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Molecular analysis of the S1 gene of vaccine strains of infectious bronchitis virus using reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism

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Abstract

Infectious bronchitis virus (IBV) is an acute and contagious viral disease of poultry that affects different systems, including the respiratory tract in particular. IBV causes major economic losses in the poultry industry globally. Due to antigenic variation of the causative agent, control of the disease is difficult. To control the disease, many vaccines that belong to different serotypes are being used in many countries, including Iran. In the present study, the S1 genes of six different IBV vaccines were analyzed. The S1 genes of IBV vaccine strains were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and the resultant PCR products were purified. Purified PCR products were digested separately with the restriction endonuclease, *Alu*I. The generated restriction endonuclease fragment length polymorphism (RFLP) patterns of the S1 gene of IBV vaccine strains were compared. The results showed that the construction of a library of RFLP patterns of the S1 gene of vaccine strains in use is beneficial. The library has the potential for use as a quick and inexpensive method for determining the genotype of future outbreaks of IBV and also assesses the degree of their similarity to the strains for which vaccines exist.

Introduction

Infectious bronchitis virus (IBV), a prototype of the *Coronaviridae* family, is an important pathogen in chickens that infects the respiratory tract, kidneys and oviducts. Infection with this organism causes reduced performance, reduced egg quality and quantity, increased susceptibility to infections, and increased mortality (Cavanagh and Naqi, 2003). The genome of IBV contains a single strand of positive-sense RNA of approximately 27.6 kb, which encoded four structural proteins: the spike glycoprotein (S), the membrane glycoprotein (M), the phosphorylated nucleocapsid protein (N), and the small membrane protein (E).

New serotypes and genotypes of IBV are emerging each year in different parts of the world (al Tarcha *et al.*, 1990; Cook 1983; Gelb *et al.*, 1991; Gough *et al.*, 1992; Jia *et al.*, 1995; Liu and Kong, 2004; Yu *et al.*, 2001; Zanella *et al.*, 2003) . A number of factors such as mutation and recombination and the wide spread use of live attenuated vaccine play an important role in increasing the number of new genetic variants (Cavanagh, 1992; Estevez, Villegas, and El-Attrache, 2003; Jia *et al.*, 1995; Kusters *et al.*, 1990; Wang, Junker, and Collisson, 1993).

Many techniques have been used for the antigenic and genetic classification of IBV. Traditionally, IBV antigenic types have been defined using virus neutralisation (VN) tests. Monoclonal antibodies that are specific for a particular antigenic type can be used in enzyme-linked immunosorbent assays (ELISAs), which are more economical than VN assays. Recently, molecular techniques have been employed for detecting IBVs and to differentiate them from each other with high accuracy. Reverse-transcription polymerase chain reaction (RT-PCR) along with restriction fragment length polymorphism (RFLP) has been used to identify IBV strains (Kwon, Jackwood, and Gelb, 1993; Mardani *et al.*, 2006). These techniques are quick and can be used to detect IBV directly from the suspicious specimens without the need to isolate the virus, which is an expensive and time consuming process.

To control IBV, vaccination is the most practical and effective strategy that is currently being used in many countries. Emergence of the new serotypes of IBV and the lack of strong cross-protection of IBV

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serotypes make it difficult to control the problem adequately by vaccination (Cavanagh, 2003). Therefore, is necessary to differentiate new isolates of IBV from vaccine strains in order to develop new vaccines if the existing vaccines are not able to control outbreaks.

The aim of this investigation was to analyse the S1 gene of IBV vaccine strains in use in Iran using the RT-PCR RFLP technique in order to obtain the RFLP profile of IBV vaccine strains and to construct a library of RFLP patterns of vaccine strains. This RFLP library will be important in the identification of the source of infection when new outbreaks of IBV occur.

Material and Methods

IBV vaccine strains

The IBV vaccine strains, H120, MA5 and 4/91, were obtained from Intervet Ltd., UK; the H52 and R120 vaccine were obtained from Razi Vaccine and Serum Research Institute, Iran, and the IB88 vaccine was obtained from Merial, France, Ltd. Vaccine strains H52, H120, MA5 and R120 belonged to the Massachusetts serotype and strains 4/91 and IB88 were from same serotype (793/B).

