



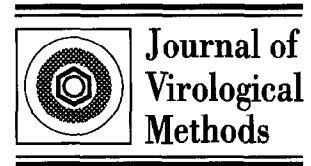
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Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction

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Abstract

A simple, sensitive and specific polymerase chain reaction (PCR) procedure was developed in order to detect infectious bronchitis virus (IBV) directly in tissue samples. Viral RNA was extracted from allantoic fluids and cell cultures infected experimentally with different strains of IBV and from tissues of naturally infected birds. Viral RNA was then amplified and identified by a nested RT-PCR assay using two sets of primers flanking a well-conserved region of the nucleocapsid gene. The selected IBV nucleocapsid sequence was detected successfully by simple direct electrophoresis of amplified material. © 1997 Elsevier Science B.V.

Keywords: Infectious Bronchitis Virus; Polymerase Chain Reaction; Diagnosis

1. Introduction

Avian infectious bronchitis viruses are members of the Coronaviridae family, enveloped viruses containing single-stranded RNA of positive polarity (Bournsell et al., 1989).

Infectious Bronchitis Virus (IBV) causes a highly contagious respiratory infection in young chickens and a significant fall in egg production in laying hens. It is endemic in chicken populations throughout the world (Cunningham, 1970; King

and Cavanagh, 1991; Pascucci and Franciosi, 1991). The disease can be controlled by vaccination of chicken flocks; nevertheless, outbreaks still occur in vaccinated flocks due to the lack of cross-protection against antigenically unrelated serotypes and variant strains of the virus (Gelb et al., 1991; King, 1988; Capua et al., 1994; Jia et al., 1995). Early diagnosis is essential for effective control of an outbreak.

IBV infection in chickens is diagnosed currently by virus isolation and identification, using embryonated chicken eggs, by serological tests, electron microscopy, tracheal organ culture, enzyme-linked immunosorbent assay (ELISA) (Lukert, 1975; O.I.E., 1992).

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The above-mentioned methods are expensive, labor 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.

This can be accomplished by the polymerase chain reaction technique (Saiki et al., 1985) which has also provided new diagnostic opportunities in veterinary medicine, (Belak and Ballagi-Pordany, 1993).

In the case of IBV, a specific genomic region of the virus was amplified by PCR by Andreasen et al. (1991) using a highly purified viral RNA. PCR for IBV diagnosis was developed further by Zwaagstra et al. (1992) and Jackwood et al. (1992), who described a rapid and sensitive method for the extraction and detection of viral RNA from IBV-infected allantoic fluid.

Kwon et al. (1993) described a PCR method for IBV detection in tracheal swabs of experimentally infected chickens and in the allantoic fluid of specific pathogen free (SPF) embryonated chicken eggs passed 4–6 times with IBV-suspected field samples.

We describe a nested RT-PCR assay (Mullis and Faloona, 1987) which can be undertaken directly on tissues of naturally infected birds, to be applied in addition to conventional infectivity assays.

2. Materials and methods

2.1. Viruses

Seven egg-adapted and one VERO cell line-adapted strains of IBV were used to calibrate the test: Ark (Arkansas serotype), D1466 and 274 (Delaware serotypes), 3794 and 843 (Italian field virus isolates) strains, were kindly provided by Dr P. Massi, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Forlì, Italy; M41, egg-adapted Beaudette (Massachusetts serotypes), Wooley (a Beaudette strain adapted to growth on Vero cell-line) strains, were part of the authors' Laboratory collection.

Egg-adapted viruses were propagated by inoculation into the allantoic sac of 9–11 day old

specific-pathogen-free (SPF) embryonated eggs and incubated for 40 h at 37°C. The eggs were kept at 4°C for 4 h before the allantoic fluid was harvested.

The Wooley strain was propagated on confluent monolayer of Vero cell line and collected from the supernatant of cell cultures 48 h after the infection.

