rapid spread among susceptible animals, high morbidity rate and its clinical signs which include depression, pyrexia, coughing, nasal discharge and neonatal losses also if EI is complicated by a secondary bacterial infection the affected animals can develop pneumonia and perish (El-Rahim and Hussein, 2004; Yamanaka *et al*., 2008; Lorono-Pino *et al*., 2010; Begg *et al*., 2011).

The equine population of Mexico comprises 11.57% of the world census, however information concerning the presence and epidemiological patterns of EI is particularly scarce in this country. Consequently, the objective of this survey was to conduct a cross-sectionals in order to demonstrate the presence of EI and to identify some factors associated with the disease in the state of Tamaulipas, Mexico.

MATERIALS AND METHODS

Area description and biological samples: The state of Tamaulipas is located in Northeast Mexico, bordering the State of Texas in the United States of America. The survey was carried out the municipalities of Güemez and Victoria in the central area of Tamaulipas (Fig. 1).

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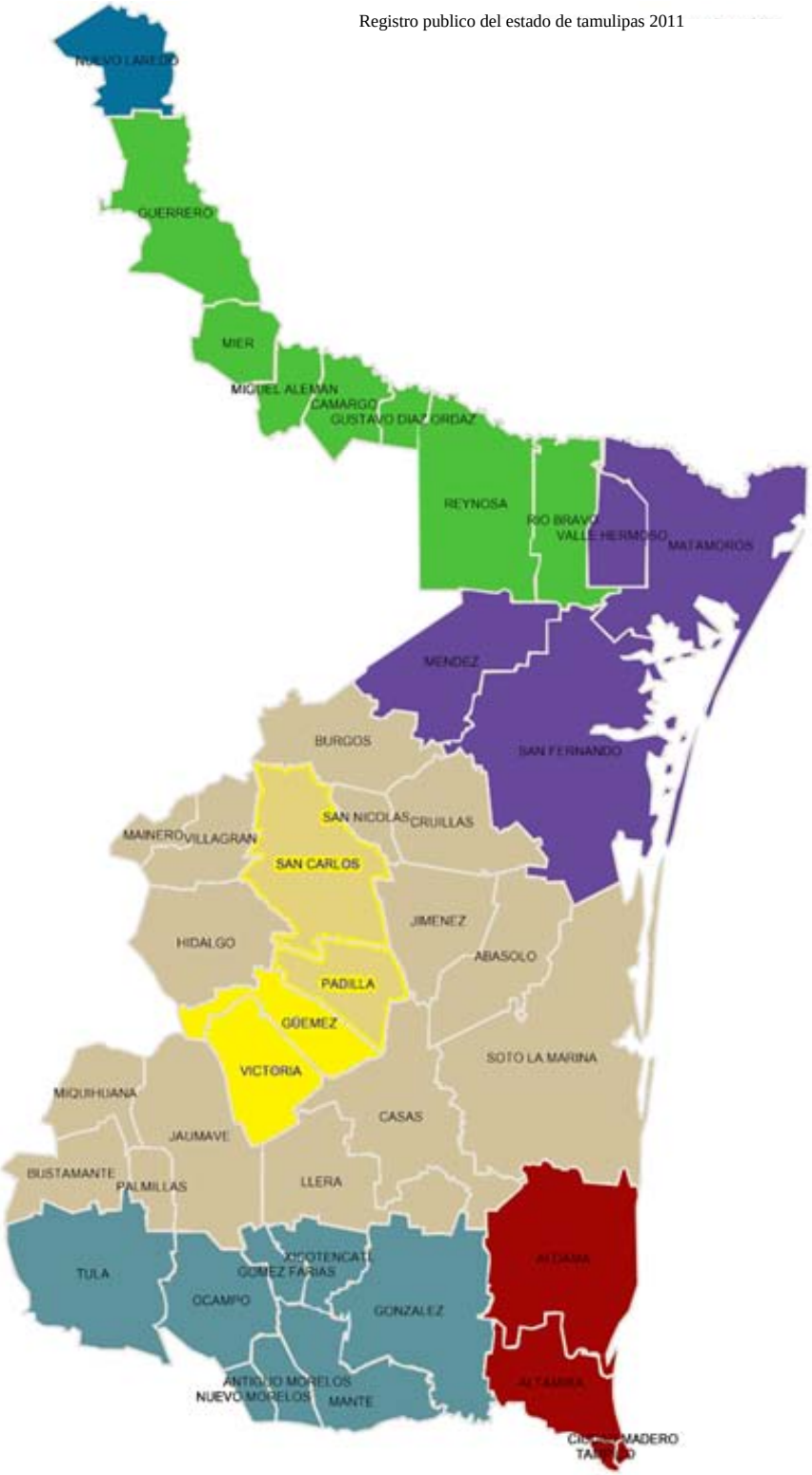

Fig. 1: Geographic location of: A) the state of Tamaulipas, Mexico; B) In solid yellow the municipalities included in the study area: a) Guemez; b) Victoria

In agreement with the last animal population census provided by the Federal Government of Mexico, the average population of horses in both municipalities was of 262 heads which are mainly utilized for sporting and cattle-handling purposes.

