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Experimental Assessment of the Pathogenicity of Avian Influenza Virus H9N2 Subtype in Japanese Quail *(Coturnix Coturnix Japanica)*

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ABSTRACT

H9N2 avian influenza A viruses are endemic in poultry of many Eurasian countries and have caused repeated human infections in Asia since 1998. It has been also reported that H9N2 can cause high mortality in commercial broiler farms in Iran previously. However there was no report of H9N2 outbreak in any other species. In order to evaluate the pathogenicity of H9N2 virus in Japanese quail, 145 Japanese quail were randomly divided into 5 separate groups (116 quails in the treatment and 29 quails in the control groups). The experimental groups infected via oral rout, eye drop, intramuscular injection and spray method at the age of 32 days with $10^{6.5}$ EID50/bird. The virus A/chicken/Iran/ZMT-101/98(H9N2) was kindly provided obtained from Razi vaccine& serum institute with EID50=10^{8}. The blood samples were experimented the day before use to show freedom from antibodies to influenza A and more specifically, the H9 subtype. The clinical signs and antibody titer of the infected chicks were also monitored. Five birds of each group were bled at 10 and 20 days post infection (DPI), and 20 birds of each group at 30 DPI were bled. The immune response to infection was measured by Haemmaglutination Inhibition (HI) test using the H9N2 virus as antigen. Feed & water consumption were recorded on daily bases before and after inoculation. Body weight of each group was also recorded on weekly bases before and after inoculation. During the current study clinical signs such as sneezing, gasping, depression observed in challenged groups followed by decreasing in laying (1-17%). High HI antibody titers of AIV subtype H9 was seen in 10 DPI. The quails exhibited no decrease in food and water consumption and all quails were growing well and did not show any abnormality.

Keywords: Avian influenza, pathogenicity, H9N2, Body weight, Immune responses, Clinical signs, Egg production.

INTRODUCTION

Variations in the pathogenicity and transmissibility of influenza viruses for different hosts have frequently caused problems in diagnosis, definition and the understanding of influenza infection in poultry (Bankowski 1982). Nardelli et al. (1970) found that quail to be infected with influenza A virus caused respiratory disease and was lethal to young quail (<3 months old)(Nardelli *et al* 1970).

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Tashiro et al. (1987) showed that quail infected with an H5N3 virus that was highly pathogenic to turkeys were resistant to disease, but could transmit the lethal virus to chickens (Tashiro *et al* 1987). The findings of Perez et al. (2003) emphasized the role played by quail in the evolution of influenza A viruses; quail provide an environment in which influenza viruses from ducks can adapt and generate variants with the capacity to infect other avian species (Perez *et al* 2003). In laboratory infections influenza viruses have tended to be either overtly pathogenic or of low pathogenic for chickens and turkeys (Alexander *et al* 1986). Variation in pathogenicity for specific viruses between hosts such as chickens, turkeys and other members of the order Galliformes are less obvious. However, several studies have suggested that variation in susceptibility to infection by influenza viruses and the pathogenicity, when infections are established, do exist with this group of closely related species (Alexander *et al* 1986). Interestingly, the natural avian reservoir of H9 viruses in Asia has not been identified. Surveillance studies in the 1970s identified H9 viruses in domestic ducks (Markwell *et al* 1982, Shortridge *et al* 1998, Shortridge *et al* 1992). Alexander et al. (1978) revealed that chickens and turkeys responded very similarly to infection by different routes, but other birds have suggested more variation in disease seen in these two hosts (Alexander *et al* 1987). Narayan et al. (1969) reported that infections of chickens with A/Turkey/Ontario/7732/66 (H5N2) tended to be less severe than infections of turkeys, although still highly pathogenic, and that the virus was less transmissible in chickens (Narayan *et al* 1969). Slemons and Easterday (1972) found considerable variation in the diseases seen in adult turkeys, quails and pheasants infected with A/ Turkey/ Ontario/ 7732/66(H5N9) by the intranasal route (Selemons & Easterday 1972), while Wood *et al.* (1985) showed that A/chicken/ Pennsylvania/83(H5N2), a highly pathogenic virus for chickens, produced only mild transient disease in pheasant (Wood *et al* 1985). During the H5N1 incident, H9N2 viruses were isolated from duck, geese, pigeon, quail, chicken and market environmental swabs. The virus appears to be widespread in chicken in Asia and may have been the donor of the 'internal' genes of the virus responsible for the H5N1 incident in Hong Kong in 1997 (Alexander 1999). H9N2 influenza A viruses are currently widespread in chickens, quail, and other poultry in Asia and have caused a few cases of influenza in humans (Peiris *et al* 1999). H9N2 viruses from Hong Kong live bird markets have receptor specificity similar to that of human H3N2 viruses (Matrosovich *et al* 2001). During 1994-99 infections of poultry with influenza viruses of H9N2 subtype appear to have been common world-wide. Outbreaks occurred in Germany in 1995-96, Italy in 1994, Ireland in 1997, South Africa in 1995, and Korea in 1996. Since 1997 serious problems associated with H9N2 virus have been reported in Iran, Saudi Arabia, Pakistan, China and other Asian countries (Alexander 1999). Quails were more susceptible than chickens to these viruses, and generation of recombinant H9 viruses by reverse genetics showed that changes in the HA gene are sufficient to initiate efficient replication and transmission in quail. Seven amino acid positions on the HA molecule corresponded to adaptation to land-based birds. In quail H9 viruses, the pattern of amino acids at these seven positions is intermediate between those of duck and chicken viruses; this fact may explain the susceptibility of quail to duck H9 viruses. These findings suggest that quail provide an environment in which the adaptation of influenza viruses from ducks generates novel variants that can cross the species barrier (Perez *et al* 2003). According to this fact, quail could transfer the pathogen, without showing obvious clinical signs; It has been previously reported that H9N2 avian influenza virus can cause high mortality in commercial broiler farms in Iran (Naeem *et al* 2003), however there was no report of H9N2