Twelve specimens, collected from naturally infected unvaccinated chicken flocks, were obtained by Dr P. Massi (Forlì, Italy). Each sample, derived from a single animal with acute respiratory disease, consisted of a pool of trachea, lung, kidney and cecal tonsils tissues.

The presence of IBV in these samples was determined by virus isolation in embryonated eggs, by an immunodiffusion test and by electron microscopy (Lukert, 1975; O.I.E., 1992).

Controls for specificity included a Canine Coronavirus (CCV), (field virus isolate, kindly supplied by Prof. Buonavoglia, Inst. Microbiology, Faculty of Veterinary Medicine, Bari), and an unrelated avian virus, Newcastle Disease Virus (NDV), (field isolate, authors' Laboratory collection).

Allantoic fluid from uninoculated embryonated eggs and a mixture of tissues homogenates from a healthy bird were used as negative control.

2.2. Oligonucleotides

A region of IBV genome, showing the highest degree of conservation between the nucleocapsid protein of different Coronaviruses (Bourne et al., 1985; Sutou et al., 1988; Zwaagstra et al., 1992) was selected at the N-terminus of the nucleocapsid gene (N). Four oligonucleotide primers (M-Medical srl/Genenco, Firenze/Italy) (IBV1 to IBV4) were designed as showed in Table 1.

The outer span, including primers IBV1 and IBV2, was 316 bp and the inner span, including primers IBV3 and IBV4, was 240 bp.

2.3. Viral RNA extraction

RNA fast™-II kit (Molecular Systems, San Diego/CA) was used to extract RNA from 300 µl of allantoic fluid from 9–11 day old SPF embryonated chicken eggs inoculated with different IBV

strains and from 10 mg of each pool of infected tissues.

Only field virus isolates were treated with a RNA binding resin, included in the kit.

RNA fast solution (900 μ l) and 0.2 ml of chloroform were added to each sample, refrigerated on ice for 5 min and centrifugated at $12\,000 \times g$ (4°C). The aqueous phase, resulting from centrifugation and containing RNA, was mixed with 0.5 vol of isopropanol and kept at –20°C for 1 h. RNA was pelleted at $12\,000 \times g$ for 10 min at 4°C; the pellet was washed with 1 ml of 75% ethanol by vortexing for 30 s and spinning for approximately 1 min.

RNA pellet was dissolved in 3 μ l of DEPC treated water (Sigma) and processed to RT-PCR reaction.

2.4. Reverse transcription

Reverse Transcription (RT) and PCR were conducted in the same PCR buffer (Perkin Elmer Cetus, Norwalk, CT.) (10 mM Tris-HCl pH8.3, 50 mM KCl), and performed in the same Eppendorf tube. A mixture of 200 ng of the ‘downstream’ primer IBV2 and 3 μ l of the RNA template was incubated at 65°C for 5 min and then cooled on ice. The RNA-primer mixture was adjusted to a final volume of 20 μ l containing PCR buffer, 5 mM MgCl₂, 1 mM each of dNTPs and 50 U of reverse transcriptase (Moloney murine leukemia virus RT) (Perkin Elmer Cetus)

Table 1
Nucleotide sequence, melting temperature and locations^a of the primers used for PCR

Primer Sequence	Melting temperature (°C)	Location
IBV1 (+) 5'-TCATG-GCAAGCGGTAAGG-3'	53	100–117
IBV2 (–) 5'-TTCAGGT-TAGCGGCTGGTC-3'	53	397–415
IBV3 (+) 5'-GCC-CCAGCTCCAGTCAT-3'	53	135–151
IBV4 (–) 5'-CCAAG-CATCTGGGACTGGT-3'	53	356–374

^aLocations of the primers in relation to the genome of the IBV Beaudette cDNA clone C5.322 (Boursnell et al., 1985).

followed by an incubation at 42°C for 40 min. The reverse-transcriptase was then inactivated by heating the mixture to 95°C for 5 min.