Considering the facilities provided by the owners for carrying out the sampling process, 212 horses which never were vaccinated against equine influenza were included in the survey with this sample size, the probability of diagnosing at least one horse as positive was 98.74% as calculated with the software Win Episcope 2.0 (Acosta-Gonzalez *et al*., 2009). From April, 2010 to November, 2011, blood samples were collected by jugular venous puncture followed by clotting at room temperature for 4 h and stored at 4°C for >3 h until their transport to laboratory.

Under laboratory conditions, the blood samples were centrifuged at 1,000 × g for 5 min at room temperature in order to obtain the serum samples required for carrying out immunological diagnostic tests. In addition, 80 nasal exudate samples were obtained using 17 inches swabs (Qosina, NY, USA) which were preserved in sterile M199 medium (Gibco®, USA) 0.5% BSA supplemented with 2×10^6 U L^{-1} penicillin G, 200 mg L^{-1} streptomycin, 2×10^6 U L^{-1} polymyxin B, 250 mg L^{-1} gentamicin, 0.5×10^6 U L^{-1} nystatin, 60 mg L^{-1} HCL ofloxacin and 0.2 g L^{-1} sulfamethoxazole. Once obtained, the nasal swabs also were stored at 4°C for >3 h until transport to laboratory where were stored at -80°C until their analysis. The total number of horses in the study was not sampled with nasal swabs because several owners considered this process a more invasive method than blood sampling and were not willing to allow for this kind of sampling.

Laboratory confirmation: The detection of non-specific antibodies against influenza A was realized at the Instituto Tecnologico y de Estudios Superiores de Monterrey in Monterrey, Mexico with an ELISA kit in agreement with the manufacturer's instructions (Virusys Corporation, Taneytown, MD, USA) each serum sample was analyzed by duplicate. Once non-specific antibodies against influenza A were detected, only the positive serum samples were further subjected to the hemagglutination inhibition assay at the Department of Infectious Diseases of the St. Jude Children's Research Hospital in Memphis, Tennessee, USA in order to detect specific antibodies against influenza A H3N8 equine/Kentucky/CA09 and influenza A H1N1 A/California/04/2009 as previously described. The use of ELISA as screening test followed by confirmation with Hemagglutination Inhibition assay (HI) has demonstrated to have 93.98 and 98.71% of epidemiological sensitivity and specificity, respectively (De Benedictis *et al*., 2010). Also, at the St. Jude Children's Research Hospital, nasal swabs were subjected to the quantitative real time reverse-transcription PCR (qPCR). The positive control utilized in the qPCR was an EI commercial vaccine with the inactivated KY/97 strain (Fort Dodge®, Iowa, USA).

Table 1: Prevalence and environmental risk factors associated with equine influenza in horses from Northeastern Mexico, near the United States-Mexico border region

Variables	Apparent prevalence	True prevalence	MH-OR* (p<0.0001)	CI (95%)
Year of diagnosis				
2010	19.01	19.12	0.617	0.548-0.694
2011	25.32	25.93	1.621	
Season of year				
Dry	19.40	19.54	1.191	0.884-0.956
Rainy	17.65	17.65	0.817	
Cold	19.30	19.43	0.919	
Municipality				
Guemez	22.45	22.83	1.270	0.76-0.816
Victoria	18.18	18.22	0.787	
Farming conditions				
Stabled	22.56	22.95	1.554	0.663-0.857
Semi-stabled	16.44	16.34	0.781	
Free grazing	5.260	4.283	0.312	
Presence of dogs	5.360	4.391	1.327	
Presence of poultry	10.27	9.688	0.652	
Landscape				
Urban	26.51	27.21	1.903	0.672-0.861
Periurban	16.00	15.87	0.761	
Rural	13.43	13.10	0.596	

