BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF AVIAN INFLUENZA VIRUS (H9N2) ISOLATES FROM IRAN

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Summary. – Three Influenza A virus (H9N2) isolates obtained from three separate broiler flocks with variable mortality rates were cloned twice in embryonated SPF chicken eggs by limiting dilution. Biological properties of these isolates were examined in 4-week-old SPF chickens and chick embryo fibroblast (CEF) cultures. The isolates neither caused mortality in the inoculated chickens nor produced CPE in cell cultures, indicating low pathogenicity. PCR products of 486 bp containing the sequences for hemagglutinin (HA) cleavage site, which were generated from the isolates, were subjected to nucleotide sequencing. Sequence analysis of the HA region containing the cleavage site of the isolates showed a similar sequence motif (PARSSRG) but different flanking regions. Phylogenetic analysis of deduced amino acid sequences revealed that the isolates were closely related to those isolated earlier, indicating a common source. Moreover, the amino acid sequences of the recent isolates were very similar to those from Saudi Arabia, Germany and Pakistan. It is postulated that, except for some Chinese isolates, the pathogenicity of Iranian isolates seems to be similar to that of other Eurasian isolates. It is possible that an elevation in mortality rate under field condition could be caused by co-infection of recent isolates with the bacteria such as mycoplasma, *Escherichia coli*, and *Ornithobacterium rhinotracheale* rather than by an emerging a pathogenic H9N2 subtype of the virus.

Key words: avian influenza; Influenza A virus; H9N2; pathogenicity; RT-PCR; phylogenetic analysis

Introduction

Influenza viruses are segmented, negative-sense, single-stranded RNA viruses that belong to the family *Orthomyxoviridae*. Influenza viruses occur in three genera, *Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus C*. Only influenza A viruses are pathogenic to chickens (Lamb and Krug, 2001). Based on the antigenic relationship of the surface glycoprotein HA and neuraminidase, influenza A viruses are divided into 15 H subtypes (H1-H15) and 9 N subtypes (N1-N9). Viral subtypes of all possible combinations have been recognized but only certain combinations are pathogenic to chickens, such as H7N7, H7N2, H7N3, H7N4, H5N9, H5N1, and H9N2. Infections of domestic or confined birds have been associated with a variety of clinical signs and variable degrees of mortality. Therefore, avian influenza (AI) viruses have been classified from highly pathogenic to low pathogenic. Infections of chickens and turkeys with viruses of low virulence result in mild respiratory signs, egg production losses and occasionally slightly elevated mortality. However, a severe disease may be seen where the influenza virus infection is associated with microbial infection or specific environmental conditions (Banks *et al*., 2000). The pathogenicity of AI viruses appears to be a polygenic trait. The principal pathogenicity factor is the HA cleavage site where the addition of multiple basic amino acids is necessary for an AI virus to become highly pathogenic (Steinbauer, 1999; Suarez, 1998).

Since early 1998 Iranian poultry industry has been affected by AI viruses. The isolated virus was subtyped as

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Abbreviations: AI = avian influenza; CEF = chick embryo fibroblast; HA = hemagglutinin; p.i. = post infection

H9N2 by Pourbakhsh *et al*. (2000). Banks *et al*. (2000) have reported low-virulence H9N2 subtypes isolated from different countries, inclusive of two AI isolates from Iran. However, field evidence suggested of a pathogenic potential for the Iranian H9N2 viruses in chickens (Nilli and Asasi, 2002). The occurrence of AI viruses (H9N2) in Iran with 20–65% field mortality rate (Vasfi Marandi and Bozorgmehrifard, 1999; Nilli and Asasi, 2002) and their high variability in genetic make-up led to the speculation that the recently isolated H9N2 viruses might have gained virulence in the field.

The main aim of this study was to determine and compare the pathogenicity of the recently isolated AI viruses from broiler flocks with high and low mortality rates according to the recommendations of the Office International des Epizooties.

