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RESEARCH ARTICLE

Molecular Detection of Avian Infectious Bronchitis Virus Serotypes from Clinically Suspected Broiler Chicken Flocks in Fars Province of Iran

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ABSTRACT

This study was conducted to detect the serotypes Arkansas, Connecticut, Beaudette and Florida in broiler chicken farms in Fars province of Iran. The samples were obtained from 32 broiler chicken farms experiencing respiratory signs with high mortality and clinically diagnosed as avian infectious bronchitis. Total RNAs were extracted from the scrapped mucosa of tracheas and RT-PCR tests were performed using different types of primers including Del1 (F, R), (XCE-1, 2), (XCE-3 & BCE-1), (XCE-3 & MCE-1), S1 OLIGO (F, R) and S1 OLIGO NEW (F, R). Infectious bronchitis virus was detected in 72% poultry farms. The serotypes 4/91 and Massachusetts were detected in 47.9 and 21.8% farms, respectively, but the serotypes in 7 farms remained unclear. The results showed that there is no evidence for the presence of serotypes Arkansas, Connecticut, Beaudette and Florida in broiler chicken farms in Fars province of Iran.

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INTRODUCTION

The infectious bronchitis virus (IBV), which causes a highly contagious disease in chickens, belongs to group III of the genus coronavirus of the coronaviridae family (Cavanagh and Naqi, 1997; Hadipour *et al*., 2011; Hafez, 2011). The virus causes a highly contagious disease in chicken with significant economic losses throughout the world and is able to spread very rapidly in non-protected birds (Wit *et al*., 2010; Shahzad *et al*., 2011). The virus affects mainly the respiratory tract, causing tracheal rales, sneezing, coughing, reduced weight gain and mortality, particularly in broiler chicken. The urogenital tract is also affected by the virus, resulting in egg production decline as well as reduced egg quality, interstitial nephritis and visceral gout. To monitor the existing different IBV serotypes in a geographical region, several tests including virus isolation, virus neutralization, hemagglutination inhibition, hybridization and ELISA have been employed (Haqshenas *et al*., 2005; Cavanagh and Gelb, 2008). Virus neutralization is currently the standard assay for typing IBV strains. The major disadvantage of serological methods is that they are rather time-consuming and the obtained results lack standardization. PCR on reverse-transcribed RNA is a potent technique for the detection of IBV. In comparison with classical detection methods, PCR-based techniques are both sensitive and fast (Zwaagstra *et al*., 1992).

The control of infectious bronchitis currently relies on vaccination with live-attenuated and inactivated vaccines. However, vaccination failure occurs frequently in chicken farms all over the world. Since the disease is caused by a variety of serotypes which are not fully cross protected, many researchers have focused on identifying local circulating serotypes in order to improve the effectiveness of the employed vaccine strains. Despite the current use of live-attenuated H120 and 4/91 vaccine strains, there are high economic losses in the broiler chicken industry in Iran due to the occurrence of clinical infectious bronchitis in the flocks. Several attempts have been made to detect circulatory IB serotypes in the country, which has led to a report of the presence of 4/91 and Massachusetts serotypes (Seifyabadshapouri *et al*., 2002; Nouri *et al*., 2003; Akbari *et al*., 2004; Haqshenas *et al*., 2005; Seifi *et al*., 2009). It has been reported that other IB serotypes also exist in the region and countries around Iran such as Dutch strains in the poultry industry in Pakistan (Ahmed *et al*., 2007) and Arkansas in Jordan (Gharaibeh, 2007). This study was conducted to investigate the molecular detection of avian infectious bronchitis virus serotypes Arkansas, Connecticut,

Beaudette and Florida in Fars province broiler chicken farms.

MATERIALS AND METHODS

Samples collection: Tracheal samples from 32 broiler farms in Fars province suffering from respiratory distress and having a mortality rate above the normal range of 5% and provisionally supposed as IB, were collected and prepared for RNA extraction protocol. The lyophilized IBV vaccine Galli vac, IB88 (Merial, No: L242746), Nobilis IB, MA5 (Intervet, DM0108806), Bioral, H120 IBV (Merial, L202560), and H120 IBV (Razi Vaccine and Serum Research Institute) containing 1000-2000 doses were reconstituted in 1 ml sterile distilled water as control positive samples.

