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RESEARCH ARTICLE

Development of a Nested Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Assay to Detect Infectious Bronchitis Virus

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ABSTRACT

Infectious Bronchitis Virus (IBV) is a highly infectious and contagious viral pathogen of chickens worldwide. IBV is a positive sense, single-stranded RNA virus that primarily targets the respiratory tract. IBV belongs to group III of the genus Coronavirus of the Coronaviridae family. In this study, viral RNA was extracted using phenol-chloroform based method. A pair of specific primers for RT-PCR and a pair of specific internal primers for Nested-PCR of the S1 region in the spike protein gene was designed and the tests were optimized for the detection of IBV. To determine the sensitivity of the test, 10 fold dilutions of virus stock (3×10^5 EID50 to 3×10^{-2} EID50/ml) were prepared in distilled water. A 531 bp fragment and a 284 bp of the spike protein gene (S1) were amplified in the RT-PCR and the Nested-PCR test, respectively. The PCR product was cloned in pTZ57R/T vector and sequenced. The sequence data confirmed the specificity of the test. The sensitivity of RT-PCR was determined to be 3×10^3 EID50 per ml. Nested-PCR was also carried out and sensitivity was increased to 3×10 EID50 per ml. Due to the high sensitivity of this test, this technique can be used as a robust and rapid diagnostic method for the detection of IBV.

Keywords: Chickens, Infectious Bronchitis Virus, IBV, Reverse Transcriptase Polymerase Chain Reaction, RT-PCR, Nested-PCR

Infectious Bronchitis Virus (IBV) is a member of the Coronaviridae family, and is an enveloped, single stranded RNA virus with an approximate genome size of 27.6 kilo base (1-3). IBV is one of the foremost cause of economic loss in the poultry industry, affecting the performance of both meat-type and egg-laying birds (4-7). The only practical means of controlling IB is vaccination, which is routinely used throughout the poultry industry (8). In general, IBV infection can be diagnosed by detection of (parts of) the IBV virus itself or the specific antibody response. There are a number of assays for diagnosing acute IBV infections. The most common, which can be used for the routine detection of IBV are virus isolation (VI), immunofluorescence assay (IFA) and immunoperoxidase assay (IPA). IBV infections are detected serologicaly by demonstrating a seroconversion, using paired serum sets or the demonstration of IBV-specific immunoglobulin M

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(IgM). Tests which are more commonly used are the haemagglutination inhibition (HI) test, the agar gel precipitation test (AGPT), and the enzyme-linked immunosorbent assay (ELISA) (9). These mentioned methods are expensive, labour intensive and often give inconclusive results. Hence, the development of an alternative diagnostic method for a sensitive, specific and more rapid detection of IBV is warranted (10). This can be accomplished by the polymerase chain reaction technique which has also provided new diagnostic opportunities in veterinary medicine (11-12). A number of PCR methods have been described previously for detection of IBV (11, 13-14), including nested-PCR (15) and RT-PCR for the *S1 gene* (16-18). Real time RT-PCR, which is likely to be the most rapid and efficient method of diagnosis of IBV, has also been applied to IBV diagnosis (10). The RT-PCR assay for IBV developed by Ramneek et al. (2005) was based on the sequences of the N-terminus of the *S1 (spike) gene* that spanned hypervariable region 1 (HVR 1), The RT-PCR assay was more sensitive than virus isolation and could be used for rapid field diagnosis of IBV infection. Furthermore, sequencing of HVR 1 allowed differentiation of field strains of IBV and indicated that the infectious attenuated vaccine strain of IBV was a likely source of some field infections.

In this study, an RT-PCR assay was described for the rapid detection of IBV which can be use directly on the tissues of infected chickens in the field.

MATERIALS AND METHODS

Virus strain

A Vaccine strain of Infectious Bronchitis Virus (H120) with a titre of 106 EID50 was provided by the Razi institute (Karaj, Iran). Field IBVs were isolated from kidney and trachea of IB suspected chickens using 10-day-old specific pathogen free embryonated eggs. After 40h at 37°C, a total of 1.5 ml of allantoic fluid was collected for RNA extraction.

