In vitro study on H9 avian influenza cytopathic effect in Al-Najaf Al-Ashraf/Iraq

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Abstract

During a period extending from July 2012 to July 2013, a total of 251 avian cases admitted for medically care unit in the veterinary hospital in Al-Najaf governorate were examined and involved in this study. The study was applied to determine risk factors affecting by avian influenza virus infection among poultry flocks in Al-Najaf-Iraq. The study revealed that poultry flocks had a considerable risk of infection with H9 subtypes according to the PCR assay. Primarily, infected birds were investigated by rapid test for the detection of type A of influenza viral antigen, based on monoclonal antibodies. Identification was also confirmed by PCR assay for the amplification of a specific region within the H9 genome. Propagation and adaptation of 5 strong positive H9 isolates on rhabdomyosarcoma cells (RD) and chick embryo fibroblast was done by cell culture techniques, for the demonstration of specific type of cytopathic effect (CPE) caused by these isolates. The observation of specific H9 cytopathic effect was also confirmed by indirect immunofluorescent antibody technique. The two types of cells involved different degrees of sensitivity for supporting the growth of H9 virus; RD cell line was sensitive enough to support the growth of the virus, followed by chick embryo fibroblast cells. The half tissue culture infective dose (TCID50) of the virus was elevated with the subsequent passages till reach its peak (10^8 TCID50/0.1ml) during 6th passage on RD cells and 10^5 TCID50/0.1ml during 7th passage on chick embryo cells. Our results indicated that H9 type is dominant in Najaf area, RD cell line is typical for culturing the virus, the addition of trypsin is very helpful for the cultivation of H9 influenza virus. Vaccination and increasing workers knowledge could prevent future infections.

Key words: Avian influenza, real-time PCR, cell culture assay, trypsin, cytopathic effects

In Anفلونزا الطيور H9 للنط المتصلي

دراسة الأثر المرضي للنمط المصلي H9 لأنفلونزا الطيور

في محافظة النجف الاشرف - العراق

خالدة كاظى ظاسب الكلابي
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الخلاصة

تعرضت هذه الدراسة لحصص 251 حالة من حالات الإصابة في الطيور، وللقرية من الشهر السابع من عام 2012 إلى الشهر السابع من عام 2013، والتي تم جلبها إلى المختبر التشريحي في المستشفى البيطرى/النجف. وقد تم تصميم هذه الدراسة للتقصي عن الإصابة بأنفلونزا الطيور في الحقل الخاص بتربية الدواجن في هذه المنطقة من العراق بشكل خاص ومن العالم عامة. وقد أظهرت هذه الدراسة وجود الإصابة بالنوع H9 وبشكل معنوي في المنطقة في الدراسة. وقد تضمنت monoclonal antibodies والمعتمد على الأصداء أحادية النسيجية rapid test للانتجبي اثبات الفحص المباشر للإصابة بالأنفلونزا الطيور، يمنع ذلك فحص العينات قبلية لمرة الحاضر A نوعية والخاصة بالنوع المستضدي A أنفلونزا الطيور ببعض هذه العينات. كما تم اتخاذ بعض العزلات (5 عينات) لدراسة الأثر المرضي الناجم عن هذا النوع من الفيروسات باستخدام نوعين من الخلايا تمثلت بخلايا cytopathic effect

