Isolation and molecular characterization of infectious bronchitis virus, isolate Shiraz3. IBV, by RT-PCR and restriction enzyme analysis

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Summary

Infectious bronchitis virus (IBV) strain 4/91, prototype of the 793/B serogroup, was recently reported from Khouzestan province, Iran. Here we isolated and molecularly characterized a virus (Shiraz3. IBV) from broilers in Fars province. Following virus propagation in embryonated chicken eggs, we extracted total RNA containing the viral RNA from allantoic fluid. cDNA of the entire S1 and nucleocapsid (N) genes were generated with AMV-reverse transcriptase and genomic specific primers. The cDNA was subsequently amplified in a polymerase chain reaction (PCR). Digestion of the PCR products representing the entire S1 gene generated restriction fragments identical to those expected from strain 4/91 and differed from the control vaccine strain of H120. The N RT-PCR products representing the entire N genes of strains Shiraz3. IBV and H120 did not show any visible difference in size. The results presented here demonstrated that isolate Shiraz3. IBV is genetically closely related to strain 4/91 of IBV and can be differentiated from the IBV vaccine strain in Iran.

Key words: Infectious bronchitis virus, IBV, Shiraz

Introduction

Infectious bronchitis (IB) is an acute, highly contagious upper respiratory disease of young chickens (Schalk and Hawn, 1931; Collisson *et al*., 1992; Cavanagh and Naqi, 1997). The disease may also affect kidney and causes renal insufficiency (Hofstad, 1984). The major clinical signs include laceration, tracheal rales, sneezing and coughing. In adult laying hens a drop in egg production and a decrease in egg quality are seen (Hofstad, 1984; Ignjatovic and Sapats, 2000). In Iran, the disease has been identified in chicken flocks with serological and virus isolation methods (Aghakhan *et al.*, 1994; Vasfi Marandi and Bozorgmehri Fard, 2000). Despite the wide use of live attenuated and inactivated vaccines to protect commerical chickens, IB still remains responsible for serious financial losses to the poultry industry of the country (Vasfi Marandi and Bozorgmehri Fard, 2000).

Infectious bronchitis virus (IBV) is the causative agent of IB (King and Cavanagh, 1991). The virus belongs to coronaviruses, a genus of the family Coronaviridae. Since the first report of the disease (Schalk and Hawn, 1931), many IBV serotypes have been identified. Recently, it was demonstrated that there exist some newly emerging IBV isolates in Iran that are not neutralized with the antiserum against the IBV Massachusetts strain (Momayez *et al*., 2002; Seify abad Shapouri *et al*., 2002). One of the major structural proteins of IBV is spike protein that is cleaved into two smaller proteins namely S1 and S2 (Kusters *et al*., 1989). S1 gene contains two hypervariable regions that are responsible for the induction of neutralizing and serotype specific antibodies (Koch *et al*., 1990; Darbyshire *et al*., 1979). Restriction enzyme analysis of either the entire S1 gene (Mondal *et al*., 2001; Smati *et al*., 2002) or a portion of this gene (Lee *et*

al., 2001) has widely been used to investigate molecular variations among IBV strains in a geographical region. In this study, we demonstrated that combination of RT-PCR and restriction enzyme analysis is a good alternative strategy to the multiplex PCR to distinguish strain 4/91 from vaccine Massachusetts strain in Iran. Moreover, we showed that the two main genes of IBV coding for the two main structural proteins of N and S1 of isolate Shiraz3. IBV did not contain any major insertion or deletion compared to the corresponding genes of isolate 4/91.

Materials and Methods

Virus, enzymes and oligonucleotides

A 2000-dose lyophilized vaccine containing strain H120 of IBV was kindly provided by Iranian Veterinary Organization (IVO). The virus is routinely propagated in allantoic fluid of embryonated chicken eggs. Restriction enzymes, AMV-reverse transcriptase and Taq DNA polymerase were purchased from Sinagen, Co. (Tehran, Iran). Oligonucleotide primers were designed using OLIGO^{TM} version 5 computer software (Cambio, UK) and ordered through Farayand Danesh, Co. (Tehran, Iran).