RNA extraction: The total RNAs were extracted from the scrapped mucosa of the tracheas using RNX ^{TM}-Plus reagent (Cinnagen, Iran). Briefly, at first, 1 ml of RNX solution was added to 100 mg of homogenized tracheal tissue or 100 µl of vaccine solution. 200 µl of chloroform was then added to the mixture. After centrifugation (12000 rpm at 4°C for 15 min), the upper phase was transferred to RNase-free tube and an equal volume of isopropanol was added. After centrifugation, the supernsatant was discarded and 1 ml of 75% ethanol was added to wash the RNA pellets. Finally, the pellets were dissolved in 50 µl of sterile distilled water and stored at -70°C.

cDNA synthesis: The extracted RNA was used in reverse transcription (RT) reaction to generate cDNA by use of lyophilized master (Bioneer, Korea). The primers used in cDNA synthesis are the same specific primers used in each RT-PCR assay. The primer pairs used in the cDNA synthesis and PCR are shown in Table 1. For synthesis of the cDNA, 5 µL of extracted RNA was mixed with 1 µL of each reverse primer and incubated at 70°C for 5 min. Then, the contents of each tube was transferred to 0.2 ml lyophilized master eppendorf tubes (containing PCR buffer, dATP, dTTP, dGTP and dCTP, $MgCl_2$ and Moloney murine leukemia virus reverse transcriptase) and the final volumes brought out to 20 µL with diethyl pyrocarbonate (DEPC). After the addition of 15 µL inorganic oil to each tube, the mixture was incubated at 42°C for 1 hour, and then incubated at 95°C for 5 min to inactivate the reverse transcriptase. The resultant cDNA was stored at -20°C for later use.

PCR: For the amplification reaction, at first, two primers, Del1 F and Del1 R were used and for the next RT-PCR (Nested RT-PCR) two primers, XCE-1 F and XCE-2 R were used in the first stage, and BCE-1 and XCE-3 or MCE-1 and XCE-3 in the second stage for detection of the 4/91 and Massachusetts serotypes, respectively (Table-1). In another RT-PCR S1 OLIGO F and R were used and in the last RT-PCR, S1 OLIGO NEW F and R to detect the S1 gene were used, according to previous reports (Kwon *et al*., 1993a; Cavanagh *et al*., 1999; Jackwood *et al*., 2005; Moscoso *et al*., 2005; Mardani *et al*., 2006). The PCRs were carried out in 20 µL volumes containing 2 µL of 10x PCR buffers, 0.2 µL Taq polymerase, 50 Mm $MgCl_2$ (0.6 µL for all primers and 1.2 µL for S1 OLIGO and S1 OLIGO NEW), 10 mM dNTPs (0.2 µL for Del1 primers and 0.4 µL for other primers), 1 µL of 10 ρmol R and F primers for all primers (except 0.5 µL for S1 OLIGO and S1 OLIGO NEW), cDNA (4 µL of for Del1, S1 OLIGO and S1 OLIGO NEW and 1 µL for other primers) and distilled water (in order for the final volumes to reach 20 µL).

Gel electrophoresis: PCR products were electrophoresed on a 1.5 % agarose gel in TAE buffer (distilled water, Tris base, concentrated acetic acid and 0.5 mM EDTA) containing ethidium bromide for 30-40 min at 80 V and visualized under an ultraviolet light transilluminator.

RESULTS

Thirty two broiler farms in Fars province suspected of IB and having a mortality rate above the normal range of 5% during the clinical disease were studied. The prevalence of the M-41 and 4/91 serotypes of IBV were about 21.8% (5 farms) and 47.9% (11 farms), respectively, and in seven farms, unknown IBV serotypes were detected. Figure 1 shows the RT-PCR results in commercial broiler flocks affected by respiratory disease. In this RT-PCR, the primer pairs of Del1 F and R, which comprised of the IBV N gene were used, and in 23 farms, IBV was detected (Fig. 1). Nested RT-PCR were conducted with the use of two primers, XCE-1 and XCE-2 in the first stage, and BCE-1 and XCE-3 or MCE-1 and XCE-3 in the second stage for the detection of the 4/91 and Massachusetts serotypes, respectively. The 154 and 295 bp fragment, including a portion of the S1 gene of the 4/91 and Massachusetts serotypes was amplified by RT-PCR using BCE-1 and MCE-1 primers, respectively (Fig. 2). Seven samples were positive for the IBV genome using a general primer, however there was no specific amplicon indicating the investigating serotypes in the nested PCR. For the amplification of the S1 gene and then the serotypes differentiation, S1 OLIGO F and R were used and lastly, RT-PCR, S1 OLIGO NEW F and R. The 1700 bp bands were not detected using these primers; only MA5 and Razi vaccines produced bands nearly 1700 bp using both primers in RT-PCR (Fig. 3).