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Introduction

Influenza is an infection and/or disease syndrome caused by type A influenza virus, a member of the Orthomyxoviridae family. Influenza A viruses are accountable for major disease problems in birds, as well as in humans and lower mammals (1). Antigenic differences based on nucleoprotein (NP) and matrix (M1) protein form the basis for the classification of influenza viruses into types A, B and C. Each type of avian influenza viruses have been categorized as type A. Two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) are antigenic determinants which further subtype the avian influenza viruses (2). The combination formed by one HA and one NA protein is used to name the virus subtype (3). Bird flu is known as H5N1 virus, being a combination of HA 5 and NA 1 proteins. There are also H7 and H9 types of bird flu. Avian Influenza viruses are also classified by their level of pathogenicity, or virulence. Highly pathogenic avian influenza (HPAI) has a high mortality rate in poultry; capable of killing between 90 and 100% of infected chickens (4). Low pathogenic avian influenza (LPAI) causes less severe symptoms. These transmembrane glycoproteins are capable of eliciting subtype-specific and immune responses which are significantly protective within, but only partly protective across, different subtypes. On the basis of antigenicity of these glycoproteins, influenza A viruses group into sixteen HA (H1–H16) and nine NA (N1–N9) subtypes (5,6). Avian influenza (AI) is a viral, highly contagious disease of domestic and wild birds and have often infected humans (2,7). The genotype (H9N2) viruses had been demonstrated to be the predominant strains circulating in poultry in this region and were responsible for most cases of human infection during the period of the survey (8,9). Influenza A viruses of subtype H9N2 are now considered to be widespread in poultry and have demonstrated the ability to infect humans (10). In Iraq, H9N2 subtype outbreaks have been frequently recorded recently (11). Some earlier studies about the genetics characterization of the gene segments have indicated that H5N1 viruses were generated by reassortment. In this regard avian influenza serotype H9N2 virus is a major donor of the internal genes including three polymerase genes (PB, 2 PB and PA) and Nucleoproteins (NP), Matrix (M) and 1 Nonstructural (NS genes). This indicates that in the presence of different serotypes of influenza viruses in the field, there is always the likelihood of generating new viruses by gene reassortment between serotypes pathogenic to birds and mammals (22,6). This signifies the potential of H9N2 as a reservoir of genes capable to cause infection in humans. The host range of H9 overlaps with H5, which presents an opportunity for H9 to acquire genetic material from strains that are virulent in humans. Thus, in southern China, H9 and H5 present the largest risk of spill-over infections to humans. While many influenza A subtypes have a strong host preference for ducks, H9 is well-adapted to chicken and quail, and H5 is adapted to all three of these dominant host species (8). The main goal of the analyses described here was to investigate the prevalence of H9 Al-Najaf live-birds, in addition to the investigation of these viral isolates via cell culture assays.
Materials and methods

Study subjects and case definition

A total of 251 avian cases suspected to be infected with avian influenza subtype H9 admitted for medically care unit in the veterinary hospital governorate were involved in Al-Najaf governorate. Data about bird flock obtained according to the statement of questionnaires including information about location and vaccination history. The Pearson $\chi^2$ test was used to compare differences between groups. Differences were considered significant if $p$ value was <0.05.(12).

Isolation and identification of H9 virus

Avian samples were collected and avian flu type A antigen (EVL corp. Netherland) was detected using rapid kit test. 60 different broiler flocks showing severe respiratory signs with mortality up 25-60%. All birds had the history of already oily vaccination against Avian influenza H9N2, Newcastle disease, attenuated vaccine Infectious bursa disease and Infectious bronchitis disease. Samples (3 samples/farm) were collected by flushing trachea from freshly dead birds with 2ml phosphate buffered saline (PBS) or by direct tracheal swabs from live birds and, centrifuged for 30 min at 1500 rpm (13) the collected supernatant mixed with antibiotics (penicillin10,000 IU/ml, gentamycin 1mg/ml, streptomycin 10,000 μg/ml) and 25μg/ml of antifungal agents (amphotericin B). Inoculum was prepared by passing through a 0.45 μm filter and incubating at 37°C for 1 h, then stored at -70°C till used for PCR and culture. A total of 20 birds from infected farm were picked up and blood samples were collected by veno-puncture of the wing vein. Serawere separated and stored at-20°C until used. Twenty serum samples per flock were tested for indirect ELISA tests.

ELISA

An indirect ELISA test kit, recommended by Swayne et al. (1998) to detect type-A group-specific antibodies was used according to the manufacturer’s (Synbionic, corp. USA) instructions(10). The plates have been coated with inactivated AI virus, type A. An EL800 ELISA reader (BIO-TEK Instruments, Inc. USA) was used. Optical-density values were transformed into titers using the ProFlock software.

RNA Extraction and PCR assay:

The extraction of RNA was performed on 150 μL of prepared sample from each flock for purification of RNA, according to the manufacturer procedure (IQeasy plus viral DNA/RNA extraction kit, intron Biotechnology). The concentration and the purity of the extracted total RNA were determined by measuring the absorbance ratio at wavelength 260 nm over 280 nm using scan drop spectrophotometer (analyticajena- Germany) about 53-243.34 ng. The assay of PCR amplification of H9 viruses genome were detected using Avian influenza A virus subtype (H9) one step Real-time PCR (H9 kit, primer design England) according to the manufacturer instructions. For each flock, 1 PCR tubes were prepared for subtypes H9. For H9 programs was followed, as one step Real-Time RT-PCR was performed using Real-Time RT-PCR system kits, according to the manufacturer instructions. Samples of PCR for H9 were amplified using the following conditions in exicycler thermal block Real-Time PCR device (Bioneer, Korea): reverse transcription cycle for 10 min at 55°C followed by 95°C for 8 min, the 45 PCR cycles at 95°C for 10 sec., 60°C for 60 sec. Positive and negative control provided by kits were used in each run.

