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Short Communication

Serological Investigation of H9N2 Avian Influenza Virus in Slaughtered Water Buffaloes (*Bubalus bubalis*) in Khuzestan, Iran

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ABSTRACT

The aim of the present was to evaluate the prevalence of H9N2-specific antibodies among water buffaloes (*Bubalus bubalis*). To this end, blood samples were obtained from 80 randomly selected water buffaloes, 40 cases of which were obtained in the winter months, and 40 cases were sampled in the spring months. The presence of H9N2-specific antibody was determined by hemagglutination inhibition method. The antibody was diagnosed in 14 buffaloes (i.e., 10 males and 4 females). There were no significant differences between the two genders and between different age groups in terms of antibody prevalence. The presence of the antibody had a seasonal pattern; in this regard, all positive cases were found in the winter months ($P<0.01$). Detection of antibody against H9N2 in water buffaloes suggests the presence of an avian-water buffalo cycle of H9N2 virus. Additional research is needed for the detection of the probable epidemiologic role of buffaloes in the interspecies transmission and pathogenesis of H9N2 avian influenza viruses.

Keywords: Serological, Influenza, H9N2, *Bubalus bubalis*

Etude Sérologique sur le Virus H9N2 de la Grippe Aviaire cheze les Buffle des Marais Abattus (*Bubalus bubalis*) dans la Région du Khouzestan, Iran

Résumé: Cetteétude visait l'évaluation de la prévalence d'anticorps spécifiques du H9N2 chez les buffles des marais (*Bubalus bubalis*). Dans ce but, des échantillons de sang ont été prélevés sur 80 buffles des marais choisis au hasard, dont 40 cas ont été obtenus pendant les mois d'hiver et 40 cas ont été échantillonnés durant les mois de printemps. La présence d'anticorps spécifique de H9N2 a été déterminée par la méthode d'inhibition de l'hémagglutination. La présence danticorps spécifique a été diagnostiquée chez 14 buffles (soit 10 mâles et 4 femelles). Il n'y avait pas de différences significatives liées ausexe et entre les différents groupes d'âge en termes de prévalence des anticorps spécifiques anti- H9N2. La présence de ces anticorps montrait un schéma saisonnier étant donné quetous les cas positifs ont été découverts pendant les mois d'hiver ($p<0,01$). La détection d'anticorps anti-H9N2 chez les buffles des marais suggère la présence d'un cycle de transmission du virus H9N2 impliquant les oiseaux, l'eau des marais et les buffles. Des recherches supplémentaires sont nécessaires pour détecter le rôle épidémiologique probable des buffles dans la transmission inter-espèces et la pathogenèse des virus de la grippe aviaire H9N2.

Mots-clés: Sérologie, Grippe, H9N2, *Bubalus bubalis*

INTRODUCTION

Influenza type A viruses, as a member of genus Orthomyxovirus, are classified into various subtypes on the basis of two surface glycoproteins, namely hemagglutinin (HA) and neuraminidase (NA). The 18 HA and 11 NA known subtypes are classified into highly pathogenic and low-pathogenic viruses. These subtypes are highly contagious viruses that have been isolated from a wide variety of host species (Ghaniei et al., 2013). Interspecies transmission of influenza virus makes it a potentially dangerous virus for mammals and birds and has resulted in the direction of considerable attention to this domain in recent years. Since the first detection of H9N2 subtype as a low-pathogenic avian influenza virus in the USA, this virus has been reported widely around the world and become the most prevalent subtype of virus in poultry during the last decade. Additionally, the isolation of H9N2 virus from humans with respiratory diseases has been reported since the late 1990s (Huang et al., 2013). In Iran, the outbreak of H9N2 virus in poultry farms was first reported in 1998, which has incurred great economic losses in the poultry industry thereafter. Today, the virus is endemic in Iran, and vaccination is practiced against this subtype (Heidari et al., 2009). It is believed that H9N2 viruses can cross the species barriers and expand their host range from avian species to mammalians (Huang et al., 2013). Since influenza viruses have been isolated from different host species, including mammals and birds, it is believed that the identification of the susceptibility of mammalian species to this virus would be helpful in developing surveillance plans or determining the risk areas. There is limited published information about influenza A virus in family Bovidae (e.g., cattle or water buffalo). A number of recent reports have addressed antibodies against influenza A viruses of human and avian origins in a range of domestic species, including cattle. These investigations have generated considerable interest both in terms of their probable role in the epidemiology of virus infection and their pathogenic potential in farm animals (Graham et al., 2002). Iranian water buffaloes (*Bubalus bubalis*) have a high economic value by providing meat, milk, and labor for local farmers (Hamidinejat et al., 2015). Water buffaloes may encounter H9N2 virus because of their extensive contact with the local birds kept in the farms and migratory birds present in the local water bodies. Furthermore, the probable interspecies transmission of influenza viruses enhances the risk of virus infection. In recent years, some studies have been conducted on influenza infection in farm animals (Reperant et al., 2009; Lv et al., 2012). Despite the great risk of exposure to influenza viruses among water buffaloes, to the best of our knowledge, there is only one report addressing the detection of influenza antibodies in water buffaloes (Graves et al., 1974). However, there is no study regarding the prevalence and epidemiological aspects of probable virus exposure in this species. With this background in mind, the present study was conducted to investigate H9N2 avian influenza antibody in slaughtered water buffaloes in Khuzestan, Iran, using serological assay.

