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Isolation of H9N2 Influenza Virus from Backyard Ducks in Egypt

Samar M. Fiala[1], Ahmed R. Elbestawy[2], Madiha S. Ibrahim[1*]
[1]Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Egypt
[2]Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, Egypt

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***Correspondence to:**
madihasalah@yahoo.com

ABSTRACT

H9N2 influenza virus has a great potential for causing severe economic loss in ducks especially in backyard ducks. The current study was conducted to isolate H9N2 influenza virus from backyard ducks and preparation of antiserum against isolated H9N2 influenza virus in rabbits and further evaluation by neutralization test. Samples from liver, spleen, brain and lung were collected from clinically infected ducks during 2014 and 2015. The viruses were isolated and propagated by inoculation of 10 days old embryonated chicken eggs via the allantoic sac. The allantoic fluid was harvested and tested for the titration of the HA activity using chicken erythrocytes. Avian influenza virus type A antigen was detected by flu detect strip test. The RNA was extracted from HA-positive allantoic fluid and identified by RT-PCR using specific primers for H9N2 virus. Antiserum against H9N2 virus was prepared in rabbits. The produced antibody was tested and titrated by HI test against H9N2 isolates from ducks and chickens as well as field samples from ducks and H5N1 isolated from chickens. Neutralization test was also done for evaluation H9N2 antibody protective titer. Ten samples from ducks were HA positive with titer values of 2^{-5} to 2^{-10}. The HA titer of H9N2 isolates from chickens ranged from 2^{-4} to 2^{-11}. Also, 16 field samples from ducks were HA positive with titer values of 2^{-3} to 2^{-7} and the HA titer of H5N1 isolates from chickens ranged from 2^{-7} to 2^{-12}. The results of RT-PCR showed that the 10 samples from ducks were positive for H9N2 HA gene. The HI results showed complete inhibition of H9N2 isolates from ducks and chickens. HI test for field samples from ducks showed complete inhibition of 16 field samples. On the contrary, HI test for H5N1 isolates from chickens showed no inhibition of 13 isolates using H9N2 rabbit specific antiserum. The neutralization index using H9N2 virus was 2^{-5}. The presence of LPAI H9N2 may add another risk factor to the poultry industry in Egypt together with the endemicity of HPAI H5N1, especially that backyard ducks and chickens are reared in direct contact allowing easy virus transmission and adds more stress to the poultry populations. Further, false negative results for the detection of H5N1 using H9 antibodies should be considered.

1. INTRODUCTION.

Avian influenza (AI) is a highly contagious viral disease affecting several species including birds. AI viruses a classified into highly pathogenic avian influenza (HPAI) virus and low pathogenic avian influenza (LPAI) virus, depending on the severity of the disease in susceptible birds (OIE, 2008). HPAI outbreaks in birds have been caused mainly by H5 and H7 subtypes, while H9 and some strains of the H5 and H7 subtypes have been characterized as LPAI (Zhou et al., 1999).Avian influenza is caused by type A influenza virus of the family Orthomyxoviridae. Type A influenza viruses are serologically categorized according to antigenic differences of 2 surface

glycoproteins into 18 HA (H1- H18) and 11 NA (N1-N11) subtypes (Tong et al., 2012, 2013).

Avian influenza virus is enveloped and pleomorphic with 80- 120 nm diameter. The genome of type A influenza is single- stranded, negative sense RNA and contains eight genome segments that encode 11 proteins (Spackman, 2008).