Clinical samples

Tracheal samples from a broiler farm showing respiratory distress were collected and suspended in phosphate-buffered saline (PBS). After clarification by centrifugation at 1500 g for 10 min, each sample was passed through a 0.45 µm filter to remove any possible bacterial contamination. One hundred microliters of each sample were injected into allantoic fluid of 5 embryonated chicken eggs at day 9 of incubation. Following inoculation, the eggs were incubated for 6 more days and then the allantoic fluids were harvested and stored at -70°C.

RNA extraction and cDNA synthesis

The lyophilized IBV vaccine containing 2000 doses were reconstituted in 1 ml sterile distilled water. An allantoic fluid sample from an embryonated chicken egg uninoculated with IBV was used as a control negative sample. The total RNA was extracted with RNx-plus reagent (Cinagen, Iran) from 100 µl of the reconstituted IBV vaccine and the allantoic fluid inoculated with the field sample. This method of RNA extraction is based on the guanidinium thiocyanate-phenol-chloroform procedure (Chomezynski and Sacchi, 1987). The RNA pellet was then dried at room temperature and resuspended in 12 µl of diethyl pyrocarbonate (DEPC)-treated deionized water. The extracted RNA was immediately used in reverse transcription (RT) reaction to generate cDNA of the S1 gene basically according to the method described earlier (Haqshenas and Meng, 2001a; Haqshenas *et al*., 2001b). Based on the nucleotide sequences of strains 4/91 (Callison *et al*., 2001, Accession No. AF093794) and H120 of IBV (Kusters *et al*., 1989, Accession No. M21970), antisense primer (RS1IBV) was designed with OLIGO^{TM} version 5.0 software (Cambio, UK). The primer sequence is available upon request from the authors. The obtained RNA was denatured at 65°C for 3 min. RT was performed at 42°C for 60 min with 1 µl of genomic specific antisense primer (0.5 µM final concentration), 1 µl of AMV-reverse transcriptase (25 IU/µl, Cinagen), 1 µl of RNase inhibitor (40 IU/µl, Cinagen), 4 µl of 5 x RT buffer, and 1 µl of 2.5 mM dNTPs. Finally, the reverse transcriptase was inactivated at 70°C for 10 min. cDNAs of the N genes were generated basically as described for S1 genes. The reverse primer (RNIBV) was designed based on the consensus sequence of the published IBV N sequences.

PCR

PCR was basically performed as described earlier (Haqshenas and Meng, 2001a; Haqshenas *et al*., 2001b). Primer RS1IBV used in the cDNA synthesis reaction was used as antisense primer in the S1-PCR. Sense primer (FS1IBV) was also designed as described for RS1IBV. PCR mixture contained 10 µl of the RT reaction, 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM each sense and antisense primers and 2.5 IU Taq

DNA polymerase (Cinagen) in a 50 µl reaction volume. The PCR thermal cycles, performed in a Corbett thermal cycler (Australia), included an initial incubation at 94°C for 4 min to dissociate RNA/DNA hybrids. This initial cycle followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 52°C for 40 sec, and extension at 72°C for 2 min, with a final incubation at 72°C for 10 min. PCR products were analyzed by electrophoresis on an 1% agarose gel and visualized under UV light after staining with ethidium bromide (0.5 µg/ml) (Sambrook and Russell, 2001).

The conditions described for the S1 gene amplification were also used to amplify the entire N genes of the viruses except that we performed the extension step of PCR for 110 sec. The N nucleotide sequences (as described above) were used to design the primers that were used in the N RT-PCR. The primers are available upon request from authors.

