

Molecular detection of infectious bronchitis and Newcastle disease viruses in broiler chickens with respiratory signs using Duplex RT-PCR

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Article Info	Abstract
Article history: Received: 01 December 2012 Accepted: 21 March 2013 Available online: 15 December 2014	Infectious bronchitis (IB) and Newcastle disease (ND) are highly contagious and the most economically important diseases of the poultry affecting respiratory tract and causing economic losses in poultry industry throughout the world. In the present study, the simultaneous detection and differentiation of causative agents of these diseases were investigated using duplex-RT-PCR. RNA was extracted from vaccinal and reference strains of infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) and then cDNA was synthesized. Using two universal primer sets for detection of IBV and NDV, the duplex-RT-PCR was developed. In order to assess the efficiency of the developed duplex RT-PCR, a number of 12 broiler farms with the symptoms of respiratory tract infection was sampled (trachea, lung and kidney were sampled from affected birds suspicious for IBV and NDV infections). After RNA extraction from tissues and cDNA synthesis, the presence of IBV and NDV genome were investigated using duplex-PCR. The results showed that three of twelve examined broiler farms were positive for IBV and two farms were positive for NDV and IBV. The results revealed that the duplex-RT-PCR is a quick and sensitive procedure for simultaneously detecting IBV and NDV in birds with respiratory infections.
Key words: Duplex-RT-PCR Infectious bronchitis virus Newcastle disease virus	

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شناسایی مولکولی ویروس‌های بیماری برونشیت عفونی و نیوکاسل در جوجه‌های گوشتی دارای علائم بالینی تنفسی با استفاده از ترانس کریپتاز معکوس و واکنش زنجیره‌ای پلی‌مراز دوگانه

چکیده

بیماری برونشیت عفونی (IB) و نیوکاسل (ND) از بیماری‌های بسیار واگیر و از نظر اقتصادی دارای اهمیت بالا می‌باشند که مجرای تنفسی طیور را تحت تأثیر قرار می‌دهند و باعث ضررهای اقتصادی در صنعت طیور در سراسر دنیا می‌گردند. در مطالعه حاضر شناسایی همزمان و تفریق عوامل ایجاد کننده بیماری‌های برونشیت عفونی و نیوکاسل با استفاده از روش ترانس کریپتاز معکوس و واکنش زنجیره‌ای پلی‌مراز (RT-PCR) دوگانه مورد تحقیق قرار گرفت. RNA ژنومی از سویه‌های مرجع و واکنس ویروس‌های برونشیت عفونی (IBV) و نیوکاسل (NDV) استخراج گردید و سپس cDNA سنتز شد. با استفاده از دو جفت پرایمر عمومی برای شناسایی IBV و NDV روش RT-PCR دوگانه ابداع گردید. به منظور ارزیابی کارایی روش PCR دوگانه ابداع شده تعداد ۱۲ گله گوشتی با علائم عفونت مجرای تنفسی نمونه‌برداری شدند (نمونه‌های نای، ریه و کلیه از پرندگان مشکوک به عفونت‌های IBV و NDV). بعد از استخراج RNA از بافت‌ها و سنتز cDNA، حضور ژنوم IBV و NDV با استفاده از روش RT-PCR دوگانه در بافت‌ها جستجو گردید. نتایج نشان داد که تعداد سه گله از دوازده گله مورد بررسی برای IBV مثبت و تعداد دو گله برای NDV مثبت بودند. نتایج بدست آمده نشان داد که روش ابداع شده RT-PCR دوگانه یک روش سریع و حساس برای شناسایی همزمان IBV و NDV در پرندگان دارای عفونت‌های تنفسی می‌باشد.