2.5. Nested PCR amplification

PCR was carried out in a two-step reaction, first with a pair of primers flanking the region to be amplified (IBV1 and IBV2) and then using a pair of primers within the amplified sequence (IBV3 and IBV4) in a total volume of 100 μ l.

Various conditions regarding the concentration of MgCl₂, primers, cycles number and annealing temperature were tested using the Beaudette strain of IBV as a reference strain. Optimisation of these parameters resulted in the following standard procedure: to the cDNA mixture, 200 ng of the ‘upstream’ primer IBV1, 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer, Cetus), PCR buffer and MgCl₂, 2 mM were added. Each sample was overlaid with 80 μ l of mineral oil (Perkin Elmer, Cetus) to prevent evaporation.

The DNA was denaturated at 95°C for 5 min and the amplification undertaken in a DNA thermal cycler 480 (Perkin-Elmer, Cetus) by a ‘hot start’ procedure (Chou et al., 1992).

The first round of amplification consisted of 30 cycles (denaturation at 95°C for 1 min, annealing at 49°C for 2 min and polymerization at 72°C for 3 min); the extension time during the final cycle was increased to 10 min to complete cDNA synthesis on all strands.

In addition, a second round of amplification of 25 cycles using the inner pair of primers was performed: a 20-fold dilution in distilled water of the first PCR product was used as a template under the same reaction conditions as in the first PCR.

2.6. Analysis and detection of products

For analysis, 4 μ l of loading buffer (0.05% [w/v] bromophenol blue, 40% [w/v] sucrose, 0.1 M EDTA pH 8.0, 0.5% [w/v] sodium lauryl sulfate [SDS]) were added to 16 μ l of the reaction mixture which was then analyzed on 1.5% agarose gel containing ethidium bromide (0.5 μ g/ μ l) in TBE buffer (100 mM Tris base, 50 mM boric acid, 2 mM EDTA) at 7 V/cm for 40 min.

Amplified DNA was detected using an ultraviolet transilluminator (Mighty Brightly, Hoefer) and photographed under UV light on Polaroid type 665 film.

3. Results

3.1. Amplification of IBV strains by PCR

Viral RNA was extracted by a rapid procedure from the allantoic fluid of SPF embryonated chicken eggs and from the supernatant of VERO cell-line inoculated with different strains of IBV.

Total extracted RNA was used as a template to produce cDNA using the primer IBV2. The cDNA of all the tested IBV strains was equally amplified with the selected primers. Amplicons appeared to be of the predicted size of 316 bp when electrophoresed with a molecular weight standard in an ethidium bromide stained agarose gel. Water control, included to monitor the possibility of cross contamination, did not show any visible band in the gel (Fig. 1A).

The primer set did not appear to amplify the aspecific RNAs, included as further controls in the test (data not shown).

3.2. Application of PCR to field virus isolates

RNA extracted from each field virus isolate was directly subjected to RT-PCR, as described.

All the samples tested showed the expected amplification products in agarose gel electrophoresis, thus, confirming the diagnosis of IBV performed using different classical techniques. Pooled tissues from a healthy animal did not show any specific band on the gel (Fig. 2A).

3.3. Second PCR amplification

The specificity of the PCR reaction was confirmed by a second PCR using inner primers within the target sequence. Nested-PCR detected the IBV-RNA sequences in all the IBV-positive samples, showing a single band at the expected site (Fig. 1B and Fig. 2B).