outbreak in any other species. At this study we want to experiment the pathogenicity of avian influenza virus H9N2 subtype in Japanese quail.

MATERIALS AND METHODS

Virus. The virus A/chicken/Iran/ZMT-101/98 (H9N2) was kindly provided obtained from Razi vaccine & serum research institute with EID50=10^8.

Bird. Quail chicks (*Coturnix coturnix japonica*) reared at Yazd industrial company. All birds were used in experiments at 5 weeks of age. Blood samples of birds were experimented the day before inoculation to show freedom from antibodies to influenza A and, more specifically, the H9 subtype.

Serological test. Serum samples were tested for the presence of antibodies to the challenge virus antigen using the HI test (Burleson *et al* 1992). Chicken red blood cells were used however, sera were also tested for agglutination of red blood cells but none was seen at titers greater than log 2. A heamagglutination Inhibition (HI) test using antigen from an H9N2 AI virus (courtesy of Iranian Razi Institution) was use for detecting anti-Influenza antibodies in field cases. All HI titers were expresses as log 2 of reciprocal of the highest dilution causing inhibition of 4 heamagglutinatinating units of virus. The HI tests were measured the day before inoculation and it also was done 3 times after inoculation (10DPI, 20DPI, 30DPI).

Experimental infections. Five-week-old Japanese quail chicks were randomly divided into 5 separate groups: group 1, eye drop challenged; group 2, oral challenged; group3, injection challenged; group 4, spray challenged; group 5 unchallenged group. The spray challenged group and control group were housed separately to prevent from trans infection. The inoculum was prepared from Iranian Razi Institution. The quail chicks were inoculated with $10^{6.5}$EID50 of virus in each group depend on the way of inoculation that was expressed before. At 10-20-30 days post inoculation (PI), 4-5 birds were bled for Antibody measurement by the HI test. Feed & water consumptions were recorded on daily basis before and after inoculation. Body weight of each groups were also recorded weekly before and after inoculation. Egg production was recorded at 20 days PI (2 weeks after the quails produced egg) to last day of experiment. The quail chicks were checked for clinical signs every day after the inoculation.

Statistical tests. The following statistical tests have been used at the current study:

1. Repeated measure ANOVA for the results of body weight.
2. One way ANOVA for the results of food & water consumptions.
3. Non parametric test for the results of antibody titers.

RESULTS

Clinical signs. Of 116 quail chicks inoculated with H9N2 virus, none of quail chicks died, but in 2nd weeks of PI 10/29 in eye drop challenged; 8/29 in oral challenged; 16/29 in injection challenged; 11/29 in spray challenged had clinical signs such as gasping, snicking and depression. No clinical signs were seen in unchallenged groups (Table 1).