واژه های کلیدی: ویروس برونشیت عفونی، ویروس نیوکاسل، RT-PCR دوگانه

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Introduction

Respiratory diseases are among the most devastating diseases in poultry industry because of their major economic losses. In most cases, there are more than one pathogen involving in the pathogenesis of the respiratory diseases.¹ Among several avian viruses with predilection for the respiratory tract, infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) are the most important viruses of poultry worldwide. Similar respiratory signs of infectious bronchitis (IB) and Newcastle disease (ND) making differential diagnosis of these two diseases difficult.²

In broilers, IBV affects weight gain and feed efficiency, and, when complicated with bacterial infections like *E. coli* or *S. aureus*, it causes high mortality and increased condemnations.³⁻⁵ IBV, the causative agent of IB is a coronavirus readily undergoes mutation in chickens resulting in the emergence of new variant serotypes and genotypes.⁶ As new strains of IBV emerge, rapid detection of IBV is useful for implementation of control measures, research purposes, and understanding the epidemiology and evolution of IBVs.⁷

Newcastle disease classified as a list A disease by the Office Internationale des Epizooties (OIE), is caused by avian paramyxovirus 1 (APMV-1) or NDV.⁸ The virus is enveloped with a negative-sense, single stranded RNA genome of approximately 15 kb encoding six proteins (nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and large protein, respectively).⁹

Several laboratory methods such as virus isolation in embryonated eggs and organ cultures and serological tests are available for detecting and differentiating avian viral respiratory infections. However, these methods are time consuming and laborious.¹⁰⁻¹² Molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR), sequencing and real time PCR, have been used for rapid and sensitive detection of IBV and NDV separately.¹³⁻¹⁷ However, those techniques detect only one specific pathogen at a time. The duplex PCR has the ability to amplify and differentiate multiple specific nucleic acids.¹⁸ The aim of the present study was to detect and differentiate two common avian viral pathogens using duplex RT-PCR for clinical diagnosis.

Materials and Methods

Virus strains. In this study for developing duplex-PCR, two standard field strains of NDV (Razi institute), a vaccinal

strain of IBV named IB88 (Merial, Lyon, France) and one field strain of IBV named 793/B (Faculty of Veterinary Medicine, Tehran, Iran) were used.

Field samples. Clinical samples including trachea, liver and kidney were collected from chickens with respiratory symptoms from 12 broiler farms. All farms had a routine history of vaccination against IBV and NDV. Six birds were collected from each broiler farm. Mucosal layer of trachea was scratched by scalpel blade. Tissues of 0.5 cm³ was removed from each liver and kidney and then crushed in a sterile mortar with one mL sterile PBS buffer.

RNA extraction. Viral RNAs were extracted using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Extracted viral RNAs were used in subsequent reverse transcription for synthesizing cDNA or stored at -70 °C for later use.

cDNA synthesis and duplex PCR. cDNA was produced from extracted RNA using reverse transcriptase enzyme. For duplex PCR two sets of universal primers were used in one reaction. The sequence and location of the primers used for amplification of M gene of NDV and 3' UTR of IBV are provided in Table 1. The duplex-RT-PCR was performed in a 25 µL total reaction volume with 0.5 µL of each primer, 2.5 µL 10x PCR Buffer, 1 mM MgCl₂, 50 µM of each dATP, dCTP, dGTP and dTTP, and 0.5 µM of each primer, 1 U SmarTaq DNA Polymerase, 3 µL cDNA. The thermal profile for duplex-RT-PCR included an initial denaturation in 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 45 sec, and a final extension cycle at 72 °C for 5 min. The duplex-RT-PCR products were separated on 1.5% agarose gel run in 0.5% TBE buffer under constant 80 V for 1 hr and the gel stained by ethidium bromide and visualized under UV light.

Results

Developing RT-PCR using vaccinal and reference strains of IBV and NDV. The specificity of duplex-RT-PCR was shown using IB88 and 793/B strains of IBV and two standard strains of NDV. The duplex-RT-PCR products visualize by gel electrophoresis was 433 bp for IBV and 121 bp for NDV (Fig. 1).

