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793/B type, the Predominant Circulating Type of avian Infectious Bronchitis Viruses 1999 - 2004 in Iran: a retrospective study

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ABSTRACT

In order to identify predominant circulating types of infectious bronchitis(IB) virus in the period of 1999-2004 in Iran, samples(trachea, lung, kidney) from 150 various flocks showing respiratory signs suspected to be related to IB infection were inoculated to SPF eggs. Then allantoic fluids studied by RT-PCR and Nested PCR using group and type specific primers to probing Massachusset, 793/B and D274 types viruses respectively. Out of 150 tested flocks approximately 72% were IBV positive in RT PCR tests. Specific nested PCRs on RT- PCR products of tested samples revealed that 57 flocks (52.7%) had been infected by 793/B type IBV alone. Massachusetts type IBV also could be detected in 18 flocks (16.6%) alone. Thirty three (30.5%) flocks had been infected by 793/B and Massachusetts type IBV in combination. Collectively, there were 90(approximately 83%) flocks that were infected by 793/B type IBV. Our results showed that the predominant circulating serotype of IBVs during the study period was 793/B type. The 274D type did not detected in any flocks during this study. The co-existence of Masachusset and 793/B type's viruses in the same times in 33 flocks revealed that Masachuset type virus vaccines have not conferred a proper immunity against 793/B type viruses.

Keywords: infectious bronchitis, 793/B type, avian viruses, Iran

INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious upper respiratory disease of chickens. IB virus a member of the family Coronaviridae is an enveloped virus that has three major structural proteins (Cavanagh 1997). In the past 50 years many new in IBV serotypes or variant have been described (Cavanagh & Naqui, 2003). These serotypes may arise either as a result of spontaneous mutation or recombination (Cavanagh 2003, Jia *et al* 1995). The nucleocapsid protein is closely associated with positive-sense virus. RNA The membrane glycoprotein is a transmembrane protein. The spike glycoprotein is post-translationally cleaved into subunits designed S1 and S2.The S1 subunit contains epitopes for virus neutralization, cell attachment and serotype specificity. Traditionally, IBV infection is diagnosed by isolation, and identification of the virus. Although the virus neutralization (VN) test in egg is the definitive test for determining the serotype of IBV isolates, it is time consuming and laborious (De Wit 2000). Reverse transcription polymerase chain

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reaction using IBV RNAs extracted from allontoic fluid been shown to be very efficient for the detection of IBV and for the identification of the IBV types (Adzhar *et al* 1996, Jackwood *et al* 1997, Handberg *et al* 1999). Outbreaks of the disease can occur even in vaccinated flocks because there is little or no cross protection between serotypes (Cavanagh *et al* 1999, Cavanagh 2003). In order to establish which IBV strains were circulating in Iranian poultry field during the years 1999 to 2004, we tested samples from 150 flocks showing clinical signs of IB using type specific RT-PCR for Massachusset, 793/B and D274 types. This allowed to learn the major circulating type in this period and also to provide possibilities for its introducing time.

MATERIALS AND METHODS

Samples. The samples of 150 flocks from 13 province were tested, including Tehran(64 herds), Ghazvin(16 herds), Azarbayejan(6 herds), Semnan(6 herds), Markazi(6 herds), Khorasan(8 herds), Esfahan(7 herds), Fars(6 herds), Kermanshah(3 herds), Lorestan(7 herds), Khoozestan(5 herds) and Hamadan(6 herds) respectively. During this study, samples(trachea, lung, kidney) from poultry flocks show in respiratory signs suspected to be related to IB infection were collected . All flocks investigated in this study were vaccinated by H120 Masachuset type vaccine. For virus isolation , organs from at least five birds of each flocks were pooled and 10-15% W/V were made in phosphate buffer saline (PH=7.2) containing 100U penicillin, 100µg streptomycin and 80 µg gentomycin per ml. two hundred µl supernatant from the suspension was inoculated into the specific pathogen free eggs(Lohman, Germany). Three to five eggs were used for each sample. After 48 to 72 hours the allontoic fluids were harvested and tested for the presence of IBV by RT-PCR and for influenza virus by haemagglutination inhibition (HI) test.

