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# Analysis of Different Diagnostis Methods of Influenza in Horses*

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**ABSTRACT**

***Background*:** Equine Influenza is a serious, acute respiratory illness with characteristical clinical signs. The disease is caused by family of Orthomyxoviridae, genera Influenza virus A by two subtypes H7N7 and H3N8. Currently, there is believe that H7N7 has been replaced as a predominant subtype with the H3N8. Horse infection with influenza virus can be detected by serological tests on paired sera using HI test. Commercial rapid tests could be used for the detection of influenza virus. Recently it is widely use a PCR method as fast and more specific methods.

***Materials, Methods & Results*:** Fifty horses and one pony, age between one and 22 years have been included in experiment. Horses were of different race, sex, and age and vaccination status. Ten out of total 51 (10/51) have been regularly vaccinated against EI. Prior to initiation of these study epidemiological survey has been performed. The clinical examination has been followed by blood sampling for blood cell and serum extraction. The serums were evaluated by HI method. Nasal swabs are taken from both nostrils twice, one was frozen for virus detection by RT-qPCR while another was used for detection of EI virus by Directi-gen™ FLU A rapid test. Analysis of titers of antibody reveled that 7 horses (14%) had specific antibodies (IgG) against subtype H7N7, while 9 horses (18%) had specific antibodies against H3N8. In the same time 4 horses had specific antibodies against both subtypes. Serological data confirmed that from 48 horses (96%) had the titer of antibodies greater than 16 against H7N7, while 40 horses (80%) had the specific antibodies (IgG) against H3N8. We found quite unexpected presence of specific antibodies (IgG) for H7N7 in horses that have not been previously vaccinated with H7N7 subtype. These horses never been utilized for sport activity and there was no legal requirements for their vaccination. Could horses with specific antibodies for H7N7 be transfected from vaccinated horses we could not confirme with scientific evidence either we could not have evidence that wild and domestic birds played a significant role especially knowing that horses are dead end of further we could confirm it. Our findings unequivocally confirmed that EI virus subtype H7N7 antibodies (IgG) were present in horses that have not been vaccinated and this is serological evidence that virus H7N7 is circulating in these geographical areas.

***Discussion*:** Fast and reliable diagnosis and isolation of suspicious horses represent the first line of defense against pandemic influenza. Recognition of clinical signs (fever, depression, sharp cough and nasal discharge) along with epizootical survey provides the basis for the early detection of infection. In some cases, cough and rapid spread of symptoms of cough in a group of horses that were either unvaccinated against influenza virus or have been in contact with influenza virus infected horses can clearly point out to the EI virus. The definitive confirmation of EI virus could be done by virus isolation on tissue culture or embrionated eggs followed by detection of virus nucleic acid by RT-PCR. Our data suggest that a substantial number of horses (90%) that have not been vaccinated or vaccinated irregularly had specific antibodies against both subtypes of EI, what suggest that those horses have been exposed to viruses sometimes during their lifetime. Additionally, despite the fact that 20% of horses had some signs of respiratory disease that resemble EI we were not able to confirm EI nucleic acid by RT-PCR while Directi-gene™ assay confirmed virus presence just in two horses. Failure to detect virus nucleic acid could be due to fact that nasal swab samples have been taken at the end of the clinical symptoms, other authors have simular PCR negative patient which displayed a significant rising titre to influenza type A.

**Keywords:** HI test, horse, influenza, PCR.

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## INTRODUCTION

Equine Influenza is a serious, acute respiratory illness caused by family of *Orthomyxoviridae*, genera Influenza virus type A. There are two subtypes of Equine Influenca virus: type 1 (H7N7) and type 2 (H3N8). Last epidemic of H7N7 subtype was confirmed in 1979 [1], and it is now circulating at low levels [17], so there is believe that H7N7 has been replaced as a predominant subtype with the H3N8 [11].

Virus is highly infectious respiratory pathogen of ungulates [6,12] and spreads rapidly in herds of seronegative ungulates. Morbidity range from 10-100% and the mortality rate is 0.5-10% [16].