In November 2011, H9N2 infection was first reported in Egypt in bobwhite Quail (El-Zoghby et al., 2012). H9N2 viruses are endemic in poultry populations causing mild to severe respiratory signs, such as coughing, sneezing, rales, rattles and excessive lacrimation (Abdel-Moneim et al., 2012; Corrand et al., 2012). It also causes reduction in egg production in breeder or layer flocks, as well as a slightly increased mortality if there are no secondary viral and/or bacterial infection or poor environmental conditions (OIE, 2008; Pillai et al., 2010). While the HPAI H5N1 viruses are 100% lethal for chickens and other gallinaceous poultry.Ducks can shed influenza virus from both respiratory and intestinal tracts while showing few or no clinical signs. They also survive infections with the highly pathogenic avian influenza strains. They are able to fly for long distances, while simultaneously being carriers of HPAI H5N1 (Truszczynski and Samorek 2008).The spread of H9N2 in Egypt can negatively affect the overall poultry health and increase the risk of infections with endemic H5N1 HPAI in Egypt. (Park et al., 2011).

Here, we aimed to isolate H9N2 virus from backyard ducks suffering from respiratory manifestations and greenish diarrhea. Further, preparation of antiserum against H9N2 virus in rabbits and testing it by HI and Neutralization tests.

1. MATERIALS AND METHODS

2.1. Sample collection and preparation

Samples from liver, spleen, brain and lung were collected from backyard reared clinically infected ducks (n= 10) of different ages (from 20 days to several months old) during 2014- 2015. These ducks suffered from greenish diarrhea, twisting of the neck and sudden death and sometimes death without any symptoms. The samples were prepared and stored at -80˚C according to OIE, 2012.

2.2. Virus isolation

Samples were inoculated in 10 days-old embryonated chicken eggs via the allantoic route using 200 µl/egg. The eggs were incubated at 37˚C for 7 days and observed daily. Eggs showing embryonic death within 24 h of inoculation were discarded. Whereas eggs showing embryonic death after 24 h and up to 7 days were chilled. Allantoic fluid from each of the inoculated eggs was harvested (OIE, 2008) and tested for hemagglutination using U-shape microtiter plate and 1% chicken RBCs as described by Alexander, DJ. 2009: OIE, 2012 and kept at -80˚C.

2.3. Detection of influenza virus antigen

Avian influenza virus type A antigen was detected using A flu detect test kit (a kind gift from Prof. K. Ikuta, Department of Virology, RIMD, Osaka University, Japan) according to Hamamoto et al., 2007.

2.4. RNA Extraction

Total RNA was extracted from the HA-positive allantoic fluid obtained after virus isolation by total RNA purification kit (QIAGEN, USA) according to the manufacturer's instructions. The purified nucleic acids were stored at -80˚C.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

H9N2 virus was identified by conventional RT-PCR. Reaction mix was performed according to the manufacturer´s instructions of Thermo Scientific Verso 1-Step RT-PCR Ready-Mix kit. PCR using H9N2 specific primers was performed according to lee et al., (2001) to amplify 488-bp fragment of H9N2 HA gene. H5N1 virus was identified by conventional RT-PCR. Reaction mix was performed according to the manufacturer´s instructions of Thermo Scientific Verso 1-Step RT-PCR Ready-mix kit. PCR using H5N1 specific primers was performed to according to (Capua and Alexander, 2004) to amplify 300-320 bp fragment of H5N1 HA gene.

2.6. Titration of H9N2 influenza virus

A total of 900 µl of diluent (PBS) was dispensed to all labeled sterile Eppendorf. 100 µl of the virus suspension was transferred to the first tube and mix with micropipette and this is the first Eppendorf of ten-fold dilution. Second ten-fold dilution was carried out with micropipette with new sterile tip. The series of ten-fold dilutions was continued in the same manner until the last Eppendorf. Inoculation via allantoic route in 5 eggs for each dilution. Eggs were incubated for 7 days at 37 °C. The end points of the virus titers were determined by Reed and Muench method (1938). ID_{50} was equal to $10^{-9.5}$

2.7. Preparation of antiserum against H9N2 influenza virus in rabbits

Immunization with H9N2 virus was performed according to Green and Manson, 1992; Cooper and Paterson, 2009. Blood samples from marginal ear vein were collected 10 days following the last booster dose.

The antiserum was collected, tested then stored in small aliquots at -20˚C. Animal handling was done according to Damanhour University ethical regulations for animal use.