Extraction of RNA, RT-PCR and Nested PCR

Viral RNA was extracted from 200 µl allontoic fluids using the High Pure Viral Nucleic Acid kit (Roche, Germany) and dissolved in 50 µl water. The RT-PCR reaction was performed using Titan one-Tube RT-PCR system (Roche).Primers S1Uni2+ (5′- CCCAATTTGAAAACTGAACA-3) from nucleotide number 31 to 47(Binns *et al* 1985) and XCE2-(5′- CCTCTATAAACACCCTTGCA-3) from nucleotide number 1170 to 1193 (Adzhar, *et al* 1997), both from S1 gene, with 1 µl RNA were used for amplification of a segment approximately 1200 base pair (bp), common to all IBVs. For the RT reaction, the mixture was incubated at 45 ˚C for 45min, and then was heated at 94 ˚C for 2 min. The PCR reaction was performed using the following condition: denaturation (94 ˚C, 30s), annealing (48 ˚C, 2 min), extinction (68 ˚C, 2min), 35 cycles followed by a final extension (68 ˚C, 10min). The amplified fragment was used in three specified nested PCRs with oligonucleotid XCE3-(5′-CAGATTGCTTACAACCACC-3′) form 1093 to 1111, which is common for all three strains. The olgonuceotides DCE1+(5′-TTCCAATTATATCAAACCAGC-3′ , 895 to 915) , MCE1+(5′- AATACTACTTTTACGTTACAC-3′ , 817 to 837), and B15′-AAGTGCCTTTAGGCCTGG-3′ , 93 to 110) that are specific for types D274, Masachusset and 793/B respectively, and generating fragments 217, 295and 972 bp length, respectively(Adzhar, 1997). The Nested PCRs were performed with 1/10 dilution of the first positive reaction in a final volume of 100µl using the following condition: denaturation (94 ˚C, 1min), annealing (48 ˚C, 2 min), extinction (72 ˚C, 90s), 25 cycles followed by a final extension (72 ˚C, 10min) the final products was analyzed by electrophoresis in a 2% agaros gel, stained with ethidium bromide and visualized by UV translumination (UVP).

RESULTS

The typical results of molecular tests were shown in figure1. The obtained results in each year have separately been presented in table 1 and readers to be referred to this table for more details. In general out of 150 tested flocks approximately 72% were IBV positive in RT PCR tests. Specific nested PCRs on RT- PCR products revealed that 57 flocks (52.7%) were infected by 793/B type IBV alone. Massachusetts type IBV also could be detected in 18 flocks (16.6%) alone. Thirty three (30.5%) flocks were infected with both 793/B and Massachusetts type IBV as a mix infections. In general, out of 108 IBV positives flocks, 90 flocks (approximately 83%) were infected with 793/B type IBV (figure 2).

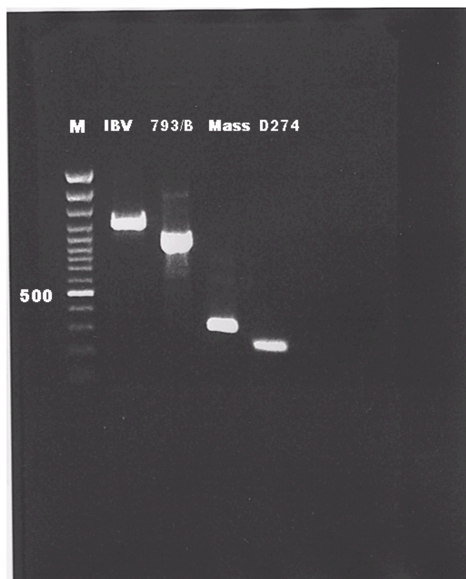

Figure 1. The typical results of molecular tests. The PCR products of 1200, 972, 295 and 217 bps length were amplified for detection of IBV, 793/B, D274 and Massachuset types respectively.

The 274D type did not detected in any flocks during this study. Of 108 flocks which were positive for IBV, 87(80.5 %) flocks were positive for H9 too (table 1). To show if the new IBV genotype had established the Exact Biomial Test was used. Even if the high expected proportion (>50%) of infection was considered our data showed the new genotype had estabilished in the country (P<0.0001).

Table 1. The results of RT-PCR, Nested PCR and HI test have been shown in each year separately and in total. Percent infection rate of 793/B, Massuchuset and Mix types infections have been calculated regarding to total number of IBV positive flocks.

year	Flocks N0	IBV infection	793/B	Mass.	Mix 793/B+Mass.	H9 (HA+)
1999	19	17	13	1	3	14
2000	19	16	10	1	5	8
2001	29	25	8	4	13	23
2002	27	16	8	4	4	15
2003	28	17	9	3	5	13
2004	28	17	9	5	3	14
Sum %	150	108 (72%)	57 (52.7%)	18 (16.6%)	33 (30.5%)	87 (80.5%)