Application of developed duplex-RT-PCR for detection and differentiation of IBV and NDV in clinical samples. The applicability of developed duplex-RT-PCR assay for detection and differentiation of IBV and NDV in the diagnosis was validated examining 12 clinical samples as showed in Fig. 2. Among five positive clinical samples belonged to five different broiler farms, three farms

Table 1. The sequences and binding sites of primers used in this study.

Primer	Primer direction	Sequence(5'-3')	Gene location	Product size (bp)	Reference
All 1-F	sense	CAGCGCCAAAACAACAGCG	3' UTR of IBV	433	13
Del1-R	Anti-sense	CATTTCCTGGCGATAGAC	3' UTR of IBV		19
AMPV1-F	sense	AGTGATGTGCTCGACCTTC	M gene of NDV	121	16
AMPV1-R	Anti-sense	CCTGAGGAGAGGCATTGCTA	M gene of NDV		16

were infected with only one virus and two farms were co-infected with IBV and NDV.

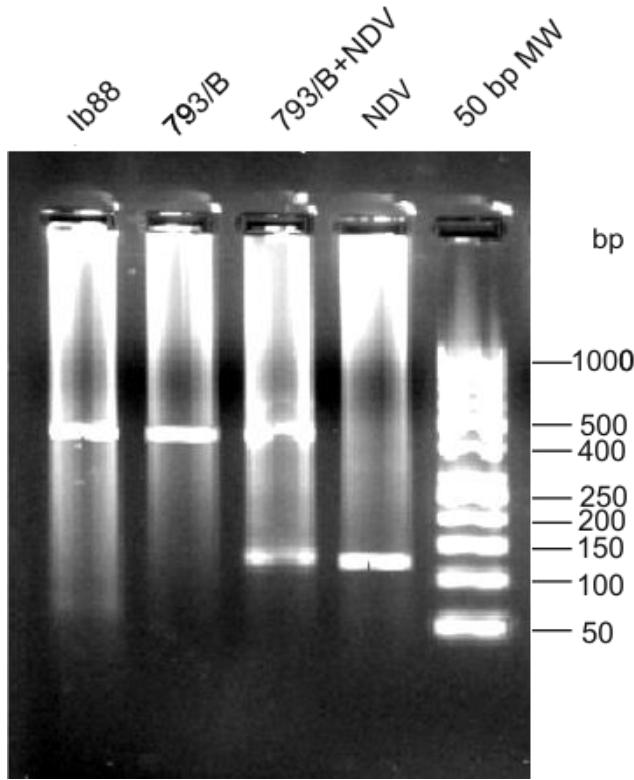


Fig. 1. The specificity of RT-PCR for differentiation of IBV and NDV. Lane 1, IB88; Lane 2, 793/B; Lane 3, 793/B+NDV; Lane 4, NDV; Lane 5, 50 bp DNA marker.

Discussion

Viral respiratory diseases are common causes of economic losses in poultry industry. These diseases cause reduction of growth rate and production, high rate of death, prevention and treatment costs. Quick detection and differentiation of causative viruses can play an important role in controlling these viruses.²⁰ IBV and NDV are the viruses that frequently affect the respiratory tract of chickens.²¹ There are several clinically similar viral diseases that can occur in intensive poultry production and require laboratory differential diagnosis. Infectious bronchitis is a global and highly infectious viral disease,²² and Newcastle disease is also an economically important viral disease in poultry industry.²³ Several studies have shown the circulating of different viral respiratory disease including IBV,²⁴⁻²⁷ NDV²⁸ and avian influenza in Iranian poultry farms.²⁹⁻³¹

The duplex RT-PCR assay which can be able to quickly identify IBV and NDV will be of great importance in the epidemiology of these viruses especially for controlling of disease transmission among poultry farms and reduction of the economic losses in poultry industries.^{32, 33} Because of high sensitivity and specificity that PCR offers, since its introduction researchers use it extensively as an indispensable diagnostic method to detect viruses. Using single PCR takes up much time. Therefore using duplex PCR can solve this restriction of PCR.³⁴