2.8. Hemagglutination inhibition test

Hemagglutination inhibition test was done using H9N2 isolates from ducks, randomly collected field samples from ducks (n=29) and influenza virus (H9N2 and H5N1) isolates from chicken (Obtained from Poultry and Fish diseases department, Faculty of Veterinary Medicine, Damanhour University) using reference antigen for H9N2 and H5N1 viruses supplied from Harbin Weike Biotechnology development company and specific antiserum against H9N2 virus. HI test was done using U-shape microtiter plate and 1% chicken RBCs against 4HA units of virus antigens as described by OIE, 2012.

2.9. Neutralization test for H9N2 and H5N1 isolates

The H9N2 rabbit antiserum was inactivated by heating at 56°C for 30 minutes. Five- fold dilutions of antiserum were reacted in equal volumes with constant viral amount (ID50 = $10^{-9.5}$**)**. Virus suspension was mixed with serum and incubated for 60 minutes at 37°C then inoculated into embryonated chicken eggs allantoic cavity. End points of the serum titers were determined by Reed and Muench method (1938). The results were expressed as a neutralization index (NI) that represents the $\log_2$ difference in the titers of the virus and the virus/serum mixtures as described by OIE Terrestrial Manual, 2013.

3. RESULTS

3.1. Virus detection by HA test

Duck samples were all HA positive (N=10) with titers ranging from 2^{-5} to 2^{-10} while field samples from ducks showed 55.1% HA positive result with titers ranging from 2^{-3} to 2^{-7}. H9N2 isolates from chickens showed HA titers ranging from 2^{-4} to 2^{-11} while H5N1 isolates from chickens showed HA titers ranging from 2^{-7} to 2^{-12}.

3.2. Detection of influenza virus type A antigen

By FLU DETECT strip test (POCTEM S influenza, Japan) rapid test for the detection of avian influenza virus type A antigen, all samples were positive for influenza virus A antigen.

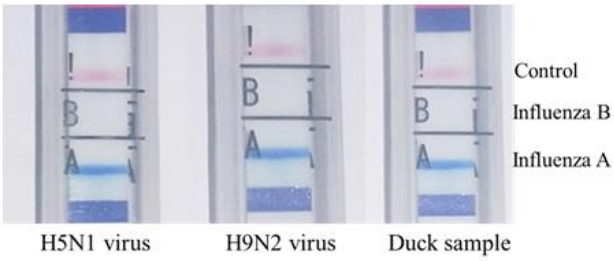

Figure (1): Influenza virus type A antigen detection by flu detect kits.

3.3. Identification of influenza virus by RT-PCR

H9N2 virus was identified by conventional RT-PCR using H9N2 specific primers to the 10 duck samples. PCR was performed to amplify 488-bp fragment of H9N2 HA gene of avian influenza virus. Identification of H9N2 virus by RT-PCR using H5N1 HA gene specific primers showed negative results. This H9N2 isolate was used for rabbit immunization.

3.4. Titration of H9N2 influenza virus

Using 100 µl of virus per well, Ten-fold serial dilutions was done. End points of the virus titers are determined by Reed and Muench method (1938). The results showed that ID_{50} of H9N2 influenza was equal $10^{-9.5}$

3.5. Hemagglutination inhibition test using H9N2 rabbit specific antiserum

This test was done using U-shape microtiter plate and 1% chicken RBCs against 4HA units of virus antigens**.** Inhibition of H9N2 viruses derived from ducks and chickens could be achieved using rabbit-H9N2 specific antiserum. Further, field samples from ducks could also be inhibited using the same antiserum.

