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Assessment of influenza A virus infection in horses of West Azerbaijan province using competitive ELISA

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Abstract Equine influenza is an extremely contagious respiratory disease of horses caused by the influenza A virus. The present study aimed to determine the prevalence of influenza A virus infection in horses of West Azerbaijan Province, north-west Iran. A total of 184 horse serum samples were collected from four localities and tested by competitive enzyme-linked immunosorbent assay to detect antibodies against the nucleoprotein of the influenza A virus. Data were analyzed for the effect of gender, age, and location on seropositivity by the chi-square, Fisher's exact, and logistic regression tests. The prevalence rate was estimated to be 5.4% (95% CI: 2.14–8.66%). Based on regression analysis, the odds of infection based on age in years was 0.581 (95% CI: 0.176–1.916) (P > 0.05), and age explained -0.544% of the variations in infection. The relative frequency of infection in male and female horses was 2.6 and 7.4%, respectively (P > 0.05), and the odds of infection in females compared with male horses was 2.72 (95% CI: 0.536–13.794). Sex explained 1.001% of infection variations. The prevalence rate in Sardasht, Urmia, Khoy, and Chaldoran was 4%, 4.8%, 6.2%, and 6.8%, respectively. A non-significant increase in prevalence was observed from the south to the north of the province (P > 0.05). Geographical location explained 0.047% of infection variations. The final summary of results indicates that exposure to the influenza A virus is very low in horses of the studied regions.

Introduction

Equine influenza, caused by the influenza A virus, is a highly contagious disease of the respiratory system in horses. The disease starts with anorexia, fever, dry cough, and nasal discharge, and in rare cases, it can lead to death due to secondary bacterial pneumonia. The incubation period of the disease is 1 to 3 days, and infected horses shed the virus from nasal secretions for 10 days. The virus is transmitted through inhalation and direct contact [24]. Influenza viruses are classified into types A, B, B, C, and D based on their nucleoprotein and matrix genes. The influenza A virus is considered the ancestor of other influenza viruses and is divided into different subtypes based on surface glycoproteins such as hemagglutinin (H) and neuraminidase (N) [17, 23, 25]. Although the highly pathogenic avian influenza virus (H5N1) has been isolated from donkeys [1], only the H7N7 and H3N8 influenza virus types circulate endemically among equids [24]. The recent subtype has spread worldwide and is the main cause of the disease, especially in areas where vaccination is not routinely performed [2]. In addition, the equine influenza virus has been able to cross the species barrier and has been isolated

from canines as well [10, 11, 20]. To date, there are no reports of natural human infection with the equine influenza virus, although there are reports of experimental infection as well as serological evidence of human infection with this virus [18, 26].

Vaccination is considered a main measure to prevent equines from being infected with the influenza A virus [28]. According to the previous recommendations, vaccination of 70% of the equine population can prevent the epidemic occurrence, but according to mathematical modeling, if the vaccine strain matches exactly with the circulating virus strain, this number can be reduced to 40%; otherwise, for complete success, up to 90% of the equine population should be vaccinated [21,22,27]. This study aimed to determine the serological frequency of influenza A virus infection in horses of West Azerbaijan province in northwestern Iran and to determine the effect of age, gender, and geographical location on the frequency of infection.

Materials and Methods

Animals

In March 2017, a random sampling was done under sterile conditions from work horses of Sardasht, Urmia, Khoy, and Chaldoran counties located from south to north of West Azerbaijan province.

Study protocol

Blood samples in the amount of 5 ml were taken from the jugular vein of horses and transferred to the immunology laboratory of Islamic Azad University, Urmia branch, in gel tubes on ice as soon as possible.

After centrifugation and separation of serum from blood, 184 samples of serum without hemolysis were transferred to sterile microtubes and kept in the freezer until the ELISA test. At the same time as blood sampling, the characteristics of horses including age, sex (76 males and 108 females), geographical location (50 from Sardasht, 42 from Urmia, 48 from Khoi, and 44 from Chaldoran), and age were recorded. To investigate the effect of age on the frequency of positive cases, the horses were divided into three groups: 4 years and younger (32 horses), 5–8 years (119 horses), and 9 years and older (33 horses).