Clinical signs are cough, pyrexia, dyspnoea, nose discharge, anorexia, depression and inappetence [7].

Vaccination is recommended and the protection is carried out via neutralizing antibodies [8].

Infection can be detected by serological tests on paired sera and increase of the specific antibodies (IgG) four or more times. Titer higher that 10 should also be considered as a positive [2]. It could be measured by: hemagglutination inhibition test (HI), single radial hemolysis (SRH), both recommended by OIE and other tests [10,14].

In Serbia, during the epidemic in 2004 almost 100% of horses seropositive to EI [15], a large number of sick horses was recorded (380), many horses was withdrawn from training and two horses died [15], it were H3N8 subtype [2]. Latest research on donkeys reveled specific antibodies in 26.5% on avian serotypes H5N1 and H7N2 [18].

Virus detection can be carried out by Directigen™ FLU-A assay [5] or virus isolation. Recently it is recommended use of PCR method [9,14].

The aim of this work is to compare the different methods of diagnosis of influenza in horses.

## MATERIALS AND METHODS

### *Animals*

Fifty horses and one pony, age between one and 22 years have been included in experiment. Horses were of different race, sex, and age and vaccination status. Ten out of total 51 (10/51) have been regularly vaccinated against EI. Two horses were previously vaccinated during their race activities but vaccination has been seized three years prior to this evaluation. Horses were kept in different ambient condition and different group size, some were kept in small groups of 1-4, some in groups of 6 horses, while horses kept in horse riding club were kept in group of 13. It is important to mention that nine horses were kept on pasture during the vegetation period and they have been in contact with variety of animal species including the: other ungulates, birds and cattle. In general all horses were exposed to birds because of being held in open or semi-closed stables.

Prior to initiation of these study epidemiological survey has been performed and the owners questioner has been prepared in regards to previous history of disease that could possible resemble EI infection. Clinical examination of horses was performed with emphasis on respiratory tract; clinical examination included adspection, auscultation and measurement of body temperature. Blood sampling for blood cell count and determination of immunological status for EI was performed by blood withdrawal from v. jugular and specific antibodies against EI were evaluated by HI method. Blood for blood cell count was collected into 1.3 mL tube with added EDTA anticoagulant and samples immediately transported to the laboratory. Blood count was performed by horse program with Idexx VetAutored Hematology Analyzer[1]. Blood samples for serology evaluation were collected into vacutainers with clot activator of 10 mL, blood was allowed to spontaneously coagulate and then was left overnight at 4°C, centrifuged at 1,448 *g* in order to separate sera. Sera were kept at - 20°C prior to evaluation for presence of specific antibodies by HI test. HI test was performed by following the guidelines of OIE [11].

### *Reference viruses*

Equine Influenza type A, A1 Prague H7N7 (A/Eqine/1/Prague/56) and Equine Influenza type A2, Miami H3N8(A/Equine/Miami/1/63)[2]. Above mention viruses have been used as reference viruses for HI testing.

### *Nasal Swabs*

Nasal swabs are taken from both nostrils and then submersed into transport media for viruses, chlamidia and mycoplasma[3]. One swab sample was frozen for virus detection by PCR while another swab sample was used to perform rapid test for detection of EI virus by Directi-gen™ FLU A rapid test[4]. Directigen™ rapid test was performed by following the manufacture guidelines.