Extraction of viral RNA from IBV vaccines

Purification of viral RNA was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Approximately 50 μl of each vaccine suspension was used for each extraction. The RNA was eluted in 30 μl RNase free water. The extracted RNA was either used for a copy of DNA (cDNA) synthesis or stored at -70°C for later use.

cDNA synthesis (reverse transcription)

For the synthesis of cDNA, firstly a premix without RNA and Oligo-dT was prepared in accordance with previous studies with minor modifications (Mardani *et al.,* 2006). Approximately 5 l of extracted RNA was mixed with 0.5 l of primer (Oligo-dT) and incubated at 100°C for 1 min; it was subsequently cooled by placing it on ice for 5 min. The sample was spun briefly, and then 19.5 l of premix was added to the RNA and primer. The mixture was mixed by pipetting and incubated at 42°C for 1 h and subsequently incubated at 100C for 5 min to inactivate the reverse transcriptase. The resultant cDNA was used immediately in subsequent PCR or stored at –70°C for later use.

Polymerase chain reaction (PCR)

PCR was performed to amplify the whole S1 gene of IBV vaccine strains. For the amplification reaction, two primers were used: POLY-F1 (5' GATTGTGCATGGTGGACAATG 3') bound to the 3' end of the polymerase gene (nucleotides 20,070 to 20,090, Beaudette strain, accession no NC-001451; Mardani *et al.,* 2006), and S1-R1 (5' CCACCAGAAACTACAAACTG 3'), which was designed in this study to bind to the 3' end of the S1 gene (nucleotides 21,857 to 21,876, Beaudette strain). These primers amplified a fragment of approximately 1.8 kb from the IBV genome. The PCR reaction was carried out in 25 l mixtures that contained 50 M each of dATP, dTTP, dGTP and dCTP, 0.5 M of each primer, 2.5 l of 10 PCR buffer (CinnaGen Co., Tehran, Iran), 2 mM magnesium chloride, 2.5 U Taq DNA polymerase (CinnaGen Co., Tehran, Iran) and 5 l cDNA as a template. Amplification was performed using 35 cycles of incubation at 94°C for 30 s, 58°C for 30 s and 72°C for 2 min, with a final extension at 72°C for 2 min. The resultant PCR products were separated in a 1.5% agarose gel and the gel photographed using ultraviolet transillumination. The molecular weight marker (MW) was phage λDNA digested with *EcoR*I and *Hind*III.

PCR product purification

Before the digestion of amplified PCR products, it was necessary to purify PCR products and remove Taq enzyme, dNTPs, magnesium chloride and other PCR reaction components. For this purpose, amplified PCR products were purified using a DNA extraction kit (Fermentas, CinnaGen, Iran). The purification procedure was performed according to the instructions of the manufacturer.

Restriction endonuclease digestion of PCR products

Purified PCR products were digested separately with the restriction endonucleases *Alu*I (Fermentas, CinnaGen, Iran) using 5 l of purified PCR product and 10 U of *Alu*I at 37°C for 2 h. After digestion, the resultant DNA fragments were separated in 2% agarose gel stained with 1% ethidium bromide.

Results

Amplification of the S1 gene of IBV vaccine strains

The first attempt to amplify the S1 gene (1.8 kb) of the three vaccine strains of IBV using oligonucleotide primers Poly-F1 and S1-R1 was successful (Figures 1 and 2). No major difference in size was detected between the PCR products from the different IBV vaccine strains. All vaccine strains examined in this study gave only one band of approximately 1.8 kb as expected.