The ELISA test was performed in a competitive way using commercial kits (ID-VET Co., France) designed to detect antibodies against the nucleoprotein of the influenza A virus in poultry, pigs, and horses. The test steps were carried out according to the instructions of the manufacturer of the kit. After reading the plate at a wavelength of 450 nm, the results were calculated based on the ratio of the optical density of the sample serum to the average optical density of the negative control serum × 100 (S/N %). Ratios ≤ 45% and ≥ 50% were considered positive and negative, respectively.

Statistical analysis

The data were analyzed using IBM SPSS Statistics 22 software (Chicago, IL, USA). Descriptive findings of the studied variables, including indicators such as mean, standard deviation, and absolute and relative frequency, were calculated and reported. The chi-square test and Fisher's exact test were used to investigate the relationship between the demographic variables (age and sex) of horses and the frequency of seropositive cases, and if there was a significant difference, the difference between the groups was determined by Bonferroni post hoc test. In addition, a multivariable logistic regression test was used to predict the probability of observing seropositive cases based on demographic variables. The permissible error for rejecting the null hypothesis (H_0) was 5%.

Results

The results indicate that out of 184 blood serum samples taken from horses in the cities of West Azerbaijan Province, 10 samples (5.4%) (95% CI: 2.14–8.66%) were positive for influenza A virus antibodies (S/N %≤ 45%). 174 samples (94.6%) (95% CI: 91.34–97.86%) were diagnosed as negative for influenza A virus infection (S/N %≥ 50%).

Two of the 32 horses 4 years old and younger (6.3%) and 8 of the 119 horses aged 5–8 years (6.7%) had positive S/N% for influenza A. Positive S/N% for influenza A was not detected in the 33 horses aged 9 years and older (Table 1).

The data from the chi-square test and Fisher's exact test showed that there was no significant relationship between the age of horses and the frequency of influenza A seropositive cases in the studied horses (X2 (2) = 2.322, P = 0.355 > 0.05).

Two out of the 76 male horses (2.6%) and 8 out of the 108 female horses (7.24%) had positive influenza A S/N% serum titers (Table 2).

According to the chi-square test, there was no significant relationship between the gender of horses and the frequency of influenza A seropositive cases (X2(1) = 1.980, P = 0.159 > 0.05).

Two of the 42 (4.8%) samples obtained from horses in Urmia city and 2 of the 50 (0.4%) serum samples from horses in Sardasht city had positive S/N% for influenza A. In addition, the results showed that 3 samples out of the 48 horse from Khoi city (6.3%) and 3 samples out of the 44 horse from Chaldoran city (6.8%) had positive S/N % for influenza A.

The output data of the chi-square test and Fisher's exact test showed that there was no significant relationship between the living area of horses and the frequency of influenza A seropositive cases (X2(3)=0.463, P = 0.945 > 0.05). The multivariable logistic regression model results were not statistically significant considering the effect of age, gender, and location (X2(3) = 3.013, P = 0.390 > 0.05), and the model had the necessary fit. The Nagelkerke R2 model explained 4.7% of the variance in influenza A disease and correctly classified 94.6% of the cases. None of the three predicting variables of age, gender, and location were significant.

Multivariate logistic regression showed that in all horses of West Azerbaijan province, the chance of infection between age in years and disease was 0.581 (95% confidence interval 0.176–1.916) (P = 372 > 0.05) and the age of horses in West Azerbaijan province explains -0.544% of the infection variance. In other words, in ages above 4 years, the probability of contamination decreases 0.58 times. Logistic regression showed that the chance of contamination of female horses was 2.72 times (95% confidence interval 0.536–13.794) that of male horses (P = 0.227 > 0.05) and gender justified 1.001% of infection variations. In addition, the results of logistic regression indicate that the living area of horses justifies 0.047% of infection variations. The chance of infection of horses in Khoy and Chaldoran (north of the province) was 1.05 times (95% confidence interval 0.253-4.339) those of horses in Urmia and Sardasht (center and south of the province) (P = 0.948 > 0.05).

Discussion

In the present epidemiological study, which was conducted for the first time in West Azerbaijan province, 5.4% of the horses were positive for antibodies against the influenza A virus. The results indicated a very low serum prevalence of influenza A virus among horses in the province, which is in line with the results of similar studies conducted in other parts of Iran. The sero-prevalence of influenza A virus infection in horses is 7.07% in Khuzestan province [13], 2.5% in Fars province [5], 7.3% in Tabriz city [14], and 8.2% in Ardabil city [15].