Materials and Methods

Virus and bacterial isolations. Virus isolation from tracheal swabs was carried out in 10-day-old SPF embryonated chicken eggs inoculated in the allantoic sac. Isolation of *O. rhinotracheale* was carried out on plates with 5% sheep blood agar containing 5 µg/ml gentamicin in 5% CO_2 at 37°C. Following 24 hrs of incubation, the colonies were subcultured and their identities were confirmed by biochemical and serological methods (rapid slide agglutination and agar gel precipitation). Isolation of *E. coli* was carried out according to standard bacteriological procedures (Lee and Arp, 1998).

Virus growth in CEF cultures. CEF cultures derived from 11-day-old SPF chicken embryonated eggs were grown to confluence in Medium 199 (Gibco-BRL) containing 5% FCS, 0.3% Tryptose phosphate broth (Difco, USA), 100 IU/ml penicillin, and 100 mg/ml streptomycin in 96-well flat-bottom microplates (Nunc, Denmark). A maintenance medium had similar composition, except for it contained 10 µg/ml trypsin but no FCS. The freshly cloned egg-grown isolates diluted 10^{-1}, 10^{-2}, and 10^{-3} were inoculated onto PBS-washed confluent cell monolayers at 37°C for 1 hr. The maintenance medium with or without trypsin was added after virus adsorption. For the second passage, this procedure was repeated with an inoculum subjected to single freezing and thawing.

Pathogenicity for chickens. Four-week-old SPF chickens were inoculated intravenously with 0.2 ml of a 1:10 dilution (approximately $10^{8.5}$ EID_{50}) of each isolate freshly propagated in embryonated eggs The chickens were observed for 10 days and then bled for detection of antibodies against the virus H9 subtype by hemagglutination-inhibition (HI) test.

Virus reisolation in embryonated eggs. The virus isolates were reisolated from the tracheas and cloacae of the inoculated chickens at day 3 post infection (p.i.). Tracheal and cloacal swabs were placed in a transport medium, clarified by a low-speed centrifugation for 5 mins, and the supernatants were filtered through a 0.45 µm membrane and inoculated into 10-day-old SPF embryonated chicken eggs via the allantoic sac route. The allantoic fluids were collected from the embryos that died between 24 and 96 hrs p.i., and subjected to HA and HI tests using a specific antiserum to the virus H9 subtype, obtained from Central Veterinary Laboratory, Weybridge, UK.

Total RNA was extracted using the Tripure Reagent (Roche, Germany). In brief, 0.1 ml of infectious allantoic fluid was mixed with 1 ml of the reagent and extracted with 0.2 ml of chloroform-isoamylalcohol (24:1). Following centrifugation, RNA was isopropanol-precipitated, washed with 75% ethanol, and finally dissolved in 15 µl of nuclease-free water.

RT-PCR. Total RNA (2 µg) was subjected to reverse transcription using MMuLV reverse transcriptase and random hexamer primers according to the manufacturer's instructions (Roche, Germany). In PCR, a 486 bp region (nt 571–1022) comprising the cleavage site of HA gene was amplified using forward and reverse primers as described previously by Banks *et al*. (2000). The reaction mixture (50 µl) contained 2 µl of cDNA, 15 pmoles of forward (5'-TATGGGGCATACAYCAYCC-3') and reverse (5'-TC TATGAACCCWGCWATTGCTCC-3') primers, 200 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$, and 1 U of Taq DNA polymerase (Roche, Germany). The PCR consisted of 30 cycles of 94°C/45 sec, 66°C/45 secs, and 72°C/45 secs, and final extension at 72°C for 5 mins. The PCR products were separated by electrophoresis in 1.5% agarose gel.

Sequence and phylogenetic analyses. The PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Germany) and sequenced from both directions, using the forward and reverse primers (MWG-Biotech AG, Germany). The nucleotide and deduced amino acid sequences were compiled and analyzed using the computer program DNASTAR. Phylogenetic analysis was carried out for 151 amino acids (aa 191–341) of HA gene of the three recent Iranian isolates as well as other isolates reported earlier using the respective sequences from the GenBank database and the Clustal W program (Table 1).

