

Detection of Avian Influenza Virus H5N1 in Horses at Assiut Governorate, EgyptMaha I. Hamed^{1*}, Omar A.K. Amen², Hassan Z. Rateb³¹Animal Medicine Department, ²Poultry Diseases Department, ³Veterinary Teaching Hospital- Faculty of Veterinary Medicine- Assiut University, Assiut- Egypt.

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Abstract

The highly pathogenic H5N1 is a major avian pathogen that intensively affects the poultry industry in Egypt, even in spite of the adoption of vaccination strategy. The virus is currently panzootic in Egyptian poultry populations and crosses species barriers to humans and animals. In February 2014, 15 horses at El-Fath center, Assiut, Egypt, started to show mild fever, dullness, restlessness, slight nasal discharge and cough. Two weeks later one of these horses died and another one became recumbent. This was associated with the spread of avian influenza cases in the backyard birds in the same area. Serum samples were collected from the diseased horses and from birds in the same area and examined by haemagglutination inhibition (HI) assay for detection of viral antibodies. At the same time, nasal swabs from horses and tracheal swabs from birds collected and examined by rapid antigen detection and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for detection of the virus. Serum samples of horses showed positive titer with HI in 13 out of the 14 diseased horses and the highest titer was 6-log₂ in the recumbent one. Meanwhile, the result of HI for birds serum samples (450) were negative for 425 samples, which indicate absence of previous vaccination or infection, while the remaining 25 samples were from balady chickens showing HI titer 6-log₂±0.84 and those birds were previously vaccinated 4 weeks before collecting the serum. Avian influenza H5N1 virus could not be detected by using rapid antigen detection strips in the nasal swabs taken from the diseased horses, but could be detected in birds in 102/150 with antigen capture immune-assay (AC-EIA) common antigen type A, 95/150 with (AC-EIA) H5, 0/150 with (AC-EIA) H7 and 0/150 with (AC-EIA) H9 of examined samples. By qRT-PCR, H5N1 virus could be detected only in the nasal swab of the recumbent horse, and in 138/150 tracheal bird swab. In conclusion, Assiut city in Egypt is a disease endemic area where the probability of intimate contact between infected backyard birds and horses is high. Therefore, the disease may be transmitted to these horses from aerosol exposure of infected birds' droppings or contaminated feeds and water or because of direct contact with infected birds. However, the moderate severity of the H5N1 in equine may be responsible for the recovery of most of the diseased horses without further complications.

Keywords: Avian influenza; H5N1; Equine flu; Assiut

Introduction

After introduction of the highly pathogenic Avian Influenza (HPAI) to Egypt in 2006, H5N1 outbreaks had seasonal incidence that is usually accompanied with bird migration (Aly *et al.*, 2008). Later on, the disease became endemic in Egypt (FAO-OIE-WHO, 2011).

Although it is known that the virus can induce interspecies infections (Marschall and Hartmann, 2008), limited studies were carried out to investigate the H5N1 situation among other animals. Al-

though equine influenza known to be caused by H7N7 and H8N3, the H5N1 could be isolated from Egyptian horses and donkeys (Abdel-Moneim *et al.*, 2010, 2011)

Horses and donkeys have a great importance in Egypt, where they are commonly housed together with poultry. Long term endemic influenza virus infections in poultry in Egypt increase exposure risks to surrounding humans and other animals and in turn, create opportunities for the emergence of human-adapted strains with pandemic potential (Matrosovich *et al.*, 1999; Webster *et al.*, 1993). We now recognize that influenza A viruses transmit relatively frequently from the avian reservoir to other birds and mammals, yet they do not typically

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establish permanent lineages in these new hosts (Webster, 2002). Nevertheless, such interspecies transmissions have resulted in the establishment of endemic influenza virus lineages in domestic poultry, pigs, horses and humans. Our study aimed to the detection of H5N1 infection in the diseased horses and in birds at the same area.

Materials and methods

Animals

A total of 15 horses from 8 to 23 years of age (13 male and 2 female) at El-Fath center- Assiut Governorate - Egypt had a history of fever, loss of appetite, reluctance to move, rapid breathing and slight nasal discharge and cough. These horses were treated with anti-inflammatory and antibiotic. Most of these horses recovered, but one horse died after two weeks of showing the clinical signs.

Samples

Horses

Serum samples were collected from the 14 diseased horses at El-Fath center, Assiut Governorate, Egypt. Nasal swabs were collected from these horses either; each swab was placed in a tube containing 0.5 ml sterile normal saline containing gentamicin sulfate solution (50 mg/ml). The swab tip was cut off in the saline and the tubes. Samples were immediately transported in an icebox to the research laboratory at the Faculty of Veterinary Medicine, Assiut University. Serum samples were stored at -20°C and inactivated at 56°C for 30 minutes before the use in hemagglutination inhibition (HI) assay. All nasal swabs were examined by Rapid antigen detection kit (type A, H5, H7 H9) and real-time reverse transcription PCR.

Fecal samples were collected from the 14 diseased horses and from the dead one in clean and dry cups, and transported directly in an icebox to the laboratory.

