Identification of *quine influenza A virus* antibodies against nonstructural protein (NS1) enables differentiation among infected and vaccinated horses

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Abstract

NS1 and NS2 are nonstructural proteins that found as two overlapping proteins encodes by the RNA segment 8 of *influenza A viruses*. NS1 is synthesized in massive amounts in the early infection and aggregates in the nucleus of infected cells. This study intends to find the tools to identify the equine influenza antibodies that derived from infected or vaccinated animals (DIVA) using indirect ELISAs, NP- ELISA and NS1- ELISA. The study was conducted between the period extended from November /2015 to March /2016. A total of 423 serum samples were randomly collected from different ages and genders horses in eight Iraqi governorates (Baghdad, Al-Muthana, Al-Najaf, Kerbala, Babel, Diyala, Wasit, and Al-Qadysia). Out of 423 samples, there were 132 (31.20 %) and 84 (19.85 %) positive serum by NP- ELISA and NS1-ELISA respectively. A significant correlation result (P< 0.01) was seen between positive and negative samples. High seropositive cases were found in males (17.73 %) by using NP – ELISA. Moreover, the NS1-ELISA also indicated the high infection rate that occurred in males (11.34%). In addition, significant (P< 0.01) results appeared for both ELISAs. A higher percentage (50.75%) of seropositive horses found in the age group of 11-15 years in NP- ELISA, however, 11-15 years age group also showed the infection rate of 50 % in NS1-ELISA with statistical differences of (P<0.01). In conclusion, the results of this study approved the possibility of using the nonstructural protein (NS1) as a differential diagnostic indicator for equine influenza virus infection.

Introduction

Equine influenza is an acute contagious respiratory infection of horses, donkeys, and mules. It is characterized by sudden onset of fever, cough, malaise, myalgia, nasal discharge, depression, inappetence and sometimes neurological complications (Daly *et al.,* 2006). It is caused by two distinct subtypes: the H7N7 (prototype A/ equine/1/ Prague/ 56), and the H3N8 (A/ Equine/ Miami/ 2/ 63). The influenza type A causative agent belongs to the genus

Influenza virus A of the family *Orthomyxoviridae* (OIE, 2000). The infection by equine influenza virus is similar to other respiratory diseases. Equine influenza causes a significant economic loss to the equine industry due to lost training days and veterinary costs. Furthermore, influenza A infections often occurs as outbreak that rapidly spread through susceptible horse populations. Inhalation of the infected particles of the influenza A virus is the main source of infection. The virus shed from the infected animals by coughing and contaminated the equipment such as feed buckets, tack, and grooming aids (Waghmare *et al.,* 2010). The serodiagnosis of the disease can be achieved by using different techniques such as: haemagglutination inhibition (HI), single radial haemolysis (SRH) (Virmani *et al.,* 2010; Gildea *et al.,* 2013), enzyme immunosorbent assay (ELISA) for both non-structural protein (NS1) and nucleoprotein (NP) (Daly *et al.,* 2004; Kirkland and Delbridge, 2011).

RNA segment 8 of influenza A virus encodes two proteins non-structural 1 (NS1) and non-structural 2 (NS2), in which the antigenic determinant(s) of NS1 is located in the C- terminal half of the protein. They are present only in the virus-infected cell (Birch-Machin *et al.,* 1997; Marc, 2014). Vaccination against equine influenza can interfere with serological surveillance, as, these vaccines induce antibodies that are indistinguishable from the antibodies produce in the natural infected horses as determined by ELISA and HI (Birch-Machin *et al.,* 1997; Ozaki *et al.,* 2001). Therefore, the efforts have been focused on developing new serological tools to allow differentiation of vaccinated and infected horses, which is commonly known as DIVA strategy. The aims of this study were to detect antibodies against equine influenza A virus nucleoprotein (NP) and non-structural protein 1(NS1) by indirect ELISAs in serum samples to differentiate vaccinated from infected horses and to study some epidemiological parameters of equine influenza.

Materials and Methods

Animals
Equine of both sex (Stallion and mare) and various age groups (1year – 25 years old) were chosen randomly from eight governorates during November / 2015 – March /2016. These horses were with the unknown status of vaccination.

Serum samples

Blood samples were collected from 423 apparently healthy horses. About 8-10 ML blood sample was collected from jugular vein with vacutainer without anticoagulant tubes. The sample held at room temperature for 20 – 30 minutes at slant position to allow the separation of the serum. Later on, the clots separated from serum by running on applicator stick around the test tube walls gently, then centrifuged at 2500 rpm for 10 minutes. Serum collected by microtiter pipette and kept in sterile Eppendorf tubes checked to be cleared from hemolysis, marked with its specific number then stored at - 20 ℃ till tested for the presence of equine influenza virus antibodies using ELISAs.