Results

Biological studies

In the course of this study, three AI virus isolates, A/chicken/Iran/772/2000 (ckIR772/2000), A/chicken/Iran/798/2000 (ckIR798/2000), and A/chicken/Iran/528/2001 (ckIR528/2001), were obtained from three separate broiler flocks. The isolates exhibited 30%, 30% and 1% mortality rate, respectively, and all were found to belong to the H9N2 subtype. The first two isolates were accompanied by *E. coli* and *O. rhinotracheale.*

Intravenous injection of the isolates induced depression, dullness, anorexia, watery diarrhoea and decreased water consumption in chickens during the days 2 and 3 p.i. These clinical signs subsided after day 4 p.i. No mortality was recorded during the course of these experiments.

Primary passage of the isolates in CEF cultures resulted in CPE in 36 hrs in the presence or absence of trypsin in the

medium. The isolates in secondary passage also produced CPE, but only in the presence of trypsin.

All the isolates were reisolated in embryonated eggs from tracheas and cloacae of the inoculated chickens on day 3 p.i. and were shown to be of H9 subtype by the HI test. The chicken sera collected on day 10 p.i. had HI antibody titers of 8–256. Control non-inoculated chickens showed no clinical signs and their sera had no detectable HI antibody titer.

Sequence analysis

The 486 bp PCR products comprising the HA cleavage site obtained from the three Iranian isolates were sequenced and the corresponding amino acid sequences (aa 191–341) were deduced. Based on these 151 aa-long sequences, the three Iranian isolates were compared with other so far published AI H9N2 isolates. This comparison revealed that (i) the three Iranian isolates differed from each other at both nucleotide and amino acid levels (Fig. 1), (ii) the numbers of amino acid differences compared to the consensus sequence were 4, 5 and 7 for the isolates ckIR772/2000, ckIR528/2001 and ckIR798/2000, respectively, and (iii) these differences were found at positions 196 (D→E), 207 (D→H), 225 (I→V), 234 (Q→M), and 282 (N→K), and (iv) the first four of these positions were specific to all three Iranian isolates.

Comparison of the HA cleavage site motifs of the H9N2 isolates (Table 1) revealed five different motifs, of which PARSSRG was common for most of them inclusive of all Iranian isolates. None of the motifs contained multiple basic amino acids. The highest amino acid identity of the Iranian isolates ckIR798/2000, ckIR772/2000 and ckIR528/2001was with the isolates ckSA99 and ckDE98 (96.7%, 96% and 94.7%, respectively).