Birds

We collect 450 serum samples from backyard birds in the same area (280 from Balady chickens, 120 from ducks and 50 from turkeys). All serum samples were checked by the HI test against H5 anti-

gen. 150 tracheal swabs were collected from freshly dead birds and diseased birds showing signs of avian influenza (cyanosed comb and wattle with hemorrhage on the shank); all tracheal swabs were examined by Rapid antigen detection kit (type A, H5, H7 H9) and real-time reverse transcription PCR.

Rapid antigen detection

All tracheal and nasal swabs were screened with rapid avian influenza detection strips using Type A, H5, H7 and H9 Kits (Anigen, 2-9 Seogu-dong, Hwaseong-si, Gyeonggi-do 445-170 Korea).

Haemagglutination inhibition assay

This assay was conducted as described earlier (OIE, 2004). The HI assay was performed on serum samples in round bottom 96-well plates using 1% chicken RBCs, two-fold serially dilutions of each serum sample were incubated at 37°C for 1 h with 4 HA units of Inactivated AIV haemagglutinin (H5) antigen was obtained from the Veterinary Laboratory Agency New Haw, Addlestone. Surrey, KT15 3NB, UK. After incubation, 25 µl of 1% chicken RBCs was added into each well and plates were incubated at room temperature for 45 minutes and the results were noted. The reciprocal of the highest serum dilution that yielded a positive result is considered as the HI titer.

Viral RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) RNA was extracted from extracts of nasal swabs from horses and tracheal swabs from chickens using a QIAamp Viral RNA Mini Kit (Qiagen, Germany, QIAGEN Strasse 1, 40724 Hilden) according to the manufacturer's instructions. The RNA samples were stored at -80°C until use.

qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction was carried out using fluorometric PCR thermocycler (Mx3000P), TaqMan® Influenza A Detection kits (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA, 94404, United States): 30 µl mixtures containing 3 µl 10x reconstitution buffer, 17 µl nuclease-free water and 10 µl RNA sample or RNA positive control or negative control into the appropriate tubes, Beads dis-

solved in 1 to 5 seconds then Run the plate in real-time (RT) thermal cyclor. Thermal profile: RT steps were carried out at 95°C for 1 min: 60°C for 15 min and 95°C for 5 min followed by 40 cycles of amplification at 95°C for 20 seconds and 60°C for 1 min.

Fecal examination

Parasitological analyses of fecal samples were done on the same day of collection. Fecal examination was carried out by using sedimentation and concentration floatation techniques, and counting of the detected eggs was done by McMaster's technique according to Soulsby (1982).

Results

Field Examination

Horses

The diseased horses showed mild fever for one to two days with loss of appetite, reluctance to move, rapid breathing, slight nasal discharge and cough. Treatment with anti-inflammatory and antibiotic, the horses return to normal, but after a few days, they started to show the same symptoms again. In most of these horses, the signs were mild and horses recovered, but in two of them, it became severe, and the horses were recumbent with shallow breathing and stop eating and one of these two horses finally recovered and the other one died after two weeks of showing the clinical signs.

Birds

The clinical signs in diseased birds varied from cyanosis of comb and wattles, nervous signs, facial edema, respiratory signs and greenish-whitish to bloody diarrhea, and decrease in egg production and eggshell quality and high mortality reach to 100%. At necropsy, subcutaneous hemorrhages, and severe congestion of pectoral and thigh muscles were observed. The liver and spleen were enlarged and severely congested. There was congestion of blood vessels of mesenchyma and intestinal serosa, whereas the intestinal lumen was filled with blood. The kidney was severely enlarged and pale with urate depositions in the ureters. In addition, congestion of the pancreas and cecal ton-

sillitis was observed.

Results of Rapid antigen detection

Horses

We could not detect avian influenza H5N1 virus by using rapid antigen detection strips in the nasal swab taken from the diseased horses.

Birds

Avian influenza H5N1 virus was detected in 102/150 with antigen capture immune-assay (AC-EIA) common antigen type A, 95/150 with (AC-EIA) H5, 0/150 with (AC-EIA) H7 and 0/150 with (AC-EIA) H9 of examining the samples.

Results of Hemagglutination Inhibition Test

Horses

Serum samples showed positive titer with HI in 13 out of the 14 diseased horses and three cases gave titer 3-log₂, five cases gave titer 4-log₂, four cases gave titer 5-log₂ and the highest titer was 6-log₂ in the recumbent horse.

Birds

The result of HI for serum samples were negative for 425 samples, which indicate absence of previous vaccination or infection, while the remaining 25 samples were from Balady chickens showing HI titer 6-log₂±0.84 and those birds were previously vaccinated four weeks before collecting the serum.

Results of Virus Detection by Real-Time Reverse Transcription PCR

Horses

Avian influenza H5N1 virus could be detected in the nasal swab of the recumbent horse, but could not be detected in the swabs from the other horses.

Birds

Avian influenza H5N1 virus was detected in 138/150 with qRT-PCR.