Restriction endonuclease digestion analysis

The RFLP patterns generated from six IBV vaccine strains by digestion with *Alu*I are shown in Figure 3. Each enzyme produced about four to seven

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easily distinguishable fragments in the range of 50 to 600 base pairs. Reproducibility was not found to be a problem with this technique, as repeated (up to three times) testing of a number of the IBV vaccine strains used in this study generated the same RFLP patterns. Three different RFLP patterns were generated using *Alu*I. The RFLP patterns for four vaccine strains, MA5, H52, H120, and R120 were identical. Two other patterns that were generated for IB88 and 4/91 were different from each other and were also different from the pattern for the other four vaccines (Figure 3).

Discussion

In this report, we present the development of a RT-PCR RFLP assay for the detection and differentiation of IBV vaccine strains. The target region for the assay was the S1 gene, which resulted in amplification of all the IBV vaccine strains tested. The enzyme chosen recognized a four base pair sequence, which increased the number of fragments for comparison (Chang *et al.*, 1997).

Figure 1: Schematic representation of the IBV genomic structure (Cavanagh *et al.,* 2002). The locations of the oligonucleotide primers POLY-F1 and S1-R1 are shown with arrows.

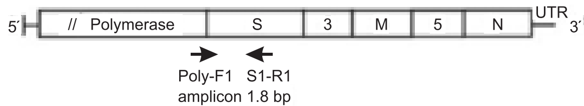


Figure 2: Electrophoresis of PCR products from three IBV vaccine strains in a 1.5% agarose gel. The molecular weight marker (MW) is phage λDNA digested with *EcoR*I and *Hind*III.

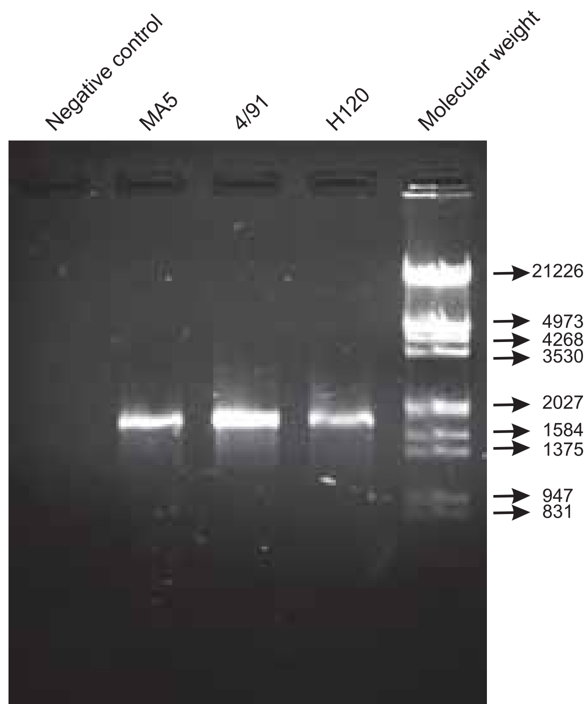
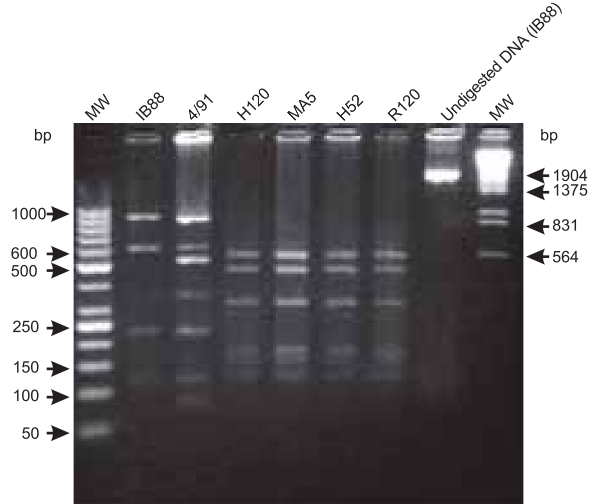


Figure 3: RFLP patterns of the PCR-amplified S1 genes from six IBV vaccine strains digested with *Alu*I. The molecular weight marker was made from bacteriophage λDNA digested with *Hind*III and *EcoR*I.