Primer designing

Oligonucleotide primers for RT reaction and subsequent PCR amplification were designed from the *S1 gene* of the virus genome using Generunner version 3.05 and Oligo5 softwares. The sequence of oligonucleotide primers of PCR and Nested-PCR are illustrated in Table1.

Table 1. Primers used for PCR and Nested PCR

Primer	Sequence	Location
IBV-S1-F	5′-GCGGTAGTTAACATTTCTAGC-3′	20509-20529
IBV-S1-R	5′-GTGATCCATCACACAAAATAA C-3′	21019-21040
IBV-S1-Nested-F	5′-ATCAGGTATGGCTTGGTCTA-3′	20628-20647
IBV-S1-Nested-R	5′-CTATGGTCTCATTAGAGGTG-3′	20892-20911

RNA extraction

Viral RNA was extracted from the vaccine and allantoic fluid using the phenol-thiocyonate based method (19). Briefly, 1 ml of RNAFAST (Genefanavaran, Iran) solution was added to 0.3 ml of virus suspension sample in a 1.5 ml microfuge tube. This was followed by 0.2 ml chloroform extraction and precipitation of supernatant by adding equal volume of cold isopropanol and subsequent centrifugation at 10000 g for 15 minutes. After, the resulting pellet was washed with 70% ethanol, dried and resuspended in 20 µl of DEPC-dH_2O. The extracted RNA was immediately used or stored at -70°C for later use.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For cDNA synthesis, 4 µl of extracted RNA, 0.25µl of reverse primer and 5 µl of DEPC-dH2O was denaturated at 70°C for 5 minutes and then cooled on ice. At this stage, 8 µl RT buffer (5x), 1µl (10 mM) dNTP, 1µl RNase inhibitor (40U) were added to the solution and incubated at 37°C for 5 min. Finally 40U reverse transcriptase (M-MuLV) and DEPC-dH_2O was added to reach a final reaction volume of 40 µl and the mixture was incubated at 37°C for 60 minutes followed by another 10 minutes at 70°C. PCR amplification of cDNA was carried out in a total volume of 50 µl containing 5 µl of 10X

PCR buffer, 0.25 µg of each forward and reverse primer, 1.5 mM $MgCl_2$, 1 µl of dNTP (10 mM), 1 unit of *Taq* DNA polymerase and H_2O to the final volume of reaction. The mixture was subjected to the following program using a thermocycler: 94°C for 3 min (1 cycle), 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec (30 cycle) and 72°C for 10 min (1 cycle). Results were observed after 7 µl of the PCR product was mixed with 2 µl of gel loading buffer and visualized after electrophoresis on an ethidium bromide stained 1% agarose gel.

Cloning and Sequencing the PCR product

The PCR product was cloned into a T-vector (pTZ57R, Fermentas) based on manufactures instructions. Recombinant plasmids were later sequenced.

Sensitivity of the RT-PCR

To determine the sensitivity of PCR, 10 fold dilutions of virus stock (3×10^5 EID50 to 3×10^{-2} EID50) were prepared in dH_2O. RNA was extracted from diluted virus suspension and was examined in RT-PCR.

Nested-PCR

Nested primers were designed from the PCR sequence and Nested-PCR was carried out. In nested-PCR 1 µl of first round PCR was used as template DNA and annealing temperature was increased to 59°C. The rest of the materials and procedures were similar to the PCR protocol described above.

Sensitivity of the Nested-PCR

Each amplicon of RT-PCR from the 10 fold dilutions of virus stock were tested in the Nested-PCR.

RESULTS

A 531 bp fragment within the *S1 gene* of Infectious Bronchitis Virus was amplified in RT-PCR and a 284 bp fragment was amplified in Nested-PCR.

Fields strains

14 of 20 allantoic fluids were positive in PCR test but in Nested-PCR 17 of 20 were positive for IBV.

