Field Study of Infectious Bronchitis Virus in Broiler Using Type-Specific RT-PCR

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Summary
The molecular detection of avian infectious bronchitis virus by use of RT-PCR and multiplex nested PCR in Fars province of Iran was investigated. Detection has been done on tracheal swab samples of 30 broiler flocks in age of 7-8 weeks. Flocks were selected from most broilers rising regions of the province. Detection was performed by primers specific for Massachusett, 4/91 and D274 serotypes. In this study 16 samples were positive for IBV 4/91 typeand 1 for Massachuset type. One swab sample showed a mixed infection of those serotypes. D274 serotype has not been detected in this study. This is the first report of the presence and a prevalence of IBV type 4/91 in Fars province.

Key words: infectious bronchitis virus, broiler, RT-PCR, Iran

Introduction

Infectious bronchitis virus (IBV) is classified in the family *Coronaviridae* genus coronavirus and causes an acute highly contagious viral respiratory disease of chicken that characterized by tracheal rales, coughing and sneezing, and kidney affection. Declines in egg production and quality in laying flocks and deep pectoral myopathy in broiler breeder may occure (Cavanagh & Naqi 1997). In Iran IB is one of the most important respiratory diseases of broiler chickens. However, only the

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Massachusset (Mass) vaccine strain is officially authorized. Despite the use of the IBV vaccine it is common to find IB problems in vaccinated chickens, causing a tremendous economic impact. Vasfi Marandi and Bozorgmehri Fard (2000) reported the presence of IBV variants in Iran and in an attempt the 4/91 variant identifed (Seyfi-abad Shapouri *et al* 2002). Typically the disease has been controlled with serotype-specific vaccines (Cavanagh & Naqi 1997). Therefore accurate serotype identification is essential in order to prevent future outbreaks. A variety of tests sach as immunofluorescence, immunoperoxidase or agar gel precipitation, using either polyclonal or monoclonal antibodies against group specific antigens can be used for preliminary identification of the virus (Cunnigham 1951). Definitive IBV serotype identification has been determined by virus neutralization (golden test) (DeWit 2000) and hamagglutination inhibition after enzymatic treatment of virus by neuraminidaes (Schuttze *et al* 1992). Some of these techniques are labor intensive and in some cases, they can yield inconclusive results. The advent of molecular biology techniques such as reverse transcriptase-polymarase chain reaction/restriction fragment length polymorphism (RT-PCR/RFLP) of S1 gene (Kwon *et al* 1993, Jackwood *et al* 1997, Falcone 1997,), type specific (RT-PCR) (Cavanagh *et al* 1999) and sequencing of S1 gene (DeWit 2000) has resulted in new opportunities for the rapid diagnosis and identification of IBV serotypes.

The purpose of this study was to determine possibly IBV variant strains in Fars province. The principal analytical tool was type-specific RT-PCR on tracheal swab samples.

Material and Methods

Swab Sample. Thirteen commercial broiler flocks in the east, west, and north and around of Shiraz in Fars province were selected. The broilers were vaccinated against IB and shown apparently health. Tracha swabbing was randomly done from

five bird of each flock. The swabs were allowed to dry at ambient temperature and stored at 4°C until RNA was extracted.The history of selected flocks indicats in table 1.

Table 1. *History of selected flocks for serotyping of infectious bronchitis virus*

Reign	No. selected flocks	Approx. age (day)
Abadeh	3	58, 55, 58
Sormagh	1	54
Marvdasht	4	56, 57, 56
Zarghan	2	40, 52
Beiza	9	49-57
Jahrom	5	50-56
Kavar	1	56, 57
Shiraz	2	59
Neiriz	1	56, 58
Khoram bid	2	56, 58
Total	30	

Extraction of RNA. Batches of five swabs were extracted by 900µl of Tripure (Roche), a commercial RNA extraction solution, in microfuge tube. Each swab was dipped into the Tripure and rotated for about 20 seconds. The swab was then discarded and the remaining four swabs processed in the same manner. The volume of the solution decreased to about 600µl and the RNA was prepcipitated according to manufacturer instruction. In this study RNA extracted from H120 (Razi Institute) and 4/91 (Intervet) live attenuated vaccines were used as control.