DISCUSSION

Currently, prevention of IB in chicken is mainly based on vaccination using live-attenuated or killed vaccines. Although live attenuated vaccines (H120 and 4/91 strains) have been widely used to control IB in broiler chicken, the disease has continued to be a serious problem in the Iranian poultry industry (Haqshenas *et al*., 2005). One of the major problems with IBV is the frequent emergence of new variants that probably cause the vaccination failure. The detection and identification of these new variants are crucial to disease control. Many systems are used for the detection and identification of IBV. Current tests are based on differentiation by clinical symptoms, cross-immunization, serology and RT-PCR. Kwon *et al.* (1993b) have developed a new IBV typing method using PCR and RFLP analysis that agrees with the VN test. The primer, S1 OLIGO 5, consisted of a sequence identical to a region near the 5' end of the S1

Table 1: Sequences, products size and specificity of the primers for IBV serotypes

Primers	Sequences	RT-PCR products size	Strains
Del1-R	5'-CAT-TTC-CCT-GGC-GAT-AGA-C-3'		All IBV strains
Del1-F	5'-GAG-AGG-AAC-AAT-GCA-CAG-C-3'	350 bp	
XCE-1	5'-CAC-TGG-TAA-TTT-TTC-AGA-TGG-3'		Massachusetts
XCE-2	5'-CTC-TAT-AAA-CAC-CCT-TAC-A-3'	464 bp	and 4/91
XCE-3	5'-CAG-ATT-GCT-TAC-AAC-CAC-3'		
BCE-1	5'-AGT-AGT-TTT-TGT-GTA-TAA-ACC-A-3'	154 bp	4/91
XCE-3	5'-CAG-ATT-GCT-TAC-AAC-CAC-3'		
MCE-1	5'-AAT-ACT-ACT-TTT-ACG-TTA-CAC-3'	295 bp	Massachusetts
S1 OLIGO- R	5'-CAT-AAC-TAA-CAT-AAG-GGC-AA-3'		Arkansas, Connecticut,
S1 OLIGO - F	5'-TGA-AAA-CTG-AAC-AAA-AGA-CA-3'	1720 bp	Beaudette and Florida
S1 OLIGO NEW- R	5'-CCA-TAA-CTA-ACA-TAA-GGR-CRA-3'		
S1 OLIGO NEW- F	5'-TGA-AAC-TGA-ACA-AAA-GAC-3'		

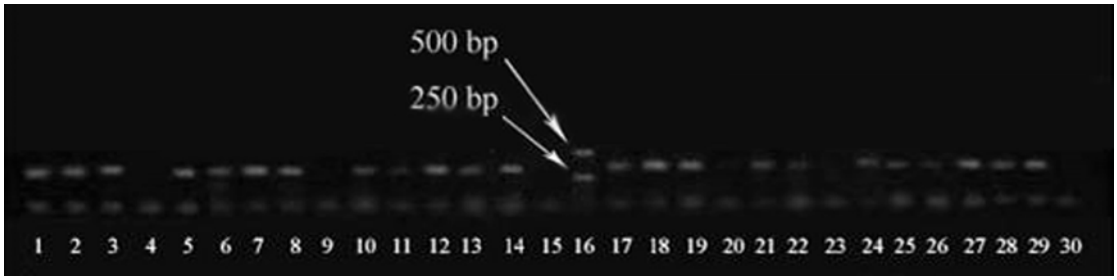

Fig. 1: Electrophoresis (1.5% agarose gel) of PCR amplified products of cDNA made RNA extracted from IBV by the Del1 F/Del1 R set of primers. Lane 16 = Gene ruler 50 bp plus DNA ladder. Lanes 1-3, 5-8, 10-14, 17-19, 21, 22, and 24-29 = positive samples (nearly 350 bp). Lanes 4, 9, 15, 20 and 23 = negative samples. Lane 30 = negative control sample.

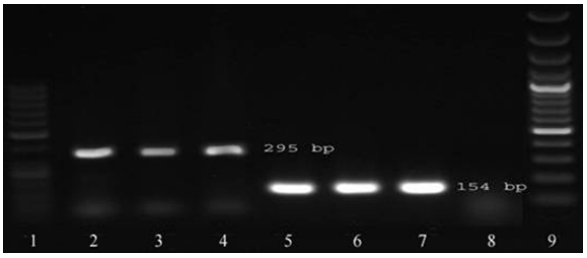

Fig. 2: Electrophoresis (1.5% agarose gel) for 4/91 and M/41 serotypes of IBV detection by the BCE-1 and MCE-1 set of primers, respectively. Gene ruler 50 bp and 100 bp plus DNA ladder were placed in lanes 1 and 9, respectively. Lane 8 = negative control sample. Lanes 2-4 = Massachusetts positive samples (with 295 bp amplicon). Lanes 5-7 = 4/91 positive samples (with 154 bp amplicon).