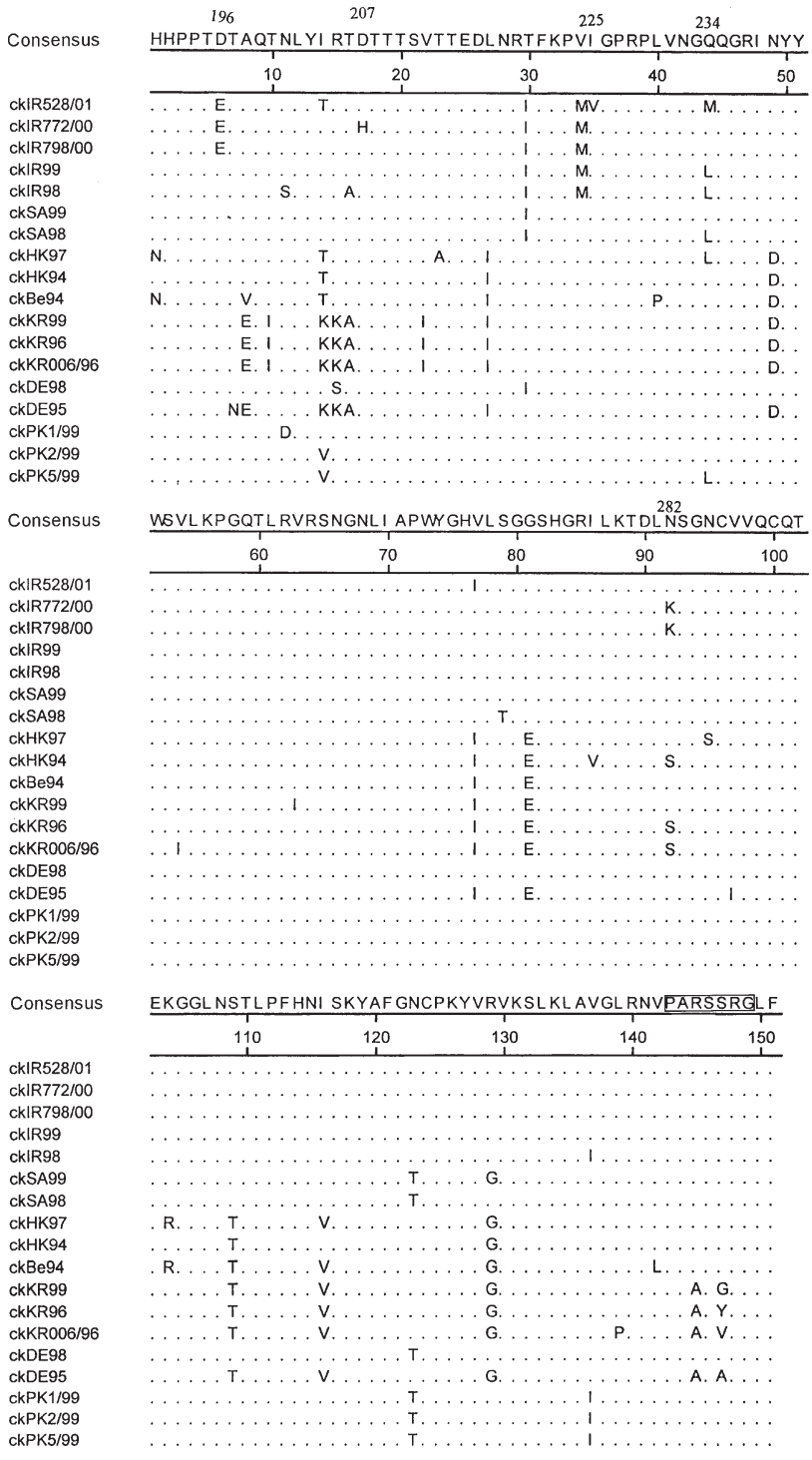

Fig. 1

Alignment of the deduced amino acid sequences of part of HA protein of AI H9N2 virus isolates

Amino acid residues differing from the consensus are indicated. The HA cleavage site motif is underlined. Amino acid positions are numbered according to the A/turkey/Wisconsin /66 (Nobusawa *et al.*, 1991).

Table 1. AI H9N2 virus isolates compared in this study

A/chicken/Iran/772/200000	ckIR772/00	PARSSRG	AJ536330[a]
A/chicken/Iran/528/2001	ckIR528/01		AJ536331[a]
A/chicken/Iran/798/2000	ckIR798/00		AJ536332[a]
A/chicken/Iran/11T/99	ckIR99		AF218112
A/chicken/Iran/16/98	ckIR98		AF218109
A/chicken/Pakistan/1/99	ckPK1/99		AF218114
A/chicken/Pakistan/2/99	ckPK2/99		AF218115
A/chicken/Pakistan/5/99	ckPK5/99		AF218118
A/chicken/Saudi Arabia/532/99	ckSA99		AF218119
A/chicken/Saudi Arabia/224/98	ckSA98		AF218110
A/chicken/Hong Kong/G23/97	ckHK97		AF156374
A/chicken/Beijing/1/94	ckBe94		AF156380
A/chicken/Hong Kong/739/94	ckHK94		AF156379
A/chicken/Germany/R45/98	ckDE98		AF218107
A/chicken/Germany/90/95	ckDE95	PAASARG	AF218099
A/chicken/Korea/25232-006/96	ckKR 006/96	PAASVRG	AF156385
A/chicken/Korea/38349-p96323/96	ckKR96	PAASYRG	AF156384
A/chicken/Korea/99029/99	ckKR99	PAASGRG	AF218111

[a]Isolates characterized in this study.

Phylogenetic analysis

Phylogenetic analysis, based on the 151 aa-long region mentioned above, was performed for the 3 Iranian isolates and 15 AI H9N2 isolates from other countries (Fig. 2). The phylogenetic tree showed two clusters. One comprised all isolates from Iran, Pakistan and Saudi Arabia, and one isolate from Germany, while the other contained all isolates from Korea and China, and one isolate from Germany. With the exception of two isolates from Germany, all isolates with common geographic origin clustered together.