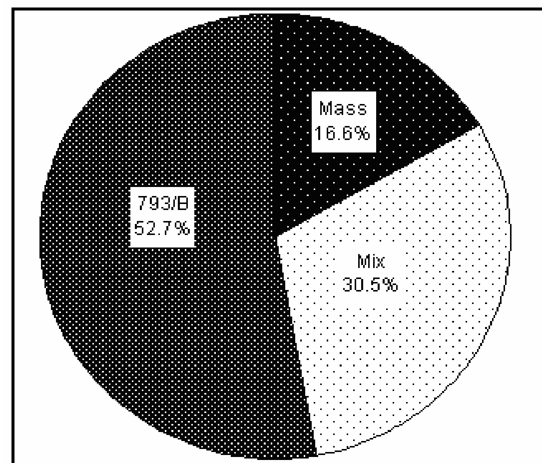

Figure 2. The overall results show that out of IBV positive flocks 53% belonged to 793/B type and 14% Masachuset type viruses respectively. Thirty three flocks were infected by both 793/B type Masachuset type viruses (Mix).

DISCUSSION

The major problem in immunization against IBV is the presence of various serotypes which do not induce a proper immunity against each other, so that the continuous monitoring of field situation has

a principal importance. Ahakhan (1994), reported presence of Masachuset type viruses as major circulating IBV in poultry industry field in the country. Introducing of new serotype was reported by following investigations which showed the existence of a new serotype, 793B (4/91) (Momayez *et al* 2003, Nuri *et al* 2003, Vasfi marandi & Bozorgmehri 2000). We believed that the present study is most comprehensive study which has been done for probing the major circulating serotype(s) in the country. The detection of extensive occurrence of 793/B serotype since 1999 is another unique data of our study. This study showed that among 150 examined flocks, 108 flocks were affected by IBV in which 793/B serotype was the major involved serotype. Eighteen flocks were just infected by Masachusset type viruses alone. A recent nucleic acid sequencing study on three isolated Masachuset type viruses showed that all belonged to vaccinal H120 IBV (Toroghi *et al* 2005).Occurrence of different infectious agents and presence of so many complicated cases make it impossible, in most occasions, to relate field problem to a unique infectious agent. Otherwise the interaction of concurrent infectious agents must be considered as the definitive cause of respiratory distresses. However regarding extensive vaccination against massachusset wild type viruses and the age of appearance of respiratory problems it seems that the major problem in our poultry industry field is the problem of introducing a new IBV serotype, as it continuously have being happened worldwide (Cavangh *et al* 1999). In company with recent studies the results of present study showed the IBV epidemiological feature as appearance of new serotype 793/B have been changed. Our result also showed that the 793/B type has been existed since 1999. According to extensive infection of this serotype in 1999, it can be easily concluded that 793/B serotype had been introduced even in earlier dates into the country. In any situation, the present study showed that the epidemiological feature of IBV, at least, had been changed since 1999. 793/B serotype was isolated in France in 1988 for the first and caused major epidemics in this country (Meulemans *et al* 2001) .In England, 793/B serotype recognized in charge of an unusual bilateral breast muscle atrophy and mortality in vaccinated flocks (Gough *et al* 1992). The extensive studies showed that 793/B serotype is a major circulating IBV virus in Europe. Nucleonic acid sequencing of recent isolated showed the similarity of these isolates to pathogenic 793/B serotypes in our country (Akbari 2003, Toroghi *et al* 2005). However just an in-vivo study can determine definitive biological role of circulating 793/B type viruses. Simplifying the cause of respiratory problems just to a unique infectious agent must be prevented, however in general speaking; it seems that 793/B type of IBV is one of major agents involved in current respiratory problems in poultry industry of the country. The present study also revealed that 80.5% of IBV positive flocks were simultaneously infected by influenza virus H9. The first occurrence report of H9N2 subtype was released in summer of 1999 (Pourbakhsh *et al* 1999). The following experimental studies showed that influenza A subtype H9N2 viruses had no obvious effect on SPF chickens(Toroghi & Momayez 2006) so that these viruses characterized as non highly pathogen subtype. However, commence of extensive respiratory and isolation of this virus from flock showing sever respiratory distress, cause that this subtype(Nili & Asasi 2003), at least partly, considered as one of major agents involving in current respiratory problems. Considering the published documents, if it is accepted that H9N2 introduced in 1999, two probabilities for the introducing time of 793/b type can be provided. The first one is the concurrent introducing of both viruses in 1999 caused beginning of such extensive respiratory problems. The second probability is that

the introducing of 793/b might happened earlier than 1999 , and introducing of H9N2 subtype in 1999 provided suitable ground for interacting of theses viruses which led to sever respiratory problem in poultry flocks. It can be concluded that these two viruses have been the major agent involving in respiratory in Iranian poultry industry field.

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