MATERIAL AND METHODS

The study was performed on water buffaloes (*Bubalus bubalis*), which were slaughtered in Ahvaz City, southwestern Iran, from December 2013 to January 2014 (two winter months) and April to May 2014 (two spring months). A total of 40 randomly selected animals were sampled in each sampling period from a slaughterhouse reserved only for buffaloes. Buffaloes were of both genders and different age groups. The age of the animals was estimated using dental characteristics. The animals were kept by local farmers and had grazed the previous three months on ranges around the city. After clinical examination, jugular blood samples were collected in plain tubes, free from anticoagulant. The blood serum was separated after centrifugation at 1800 g for 10 min, and then stored at -20 ºC until analysis. Presence of antibody against H9N2 avian influenza virus in the serum samples was evaluated using the

hemagglutination inhibition (HI) assay. The HI assay was performed on serially diluted sera in a U-bottom 96-well microplate according to the standard method (OIE, 2012). Prior to use, the samples were brought to ambient temperature, and then incubated at 56 °C for 30 min to destroy the non-specific inhibitors. The specific antigens for chicken H9N2 subtype (Pasouk Co, Iran) were used for HI test (OIE, 2012). After 30 min of incubation at ambient temperature, the plates were inspected visually for agglutination, and the highest dilution of each sample, which caused the inhibition of HI was considered as HI titer.

Statistical analysis. Statistical analysis was performed using SPSS software, version 17 (Illinois, Chicago). Based on the age, the buffaloes were divided into three groups of $G_1 < 2$ years, $2 \leq G_2 < 4$ years, and, $G_3 \geq 4$ years. The Chi-square test was used for the comparison of the affected rates among the different age groups. Furthermore, the Fisher's exact test was run to compare the two genders and two sampling periods. Comparison of the mean HI titer between the two genders and different age groups was accomplished using paired sample *t*-test and analysis of variance test, respectively. Paired sample *t*-test was also used for the comparison of the two genders in terms of age. P-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In the current study, sampling was performed on 54 male and 26 female buffaloes. In the preslaughter examination, the animals were clinically healthy. The mean ages (mean±SEM) of the male and female buffaloes were 2.15±0.1 and 2.67±0.3 years, respectively. Table 1 shows the prevalence of H9N2-specific antibody in the serum samples. The data were presented based on gender, age, and season. In the current study, the HI titer of > 3 log_2 was considered a positive result. The mean antibody titer was 6.88±0.53 log_2 (range: 3-11 log_2). Overall, 14 (17.5%) serum samples (i.e., 10 males and 4 females) were positive for H9N2 antibody (95% CI: 10.7-27.3). All positive sera were collected in the winter; accordingly, no positive case was detected in the spring samples (P<0.01). There was no significant difference between the two genders and between different age groups regarding the rate of positive serum samples and mean antibody titer.

Table 1. Prevalence and mean titer of H9N2 antibody in two genders, different age groups, and different seasons among water buffaloes

	Number of buffaloes	Number of positive buffaloes (%)	Mean antibody titer (Mean±SEM)
All sampled buffaloes	80	14 (17.5%)	6.88±0.53
Male buffaloes	54	10 (18.5%)	7.05±0.59
Female buffaloes	26	4 (15.4%)	6.5±1.2
G_1 (<2 years)	31	6 (19.4%)	7.5±0.56
G_2 (2≤and<4 years)	35	5 (14.3%)	6.2±0.84
G_3 (4 years≤)	14	3 (21.4%)	7.0±1.6
Summer months	37	0 (0%)	0
Winter months	43	14 (32.5%)	6.88±0.53