Specificity of the RT-PCR

The nucleotide sequence for the PCR product was determined after cloning in PTZ57R T vector and results were compared with other sequences in the National Center for Biotechnology Informations (NCBI) using online Blast program and the specificity of the test was confirmed.

Sensitivity of the RT-PCR

Serial 10 fold dilutions from the virus stock were prepared and RNA extraction followed by RT-PCR was carried out. Positive DNA bands of expected size were detected in ethidium bromide stained agarose gel covering a range from 3×10^5 to 3×10^3 EID 50/ml. No signal was observed when RNA extracted from samples with lower dilutions or negative controls were analysed (Figure 1).

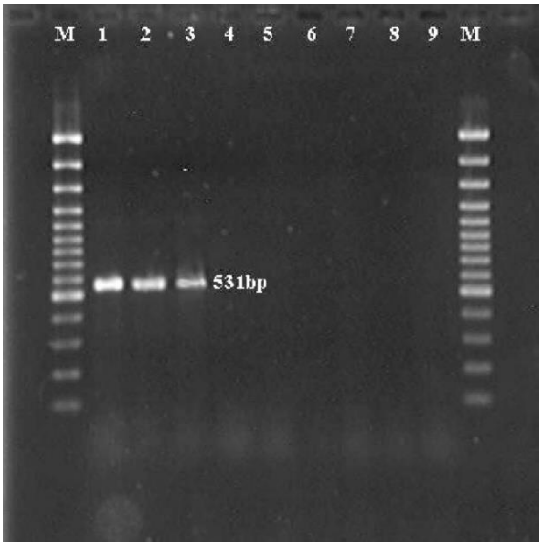

Figure 1. Sensitivity test for RT-PCR on 1% agarose gel. M is 100 bp DNA ladder, lines 1 to 8 are dilutions of virus from 3×10^5 to 3×10^{-2} (EID50) respectively, and line 9 is blank (without template).

Sensitivity of Nested-PCR

Each PCR product from the 10 fold dilutions of virus stock was tested in Nested-PCR. A 284bp DNA fragment was observed in 3×10^5 to 3×10^1 EID 50/ml. DNA was not amplified from lower dilutions or PCR and Nested-PCR negative controls. Nested-PCR was found to be 100 times more sensitive than PCR since the viral genome was detectable in samples with lower dilutions (Figure 2).

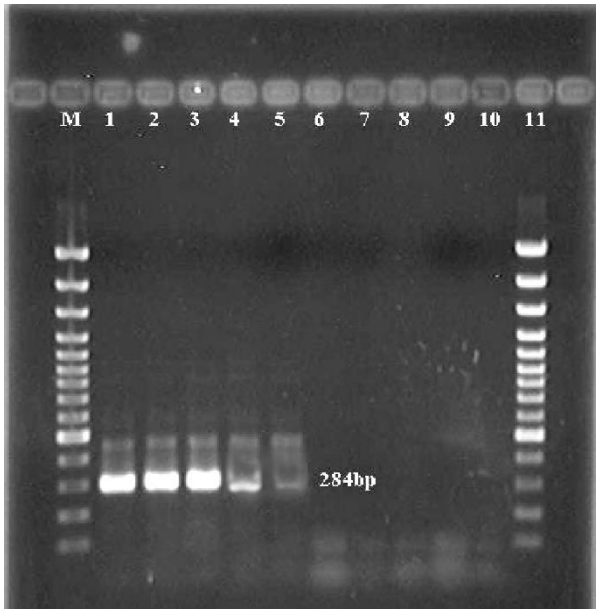

Figure 2. Sensitivity test for the Nested-PCR on 1% agarose gel. M is 100 bp DNA ladder, lines 1 to 8 are dilutions of virus from 3×10^5 to 3×10^{-2} (EID 50) respectively. Line 9 is RT-PCR blank (without template), and line 10 is Nested-PCR Blank (without template).

Sequencing Data

531 bp fragment was sequenced and data were compared to that of the IBV strains using the BLAST program of NCBI and the result confirmed the data as part of the IBV genome. Sequence were submitted to NCBI with the accession number AY921572 (Sequence 1).