In vitro cell culture assay

Two types of cell culture media were used in this assay: growth media (GM), and maintenance media (MM),their composition was prepared as mentioned by WHO, (1997) with a modification (10% instead of 5% of FCS in GM), (29,15). Two types of cells monolayer (rhabdomysarcoma and chick embryo fibroblast) were used for the viral propagation and revealing of cytopathic effects. The medium and non-adherent cells were decanted. Then the cell layer was washed twice with PBS, using approximately 10 ml per 25 cm$^2$ of surface area. The original cells was treated with trypsin-versene mixture solution (1:1) at 37°C by
gradual pealing of cell from the surface of flask, and the infectivity was also confirmed by the using of indirect fluorescent assay.

**Results**

Out of the 60 chicken flocks tested by ELISA, 43 (60.2%) showed positive anti-NP antibodies. Significantly higher sero-prevalence was found in old flock infection compared to recent flock infection. In addition, AIV sero-positivity was significantly associated with low biosecurity measures. Out of the 251 avian cases, a total of 135 (60 strong and 75 weak) positive, while 116 cases were negative as detected by using rapid avian flu type A antigen kit test (EVL corp. Netherland). Grossly, the lesions appeared as serous, catarrhal or caseous exudates in the trachea, cloudy air sacs which may contain yellow caseous exudates, the nasal passages and sinuses filled with mucopurelant exudates, in addition to intestinal congestion. By using the RT-qPCR, all the 60 different broiler flocks with severe respiratory signs were showed 100% positive infection with avian influenza subtype H9 (fig. 1).

(Fig.1): Showing the PCR fluorescence data (FAM) collection during 60°C extension for H9 virus, their curves higher than threshold line were positive results and the negative result the curves under than threshold line in rRT-PCR for H9 detection.

**Detection of Avian influenza subtype H9 virus in cell culture**

The demonstration of specific Avian influenza subtype H9 virus antigen in infected cells at each passage was accomplished by IFAT on Lab-Tek slides, which had been carried out after 48 hrs PI. The character was increased with subsequent passages represented by the appearance of fluorescent cells with bright cytoplasm fluorescence which occupied most of the cytoplasm in severe extensive infection of passages with the peak of TCID50 of each type of cells used (Figures E-F). The main characteristic of CPE on RD cells was firstly observed at the 4th day post infection (PI) in the first passage was cytopathic granulation, rounding, and sloughing of the cells, followed by formation of syncytial cells and large empty plaques two days later. After that the dead cells began to detach from flask surface and float in media. The CPE is generalized to involve large areas of monolayer sheet two days later, while the control flasks remained unchanged, and showed no CPE (Figure2-A). After 80-100% CPE appearance (Figure 2-B), infected cell cultures were frozen at -30°C for further passages. On the subsequent passages of the virus on the same cell lines, the development of the CPE began to appear much more rapid than the first passage. In the second passage CPE appeared more clear in the 3rd day PI, which were harvested and frozen at -30°C, and on the fourth passage, CPE appeared within 48 hrs. Ninety percent of cells were detached from the surface of the falcon in the sixth day PI. The peak of tissue culture infective dose (TCID 50=10⁸/0.1 ml) was seen in sixth passage. Chick embryo fibroblast cells showed slower appearance of CPE. The main characteristic of this CPE firstly observed on 6th day post infection (PI) was swelling and rounding of few numbers of cells which were increased by the 8th day PI, accompanied by syncytial cells, granulation, and rounding with the flotation of many dead cells in the culture media (Figure 2-D). These characteristics appeared much more rapid in the subsequent passages till they reached the peak of TCID50 (10⁵/0.1 ml) in the 7th passage.