The interspecies transmission of influenza A viruses is an important part of the epidemiological characteristics of influenza viruses. There is evidence regarding the susceptibility of mammalian species, such as members of the family Bovidae, to human and poultry influenza viruses (Kalthoff et al., 2008). It has been documented that some H9N2 viruses have switched their receptor binding specificity and are able to infect and replicate in mammalian hosts (Zhang et al., 2013). Identification of the susceptibility of mammalian species to poultry influenza virus would be helpful in developing new surveillance plans and determining high-risk regions. On the other hand, the phylogenetic analysis of gene segments has shown that the mammalian subtypes of influenza are closely related to avian influenza viruses and are probably transmitted from birds to mammalians as important zoonotic pathogens (Reperant et al., 2009). It has been suggested that influenza A viruses currently circulating

in avian species are a potential source of viruses that can infect the mammals and a source of new pandemic strains in human (Hadipour, 2010). As a result, there are some concerns regarding the evolvement of H9N2 virus, as the most prevalent subtype of virus in poultry, into a pandemic strain in humans (Lv et al., 2012). Additionally, due to the close contact between people and animals in many countries, domestic animals may serve as an intermediate host for influenza virus infection. Therefore, the detection of host ranges, transmission methods, and epidemiological aspects of interspecies transmission of the virus seems necessary. Graves et al. (1974) reported the presence of antibody against the human influenza virus strains (H3N2) in cows and water buffaloes in Nepal. However, there is no study examining the prevalence, seroprevalence, and epidemiological aspects of avian influenza virus in water buffaloes. In the current study, the buffaloes had grazed on ranges around the city and in local water bodies, in which the animals were in contact with migratory birds. Additionally, the animals were in contact with local birds around the farms and the local people. Despite the availability of little information about the circulation of influenza virus in migratory water birds in Iran, there is evidence regarding the consequential role of water birds as viral reservoirs in different regions across the world (Nickbakhsh, 2016). According to our results, the antibody against H9N2 virus was detected in water buffaloes only in the first sampling period (i.e., winter months), and no positive case was found in the second sampling period (i.e., spring months). In the current study, the first sampling stage was after the wet period and during the migration of the birds in the region, in which millions of migratory birds (e.g., water birds) live around the local water bodies. Accordingly, it seems that the H9N2 had been transmitted from birds to the tested buffaloes. It also appears that there is a short-term presence of antibody in water buffaloes following the exposure to the virus in the migratory birds. Influenza vaccine for poultry is administrated in Iran; however, the vaccine is inactivated, and there is no chance for the transmission of the virus from the vaccinated local birds to mammalians. It is believed that inoculation with a high titer of influenza virus may cause asymptomatic shedding of virus by infected animals and subsequent seroconversion. Even low levels of virus may be sufficient to induce a detectable antibody response (Kalthoff et al., 2008). The experimental infection of different mammals, such as cattle, dogs, and cats, with human and avian influenza viruses reportedly resulted in no clinical disease development (Kalthoff et al., 2008; Reperant et al., 2009). On the other hand, in a study, influenza A virus infection showed a correlation with reduced milk yield and respiratory symptoms in dairy cows (Brown et al., 1998). In the same vein, a number of authors believed that avian influenza virus infection in mammals results in respiratory diseases. In the current study, the clinical examination of the sampled water buffaloes revealed no clinical signs. It seems that the different titers of inoculated influenza virus may result in different stages of infection in mammals. Some H9N2 strains are able to infect and efficiently replicate in mammals; however, there is no evidence regarding the transmission of avian influenza virus between mammals (Kalthoff et al., 2008; Huang et al., 2013). Our results indicated that age and gender had no effects on the risk of exposure to H9N2 in buffaloes. Nonetheless, it should be noted that the group of sampled animals in this study was not a true cross-section of the normal buffalo population due to the low number of female animals and the animals in G_3 group. In birds, the replication of low-pathogenic influenza viruses mostly occurs in the respiratory and intestinal tracts; accordingly, the virus is transmitted through the respiratory and fecal-oral routes. Nevertheless, influenza viruses mainly replicate in the respiratory tract of mammals and are transmitted primarily through the respiratory route. There is no information regarding the precise route of mammalian exposure to avian influenza virus. However, direct physical contact with bird reservoirs, including the ingestion of infected bird carcasses, indirect contact with bird feces and aerosols or a contaminated

environment, and ingestion of contaminated food or water have been proposed as the probable routes for the cross-species transmission of the virus (Chan et al., 2015; Reperant et al., 2009). Diagnosis of specific antibodies, isolation of virus, and molecular assays are indicative of exposure to influenza virus. The HI test is more specific than other serological methods and is the most commonly used assay in the detection of infection and surveillance programs (Ghaniei et al., 2013). Detection of antibody against H9N2 in water buffaloes suggests that there may be an avian-water buffalo cycle of H9N2 virus. However, in the current study, only HI assay was applied for serological test; therefore, mild or asymptomatic infections may have remained undetected. The confirmation of a cross-species circulation requires virus isolation. The findings of the present study provided further evidence regarding the probable role of water buffaloes in H9N2 virus circulation. Our results also suggested that the transmission of H9N2 virus from avian species to water buffaloes may allow viral adaptation to mammals. This issue highlights the importance of giving more close attention to this domain. Consequently, it is required to further research to prove this hypothesis and investigate more epidemiological aspects and probable pathogenesis of the cross-species transmission of the virus and its related risk factors.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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