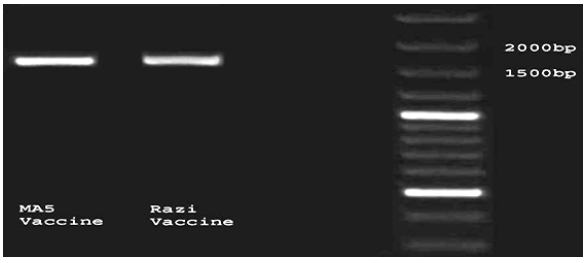

Fig. 3: Electrophoresis (1.5% agarose gel) of MA5 and Razi vaccines of IBV by using S1 NEW OLIGO F/ S1 NEW OLIGO R set of primers. Gene ruler 1000 bp plus DNA ladder. These two vaccines showed 1700 bp bands.

glycoprotein gene; the primer, S1 OLIGO 3' consisted of a sequence complementary to a region at the 5' end of the S2 glycoprotein gene. Both IBV primers were 20 bases long and flanked a 1720-base sequence containing the whole S1 glycoprotein gene. For each of the IBV strains, the size of the amplified product appeared to be identical (Kwon *et al*., 1993b). This technique was also used by other Iranian researchers (Seifyabadshapouri *et al*., 2002; Nouri *et al*., 2003; Akbari *et al*., 2004; Haqshenas *et al*., 2005; Seifi *et al*., 2009) and showed a field circulation of Mass and 4/91 serotypes and the absence of Dutch strains in the poultry industry. Shoushtari *et al*. (2008) revealed that the predominant circulating type of avian infectious bronchitis viruses in Iran during 1999 - 2004 is 793/B type IBV. No one has investigated the entity of Arkansas, Connecticut, Beaudette and Florida serotypes in the country.

In this study, the set of primers, S1 OLIGO 5' and 3' were used for the amplification of a 1720 bp fragment containing the entire S1 subunit of the spike gene of the samples that were positive for Del1 5' and 3' (showing 350 bp amplicons), but none of the positive samples showed amplicons. Jackwood *et al*. (1997) and Lee *et al*. (2000) designed new forward and reverse primers in response to changes in the virus in 1997 and 2000, respectively. The new primer was named S1 NEW OLIGO 5' and S1 NEW OLIGO 3'. Thus, these primers were used to investigate four suspected serotypes, but no amplicon has been seen yet. Although we have used a specific primer to detect IBV in clinically suspected samples, it has been deduced that some incorrect provisional diagnosis or clearance of the virus from the trachea is responsible for the lack of amplification. The other etiologic agent that can produce similar signs and lesions in co-infections with bacteria in suspected flocks is the H9N2 avian influenza virus, which might be prevalent in some broiler chicken flocks (Seifi *et al*., 2009) and cannot be ruled out.

This study was conducted to monitor the existence of these serotypes of infectious bronchitis virus which have been reported, in part, from the countries around Iran, in commercial broiler chickens in Fars province. From thirty-two commercial broiler farms clinically suspected of IB studied here, twenty three farms were positive using

the general primer of the IB gene. Comparison of these positive samples by serotype specific primers showed that M41and 4/91 were prevalent in 5 (21.8%) and 11 (47.9%) out of 32 flocks, respectively. Ahmed *et al.* (2007) has shown that the commercial broiler and layer flocks in Pakistan were seropositive with M-41 (88%), D-274 (40%), D-1466 (52%), and 4-91 (8%) strains. There was no evidence of the existence of Arkansas, Connecticut, Beaudette and Florida serotypes in our samples. In seven farms unknown serotypes in RT-PCR were detected using Del1 R & F primers, but they had no amplicon by use of MCE-1 and BCE-1 primers. Although it was reported that partial sequencing of isolates from Iran and Saudi Arabia, isolated in 2000, revealed approximately 95% nucleotide identity with European isolates, including the two live 793/B vaccinal strains (Cavanagh *et al*., 2005), it seems some variations have occurred recently. So it is suggested that the unknown detected viruses belong to serotypes with genetic changes. This genetic variation may address, at least in part, vaccination failure in the country despite the extensive use of live attenuated and inactivated vaccines including M41 and 4/91 serotypes. It is reported that most neutralizing antibodies are induced by a small number of dominant epitopes, so a small number of amino acid changes in these regions is sufficient to change the serotype, which may affect weak cross protection (Cavanagh *et al*., 2005). Therefore, it is imperative to know the prevalent status of IBVs and the major circulating strains in a region or country in order to select the appropriate vaccine to prevent the disease. Further research on the sequence analysis of the S1 gene of the prevalent serotypes and experimental challenge are needed to elucidate the problem of such vaccine failures.

Future work should include the isolation & sero-typing of IBV in the region in order to adopt a suitable vaccination program, using the common field serotypes as vaccines, to protect against IBV caused disease.

Conclusions

Infectious bronchitis virus was detected in 23 poultry farms. The serotypes 4/91 and Massachusetts were detected in 11 and 5 farms respectively, but the serotypes in 7 farms remained unclear. The results showed that there is no evidence for the presence of serotypes Arkansas, Connecticut, Beaudette and Florida in broiler chicken farms in Fars province of Iran.

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