Detection of the EI virus RNA in nasal swabs was performed by utilizing in-house method based on the methodology of single-TaqMan reverse transcription reaction - polymerase chain reaction in real-time TaqMan based on one step reverse transcription real-time PCR (RT-qPCR) that is amplifying part of the M gene coding for the synthesis of matrix proteins of all influenza A viruses regardless of the type of host (influenza A viruses of birds, horses, pigs, humans etc. Viral RNA was extracted using a commercial kit ISOLATE II RNA Mini Kit[5] according to the manufacturer's instructions. Reaction one-step RT-qPCR was conducted using a commercial kit RNA UltraSense™ One-Step qRT-PCR System[6] with the primers (forward M gene primer SpaMgen F: 5'-AGA TGA GTC TTC TAA CCG AGG TCG-3 'and reverse M gene primer SpaMgen R: 5'-TGC AAA AAC ATC TTC AAG TCT CTG-3') and probe (M gene probe SpaMgen P: 5'-6-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3') that target the matrix protein gene regions of all influenza A viruses [10]. Briefly, each reaction contained 15 µL of reaction mix containing 1 X RNA UltraSense reaction mix, 20 µL of each primer, 10 µM of WNproC probe, 1X ROX reference dye and 1 µL of RNA UltraSEnse enzyme mix., 5 µL of nucleic acid extract samples were added to make a final reaction volume of 20 µL. The termocycling conditions were: reverse transcription 15 min at 52°C, 2 min at 95°C for DNA denaturation and reserve transcription deactivation, followed by 50 cycles of denaturation of 15 seconds at 95°C and product anneling/extention for 50 s at 60°C. All TagMan RT-qPCR reactions were done by 7500 Real Time RT-PCR System Instrument[6]. Quality Controls were included in each reaction. As positive virus control both Influenza H3N8 (A/equine/Miami/1/1963/) and Influenza strain H7N7 (A/Equine/Prague/1/1956) have been used while RNA/DNA free water was used as a negative control.

## RESULTS

During the first clinical examination of horse's clinical signs of disease were diagnosed in 10 animals, clinical signs were slight serous discharge from the nose or mucopurrulent discharge, crepitus, cough, conjunctivitis and enlargement of submandibular lymph nodes. In the second examination these symptoms disappeared or were less pronounced. Displayed results in Table 1 reflect only animals that exhibited one or more clinical symptoms.

**Table 1.** Results of clinical examination of horses.

| No. of animals with clinical symptom | Clinical symptoms | | | | |
|---|---|---|---|---|---|
| | Serous discharge | Mucopur. discharge | Crepitus | Cough | Enlargement of submandibular ln. glands |
| 1 | + | | | + | |
| 2 | + | | | | |
| 3 | + | | | | |
| 4 | + | | | | |
| 5 | | | | + | |
| 6 | | | | + | |
| 7 | | | | + | |
| 8 | + | | | | |
| 9 | | + | + | + | + |
| 10 | + | | | | |
| % of total number of horses | 11,76 | 1,96 | 1,96 | 9,80 | 1,96 |

*Hematological evaluation*

Hematology testing showed that 25 horses had some of the blood parameters outside of the reference values, some horses showed presence of anemia, lymphopenia, leukopenia, leukocytosis, monocitosis or leukocytosis with neutrophilia. Total of 16 horses had haematological parameters outside of normal value. The numbers of animals which had hematological values outside of reference values are shown in the Figure 1.

In figure 1, it can be seen that 4 horses were diagnosed with mild anemia, while leukopenia was diagnosed in 7, leukocytosis in 3, lymphopenia in 13, lymphocytosis in 4, granulocytosis in 7, leukocytosis with neutrophilia in 7.