Table1. Clinical signs following infection with H9N2 virus

Clinical signs	Treatment[1]				
	A	B	C	D	E
Gasping	3[f]	3	6	5	-
Snicking	3	2	4	-	-
Depression	4	3	6	6	-

[1]Treatments: A= eye drop challenged group; B= oral challenged group; C= injection challenged group; D= spray challenged group; E= control challenged group (received no virus), f=number of bird in each group.

Serological response. HI test was used to measure the antibody titer against H9N2 in the blood samples collected on days 10, 20 and 30 PI. As shown in Table 2, the mean antibody titer was increased at 10 DPI and reached to 9-10 at 10 DPI in the experimental group. All challenged groups had HI titers at 42 days of age (10 DPI). Antibodies

to AI virus were some positive in the unchallenged (Table2).

Egg production. In the present study, there was decrease in Egg production about 1-17% in challenged groups (Table 3). It should be check for few weeks, from beginning of egg production until the end of experiment. As the recording of egg production wasn't proposed for this study, then it mentioned lately during the mentoring of birds, because of getting a good result as a helping factor to assess the pathogenicity of virus better, so there wouldn't re mark any statistical test for it.

Table 2. Haemagglutination inhibition titers[1] of birds infected by different routes with influenza virus of H9N2 subtype.

Treatment[2]	Mean ± SD		
	10 days PI	20 days PI	30 days PI
A	0.577±9.66	6.00± 0.00	4.55±1.14
B	0.577±9.66	6.33± 0.577	3.95±1.46
C	0.577±10.66	7.00± 0.00	3.40±0.75
D	0.00±10.00	6.33± 0.577	5.10±1.13
E	0.577±3.66	2.00± 0.00	2.05±1.09

1: Titers are expressed as log 2 of the reciprocal of the highest dilution of serum inhibiting 4HAU of the infecting virus.
2: Treatments: A= eye drop challenged group; B= oral challenged group; C= injection challenged group; D= spray challenged group; E= control challenged group (received no virus). ($p<0.05$)

Water & feed consumption-body weight. In the present study quail chicks exhibited no decrease in food and water consumption. All quail chicks were growing well and did not show any clinical abnormality.

At the end of the study all quail chicks were killed for necropsy that none of challenged and unchallenged showed any gross lesions.

DISCUSSION

In the present study we have evaluated the pathogenicity of A/chicken/Iran/ZMT-101/98 (H9N2) isolation in quails. Slemons and Easterday (1972) reported that quail to be more resistant than chickens and turkeys to infection with A/Ontario/7732/66(H5N2), but they noted high serological responses in 17/20 birds and 3/20 deaths in adult quail given the virus by intranasal route (Selemons *et al* 1972). Viruses of: A/Chicken/Pennsylvania/1370/83(H5N2), a/Tern/ South Africa/61(H5N3) and A/Chicken/Scotland/59 (H5N1) were able to infect quail without death or in some cases sickness (Alexander *et al* 1986). It seems reasonable to extrapolate these finding to suggest the possibility that viruses of high pathogenicity for chicken and turkeys may be carried by wild Galliform bird such as quail and pheasant (Wood *et al* 1985). However, MPAI viruses produced mostly respiratory diseases, but mortality rates have been very high in the field, while experimental studies reported no or low mortality rates with the current outbreak of H9N2 in Iran;

Table3. Mean egg production following infection with H9N2 virus

Days post infection(DPI)	Age (days old)	Treatment [1]				
		1^A	2^B	3^C	4^D	5^E
		Number of eggs				
20	53	4	4	4	5	7
21	54	7	4	4	6	8
22	55	6	3	4	5	8
23	56	5	3	7	4	8
24	57	3	3	7	8	7
Number of female quail		10	9	16	14	15
Mean egg production ± SD		50±15.8	35.55±4.9	32.46±10.22	39.98±10.82	50.62±3.6