Diagnostic Kits

Influenza A Virus Antibody Test Kit

The influenza A test kit is an enzyme immunoassay for the detection of antibody to *influenza A virus* nucleoprotein in horse serum (IDEXX). This test kit used following the manufacturer's instructions.

NS1- ELISA Kits

Blocking enzyme immunoassay (B – ELISA) for detection antibodies against non-structural protein *influenza A virus* in horse serum (Sinobiological Inc) was used according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed statistically by Chi-Square test (X^2) and ANOVA by SAS (2012) program.

Results and Discussion

Antibodies against equine influenza viral nucleoprotein were found in 132 (31.20 %) out of 423 tested sera by using NP- ELISA, while 291 (68.79%) out of 423 serum samples revealed the negative result. The result findings showed a significant difference (P < 0.01) between positive and negative samples (Table. 1). The results of this study showed relatively high percentage (31.20%) of seropositive animals in compare with the previous studies (Mavadiya *et al*., 2012; Algezoli and Kheir, 2014; Badiei *et al*., 2014). Badiei *et al.,* (2014) examined six hundred equine sera to detect specific antibodies for influenza virus- using competitive ELISA in the south of Iran during the period extended from 2011 to 2012. Totally, 2.5 % of horses revealed a positive reaction, which was confirmed to be due to natural exposure to *equine influenza virus*. However, their study showed variations in the percentage of seropositive animals according to the season. The seropositive animals were, 1.94 % and 5.47 % in autumn and winter respectively . The percentage of the seropositive animals that reported in the present study was lower than previous studies. Ataseven and Daly, (2007), Blitvich *et al.,* (2010) and Boukharta *et al.,* (2012) percentages were 41.80 %, 39%, and 41.33% respectively.

The relatively high percentage of seropositive equine sera that reported in the present study might be related to different predisposing factors. These factors are climate, management, stress factors, illicit movement and cross – border trading of animals, which were also reported by other studies (Gross *et al.,* 1998; Firestone et *al.,* 2012; Sooryanarain and Elankumaran, 2015). Non-structural protein 1 is synthesized in large amounts early in infection and accumulates in the nucleus of the infected cell. Furthermore, NS1 has also been found in association with cellular RNA in the form of paracrystalline inclusion bodies within the cytoplasm of infected cells (Shaw *et al.,* 1982). In order, to use antibody response to NS1 in routine diagnostic setting, it will be necessary to develop NS1- ELISA for differentiation the infected and vaccinated animals (Talazadeh *et al.,* 2013).

Out of 423 serum samples collected randomly from horses, 84 (19.85%) and 339 (80.14 %) were seropositive and negative respectively for NS1 (Table. 1). The result was statistically significant with the probability of (P< 0.01). The 19.85% indicated the infection rate since the NS1 is expressed in influenza infected cells (Marc, 2014).

The results of this study showed that Baghdad governorate revealed the higher seropositive rates (41.78%) followed by AL-Qadysia (20 %) by NP-ELISA. However, by using NS1-ELISA, the higher percentage of positive horse sera (infection rate) was also in Baghdad (26.78%) followed by AL-Najaf (11.53%). Moreover, Kerbala, Babel, and Wasit, also showed seropositive equine sera against viral NP with percentages 11.11%, 15.38 %, 13.33 % respectively. Whereas AL-Muthana and Diyala revealed seronegative results against equine influenza nucleoprotein and non- structural protein. Kerbala, Babel, Wasit and Al-Qadysia also showed positive anti-NS1 antibodies with significant interaction between the positive and negative sera from governorates in both ELISAs (Table 2). All the governorates demonstrated positive horse sera against NP equine influenza. These results explain that the horses might be infected or may be vaccinated, except AL-Muthana and Diyala which reported no anti–NP and anti-NS1 antibodies with ELISAs. The high seropositivity in Baghdad and AL-Qadysia might be due to considerable influence of distribution, density and the management mode of the horse population. Besides, the weather condition, exercise and the close contact between horses were played important roles in the maintaining of the infection and spread of the disease. These factors also were reported by previous studies (Boukharta *et al.,* 2012; Sooryanarain Elankumaran, 2015). Although the number of horse populations in AL-Muthana and Diyala, as recorded in Ministry of Agriculture (2014) were 150 and 159 horses respectively, negative results were noticed. This may be explained because the samples were collected from single animals on the farm and not from grouped horse population (Barquero *et al.,* 2007). In AL-Najaf, both types of ELISA showed the similar percent 11.53 % that explain infective horses but not immunized.