Results of fecal examination

Strongylus vulgaris eggs were detected in the examined fecal samples. By counting of the detected eggs, we found that it was a mild infestation (below 100EPG feces) in all horses, but was a severe infestation (more than 1000 EPG feces) in the recumbent dead one.

Discussion

The highly pathogenic H5N1 is a major avian pathogen that crosses species barriers and seriously affects humans as well as some mammals (Abdel-Moneim *et al.*, 2010). The investigation of possible hidden or new sources of the virus is necessary for the performance of efficient control program in Egypt (El-Sayed *et al.*, 2013).

Our study showed that horses was suffering from mild fever, loss of appetite, rapid breathing and slight cough; two horses did not respond to treatment with antibiotic and anti-inflammatory and one horse died. The inhibition induced by antibiotic may be due to the possible secondary bacterial invasion, besides the moderate severity of the H5N1 in equine may be responsible for the recovery of the other horses without further complications (Abdel-Moneim *et al.*, 2010)

Although H5N1 crosses species barriers and affects horses, it cannot lead to serious disease in horses may be due to absence of its specific receptors in this host, which explained before by Chambers *et al.* (2013), who demonstrated that the equine tracheal explant model is a poor predictor of AIV infectivity in the upper respiratory tract of live equines. Also, Connor *et al.* (1994) studied the correlation of receptor specificity of influenza virus with species of origin, and they found that in case of equine viruses, the presence of potent glycoprotein inhibitor $\alpha 2$ macroglobulin in equine serum represents a potential selective pressure that would account for the extreme insensitivity to inhibition by horse serum.

In addition, (Abdel-Moneim *et al.*, 2010) reported that the H5N1 virus induced only mild to moderate disease in donkeys. This would suggest that equines might harbor genetic factors that enable them to prevent the fatal consequences or that the virus has not yet fully adapted to infect domestic donkeys (Abdel-Moneim *et al.*, 2011). So most of the affected horses responded to treatment and

recovered except the recumbent horse. We tried to figure why the condition was getting worse in this horse and led to its death, and by fecal examination of the stool samples of these horses, we found infestation with *Strongylus vulgaris* eggs. It was a mild infestation (below 100EPG feces) in all horses but the recumbent dead one was severely infested (more than 1000 EPG feces). Therefore, this may be caused the condition of that horse getting worse and finally died.

On the other hand, the H5N1 virus is devastating disease in chicken and turkeys. The Birds in the same area around these horses showed the characteristic clinical signs and necropsy finding of the disease. Assiut governorate as well as the rest of Egypt is a disease endemic area (ECDC, 2007) where the probability of intimate contact between infected backyard birds and horses is high. Therefore, the disease may be transmitted to these horses from aerosol exposure of infected birds' droppings or contaminated feeds and water or because of direct contact with infected birds (Abdel-Moneim *et al.*, 2010)

HI test is the standard test for diagnosis of avian influenza infection (El-Sayed *et al.*, 2013). In our study, we did test all the diseased horses by HI test that give positive results on all of them, thus proved the infection of these horses with H5N1 virus. On the other hand, examination of the nasal swabs by using RT-PCR were positive only in one sample taken from the recumbent horse. This agreed with the results of Chambers *et al.* (2013), who found that most nasopharyngeal swabs from experimentally infected horses were negative by qRT-PCR.

Regarding result of HI test from unvaccinated birds they didn't have any levels of antibodies, which explain absence of previous vaccination nor infections, while samples collected from vaccinated birds showed reasonable levels of HI titers between 6-log₂, which should be protective levels of the antibodies as described by Swayne (2006). The Serological Surveillance of H5 In the backyard confirm improper vaccination of the backyard in frequency, route, and preservation of the vaccine. Although there was an obligatory program to vaccinate all backyard flocks for free, but obviously it didn't serve the potential purpose.

Those backyard birds complicate the problem of controlling avian influenza, through becoming susceptible to infection and surviving the avian influenza viruses and maintain it in the environment

to infect another host because the viruses cannot stay viable for a long time in the environment especially in high temperature. In addition, duck can shed the virus for a long time after infection. In addition to the vaccinated birds with low level of antibodies can accelerate the incidence of antigenic drifts through the selection pressure (Lee *et al.*, 2004).

Regarding result of virus detection, the comparison between the different detection systems for avian influenza viruses from tracheal swabs revealed the sensitivity and specificity for a commercial antigen capture enzyme immunoassay (AC-EIA) is less than nucleic acid detection test real-time RT-PCR (RRT-PCR). Similarly, data obtained from both experimental and field studies suggested a higher sensitivity of the PCR-based methods compared with the AC-EIA, and the economical and practical implications of using one of the rapid tests as an alternative to virus isolation during an avian influenza epidemic are very important (Cattoli *et al.*, 2011).

Conclusion

We demonstrated that H5N1 might be transmitted from poultry to horses. These findings extend the host range of the H5N1 influenza virus, and highlight the need for the detection of H5N1 virus in animals near backyard poultry units, especially in the endemic areas like Egypt.

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