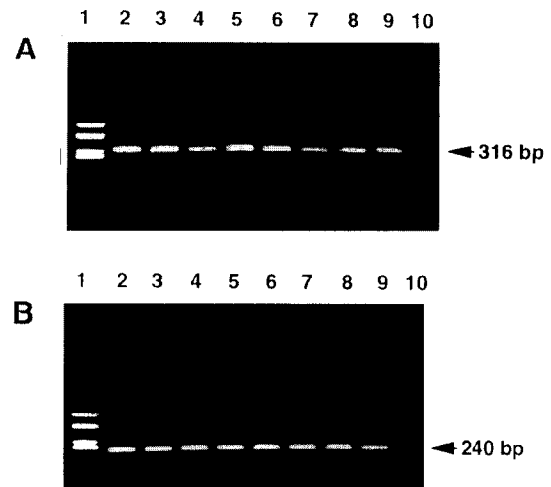


Fig. 1. Double PCR amplification of IBV cDNA from allantoic fluid of SPF embryonating chicken eggs inoculated with different strains of IBV: Arkansas (2), D 1466 (3), D 274 (4), 3794 (5), M 41 (6), 843 (7), Beaudette (8), from supernatant of Vero cells infected with Wooley strain of IBV (9). Water control (10). DNA-size marker pUC 18/ *Hae*III digest is seen in position (1). A. Electrophoresis of PCR products from the first round of amplification using primers IBV1 and IBV2. B. Electrophoresis of PCR products from the second round of amplification using primers IBV3 and IBV4.

4. Discussion

IBV diagnosis is quite complex and time consuming: several passages in chicken embryos are required before field strains of IBV can produce typical lesions (King and Cavanagh, 1991).

To address this issue, a rapid and simple RNA extraction procedure combined with a reverse transcription and a double-PCR amplification protocol using primers homologous to a highly conserved region of the IBV genome was developed.

The final assay protocol aimed at being as simple as possible to allow its use on a large number of field samples and non isotopic.

In practical terms, this novel technique allows a marked reduction in the handling of the specimens and reduces the time required for the diagnosis by eliminating the complex adaptation of field isolates to the growth in intermediate substrates.

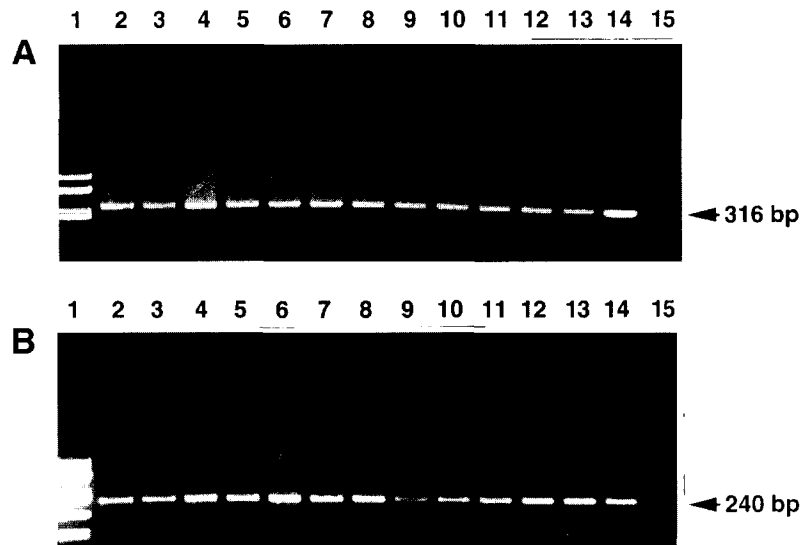


Fig. 2. Double PCR amplification of IBV cDNA from infected organ homogenates: (2–13) different IBV-infected samples, (14) Beaudette strain, (15) uninfected tissues. DNA-size marker pUC18 *Hae*III digest is seen in position (1). A. Electrophoresis of PCR products from the first round of amplification using primers IBV1 and IBV2. B. Electrophoresis of PCR products from the second round of amplification using primers IBV3 and IBV4.

The whole procedure, including reverse transcription, cDNA synthesis, and DNA double amplification, can be accomplished within 12 h.

The agreement of the results with those obtained by conventional diagnostic procedures showed that the detection of IBV genome by PCR amplification is both specific and sensitive.

The specificity, sensitivity, ease of use and reduced time needs of the IBV RT/PCR test indicate its potential for application as a rapid laboratory test for routine diagnostic purposes.

PCR-amplified products could be further analyzed by means of restriction fragment length polymorphism or by sequencing in order to identify different serotypes for epidemiological studies and surveillance programs.

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