The results of this study also showed a variation in the seropositive animals according to the gender (Table. 3.). The percentage of the seropositive cases were higher in males.

Table.1. Shows the percentage of positive equine influenza serum samples by NP - ELISA* and NS1- ELISA *

Number of tested serum samples	Positive samples (%) of NP	Negative samples (%) of NP	Positive samples (%) of NS1	Negative samples (%) of NS1
423	132 (31.20)	291 (68.79)	84 (19.85)	339 (80.14)
Chi-square P-value	0.283 ** 0.00215		13.048 ** P- 0.0001	

*Detected antibodies against the viral NP and NS1 of equine influenza virus A
** (P<0.01)

17.73% than females 13.47% in all governorates by using NP – ELISA. On a contrary, AL-Najaf and kerbala were the only governorates that showed higher seropositive sera in females. The statistical analysis of the results revealed that there were significant differences (p < 0.01) between males and females in all governorates tested by NP-ELISA. Moreover, AL-Najf, Kerbala, Babel, and Wasit were also revealed significant differences of (P < 0.05) between both sexes. The high level of infection rate was found in males 11.34%, but in females 8.51% in all governorates by NS1- ELISA. The test

approved that the highest infectivity rate in males was found in Baghdad with the significant difference as compared with Babel, Wasit, and AL-Qadsyia. Moreover, AL-Najaf and Kerbala showed high infectivity rates in females 11.53% and 5.55% respectively. No significant differences appeared between sexes in each Governorate except AL-Najaf with a probability of (P< 0.01).

Table.2. Shows the percentage of positive equine influenza serum samples according to Governorate by NP- ELISA* and NS1-ELISA*

Governorate	Total tested serum samples	Positive NP samples (%)	Negative NP sample (%)	Chi-square	Positive NS1 samples (%)	Negative NS1 sample (%)	Chi-square
Baghdad	280	117 (41.78)	163 (58.21)	6.795 **	75 (26.78)	205 (73.21)	10.52 **
Al-Muthana	21	0 (0)	21 (100)	15.00 **	0 (0)	21 (100)	15.00 **
Al-Najaf	26	3 (11.53)	23 (88.46)	13.942 **	3 (11.53)	23 (88.46)	13.64 **
Karbala	18	2 (11.11)	16 (88.88)	13.971 **	1 (5.55)	17 (94.44)	14.59 **
Babel	13	2 (15.38)	11 (84.61)	13.755 **	1 (7.69)	12 (92.30)	14.24 **
Diyala	20	0 (0)	20 (100)	15.00 **	0 (0)	20 (100)	15.00 **
Wasit	15	2 (13.33)	13 (86.66)	13.794 **	1 (6.66)	14 (93.33)	14.42 **
Al-Qadsyia	30	6 (20)	24 (80)	13.25 **	3 (10)	27 (90)	14.18 **
Total	423	132 (31.20)	291 (68.79)	10.283 **	84 (19.85)	339 (80.14)	13.27 **
Chi-square	---	12.581 **	12.581 **	---	8.593 **	8.593 **	----

*Detection antibodies against viral NP and NS1 of type A influenza virus
** (P<0.01)

Many previous studies reported that the disease spread quickly and soon involved both sexes with non-significant differences in its susceptibility (Uppal and Yadav, 1987; Abd El-Rahim and Hussein, 2004; Boukharta *et al.,* 2012). However, other studies showed that positive cases were seen in females more than males (Algezoli and Kheir, 2014; Badiei *et al.,* 2014). The present study was compatible with the study of Mavadiya *et al.,* (2012), who reported high seropositive cases 11.95% in males by using HI test. In the present study, the percentage was 11.34%. Although, there was no particular reason for this occurrence. The reasons may be related to poor management and higher exposure to exercise. Furthermore, it was noted that traditionally in the area, females usually receive more attention regarding proper feeding, keeping condition due to breeding purposes and this may also contribute to significant differences in the rate of infection between the both sexes. All age groups were positive for equine influenza A nucleoprotein. However, the higher percentage of seropositive horses has been found in the age group 11-15 years (50.75%). Statistically, significant differences (P < 0.01) observed between the five categories of ages. The findings on using the NS1-ELISA also revealed that all age groups infected with equine influenza. Moreover, the age group 11-15 years showed the higher percentage (50 %). The current finding showed significant differences between all age categories with (P<0.01) (Table. 4).