Sequence 1: Nucleotide sequence of the PCR product (530 bp), which was submitted to NCBI with the accession number "AY921572" as a part of IBV S1 gene

TGCGGTAGTT AACATTTCTA GCGAATCTAA
TAATGCAGGC TCTTCATCTG GGTGTACTGT
TGGTATTATT CATGGTGGTC GTGTTGTTAA
TGCTTCTTCT ATAGCTATGA CGGCACCGTC
ATCAGGTATG GCTTGGTCTA GCAGTCAGTT
TTGTACTGCA TACTGTAACT TTTCAGATAC
TACAGTGTTT GTTACACATT GTTACAAACA
AGTTGGGTGT CCTATAACTG GCATGCTTCA
ACAGCATTCT ATACGTGTTT CTGCTATGAA
AAATGGCCAG CTTTTTTATA ATTTAACAGT
TAGTGTAGCT AAGTACCCTA CTTTTAAATC
ATTTCAGTGT GTTAATAATT TAACATCCGT
ATATTTAAAT GGTGATCTTG TTTACACCTC
TAATGAGACC ACAGATGTTA CATCTGCAGG
TGTTTATTTT AAAGCTGGTG GACCTATAAC
TTATAAAGTT ATGAGAGAAG TTAGAGCCCT
GGCTTATTTT GTTAATGGTA CTGCACAAGA
TGTTATTTTG TGTGATGGGA

DISCUSSION

Isolation and identification of IBV is needed for positive diagnosis but it cannot be made on clinical signs alone, since other avian pathogens such as Newcastle disease, infectious laryngotracheitis and avian pneumoviruses can produce similar clinical signs. Isolation and identification of IBV is to passage a sample in embryonating specific-pathogen-free chicken eggs (20). Since several passages in chicken embryos are required before field strains of IBV, it can produce typical lesions; make IBV diagnosis quiet complex and time consuming (21).

RT-PCR is a potent technique for detection of IBV. In comparison with classical detection methods, PCR-based techniques are both more sensitive and fast. One of the major problems with IBV is the frequent emergence of new variants. The detection and identification of these new variants is crucial to disease control (14). In this study, the RT-PCR method was used that is sensitive to any specific viral strains. Previous studies have shown that the 3' end of this gene is not the same in all strains of this virus (22), so it can be used for specific strain

detection. But our aim was to detect all strains of this virus and therefore the primer designing was focused on an estimated 1000 nucleotide zone of the 5' end of the *S1 gene* in order to cover and detect all strains.

For better detection of viral genome, a reliable procedure to viral RNA extraction is very important because the degradation of RNA molecules during extraction phase may affect the final results. Pang et al (2001) designed a pair of primers which amplified a 1720 fragment of IBV. The Pang primers were also tried to detect IBV but the results were not encouraging which could be because coronaviruses have the largest known RNA genome (23) and the breakdown of this Genomic RNA to smaller fragments during RNA extraction is expectable. Therefore, new primers were designed for this study that amplified smaller fragments (531 fragments) and gave better results which could be related to their smaller amplicon size and was also time-saving because of the smaller apmlicon size.

Nested-PCR can be much more sensitive than conventional RT-PCR because of the addition of a second amplification step.

The sensitivity of RT-PCR was determined to be 3×10^3 EID50/ml and 3×10^1 EID50/ml in Nested-PCR. This sensitivity might be good enough for the detection of IBV genome in the clinical samples. However, the detection limit of nested-PCR was found to be 10 EID50/ml which was 100 folds more sensitive than PCR.

Because of the low virus titre in some samples, the use of RT-PCR alone may not show good results and nested-PCR can help for a better detection of infection.

The procedure used in this study was rapid (less than eight hours), reproducible and could be practiced without using extra purification steps which are usually tedious or time consuming.

In this study, a rapid and simple RNA extraction procedure combined with a reverse transcription and a double-PCR amplification protocol was developed. Due to the high sensitivity of the test, this technique can be used as a robust and rapid diagnostic method for the detection of IBV.

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