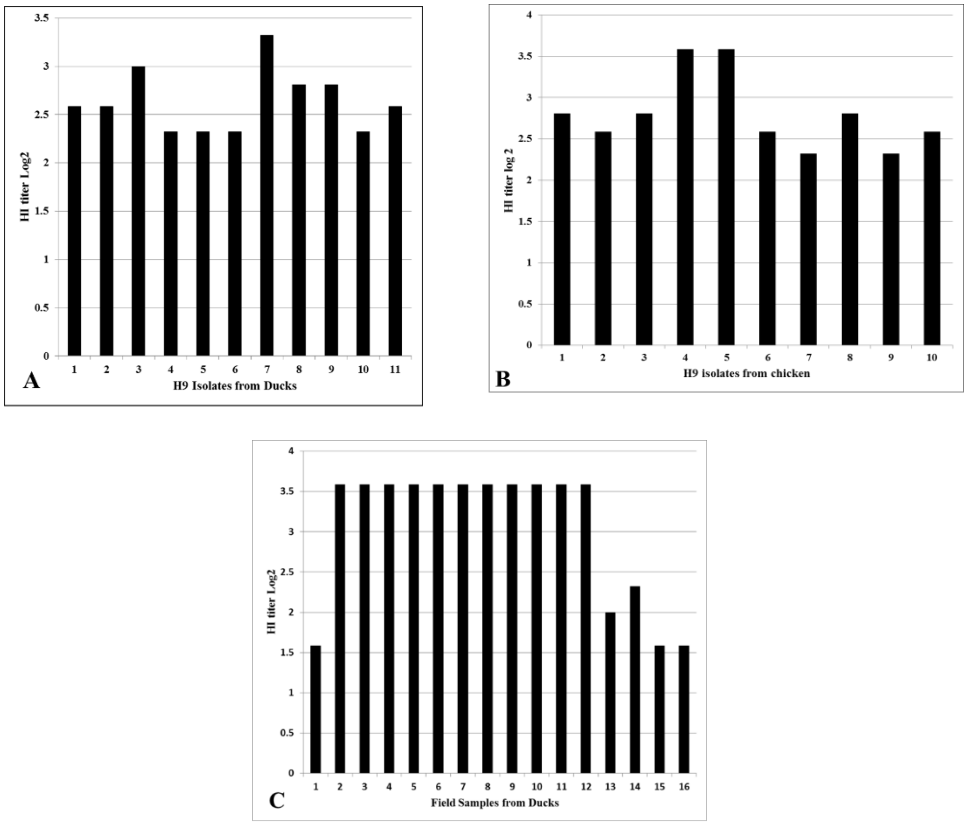

Figure (2): HI test using rabbit H9 specific antiserum. H9 isolates from ducks (A); chickens (B) and field samples from ducks (C). No. 11 in A is the reference antigen for H9N2 virus.

Figure. 2 showed complete inhibition of 10 H9 isolates from ducks (A), complete inhibition of 10 H9 isolates from chickens (B) and complete inhibition of 16 field samples from ducks (C) using rabbit- H9N2 specific antiserum.

On the contrary, no inhibition could be detected with 13 H5N1 isolates from chickens using rabbit H9N2 specific antiserum.

3.6. Neutralization test for H9N2 and H5N1 isolates using anti-H9N2 rabbit antibodies

Neutralization index was equal to 2^{-5} with H9N2 virus (OIE Terrestrial Manual, 2013). However, the H9N2 rabbit antibodies were not protective against H5N1 virus.

4. DISCUSSION

The H9N2 virus is a notable member of influenza A genus as it can infect not only avian species but also, although sporadically, mammals such as pigs and humans **(**Butt et al 2005). H9N2 viruses of gallinaceous poultry spread from Asia to most Eurasian countries during the 1990s (Aamir et al., 2007 and Alexander, 2007). It has been detected in domestic poultry in several neighboring Middle Eastern countries (Brown et al., 2006; Perk et al., 2006; Aamir et al., 2007; Roussan et al., 2009 and Moosakhani et al., 2010).