mortality rates of up to 65% have been reported (Nili & Asasi 2002). Other experimental studies with virus alone have shown all H9N2 AI viruses from Middle East to be non HP (i.e. causing low or no mortality in chickens in pathotyping test) (Banks *et al* 2000). No mortality was observed in the current study. Previous study showed high HI antibody titers of AIV subtype H9, 2 weeks following the infection in field study and 21 days post challenge in chicken inoculated experimentally with H9N2 virus (Nili & Asasi 2002). Vasfimarandi *et al*. (2002) in field study showed that antibodies against A/chicken/Iran/ZMT-101/98 (H9N2) strain of AIV antigen were increased from 0 to 9 $\log_2$ titers in sera prepared at 1,2,3,4,5 and 6 weeks after acute phase of disease occurred in 32 week-old layer and 38 wk-old breeder flocks (Vasfi Marandi *et a*l 2002). Slemons and Easterday (1972) identified high serological responses with H5N1, H5N2 and H5N3 (Selemons *et al* 1972). In the current study with A/chicken/Iran/ZMT-101/98(H9N2) in quails showed high serological responses 10 DPI in all inoculated groups. At the present study, all challenged groups were shown a significant increase in antibody titers. This increase showed at 10 DPI in all challenged groups clearly. The procedure of decreasing of antibody titers in injection challenged group was more than other treatment groups. The procedure of decreasing antibody titers in other challenged groups was lower than injection challenged group at 20 DPI to 30 DPI, on the other hands the titers were stayed for a long time at 20 DPI to 30 DPI in all challenged groups except injection challenged group. In injection challenged group, there is no place for viruses to localize in order to growing, so they enter to blood stream very rapidly than other treatment groups, on the other side there are no specific obstacles or immunity surface to prevent the viruses to settle. As we see in table 2, the highest antibody titers belong to injection challenged group. The viruses in other treatment groups face at least to an obstacle, which it prevents them to settle, but after localizing, there would be a place to let the viruses to grow, for example in oral challenged group the virus at first face to mucous of oral cavity, and then the low pH of proventriculous and gizzard. In eye drop challenged group the virus should pass from eye mucosa and in spray challenged group the virus after passing the nasal cavity should traverse the mucocilliary of trachea, which not let them to localize in trachea. After localizing, the body system produces many virus particles, and then they moved to blood stream, so after that the antibody titers increase very lately rather than injection challenged group, so the durability of the antibody titer in body will be more than injection challenged group. A suitable place to provide a media for growing can probably play an important role, which caused a long time staying antibody titers, for example in eye drop challenged group and spray challenged groups “the trachea”, let the the virus to grow, In oral challenged group the virus probably didn’t find a suitable place to grow, and then excreted from body through stool, so we can see significant reduced antibody titers after injection challenged group during 20 DPI and 30 DPI. The mild pathogenic AI in addition to clinical signs (particularly depression, respiratory and enteric signs) and variable mortality (2-10%) includes also decrease in laying (5-50%) for about 5-10 weeks in chickens (Zanella *et al* 2002). Naeem *et al.* (1997) showed in his research that the flock-level morbidity rates ranged from 13.9 to 86.7% and within flock mortality ranged from 51 to 100% (Naeem *et al* 1997). This study revealed that mild clinical signs in 14 days PI such as gasping, snicking and depression was seen (table 2) but there was no mortality in all infected groups. Mutinelli showed that the oviduct wall appeared thickened due to edema, with minimal diffuse hetrophil infiltrated within epithelium, the latter were also occasionally found in the lumen after infection with MPAIs (Mutinelli *et al* 2003). Prior experiment revealed that

seroprevalence of AI virus had relation with increased mortality and decreasing egg production in chicken (Narayan *et al* 1969). In1996, AI outbreak was reported in breeder broiler farm in Korea with 20-40% mortality and dramatic (80%) drops of egg production. The viruses isolated were identified as non-highly pathogenic H9N2 AI virus (Mo *et al* 1997). At the present study in addition to clinical signs without mortality there was also decrease in laying (1-17%). Nili and Asasi (2002) showed Between 8 and 14 days post challenge with the field inoculums the infected chicken, exhibited decrease food and water consumption followed by depression, respiratory disease, swelling of the head and nasal and ocular discharge (Nili & Asasi 2002). The recent experimental infection of chickens with H9N2 subtype showed significant clinical signs and recovered gradually without any deaths. However, the virus induced mild influenza in layers, and eliciting severe drops of egg production. Although, 20-60% mortality in broiler flocks were seen during the outbreak of AI, but the mortality was closely associated with the improper disease prevention practiced and was not entirely caused by AI. This is due to secondary infections and is related to the greater bird density, the poorer hygienic measures and air quality of confinement conditions (Pourbakhsh *et al* 2000). In the current experimental study, the lack of mortality, decrease food and water consumption was observed in all groups and all quail chicks had normal body weight.

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References

Alexander, D.J. (2000). A review of avian influenza. Symposium on Animal Influenza in different bird species. *Veterinary Microbiology* 74:3-13.