*Mantel-Haenszel adjusted odds ratio

Statistical analysis: The categorical variables of the diagnosed horses were the year of diagnosis, those variables related with the environment determinants under which the horses were exposed, i.e., season (dry, rainy and cold seasons), geographical origin (Güemez and Victoria municipalities), management conditions (stabled, semi-stables and free-grazing, permanent presence of dogs and domestic poultry in stables), landscape (urban, periurban and rural) and main use given to horses (racing, ridding, cattle handling, jumping and police horses) (Table 1). Also, there were variables which were statistically analyzed related to the host's determinant, i.e., gender (female, male and gelding), age (foal any gender, until 3 months), yearling (any gender from 4-24 months), filly (female, from 25-36 months), colt (male from 25-36 months), mare (adult female, 37 months and over), stallion (adult male, 37 and over) and gelding (orchidectomized male, 37 months and over)) and breed (undefined breed, Quarter horse, cross Quarter horse and cross thoroughbred) (Table 2).

In order to determine the apparent and true prevalence values of equine influenza, the population at risk considered was the total number of horses in each of the aforementioned variables. The association between diagnosis results and the aforementioned variables was

Table 2: Prevalence and host-related risk factors associated with equine influenza in horses from Northeastern Mexico, near the United States, Mexico border region

Variables	Apparent prevalence	True prevalence	MH-OR* (p<0.0001)	CI (95%)
Gender				
Female	33.82	35.10	0.300	0.107-0.843
Male	45.24	47.42	1.239	
Gelding	66.67	70.54	2.136	
Horse age				
Yearling	14.29	14.03	0.191	0.77-0.986
Filly	37.50	39.07	0.872	
Colt	70.00	74.13	1.756	
Mare	36.96	38.48	0.580	
Stallion	62.50	66.04	1.542	
Gelding	66.67	70.54	1.920	
Breed				
Undefined	50.00	52.55	1.367	0.478-0.629
Quarter horse	34.34	36.66	0.548	
Quarter horse cross	58.02	61.20	1.565	
Thoroughbred cross	42.86	44.85	0.815	
Use				
Racing	34.78	36.13	0.874	0.827-0.925
Ridding	47.96	50.35	1.357	
Cattle handling	27.78	28.58	0.830	
Jumping	50.00	52.55	1.325	
Police horse	71.88	76.16	0.692	

*Mantel-Haenszel adjusted odds ratio

determined using a stratified cross-sectional design this design was used in order to avoid the effect of confounding variables, i.e., horses' age, gender and geographical origin. The association was obtained with the Mantel-Haenszel statistical method for calculating the adjusted Odds Ratio (MH-OR). The analysis was carried out with the software Win Episcope 2.0 at a 95% level of confidence, thus the Type I error was 0.05.

RESULTS AND DISCUSSION

The main features of the sampled horses were the following: the age range was 12-240 months (76.66±53.01 months) with 32.08, 19.81 and 39.62% of females, males and geldings, respectively. Concerning the farming conditions, the completely stabled individuals predominated (62.74%) followed by semi-stabled horses (34.43%) and animals that were under free grazing conditions (8.96%). The quarter horse breed predominated (46.7%) and was followed by quarter horse cross breed (38.21%), undefined breed (17.92%) and thoroughbred cross breed (3.3%). The main use of these horses was for riding (46.23%), racing (32.55%) and police horses (15.09%); a lesser percent of the horses were used for cattle handling (8.49%) and jumping (3.77%).

The overall seroprevalence of non-specific antibodies against influenza A H3N8 was 45.78%. When the positive samples were analyzed, the seroprevalence of specific antibodies against influenza A H3N8 Equine/ Kentucky/CA09 was 19.11% which was higher in the year 2011 (25.32%) for the municipality of Guemez (22.45%) and for geldings (26.19%), however the seroprevalence values slightly varied when was recalculated using the diagnostic confidence limits reported by De Benedictis *et al.* (2010) (Table 1). The higher seroprevalence of influenza A H3N8 Equine/Kentucky/CA09 antibodies was mainly associated with the dry season (MH-OR = 1.91; p<0.0001) completely stabled horses (MH-OR = 1.554; p<0.0001) under urban landscape (MH-OR = 1.903; p<0.0001) and with the permanent presence of dogs in the stables (MH-OR = 1.327; p<0.0001).