Therefore, this enzyme had many recognition sites in the 1.8 kb PCR product of the S1 gene of IBV genomes and consequently generated a greater numbers of fragments to analyze. The results obtained from this assay indicated that IBV vaccine strains H52, H120, MA5 and R120 all from same serotype (Massachusetts) cannot be differentiated from each other using RT-PCR RFLP procedure described here. However, two vaccine strains 4/91 and IB88, which were not from the Massachusetts serotype but were from same serotype, gave different RFLP patterns and can be differentiated from each other. Therefore this procedure is highly specific and provides sensitive results in a timely manner for the detection of different IBV strains; it also differentiates between strains that belong to different serotypes and sometimes even from the same serotype.

However, whilst the RT-PCR and RFLP technique is very quick and easy to perform, it has some drawbacks. Single mutations can modify the restriction site and therefore change the restriction profiles of genetically related strains. In addition, mutations beside the restriction site do not change the restriction profiles but may change the properties of the virus, which this technique cannot discover.

In a study by Kwon *et al.* (Kwon, Jackwood, and Gelb, 1993), they demonstrated the utility of RT-PCR and RFLP analysis of S1 gene for detection and identification of the IBV serotypes. In another study, the Arkansas and Massachusetts serotypes of IBV were detected in tracheas and cecal tonsils by RT-PCR RFLP (Alvarado *et al.*, 2006).

Mardani *et al*. showed in a study on RT-PCR RFLP analysis of 7.5 kb PCR products from different Australian IBV strains in 2006 that IBV strains that

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belonged to the same serotype grouped in the same cluster. This concurs with the results of our present study.

When designing a vaccination program for IBV, it is important to identify the serotype of field strains, as cross-protection may be limited between different serotypes of the virus. Different methods such as the hemagglutination inhibition test, serum neutralization test, tracheal organ cultures and monoclonal antibodies (Clewley *et al.,* 1981; Hopkins, 1974) have been used to group IBV isolates. These methods are time consuming and have produced conflicting results. It has been reported that IBV typing method using PCR and RFLP analysis agrees with the VN test (Kwon, Jackwood, and Gelb, 1993). However in this study two vaccine strains 4/91 and IB88 that belong to the same serotype generated different RFLP patterns, which indicate the variation in the spike protein within the same serotype as reported by Cavanagh *et al.* (2005). Two primers, designated PolyF1 and S1-R1, which flanked the 3' end of polymerase and S1 glycoprotein genes of IBV, were designed. The PCR was used to amplify the S1 glycoprotein gene of the majority of recognized IBV vaccine strains in Iran. For each of the IBV strains examined, the size of the amplified product appeared to be identical following agarose gel electrophoresis. Using *Alu*I restriction enzyme, we grouped six IBV vaccine strains into three genotypes. The H120, H52, MA5 and R120 strains could not be differentiated from each other, as their RFLP patterns were identical, but the 4/91 and IB88 strain were differentiated from other strains by digestion with *Alu*I. The RFLP pattern of 4/91 and IB88 vaccine strains were also different from each other. The reason for different RFLP patterns for strains that belong to same serotypes may be due to the fact that a single base change in the endonuclease restriction site can affect the RFLP pattern without any change in the antigenic properties of that virus and vice versa. This is one of the drawbacks of the RFLP analysis as discussed above. The total amount of PCR fragments of the 4/91 strain seemed to be more than 1.8 kb, which indicates the possibility of the existence of more than one IBV strain in this batch of vaccine. However, examination of two different batches of the 4/91 vaccine strain gave the same RFLP pattern, as was described before. The probability of the DNA contamination existed, but this probability was very low.

In summary, we have demonstrated the utility of PCR and RFLP analysis for the detection and identification of different IBV vaccine strains. In addition, the method can be used to identify variant virus strains that are antigenically different from any of the known IBV vaccine serotypes. Currently, the procedure can be completed in less than two days, barring complications during the amplification of the viral genome. It is feasible that the routine identification of IBV serotypes with the use of this method could be conducted in poultry diagnostic laboratories.

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