RT-PCR by common primers. The RNA pellet was dissolved in 9.5µl of DEPC (Sigma) treated double distilled water and mixed with 1µl of each of XCE1+ and XCE2- primers (50pmol/µl), common to all three types of IBV S1gene (Adzhar *et al* .1997). The mixture was denatured at 65°C for 10min. After quickly standing on ice for 10min, 4µl 5X RT buffer, 2µl 0.1M DTT, 1µl dNTPs mix (10mM), 0.5µl

RNasin (ribonucleas inhibitor) and 1µl (50 units) Expand RT enzyme (Roche) were added and cDNA was synthesized at 42°C for 45min. The reaction was stopped by incubation at 94°C for 2min. The cDNA obtained from RT reaction was amplified by PCR. 5µl cDNA, 5µl 10X PCR buffer, 2µl dNTPs mix (10mM), 0.5µl Taq polymerase (5U/µl, CinaGen), 1µl of each of XCE 1+ and XCE2- primers (50 pmol/µl) were mixed to a final volume of 50µl. Amplification was performed using a Corbet Research thermal cycler for 35 cycles of denaturation at 94°C for 30 Sec, annealing at 48°C for 30 Sec and polymerization at 72°C for 2 min. The predenaturation step was at 94°C for 2 min and post-polymerization step was 72°C for 7min. The product were analyzed on a 2% agaros gel containing ethidium bromide (0.5µg/ml) using an ultraviolet transillumintor.

Nested Multiplex PCR. For serotype identification, a type-specific nested multiplex PCR was conducted. Oligonucleotide primers included MCE1+, DCE1+ and BCE1+ respectively specific for a hypervariable region in the S1 genes of serotypes Massachusetts, D274 and 4/91 (793/B), and primer XCE3- common for the three serotypes (Adzhar *et al* 1997, Cavanagh *et al* 1999). The primers have been designed to generate cDNA of 295, 217 and 154bp for the serotype Massachusetts, D274 and 4/91, respectively. The multiplex PCR reaction contained 0.5µl RT-PCR product of last positive reaction, 0.5µl Taq polymerase, 2µl dNTP, 5µl X10 PCR buffer and 4µl mixed type-specific oligonucleotide primers. A 50µl total reaction volume was obtained by adding PCR grade water. The thermal cycler parameters were the same as described above and the obtained products were electrophoresed on 2% agaros gel to determine the size of the products.

Restriction enzyme digestion of PCR products. To further confirm the identity of DNA bands which were identified as the type 4/91 by nested multiplex PCR, RT-PCR products of this type were digested by enzyme *Hpa*I (Roche) as the manufacture instructed. Based on the sequences reported by Adzhar *et al* (1997), the

enzyme cut the 4/91 RT-PCR product after base 106, specifically and did not affect the RT-PCR of Mass serotype.

Results

Amplification of an expected DNA band (466 bp) from positive control as well as IBV-positive swab samples, indicating that the RT-PCR reaction has been performed correctly (Figure 1).

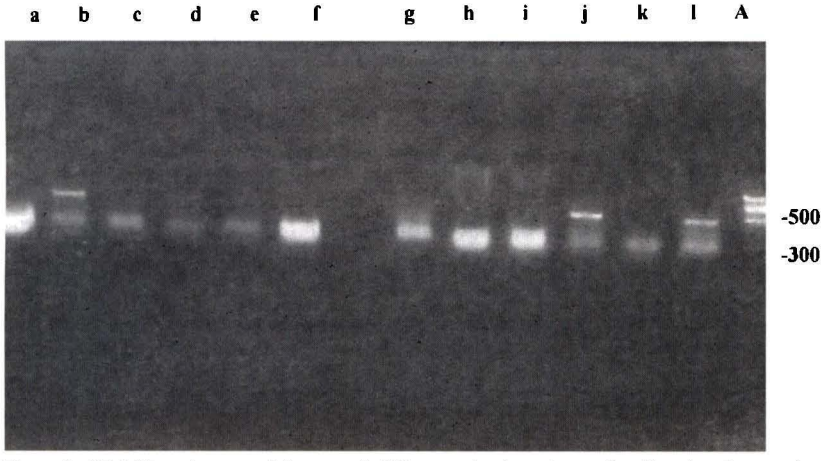

Figure 1. *RT-PCR to detect and distinguish IBV in tracheal swab sample of broiler. Positive lanes are b, c, d, j and l. Lane A showing DNA size marker with the size at the left side The size of amplified oligonucleotide sequences are 466bp*