The presence of seropositive horses also was associated with males and geldings (MH-OR = 1.239 and 2.136, respectively p<0.0001) of undefined breed and quarter horse cross breed (MH-OR = 1.367 and 1.565, respectively; p<0.0001) and those animals which were used for ridding and jumping (MH-OR = 1.274 and 1.244, respectively; p<0.0001). No positive results were obtained when serum samples were subjected to HI to detect antibodies against the reference strain H1N1 A/California/04/2009 nor when the nasal swabs were subjected to qPCR in order to detect Influenza A non specific strain. EI is considered a highly pathogenic disease for equines and it results in severe economic losses mainly due to cancelation of sporting and racing events which has been demonstrated in several outbreaks around the world (Yamanaka *et al.*, 2008). In this century, EI outbreaks have occurred in Croatia, Japan (Nishiura and Satou, 2010) India, Mongolia, China (Virmani *et al.*, 2010). In agreement with the World Organization of Animal Health (OIE), the last EI outbreak in Mexico was recorded in 1997 and after this year, the presence of the disease is suspected but not confirmed by the animal health authorities.

Some epidemiological patterns of EI have been determined during outbreaks, however because the aforementioned analysis have been done using a purposive sampling that involves diseased animals, the generated information could not be representative of the general population and the findings only can be extrapolated to equines with clinical signs. In spite of this, the seroprevalence determined in this study was <22.85% reported for outbreaks in India (Virmani *et al.*, 2010), 37% in Hong Kong (Lai *et al.*, 1994) 56.5% in Canada (Diaz-Mendez *et al.*, 2010) and 5.6% in Egypt (El-Rahim and Hussein, 2004) however, the results of this study can be compared and were similar to the 19.4% reported by Yamanaka *et al.* (2008) whom analyzed subclinical healthy race horses in an outbreak in Japan but when compared with the results of race horses in this study were considerable higher than the aforementioned

study. On the other hand, the seroprevalence rate of this study was >13.6% in Tunisia (Boussetta *et al*., 1994) and 9.07% in the United States of America (Pusterla *et al*., 2011) reported in random surveillance surveys. In Mexico, previous surveys have reported seroprevalence rates of 28% (Lorono-Pino *et al*., 2010) and 38.8% (Blitvich *et al*., 2010) both seroprevalence values are higher than the reported in this study. In addition in the aforementioned reports, the seroprevalence was not associated with risk factors.

Concerning risk factors there is a great lacking of information, probably due to the urgency for controlling outbreaks rather than generating epidemiological information. In spite of this, the presence of EI has been associated with the animals' age. In the United Kingdom, Newton *et al*. (2006) reported that infections among 2 years old horses were lower than in older animals which is in agreement with the risk factors determined in this study. In this study, older animals were also associated with the presence of antibodies against H3N8 equine/Kentucky/CA09 which is in disagreement with the report of Goto *et al*. (1993) whom reported that in Japan the presence of reactors decreased in older horses.

Racing horses also has been previously associated with EI (Lany *et al*., 1997), however in this study, the aforementioned horses' activity was not determined as a risk factor but ridding and jumping horses were strongly associated with the disease because under local conditions hundreds and thousands in some particular circumstances, of horses are in close contact in these activities. Another factor associated with EI has been the stabled horses (Powell *et al*., 1995) which is in agreement with the results of this study horses that were located in periurban locations also are associated with high seroprevalence rates (Cowled *et al*., 2009) similarly to the horses located in urban locations in this study. Also, Yamanaka *et al*. (2008) in Japan and Blitvich *et al*. (2010) and Lorono-Pino *et al*. (2010) in Mexico, determined differences in morbidity rates in geographical location as in this study.

Recent evidence exists showing that dogs can be infected by different EI virus strains when they are in close contact with infected horses (Daly *et al*., 2008; Hayward *et al*., 2010; Kirkland *et al*., 2010; Crispe *et al*., 2011). Despite the fact that the role of dogs has not been clearly demonstrated in the epidemiology or perpetuation of the disease this study reports an association between EI in horses that were permanently in close contact with dogs. Also, wild birds are considered a natural reservoir of EI virus (Daly *et al*., 2008) and these could transmit the disease to horses (Spokes *et al*., 2009) and domestic poultry, however the permanent presence of domestic poultry in the analyzed stables was not associated with the reported seroprevalence rates. Despite the use of effective control measures, i.e., vaccinations programs, movement restriction, isolation and quarantine of infected horses or stables (Nishiura and Satou, 2010) when outbreaks take place, EI due to the influenza is still a significant health problem worldwide. Continuous surveillance is necessary in order to detect clinical or subclinical cases or antigenic drift of the virus. Thus, this study emphasizes the need to conduct epidemiological surveys in randomly selected populations of equines in order to determine not only risk factors but also to implement early warning systems for the prevention and control of outbreaks as well as implementing the appropriate eradication programs.

CONCLUSION

No positive results were obtained when serum samples were subjected to HI to detect antibodies against the reference strain A H1N1 A/California/04/2009.

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