In the present study, developed duplex RT-PCR was able to detect and differentiate two important viral respiratory diseases of poultry and more importantly the

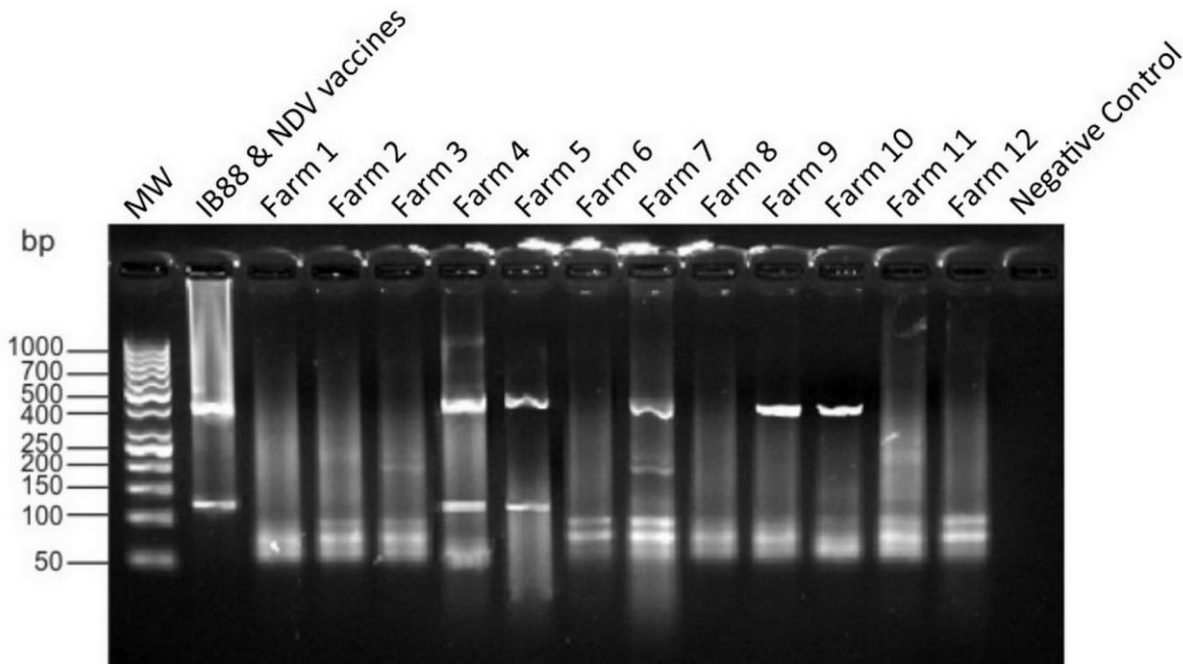


Fig. 2. Evaluation of RT-PCR developed in the present study using clinical samples. Lane 1, 50 bp DNA marker. Lane 6 and 7 co-infection with IBV and NDV and lane 9, 11, 12, positive samples for IBV.

technique was able to simultaneously detect infected birds with both viruses. Since the rapid detection of viral infectious agents in intensive poultry production system is very important, this procedure will be useful to detect more than one infectious agent in the infected farms reducing the time and also costs involved.

Because of the importance of avian respiratory pathogens, many researches have undertaken the detection and differentiation of these pathogens especially AIV, IBV and NDV.³⁵⁻³⁷ A duplex RT-PCR was developed to detect class I and class II strains of NDV. It was shown that this method had high specificity and high sensitivity.³⁸ In another study, Chaharaein *et al.* used duplex RT-PCR for detecting H5, H7 and H9 subtypes of avian influenza viruses.³⁹

In the present study two farms were co-infected with IBV and NDV viruses. It was concluded that the developed duplex RT-PCR could be a rapid and economic procedure for detection of IBV and NDV in poultry farms. Using this procedure for detecting these viruses in wild birds is also recommended.

Acknowledgments

This research was supported by the Research Council of Urmia University. We thank Dr. Heman Aminzadeh for her technical assistance.

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