Considering the low percentage of infection with the influenza A virus in different regions of Iran and the lack of reports of disease outbreaks, it seems that horse influenza is an endemic problem in Iran. Of course, the role of imported horses or migratory birds in the transmission of infection should also be considered. For example, the studies conducted in the countries of Turkey and Iraq [3], which share borders with the studied province, have reported 31.1% and 31.2% infection in horses, respectively. In a study conducted in Spain, they

Table 1. The results of Pearson's chi-square test for the different S/N% diagnostic criteria for influenza A and the age of horses.

Variables	Influenza A positive S/N ≤ 45%	Influenza A negative S/N ≥ 50%	Chi-square index value	Degree of freedom	P-value
≤ 4 years old	2 (6.3%)	30 (93.8%)	2.322	2	0.355
5–8 years old	8 (6.7%)	111 (93.3%)			
≥ 9 years old	0 (0.0%)	33 (100%)			

Table 2. Pearson's chi-square test results for the different S/N% diagnostic criteria for influenza A and gender of horses

Variable	Influenza A positive S/N ≤ 45%	Influenza A negative S/N ≥ 50%	Chi-square index value	Degree of freedom	P-value
Male	2 (2.6%)	74 (97.4%)	1.98	1	0.159
Female	8 (7.4%)	100 (92.6%)			

Table 3. Pearson's chi-square test results for the different S/N% diagnostic criteria for influenza A and the living area of horses

Variables	Influenza A positive S/N ≤ 45%	Influenza A negative S/N ≥ 50%	Chi-square index value	Degree of freedom	P-Value
Urmia	2 (4.8%)	40 (95.2%)	0.463	3	0.945
Sardasht	2 (4.0%)	48 (96.0%)			
Khoi	3 (6.2%)	45 (93.8%)			
Chaldoran	3 (6.8%)	41 (93.2%)			

also witnessed a significant frequency of infection in the horse population located around Donana National Park, which is one of the important habitats for waterfowl and migrant birds from Africa in western Andalusia [16]. It is noted that Lake Urmia is also a temporary habitat for many migratory birds that can be effective in the transmission of infection.

In reviewing the results of studies conducted in other countries, we see a higher prevalence of the influenza A virus in horses from 12.3% in Croatia [6] to 67.7% in England [9]. However, comparing the level of infection between studies should be done while keeping in mind many factors, such as the differences in antibody measurement methods, sample size, host factors such as age, sex, breed , sampling time, weather, and health and management measures. The disease is associated with high prevalence in susceptible populations, but the infection rate is lower in populations that have already been infected or vaccinated [4, 7, 8, 12].

In this study, there was no significant relationship between age and the frequency of infection although in ages above 4 years, infection odds decreased by a factor of 0.58. This result may be due to the sensitivity of young horses. It seems that the contamination in the

province had happened recently because no infection was observed in horses over 9 years old. A similar result was obtained by Badiei et al. in Fars province [5], where infection was rarely reported in horses over 10 years old, but in a study conducted in 2017 in Khuzestan[13], it was reported that with each year increase in age, the probability of infection increased by 6%, and the highest level of infection, 9.17%, was reported in horses over 10 years old. Comparing the results, it can be said that the history of infection in Khuzestan province is probably older than in West Azerbaijan province.

In this study, although the relative frequency of seropositive cases of the influenza virus was higher in female horses than in males, the difference was not statistically significant. In the surveys carried out in Fars [5], Khuzestan [13], and Tabriz districts [14], the difference in infection rates in female horses has been non-significant. However, other studies have found infection to be more common in males than in females.

Considering the absence of vaccination programs against horse flu in Iran, the antibodies against the influenza A virus in the horses of the province can be the result of natural exposure to this virus. Therefore, to prevent the occurrence of influenza epidemics by carrier horses, the transmission of the virus to other animal species, and the creation of possible dangers and threats to human societies through the emergence of new strains of human influenza caused by contact with infected animals, the effective implementation of monitoring and control measures such as vaccination is strongly recommended. Identifying the birds that are potential reservoirs of the virus and the circulating influenza A virus types and strains in horses in the province will also be extremely beneficial.

Conclusion

The final summary of results indicates that exposure to the influenza A virus is very low in horses of the studied regions.

Acknowledgements

Not applicable

Conflict of interest

The authors declare that they have nothing to declare.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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