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Short Communication

Avian Influenza Survey in North-West of Iran from January to March 2008, Using RT-PCR

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Influenza virus types A, B and C all belong to the Family of Orthomyxoviridae and therefore have many common biological properties. Influenza B and C are predominantly human pathogens with some exceptions; Influenza B and C have been isolated from seals and pigs, respectively. Influenza A viruses have been isolated from many species including humans, pigs, horses, marine mammals and a wide range of domestic and wild birds (1, 2). It is generally accepted that in human influenza pandemics from the last century and numerous recent outbreaks in domestic and wild animals, interspecies transmission of Avian Influenza viruses (AIVs) have caused major concern. Predominantly wild aquatic birds are the most important reservoir of influenza A viruses in nature. So far, all sixteen recognized antigenic subtypes of the virus haemagglutinin antigen and all nine subtypes of neuraminidase antigen have been isolated and identified from these bird species. AIVs preferentially infect cell lining of the intestinal tract of birds and are excreted in high concentrations through feces. The transmission of influenza viruses between birds is thought to occur primarily via the fecal-oral route, whereas AIVs are generally nonpathogenic in their natural hosts, they may cause significant morbidity and mortality upon transmission to other species (3, 4).

Western Azerbaijan province in northwestern portion of Iran is one of the centers of mother chicken meat production in Iran, having 41 breeding units and is ranked second in the country. Therefore the condition is right for emergence of avian influenza. These areas represent a stop-over sites for a wide variety of migrating birds species from central Asia, Siberia, western Europe and the Mediterranean countries and also these are parts of two flyways: Eastern Asia-Western Africa and Black sea-Mediterranean routes (5, 6).Therefore, these areas are considered as a prime site for risk of introduction and transmission of bird-borne pathogens. There is a strong potential to introduce AIVs from countries such as Iraq, Turkey and Azerbaijan to Iran, particularly during annual migration of wild aquatic birds (7). Numerous cases of death among birds in the form of a collective and scattered in these regions have been reported. The most important of cause of death of the birds with high mortality rate has been reported in March of 2005 in the Aras Dam Lake. About 2500 pieces of various kinds of aquatic wild migrant birds that included mostly common Coot and Mallard were killed. A total of 100 villages in direct connection with the lake were defined as red areas (8). Also, in North of Iran in recent years two events of the outbreak and mortality in the wild and domesticated birds were due to the deadly H5N1 bird flu (9). Since water-associated wild

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Fig. 1: The map of North-West of Iran, indicating the sample collection sites: Aras Dam Lake (A), Kani Brazan wetland (B).

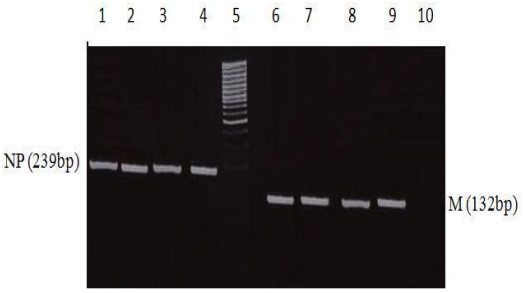

Fig. 2: Gel electrophoresis of RT-PCR products of NP and M genes from 4 positive cloacal swabs. The expected sizes of RT-PCR products are shown in parentheses. Lane 1–4: products of M gene of clinical samples, Lane 5: Gene Ruler 100 bp (CinnaGen, Iran), Lane 6-9: products of NP gene of clinical samples, Lane 10: negative control.

birds such as ducks, geese, gulls and shorebirds are the most important reservoir of influenza A viruses in the nature, surveillance and monitoring of these birds are crucial. Through the continuous surveillance of wild birds around the world, prevalence of AIVs in the environment can be monitored and pathogenesis of the circulating viruses can be determined as well. In addition, such studies will ensure that panels of reference reagents required for testing of animals and humans be updated continuously and that the virus isolates with the appropriate antigenic properties will be available for propagation of potential influenza vaccines (1, 3).

In this study the potential arrival and circulation of AIVs monitored in the North-West of Iran during the winter of 2008 by combined different data sets including; wild migratory census birds, death surveillance and Molecular detection were determined. The data collected in this study can be used by scientists to develop a better understanding of the transmission of avian influenza viruses among wild and domestic animals, improve risk analyses and plan monitoring strategies to track future avian influenza spread.

The best molecular method recommended by WHO and OIE for identification and characterization AIVs is RT-PCR assay. Therefore, in this study we used RT-PCR assay for detection of AIVs in collected samples employing routine kits and reagents. RT-PCR assay could be completed within one day with high sensitivity and specificity in comparison to classical virus propagation in eggs and cell cultures and provides a good alternative for rapid and efficient screening of the virus in wild birds (6, 10). Out of the samples collected between January and March of 2008, 73 fresh fecal samples from wild migratory aquatic birds in 2 geographical sites of: North (Aras Dam Lake) and Center (Kani Brazan wetland) of Western Azerbaijan province in North-West of Iran (Figure 1) were collected randomly, using polyester swabs. The samples were delivered in Viral Transport Medium (VTM) to the laboratory and stored at –20°C for further testing. VTM was prepared with PBS/glycerol (1:1) solution (pH=7.2) including antibiotics: 2x106 IU/liter Benzyl Penicillin- 200 mg/litre streptomycin, 2x106 IU/liter Polymyxin B, 250 mg/liter Gentamicin, and 0.5x106 IU/liter Nystatin (16). Fecal sampling is used extensively in monitoring studies for AIVs in wild bird populations. The principal advantages of this method are that the cost and effort of capturing birds are avoided and large of sample can be quickly and easily obtained. It is also a good method to determine the presence or absence of virus in bird populations present at a specific location. Infectivity of the virus is maintained up to 4 days in wet feces at 25^{o}C. Best analytical