Discussion

In recent years, AI has caused major economic losses to the Iranian poultry industry.

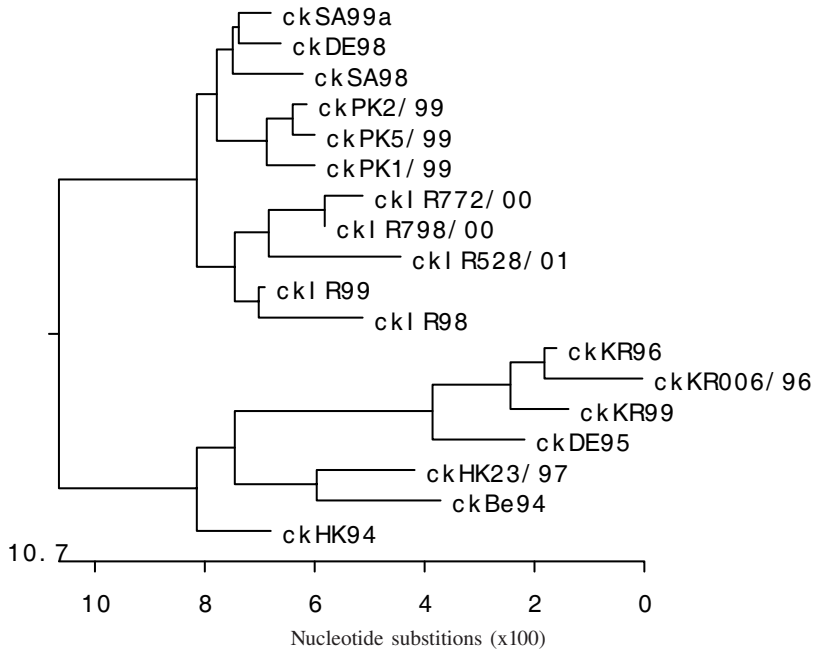

Fig. 2

Phylogenetic tree of AI H9N2 virus isolates based on the deduced amino acid sequences of a part of HA

The latest Iranian H9N2 isolate has been reported to be low pathogenic for SPF chickens (Pourbakhsh *et al*., 2000). However, recent AI H9N2 outbreaks have caused up to 65% mortality rate (Nilli and Asasi, 2002). Biological and molecular characterization of the three AI H9N2 isolates from broiler flocks with different mortality rates, described in this paper, was carried out according to the recommendations of OIE for investigation of the effect of viruses on mortality. The results of this study did not identify the three Iranian AI isolates as highly pathogenic.

In spite of different mortality rates in the field, these isolates were of low virulence and did not produce mortality among SPF chickens. Depression, dullness, watery diarrhoea, and decreased food and water consumption were the only clinical sings observed in the inoculated chickens during the second and third days of the experimental infection. *E. coli* and *O. rhinotracheale* were found along with the two AI isolates in the broiler flocks with high (30%) mortality rate. The third AI isolate from the broiler flock with low (1%) mortality was not accompanied by pathogenic bacteria. These results suggest that the co-infection of the AI isolates with *E. coli* and/or *O. rhinotracheale* might have caused an elevation in mortality rate. *O. rhinotracheale* has been isolated from 65.4% of H9N2-affected commercial poultry flocks in Iran (Banani *et al*., 2002). It has been shown that the association of bacterial agents with H9N2 viruses is necessary to produce mortality in susceptible chickens (Bano *et al*., 2003). Moreover, Kishida *et al.* (2004) have reported enhanced pathogenicity and replication of a H9N2 virus in chickens co-infected with H9N2 virus and either *Staphylococcus aureus* or *Haemophilus paragallinarum*. Thus pathogenic potential of AI H9N2 viruses can vary in dependence on other factors.

The virus reisolation and the elicitation of antibody response in the inoculated chickens without mortality indicated that H9N2 viruses alone were not virulent. Banks *et al.* (2000) have also shown that representative H9N2 viruses from some countries are not highly pathogenic. In contrast, Guo *et al*. (2002) have reported an exclusive H9N2 isolate causing as high as 80% mortality in chickens.