Restriction enzyme analysis

The S1 gene PCR products, without any further purification, were directly used in restriction analysis. For each reaction, 10 µl PCR product, 8 µl ddH20, 2 µl 10x enzyme buffer and 1 µl (10IU) *Hae*III enzyme were added and incubated at 37°C for 2 hrs. The resultant restriction fragments were analyzed onto a gradient acrylamide gel (4% to 20%) prepared in Tris-borate-EDTA (TBE) buffer and stained with ethidium bromide as described above.

Results

Virus isolation

To show if the allantoic fluid contained IBV particles, we used a Western blot analysis established in our laboratory (Haqshenas *et al*., unpublished data). In our assays we exposed the allantoic fluid samples to IBV positive and negative antiserum samples and confirmed that the sample was positive for IBV antigen (data not showed). Control vaccine strain and an IBV-uninoculated allantoic fluid were used as our control positive sample and control negative, respectively. The virus isolated in this study was designated as Shiraz3. IBV.

RT-PCR and restriction enzyme analysis

Following the generation of cDNA of IBV strain H120 and the field isolate with genomic specific primers, we performed PCR to amplify the cDNA. The amplified fragments were observed on the gel in lanes related to the positive control and the field sample with an approximate size of 1.6 kb (Fig. 1). No band was observed when we used an uninoculated allantoic fluid sample as the control negative. On the gel, the amplified S1 genes of strain H120 and the field isolate did not differ in size. The RT-PCRs amplifying the N genes of strain H120 and the field isolate also resulted in two DNA bands on the gel with the same expected size of 1.2 kb (Fig. 1). When the N RT-PCR was performed on an RNA preparation from an uninoculated allantoic fluid sample no band was observed (Fig. 1).

Fig. 1: RT-PCR amplification of S1 (2, 3) and nucleocapsid (5, 6) genes IBV strains H120 and Shiraz3. IBV. An IBV-uninoculated allantoic fluid (1,4) was used as control negative. The amplified fragments were analyzed on a 1% agarose gel and stained with ethidium bromide. Gene Ruler TM 100 bp ladder mix (Fermentas, UK) was used as DNA size marker (M). The photo has been inverted

HaeIII enzyme worked well on the crude preparation of PCR products and none of the PCR components interfere with the enzyme. When we digested the PCR product of Shiraz3. IBV, three bands with the approximate sizes of 1000, 500 and 100bp were observed. Digestion of the PCR product of the vaccine strain also resulted in three bands with the approximate sizes of 900, 400 and 300 pb (Fig. 2). Using gradient acrylamide gel, as can be seen on the lane of the control positive, the restriction fragments moved slightly slower than what we expected. When we eluted the PCR products from the gel and subjected to enzyme digestion we did not observed any difference in the band sizes (data not showed). However, the overall sizes of the DNA bands were in agreement with our prediction from computer sequence analysis. Using DNAstar software, computer analysis of the published S1 genes of strains 4/91 and H120 predicted that the S1 gene PCR products of strain.

(Accession No. AF093794) and H120 of IBV (Kusters *et al*., 1989, Accession No.M21970), H120 (Accession No. M21970) are cleaved by HaeIII enzyme into three fragments with the sizes of 906, 415 and 290 bp while the S1 gene PCR products of strain 4/91 (Accession No. AF093794) are cleaved into three fragments with sizes of 1030, 483 and 104 bp (Fig. 3).

Fig. 2: Restriction enzyme analysis of the PCR-amplified S1 genes of isolate Shiraz3. IBV (1) and strain H120 (2). The resultant restriction fragments were analyzed on a gradient acrylamide gel (4% to 20%) stained with ethidium bromide. Head arrow indicates primer dimers. Gene Ruler TM 100 bp ladder mix (Fermentas, UK) was used as DNA size marker (M). The photo has been inverted

Discussion

In a recent report from Khouzestan province it was demonstrated that multiplex PCR was successfully used to differentiate strain 4/91 from the vaccine strain of IBV (Seify abad Shapouri *et al*., 2002). Here, we report an alternative method to multiplex PCR to differentiate these two IBV strains in Iran. We successfully isolated shiraz3. IBV from Fars province in embryonated chicken eggs and demonstrated that the virus is closely genetically related to strain 4/91. By the use of restriction enzyme analysis we demonstrated that *Hae*III restriction enzyme generated a suitable restriction pattern to differentiate the IBV vaccine strain from Shiraz3. IBV circulating in Iran. We also showed that the N and S1 genes of isolate