Alexander, D.J., Pearson, G., Manvell, R.J. (1986). Experimental assessment of the pathogenicity of eight avian influenza A virus of H5 subtype for chickens, turkeys ducks and quail, *Avian path*ology 15:647-662.

Alexander, D.J., Allen W.H., Parsons, D. and Parsons, G. (1978). The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. *Research in veterinary science* 24:242-247.

Bankowski, R.A. (1982). Introduction and objectives of the symposium proceeding of the first international symposium on avian influenza. Richmond, USA: *carter Company Corporation* Pp vii-xiv.

Banks, J., Speidel, EC, Harris, P.A. & Alexander, D.J. (2000). Phylogenic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian pathology. 29: 353-360.

Burleson, F.G., Chambers, T.M. and Wiedbrauk, D.L. (1992). *Virology, a laboratory manual*. London, United Kingdom, Academic Press, Inc., Pp: 86-92.

Markwell, D. D. and Shortridge K. F. (1982). Possible waterborne transmission and maintenance of influenza viruses in domestic ducks. *Applied Environmental Microbiology* 43:110-115.

Matrosovich MN, Krauss S, Webster RG. (2001). H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* 15; 281(2):156-62.

Mo, L.P., Song, C.S., Kim, K.S. and Rhee, J. (1997). An occurrence of non-highly pathogenic avian influenza in Korea. *Proceeding of fourth international symposium on avian influenza* 379-383.

Mutinell,i F, Capua, I., Terregino, C., Cattoli, G. (2003). Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenicity avian influenza epidemics in Italy during 1999 and 2000. *Avian Disease* 47(3):844-848.

Naeem, K., Naurin, M., Rashid, S., Bano, S. (2003). Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. *Avian pathology* 32(3):285-289

Naeem, K. (1997). The avian influenza H7N3 outbreak in south central Asia. *Proceeding of fourth international symposium on avian influenza* Pp: 31-35.

Narayan, O., Lang, G. and Rouse, B.T. (1969). A new influenza A virus infection in turkeys .IV. Experimental succeptibility of domestic birds to virus. strain A/ty/Ontario/7732/1966. *Archiv für die gesamte virusforschung* 26:149-165.

Nardelli, L., A. Rinaldi, H. G. Pereira, and G. Mandelli. (1970). Influenza virus infections in Japanese quails.

Archiv Experimentelle Veterinarmedizin 24:231-249. (In German.).

Nili, H. & Asasi, K. (2002). Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran .*Avian pathology*. 31:247-252.

Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Ip, P.L.S., Lai, R.W.M., Orr,W.K. & Shortridge, K.F. (1999). Human infection with influenza H9N2. *Lancet* 354, 916-917.

Perez, D.R., Lim, W., Seiler, J.P., Yi, G., Peiris, M, Shortridge, K.F., Webster, R.G. (2003). Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. *Journal of Virology* 77(5):3148-56.

Pourbakhsh, S.A., Khodashenas, M., Kianizadeh, M. and Goodarzi, H. (2000). Isolation and Identification of Avian Influenza Virus H9N2. *Archives of Razi Institute* 51: 27-38.

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Selemons, R.D. and Easterday, B.C. (1972). Host response differences among five avian species to an avian influenza virus. A/turkey/Ontario/7732/66(Hav5N?). *Bulletin of the world Health Organization* 47:521-525

Shortridge, K. F., Zhou, N., Guan,Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K. G., Norwood, M., Senne, D., Sims, L., Takada, A., and Webster, R. G. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* 252:331-342.

Shortridge, K. F. (1992). Pandemic influenza: a zoonosis? *Seminar of Respiratory Infestation* 7:11-25.

Tashiro, M., M. Reinacher, and R. Rott. (1987). Aggravation of pathogenicity of an avian influenza virus by adaptation to quails. *Archive of Virology* 93:81-95.

Vasfi Marandi, M., Bozorgmehri Fard, M.H. (2002). Isolation of H9N2 Subtype of Avian Influenza Viruses during an Outbreak in Chickens in Iran, *Iranian Biomedical Journal* 6(1):13-17.

Wood, J.M., Webster, R.G. and Nettles, V.F. (1985). Host range of A/chicken/Pennsylvania/83(H5N2) influenza virus. *Avian disease* 19:198-207.

Yazd industrial company, *Maybod, IRAN*.

Zanella, C., Tassi, A. Volorio. (2002). Viral diseases related to egg laying problems. *World poultry* 18 (11):34-39.