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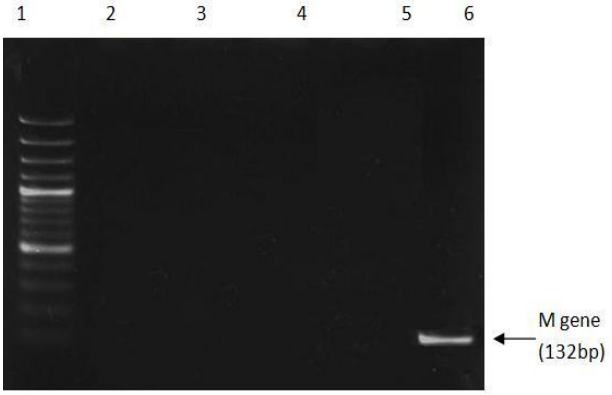

Fig. 3: Gel electrophoresis of RT-PCR products of M genes. Lane 1: Gene Ruler 100 bp (CinnaGen, Iran), Lane 2: negative control, Lane 3-4-5: negative results, Lane 6: positive control (M gene).

results come from fresh fecal samples that are either processed quickly or frozen until processing. By restricting fecal collection to fresh samples, it allows for population census data to be collected, and the source of the contamination determined (1). In addition the sample size selected for testing needs to be large enough to detect infection if it were to occur at a predetermined minimum rate. Despite our effort to sample a large number of birds, the chosen sample size is only appropriate for the geographic target sites mentioned in this survey.

Extraction of total RNA was performed following manufacturer's protocol from QIAamp Viral RNA Mini Kit (Qiagen, USA) using all necessary safety precautions. Briefly, Buffer AVL containing carrier RNA was prepared in a 1.5 ml microcentrifuge tube. A 140 µl of fecal sample was added to the tube. After Incubation at room temperature (15–25°C) for 10 min. 560 µl of ethanol (96–100%) was added to the mixture and then in two steps, 630 µl of the solution was added to the QIAamp Mini column and centrifuged at 6000g for 1 min. Washing and elution was done according to the manufacturer's instructions by AW1 and AW2 buffers. The resultant RNA was dissolved in 60 µl of Buffer AVE.

For reverse transcription, 2 µl of Uni-12 primer (20 pmol) were added to 10 µl RNA solutions and incubated at 70°C for 50 min. The Uni-12 primer sequence was: 5- AGCAAAAGCAGG -3 (2). Then 200 U RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Canada), 20 U RiboLock™ Ribonuclease Inhibitor (CinnaGen., Inc, Iran), 4 µl of 5x RT buffer, 2 µl 10 mM dNTPs were added to the mixture which was heated to 42°C for 60 min and 70°C for 10 min, and subsequently chilled on ice.

RT-PCR assay was optimized to detect Influenza A virus with 4 positive cloacal swabs from poultry with influenza symptoms (Figure 2). 1.25 U Taq DNA polymerase (CinnaGen, Iran), 2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTPs, 1 µl sense and antisense primers (10 pmol) and 16.5 µl double distilled water were added to 2.5 ml cDNA mixture. Influenza type specific primers were designed considering the conserved regions of influenza A matrix (M) and nucleoprotein (NP) genes (Table 1). The properties of the primers were analyzed with BioEdit, Clastal X and Generunner softwares. The amplification protocol was: One step of denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 second - annealing at 50°C for 45 second - extension at 72°C for 45 second, and one step of final extension at 72°C for 10 min. Samples with RNase free sterile water instead of specific template were used as a negative controls and RNA extracted from 4 positive cloacal swabs from poultry with influenza symptoms used as

Table 1: Influenza A primers used for RT-PCR

PCR primer	Sequence	Location	Size(bp)
M_F	5 -GACTCAAATGTCAAGAACCTTTA-3	35-59	
M_R	5-CCACTTATTTCCTCTCTGTTTAG-3	145-167	132
NP_F	5-GTTTGAGTCTGTTGCTTGGTC-3	1179-1199	
NP_R	5-TGATAGTGTCTGTTATTATGCC-3	1487-1508	329

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a positive control. The products were detected by 2% agarose gel electrophoresis.

No AIVs was detected in any of 73 samples collected from wild migratory aquatic birds of common species in two sites: North (Aras Dam Lake) and Center (Kani Brazan wetland) of Western Azerbaijan province in Iran from January and March of 2008 (Figure 3). There is no evidence of influenza type A viruses in North West of Iran, based on surveillance of wild birds and investigations of bird deaths, and we did not observe any dead or sick bird and did not notice abnormal population size reductions during the study period.

Results from the on foot census of wild migratory birds during the study period and also further important information provided by the analysis of the long-term monitoring data of wintering wild migratory aquatic birds in regions indicated that there was no abnormal population reduction.

On based achieved data of OIE, there was no report of outbreak and mortality of wild migratory aquatic birds from avian influenza viruses in adjacent countries including Azerbaijan, Armenia, Iraq and Northeastern regions of Turkey during the study period. However, there are reports of outbreak and mortality in Turkey provinces neighboring Black sea including Zonguldak, Sakarya, Sinop and Samsun.

Therefore these data sets concluded that circulation of AIVs during the winter of 2008 in these regions was either non-existent or negligible, and there is no introduction and spread of the type A influenza viruses to the regions from wild migratory aquatic birds to native and industrial poultry.

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