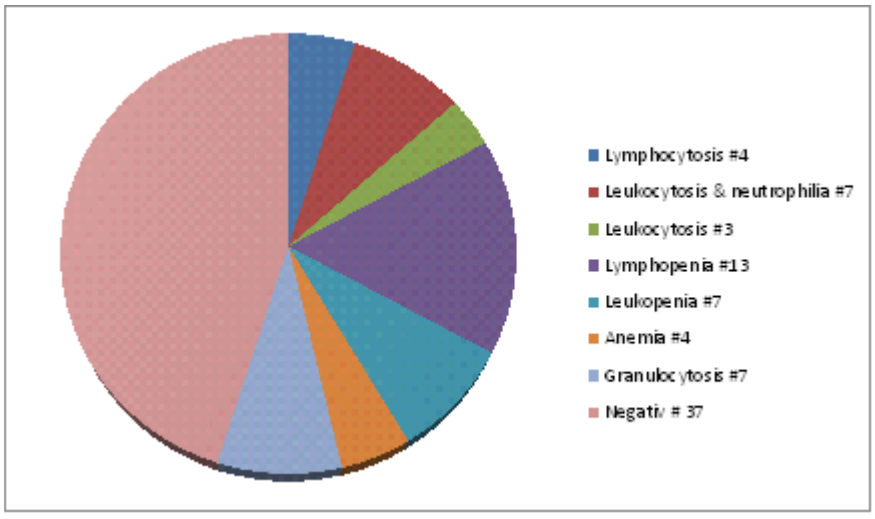


**Figure 1.** Distribution of non physiologycal hematological values.

*Serological evaluation*

Hemagglutination inhibition test revelead that nine horses (18%) were diagnosed with four-fold or greater increase in IgG antibody titer against H3N8 subtype for influenza virus. Additionally, seven horses (14%) had four-fold increase in IgG antibody titers against H7N7 subtype of influenza virus. It is important to notice that four horses (8%) had specific antibodies for both subtypes - H3N8 and H7N7. The results of HI titres are shown in Figure 2.

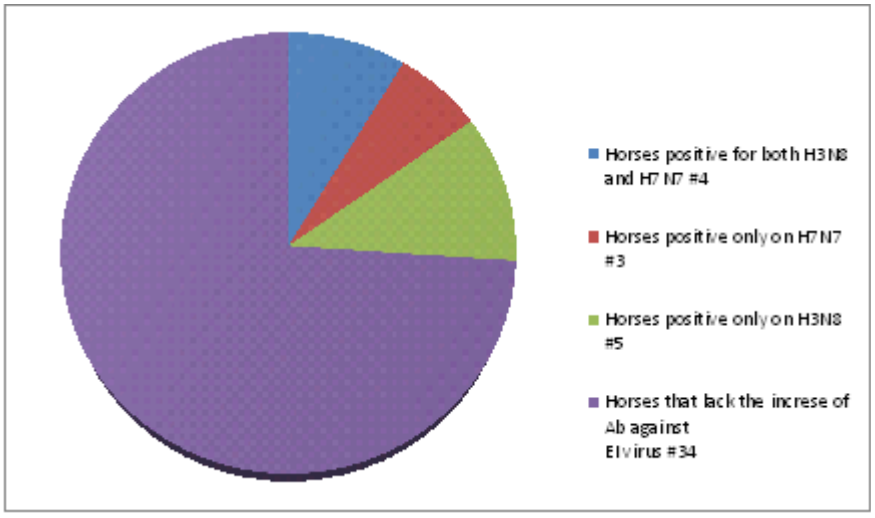


**Figure 2.** Resuls of HI tests on horses.

We followed recommendations of OIE regarding the set test values for HI test and titer of antibodies ≥10 was regarded as a positive. It was concluded that 48 horses (96%) had specific IgG antibodies for H7N7 and 40 horses (80%) were positive for H3N8. It is important to point out that 36 horses (72%) were positive for both virus subtypes; H3N8 and H7N7. The highest HI titer for the H3N8 was 128 while for H7N7 were 64. The standard deviation and the arithmetic mean of antibody titers are shown in Table 2.

All examined swabs were negative on PCR testing.

**Table 2.** Standard deviation and the arithmetic mean of antibody titers in the sera of horses.

| Variable | Descriptive Statistics (Antibody titers) | | | | |
|---|---|---|---|---|---|
| | Valid N | Mean | Minimum | Maximum | SD |
| H7N7 | 51 | 29,25490 | 4,000000 | 64,0000 | 18,52981 |
| H7N7 pared | 51 | 28,62745 | 2,000000 | 64,0000 | 19,95391 |
| H3N8 | 51 | 18,78431 | 2,000000 | 128,0000 | 21,35070 |
| H3N8 pared | 51 | 15,17647 | 2,000000 | 64,0000 | 13,97813 |

## DISCUSSION

Fast and reliable diagnosis and isolation of suspicious horses represent the first line of defense against pandemic influenza outbreaks in horses. Recognition of clinical signs (fever, depression, sharp cough and nasal discharge) along with epizootical survey provides the basis for the early detection of infection. In some cases, cough and rapid spread of symptoms of cough in a group of horses that were either unvaccinated against influenza virus or have been in contact with influenza virus infected horses can clearly point out to the EI virus [7]. The definitive confirmation of EI virus could be done by virus isolation on tissue culture or embrionated eggs followed by detection of virus nucleic acid by reverse–transcription polymerase chain reaction (RT-PCR).