According to tested swab samples IBV was detected in 18 out of 30 flocks. The multiplex nested PCR was only performed on RT-PCR product of the samples in which IBV genome had been detected. This experiment generated different-size products, which were easily distinguishable (Figure 2). Swab samples of sixteen flocks out of 30 flocks were positive for 4/91 serotype, one were positive for Mass serotype (isolated from Shiraz) and in one case; a mixed infection by these two serotypes (isolated from Marvdasht) was detected. D274 serotype of IBV was not detected in this study.

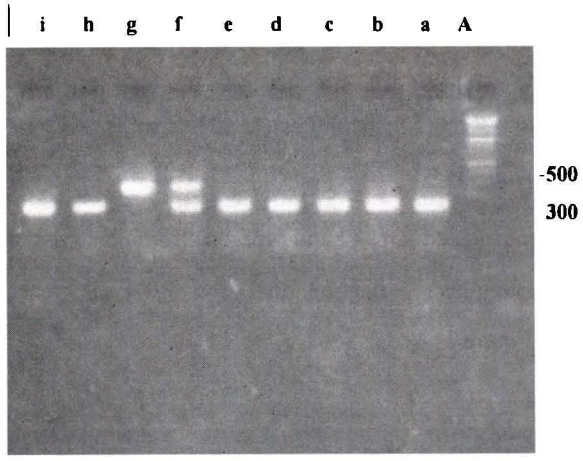

Figure 2. *Multiplex nested RT-PCR with RNA extracted from tracheal swabs taken from broilers in field (lanes a-j) showing detection of types massachusttes and 4/91. Lane f showing detection of two types in mixed infection. Lane A showing DNA size marker with the size at the left side*

Restriction enzyme *Hpa*I cut the sequence of GTTAAC, which specifically presents in RT-PCR amplified sequence of 4/91 serotypes. The result suggested that digestion only occured on RT-PCR products that were correspond to type 4/91

Discussion

The result of this study indicates the presence of 4/91 serotype of IBV in Fars proviance. Isolation and molecular identification of the serotype in other parts of Iran has been reported (Seify-abad Shapouri *et al* 2002). Detection of 4/91 serotype has been reported from Saudi Arabia, Japan, Denmark, Poland, France, Italy, and Argentine (Cavanagh *et al* 1998, Cook *et al* 1993).

Flocks that subjected to sampling have been vaccinated at one day of age with Mass serotype but the 4/91 was the most prominent serotype (94%) that detected on 6-8 weeks of ages. Because of using a common RT-PCR reaction for types Mass, 4/91 and D274, and the fact that all of positive samples were 4/91 or Mass serotypes and/or both it can be concluded that D274 serotype is not currently present in Fars proviance.

Mass type IBVs, isolated and identified in Iran, have been postulated to be the vaccine strains because of their rapid replication upon chicken emberyonated egg inoculation. (Seify-abad Shapouri *et al* 2002). Some immune responses including non-specific ones to the Mass vaccine virus would impede replication of the 4/91 types in live young birds (Cavanagh 1999). So in isolation attempts high frequent of Mass types detection is predictable. The high rates of 4/91 type detection on age of 7-8 weeks have also been experienced by Cavanagh (1999). He showed that infection with type 4/91 had commenced late in the life of the flock vaccinated by Massachusetts's vaccine strain in earlier ages.

In this study, a high frequency of 4/91 type detection on healthy birds reveals a low pathogenesity of the IBV type and a high incidence of infection. There was no relationship between the geographical locations of the flocks in this study and the detection of IBV type 4/91. For evaluation of IBV type 4/91 infection through the life of broiler flocks to find out the exact role of the virus on losses attributed to avian infectious disease further studies will be necessary.

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