**Discussion**

Respiratory diseases in poultry have been reported to be caused by multiple or single
Fig. (2): Cytopathic effect of avian influenza infection on RD and chick embryo fibroblast cells: A-Normal RD cells B- Infected RD cells after 48 hrs. PI C-Normal chick embryo fibroblast cells; D- Infected chick embryo fibroblast cells after 48 hrs.; E and F: avian influenza infection as detected by IFAT both on RD and chick embryo fibroblast cells respectively.
infections with several agents that molecular techniques such as RT-qPCR help in rapid and accurate identification of the etiological agents responsible for an infection (16). RT-qPCR has the ability to even detect a single virus particle, whether active or inactive (17). In our study all the 60 different broiler flocks with severe respiratory signs were showed 100% positive infection with avian influenza subtype H9 by the RT-PCR tests. The RT-PCR tests revealed significantly higher numbers of AI positive samples as compared to ELISA test. Avian influenza (AI) is a highly contagious disease of poultry widely distributed all over the world. An obvious outbreak of LPAI (H9N2) have emerged in Najaf, in June to December 2008, (11) and were associated with drastic mortality up to 80% in infected chickens. The avian influenza virus subtype H9 virus are the major cause of a respiratory tract infection of broiler chickens and every year bring about high morbidity and mortality in flocks in province of Najaf, Iraq. The high rates of AIV infections in broiler flocks suggested that AIV are the most important causes of respiratory disease in this study. A total of 60 commercial broiler flocks with a history of respiratory disease were received. The results show that all of the samples (60 farms 100%) were positive. Several diagnostic methodologies are currently available for the detection of AI infection, with viral infection in eggs and cells culture universally recognized as the gold standard, however these methods are time consuming and requires. Recently, molecular diagnostic tests have proven themselves to be invaluable as a first step in the identification and control of disease outbreaks. RT-qPCR have been applied successfully to the diagnosis of this disease(18). In this study, we present data on the development and validation of a real-time hydrolysis probe-based RT-PCR assay for the simultaneous detection of AI viruses belonging to subtypes H9. To detect and partially characterize influenza A viruses from different animal species, RT-qPCR has earlier shown to be sensitive and specific for the detection of human, avian and swine influenza A viruses (19). The high percentage of AIV reported in this study addresses the strong need for more aggressive monitoring and vaccination of the susceptible and already vaccinated poultry flocks. Therefore, the present study is conclusive with this fact that the etiology of respiratory organisms is very complex and it usually involves more than one pathogen (20,21,22). Recently, H9N2 of avian influenza was identified in Najaf/Iraq , H5, H7, H9 in various countries in the Middle East and South East Asia. This history give a continuous threat for the emergence of more pathogenic strains of influenza viruses. The isolation of H9N2 serotype from poultry, in this part of the world, signifies its pathogenic potential and therefore, suggests these viruses to be a possible candidate for future human pandemics originating in Asia. It, therefore, will be appropriate to launch comprehensive surveillance of live bird markets in the region using RT-qPCR techniques, so as to assess the burden of various serotypes of AIV in a particular area. In this study, our results indicates that it is vital that all bird keepers in the Najaf province continue to practice the highest levels of biosecurity and be vigilant for any signs of disease. Early reporting, rapid action, biosecurity, culling and surveillance remain the most effective way of disease prevention. It is common practices in Najaf government, to vaccinate broiler flocks against H9N2. The vaccine seed strain evaluation should include field viruses from all relevant geographical regions and production sectors, and sequence analyses of such viruses to identify genetic variants that can be further evaluated for antigenic change that may reduce the efficacy of the vaccine in use. Avian flu virus subtype H9 has been reported in commercial chicken in different countries in Asia, Pakistan (23), Iran (13), United Arab Emirate (24), Saudi Arabia (25), Korea (26), and Jordan (27). Avian influenza viruses (AIV) of serotype H5, H7 and H9 have attained great significance in recent years due to emergence of pathogenic form of these viruses in poultry and also due to
their zoonotic potential (28,23). Early after infection, viruses are capable of triggering a series of intracellular events which may be accompanied by changes in host gene expression and activation of a variety of intracellular signaling pathways that are in part exploited by the virus to ensure efficient replication (29,30,31). Our results indicate that the addition of trypsin (0.5% of maintenance media) is very helpful for the cultivation of H9 influenza virus on chick embryo fibroblast and RD cells, these results demonstrate that these cells can support growth of the H9 with trypsin supplementation in the medium after infection, this came in agreement with findings of Jiang et.al, 2013 who demonstrated that addition of trypsin to medium was essential for obtaining high virus titers (30), and the finding of Herman et al.,2005, which indicates that the protocol of trypsin usage should continue until better method is discovered(14). This result suggests that LPAIV cannot replicate very well in chick embryo fibroblast and RD cells without exogenous trypsin. This is consistent with previous reports that report a need for trypsin-like proteases to cleave the hemagglutinin protein into the HA1 and HA2 subunits, which is required for the virus to be infectious (32,33).

References