The CEF culture results of this study revealed that the isolates, if passaged, did not propagate in the absence of trypsin. However, a CPE was observed and virus presence was confirmed by the neutralization test in the first passage (data not shown). It has been shown that all influenza viruses yield infectious particles in embryonated chicken eggs and their HAs are cleaved (Klenk *et al*., 1975).

In case of non-highly-pathogenic AI viruses, CEF cells produce viruses of low infectivity that can be cleaved by trypsin to yield viruses of high infectivity. The presence of host proteases or exogenous trypsin is necessary for infectivity of AI viruses (Steinhauer, 1999). Thus, it is very likely that the observation of CPE in the primary passage was due to the fact that the viruses were propagated in embryonated chicken eggs.

To determine the epidemiological characteristics and the HA cleavage site motifs of the three Iranian AI (H9N2) isolates, the corresponding 486-bp PCR products comprising the HA cleavage site were sequenced and compared to those from 15 other AI H9N2 isolates.

The three isolates displayed a high level of overall identity, although individual differences were detected at both nucleotide and amino acid levels indicating mutations. Sequence analysis showed differences in four amino acids unique to the Iranian isolates compared to other isolates. It is possible that these differences represent mutations that could have occurred during field passages. They had no significant effect on their pathogenicity, since all the three isolates had low pathogenicity indices like other isolates under comparison.

Comparison of the HA cleavage site motifs of all the isolates indicated a common motif (PARSSRG) with the exception of 3 Korean and 1 German isolate. None of the altogether 5 different motifs contained multiple basic amino acids. The H9N2 isolates that were capable of producing high mortality (Guo *et al*., 2000) or had wider tissue tropism (Lee *et al*., 2000) did not contain multiple basic amino acids at their cleavage site motifs.

The HA0 protein of non-pathogenic influenza viruses can be cleaved extracellularly by trypsin-like enzymes originating from co-infecting bacteria or host inflammatory response induced by respiratory tract infections. Influenza viruses can also predispose the host to secondary bacterial infections by causing tissue damage, inhibition of bacterial clearance, promotion of bacterial adherence, and interference with non-specific immunity (Steinhauer, 1999). We assumed that co-infections play a critical role in pathogenesis of AI viruses in Iran. Indeed, the majority of the AI infections with high mortality in commercial flocks examined in our laboratory have been associated with one of the bacterial or viral infections (R. Toroghi, unpublished data).

The phylogenetic analysis based on the amino acid sequence of a HA region showed that the isolates from Iran, Pakistan and Saudi Arabia clustered together, indicating a close relationship. Interestingly, the isolates from each particular country formed a homogenous group, revealing that these viruses probably had a single source. The fact that the Korean and Chinese isolates formed separate groups within one cluster suggests that the AI outbreaks in these countries resulted from different AI H9N2 viruses. Interestingly, the German ckDE95 isolate was more closely related to the three Korean isolates (which had mutations within their motifs) than to the other German isolate (ckDE98).

A maximum identity was observed for two isolates, from Saudi Arabia (ckSA99) and Germany (ckDE98). Interestingly, Banks *et al*. (2000) have shown that ckDE98

is the only European AI H9N2 isolate that has a very close genetic relationship to and could also be grouped with Asian H9N2 isolates.

In order to identify the source of the Iranian AI H9N2 outbreaks it is necessary to study the nucleotide sequence of the HA gene or other genes of other Iranian H9N2 isolates from different sources including backyard flocks, waterfowl or migratory birds. Recently, AI H9N2 viruses have been isolated from migratory birds in Iran (Ferradoni *et al*., 2005).

Overall, on the basis of *in vivo* and *in vitro* experiments, and nucleotide and amino acid sequences of the HA gene/protein, the Iranian AI virus isolates could be characterized as non-highly-pathogenic. The fact that infection with these viruses has been associated with high mortality in the field condition suggests that co-infections, secondary pathogens and environmental conditions may play a major role in exacerbation of the infection in chickens. The significance of these factors in the observed mortality remains to be determined.

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