Fig. 3: Schematic HaeIII restriction maps of IBV strains H120 and 4/91. Based on the results presented in this paper we propose a same restriction map for strain 4/91 and isolate Shiraz3. IBV

Shiraz3. IBV did not contain any major insertion or deletion compared to the corresponding genes of isolate 4/91 (Callison *et al*., 2001; Cavanagh *et al*., 1998).

Prevention of IB in chicken is based mainly on vaccination against the virus. In Iran, strain H120 of IBV has been used for a long time in broiler chicken flocks to achieve this goal. A vaccination program against IB fails when new strains of IBV emerge in a geographical region. Therefore, routinely monitoring of the existing IBV strains in a geographical region has been suggested to choose a suitable virus strain for vaccination. To do this, several tests including virus isolation (Hofstad and Yoder, 1966), virus neutralization test (Chomiak *et al*., 1963; Darbyshire *et al*., 1979), haemaggltination inhibition test (Bingham *et al*., 1975; King and Hopkins, 1983), hybridization (Nagano *et al*., 1993) and enzyme-linked immunosorbent assay (Marquardt *et al*., 1981) have been developed. Molecular epidemiology is now recognized as a valuable tool to study IBV genotypes. Multiplex PCR has been recently introduced to distinct different IBV strains and was recently used by Seify abad Shapouri *et al* (2002) to detect vaccine strain from isolate 4/91 in Iran. In this study on the Iranian IBV isolates only a very small portion of the S1 gene was amplified and subjected to nucleotide sequencing. The RT-PCR reported in this study has the advantage of amplifying the entire S1 gene of the virus that can directly be used to determine consensus sequence of the complete nucleotide sequence of S1 gene. It has been demonstrated that PCR and restriction analysis results are in agreement with the virus neutralization test (Kwon *et al*., 1993). In our study we employed RT-PCR and restriction enzyme analysis to molecularly characterized Shiraz3. IBV variant of IBV.

Amplification of the entire S1 and N genes of isolate Shiraz3. IBV did not show any detectable size difference between these two genes and corresponding genes of strains 4/91 and Massachusetts (vaccine strain) of IBV. However, in the restriction enzyme analysis we observed a restriction pattern that we expected from strain 4/91. In order to confirm our RT-PCR and restriction analysis results we determined the partial nucleotide sequences of both 5' and 3' ends of S1 gene of isolate Shiraz3. IBV. Comparison analysis demonstrated that the partial nucleotide sequences were almost identical to the published sequences of the IBV isolates of the 793/B serogroup including isolate 4/91 (data not showed).

Kwon *et al*., (1993) demonstrated that *Hae*III restriction enzyme could be used to differentiate some of IBV genotype. In another study, Smati *et al*., (2002) characterized three novels IBV isolates from Canada by the use of *Hae*III restriction enzyme. Moreover, as it was showed in the Results, computer analysis of the S1 genes of strains H120 and 4/91 showed that *Hae*III enzyme would create restriction patterns suitable to distinct these two strains. Therefore we decided to use this restriction enzyme in our study. From the results presented here we suggest to use this restriction enzyme to differentiate the IBV vaccine strain from isolate 4/91 in the future study.

In this study we also amplified the N gene of isolate Shiraz3. IBV to investigate any possible deletion or insertion. As it was seen in the results, we did not notice any detectable difference in the size of this gene with the other publiched sequences. Therefore, in this study it was demonstrated that the two main genes of isolate Shiraz3. IBV did not contain any major insertion or deletion. We are currently sequencing the entire S1 gene of isolate Shiraz3. IBV to determine its phylogenetic relationship with the other IBV isolates.

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