It should be pointed that extensive research has been done on seroprevalence of EI in ungulates. In recent years, other species of equine have been evaluated such as donkeys, mules and ponies. It has been recorded that prevalence of EI among ungulates in Pakistan is around 12% [13], while in Turkey is around 9.4% [1].

In our studies virus detection was performed from nasopharyngeal swabs which have been taken from horses that have been suspicious or had some of the clinical signs that resemble EI disease. Unvaccinated horses or horses without protective antibodies once infected with EI virus, will produce large amounts of the EI virus in their respiratory secretions from 4 to 10 days, while the horses that had previously been exposed to EI viral antigens, either through vaccination program or natural infection with wild strains of influenza virus, produce small quantities of the specific subtype of virus and for a shorter period of time [1]. Literature reveled that PCR is the most sensitive test and widely used [9]. Some authors [4] argue that the virus isolation is more sensitive than ELISA, while other authors [3] had difficulties in isolation of the virus during the epidemic outbreaks, while the ELISA demonstrated rise in specific antibodies for EI virus.

Hematological findings showed that of the total number of horses and ponies 25 had some of the parameters that are outside the reference values. Hematological findings showed the existence of anemia, lymphopenia, leukopenia, leukocytosis, monocitosis or leukocytosis with neutrophilia. Total of 16 horses at least had one hematologhical parameter outside the reference values.

Clinical signs that resemble EI infection has been diagnosed in 13 horses during the first examination, clinical signs quite vary; as a slight serous discharge from the nose or mucopurrulent discharge, crepitus, cough, conjunctivitis and enlargement of submandibular lymph nodes. During the second examination of horses 15 days later symptoms have either disappeared or were less pronounced.

Analysis of titers of antibody reveled that 7 horses (14%) had specific antibodies (IgG) against subtype H7N7, while 9 horses (18%) had specific antibodies against H3N8. In the same time 4 horses had specific antibodies against both subtypes. Serological data confirmed that from 48 horses (96%) had the titer of antibodies greater than 16 against H7N7, while 40 horses (80%) had the specific antibodies (IgG) against H3N8. We found quite unexpected presence of specific antibodies (IgG) for H7N7 in horses that have not been previously vaccinated with H7N7 subtype. These horses never been utilized for sport activity and there was not legal requirements for their vaccination. Questioner also reveled that owners owned horses for a minimum of three years and that they did not vaccinate them. Could horses with specific antibodies for H7N7 be transfected from vaccinated horses we could not confirm with scientific evidence either we could not have evidence that wild and domestic birds played a significant role especially knowing that horses are

dead end of further we could confirm it. Our findings unequivocally confirmed that EI virus subtype H7N7 antibodies (IgG) were present in horses that have not been vaccinated and this is serological evidence that virus H7N7 is circulating in these geographical areas.

## CONCLUSION

In conclusion our data suggest that a substantial number of horses (90%) that have not been vaccinated or vaccinated irregularly and kept free on pasture had specific antibodies against both subtypes of EI (H7N7 & H3N8) what suggest that those horses have been exposed to viruses sometimes during their lifetime. Additionally, despite the fact that 20 % of horses had some signs of respiratory disease that resemble EI we were not able to confirm EI nucleic acid by RT-PCR while Directi-gene™ assay confirmed virus presence just in two horses. Failure to detect virus nucleic acid could be due to fact that nasal swab samples have been taken at the end of the clinical symptoms, other authors [19] have simular PCR negative patient which displayed a significant rising titre to influenza type A.

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