Low pathogenic AI H9N2 was first reported in Egypt in November 2011; the isolated virus was closely related to viruses of the G1- like lineage isolated from neighboring countries, indicating possible epidemiological links (El-Zoghby et al., 2012). AIV subtype H9N2 was very common among domestic poultry in Egypt. The highest percentage of AIV detected was among turkeys and ducks that appeared to be healthy. Ducks in Egypt, like those in other regions, play a key role in AIV transmission (Kim et al., 2009). The positive H9N2 cases recorded during winter more than that in the summer, that supports the theory of increasing the activity of H9N2 AIVs by low temperature and it agreed with Naeem et al. (1999)and (2003) who found that AIV H9N2 caused lesions and mortalities during winter.

H9N2 viruses, although of low pathogenicity, are correlated with increased severity because of co-infection with other poultry viruses (Bano et al., 2003; Kishida et al., 2004; Haghighat- Jahromi et al., 2008) thus, they indirectly might lead to economic losses for the poultry industry.

The present study described H9N2 virus isolation from backyard ducks that suffered from respiratory manifestation and greenish diarrhea during the period from 2014 to 2015.

The allantoic fluid was harvested and tested for the presence of HA activity titration using chicken erythrocytes (Kilany, 2007 and Safwat, 2012). Ten samples from ducks were found positive for microtiter plate HA test with titer value of 2^{-5} to 2^{-10}. The HA titer of H9N2 isolates from chickens ranged from 2^{-4} to 2^{-11}. Also, 16 field samples from ducks were found positive with HA titer values of 2^{-3} to 2^{-7}. The HA titer of H5N1 isolates from chickens ranged from 2^{-7} to 2^{-12}.

Influenza type A antigen was detected in samples using POCTEM S influenza kit. It has type-specific high sensitivity and specificity for the detection of influenza A (Hamamoto et al., 2007) and B viruses (Hara et al., 2005, Takahashi et l., 2005, Hamamoto et al., 2007), however, it does not discriminate between the influenza A virus subtypes. Even though POCTOM S influenza kit cannot discriminate between avian influenza viruses, it can with high specificity identify influenza viruses of avian origin making it a kit of choice for rabid field diagnosis.

H9N2 virus was identified by conventional RT-PCR using H9N2 specific primer (lee et al., 2001). The results showed that 10 samples from ducks were positive.

H9N2 virus was identified by RT-PCR using H5N1 specific primer before preparing antiserum in rabbits. The result of RT-PCR for this isolate was negative for H5N1 virus as agreed with Capua and Alexander**, (2004)** who showed amplification of 300-320 bp fragment of H5N1 HA gene and the PCR products were separated in 2% agarose gel and visualized under ultraviolet light.

Antiserum for H9N2 virus was prepared and the produced antibody was tested and titrated by HI test (Alexander, DJ.2009: OIE, 2012) for H9N2 isolates from ducks and chickens and the results showed complete inhibition of the isolates so H9N2 rabbit antiserum is specific to H9N2 isolates. HI test was performed for field samples from ducks and the results showed complete inhibition of 16 field isolates using H9N2 rabbit antiserum. In addition, HI test for H5N1 isolates from chickens was done and the results showed no inhibition of 13 isolates using the prepared antiserum so H9N2 antiserum is not specific to H5N1 isolates

Neutralization test for H9N2 and H5N1 viruses was done using anti-H9N2 rabbit antibodies. The results showed that the neutralization index for H9N2 virus was 2^{-5} which indicates H9N2 virus was homologus to H9N2 rabbit antibodies. On the contrary, the results showed that embryonated chicken eggs inoculated with H9N2 antiserum and H5N1 isolate died within 12hrs so the H9N2 rabbit antibodies was not protective against H5N1 virus.

The presence of H9N2 in backyard ducks may add another risk factor to the poultry industry in Egypt, especially with the endemic situation of HPAI H5N1 and the presence of other pathogens with low biosecurity levels. Vaccination however, cannot be used alone for the control of AI and must be accompanied by other control measures including quarantines, controlled depopulation and increased surveillance. Surveillance approaches depending on serodiagnosis using H9 antiserum should consider the difference in the reactivity and the possibility of false negative results.

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