The results of this study approved the susceptibility of different age group of horses to equine influenza A as shown in the table (4). These results are in agreement with previous studies (Abd-Rahim and Hussein. 2004; Boukharta *et al.,* 2012). The risk analysis revealed that the higher seropositive age group was the 11-15 years among other horses aged categories. Previous studies reported that the older horses are at less risk for equine influenza A virus in the enzootic area because of the acquired immunity from previous exposure (Morley *et al.,* 2000; Yondon *et al.,* 2013). In this study, the bias in the collecting of the serum samples from different age categories might occur because the owners did

not allow to collect the samples from younger animals. The variations in the susceptibility of horses according to age group had also been reported by previous studies that showed its effect on the epidemiology of the disease (Mavadiya *et al.,* 2012; Algezoli and Kheir, 2014). A previous study of Nyaga *et al.,* (1980) demonstrated that horses of category 7-10 years showed a significant low and sparing risk. Whereas horses less than two months or over ten years, as well as those in ages from six months to 7 years had non- significant low risks to the epidemiology of infection.

Table.3: Shows the percentage of positive equine influenza serum samples according to gender in different governorates by NP ELISA and NS1- ELISA ***

governorate	Total sample	Male	Male NP +ve (%)	Female	Female NP+ve (%)	Total NP+ve (%)	Chi-square	Male NS1 +ve (%)	Female NS1+ve (%)	Total NS1 +ve (%)	Chi-squre
Baghdad	280	149	68 (24.28)	131	49 (17.5)	117 (41.78)	2.35 NS	44 (15.71)	31 (11.07)	75 (26.78)	4.27 *
Al-Muthana	21	12	0 (0)	9	0 (0)	0 (0)	0.00 NS	0 (0)	0 (0)	0 (0)	0.00 NS
Al-Najaf	26	11	0 (0)	15	3(11.53)	3(11.53)	5.27 *	0 (0)	3 (11.53)	3(11.53)	7.25 **
Kerbala	18	4	0(0)	14	2(11.11)	2(11.11)	5.27 *	0(0)	1 (5.55)	1(5.55)	2.36 NS
Babel	13	8	2 (15.38)	5	0(0)	2(15.38)	5.44 *	1(7.69)	0(0)	1(7.69)	2.41 NS
Diyala	20	9	0 (0)	11	0 (0)	0 (0)	0.00 NS	0 (0)	0 (0)	0 (0)	0.00 NS
Wasit	15	12	2 (13.33)	3	0 (0)	2 (13.33)	5.61 *	1 (6.66)	0 (0)	1 (6.66)	2.15 NS
Al-Qadsyia	30	15	3 (10)	15	3 (10)	6 (20)	0.00 NS	2 (6.66)	1 (6.66)	3 (10)	0.00 NS
Total	423	220	75 (17.73)	203	57 (13.47)	132 (31.20)	0.62 NS	48 (11.34)	36 (8.51)	84 (19.85)	0.77 NS
Chi-square	---	---	8.94**	---	6.82**	10.74 **		6.22 **	8.96 **	9.52 **	----

* (P<0.05)
** (P<0.01), NS: Non-significant
***Detection antibodies against viral NP and NS1 of type A influenza virus

Table.4: Shows the percentage of positive equine influenza serum samples according to ages of horses by NP- ELISA* and NS1 – ELISA *

Test	Age group (year)				
	1-5	6 – 10	11 -15	16 – 20	21 – 25
+ ve NP	1 (0.75%)	30 (22.72%)	67 (50.75%)	32 (24.24%)	2 (1.51%)
+ ve NS1	1 (1.19%)	21 (25%)	42 (50.%)	18 (21.42%)	2 (2.38%)
Chi-square P –value for NS1	11.084 ** 0.00031				
Chi-square P-value for NP	12.094** 0.00267				

*Detection of antibodies against the viral NP of *equine influenza A*
**(P<0.01). NB: The values of age groups were calculated from total positive serum samples by Np-ELISA and NS1-ELISA.

In conclusion, this study approved the equine influenza A seropositive for DIVA horses in Iraq. The results of this study validated the ability of NS1–ELISA as an additional test for serological diagnosis of EIA that can be used to distinguish the naturally infected from immunized horses with inactivated vaccine. Moreover, the result of this study approved that the stallions were more susceptible to equine influenza A, in addition to the susceptibility of all ages.

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