Genotypic characterization of infectious bronchitis virus from clinically suspected broilers in Basrah, South of Iraq

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ABSTRACT

Infectious bronchitis virus (IBV) is a causative agent of an important respiratory disease (infectious bronchitis) that infects chickens. The disease is being controlled through immunization of birds using S1 subunit vaccine. However, such a vaccine has been no longer protected due to the occurrence of new variants. The aim of the present study was to detect and subtype the IBVs by reverse transcriptase – polymerase chain reaction (RT-PCR) technique. Eight commercial broiler flocks were examined, located in the east (Shat Al Arab), west (Zubair) and north (Qurna) of Basrah city/ south of Iraq during the period from September 2014 to June 2015. The selected birds in all flocks were between 2 to 6 weeks of age and they all had respiratory signs. All birds in the farms were vaccinated against IBV during the first week of age. The birds were killed and samples were collected from the tissues of the kidneys and trachea to extract viral RNA. Specific universal primers XCE2- and XCE2+ for all IB virus serotypes were used in the first round of PCR to detect the virus. A type specific nested PCR was performed to identify the virus serotype using type specific oligonucleotide primers including the reverse primer MCE1+, DCE1+ and BCE1+ respectively specific for a hypervariable region in the S1 gens of serotypes Massachusetts, D274 and 4/91, and the forward primer XCE3- which is common for all three serotypes. The results showed that 7 out of 8 (87.5%) of the sampled flocks were positive to IBV by RT-PCR. The results of virus serotyping showed that the prevalence of Massachusetts serotype was about 71.4% (5 farms) and in two farms, unknown IBV serotypes were detected. These results suggest that vaccination against infectious bronchitis virus is no longer efficient because of the prevalence of different virus serotypes in Basrah city/ south of Iraq.

Keywords: primer XCE3, Infectious bronchitis virus (IBV), broilers

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الملخص باللغة العربية

يعتبر فيروس التهاب القصبات المعدي (IBV) العامل المسبب لمرض التهاب القصبات المعدي الذي يصيب فروج اللحم. وعادة ما يتم السيطرة على المرض من خلال تحصين الطيور باستخدام لقاح وحيدات س1. مع ذلك فإن هذه اللقاحات لا تحمي الطيور من العتر الجديدة.
هدفت هذه الدراسة إلى الكشف عن فيروس التهاب القصبات المعدي وتحديد النوع بواسطة تقنية الناسخ العكسي – لسلسلة تفـاعلات البلمـرة (RT-PCR). تم دراسة ثمانية حقول لفروج اللحم، تقع في الشرق (شط العرب)، الغرب (الزبير) والشمال (القرنة) من مدينة البصرة / جنـوب العراق وذلك خلال الفترة من شهر أيلول / سبتمبر 2014 إلى شهر حزيران /يونيو 2015. كانت الطيورالمنتقات في عمر 2–6 أسابيع، ومصابة بأعراض تنفسية. تم تلقيح جميع الطيور في الحقول ضد فيروس التهاب القصبات المعدي خلال الأسبوع الأول من العمر. بعد قتل الطيور تم جمع عينات من أنسجة الكلى والقصبة الهوائية لاستخلاص الحامض النووي الفيروسي. واستخدمت البادئات XCE2− وXCE2 + لجميـع الأنمـاط المصلية لفيروس التهاب القصبات المعدي في الجولة الأولى من PCR للكشف عن الفيروس. ثم إجراء nested PCR لتحديد النمط المـصلي للفيروس باستخدام بادئات خلفية محددة + MCE1، DCE1 + وBCE1 + لتحديد الأنماط المصلية ماساتشوستس، D274 و91/4، على التوالي، أما البادئ الأمامي XCE3− فهو لجميع الأنماط المصلية الثلاثة. وأظهرت النتائج أن 7 من أصل 8 (87.5٪) من عينات الحقول كانت إيجابيـة لفيروس التهاب القصبات المعدي بواسطة RT-PCR. وأظهرت النتائج أن انتشار النوع المصلي ماساتـشوستس Massachusetts بلـغ نحـو 71.4٪ (في 5 حقول)، وتم الكشف عن الأنماط المصلية مجهولة لفيروس التهاب القصبات المعدي في حقلين. وتشير هذه النتائج إلى أن التطعيم ضد فيروس التهاب القصبات المعدي لم يعد فعالا بسبب انتشار فيروس بأنماط مصلية مختلفة في مدينة البصرة / جنوب العراق.

INTRODUCTION

Infectious bronchitis, which is commonly known as (IB) is a highly transmissible, febrile viral respiratory disease of chickens (1-3). The virus primarily infects the respiratory tract, kidneys and oviduct (4). The main clinical signs include tracheal rales, coughing and sneezing. In addition, other manifestations such as decline in egg production, enteritis, kidney damage, and occasionally pectoral myopathy may be observed (5). The disease is common worldwide and has significant economic consequences (6).

Infectious bronchitis virus which is referred as (IBV) is belonging to the genus *Coronavirus* of the family Coronavirdae. The virus is enveloped with a single stranded RNA of positive sense. The most conserved gene of the virus is responsible for coding the nucleocapsid protein, whilst the most variable gene is responsible for coding the spike 1 (S1) subunit of the spike protein (4). The S1 subunit is responsible for prompting neutralizing and serotype-specific antibodies. Mutations that may generate in this genome region may result in the emergence of new variants against which vaccines are no longer protective (7). Lots of genotypes and serotypes of the virus have been identified, and many more will surely be detected in the future. The occurrence and emergence of multiple serotype of the virus will complicate the control of the disease by vaccination (8). The emergence of new variants is associated with outbreaks in many countries (9-11). In Iraq, IB is considered an important viral disease of chickens. Although the vaccination is a good tool to protect from the disease, outbreaks occur frequently and the infection is not prevented by the existing vaccines, due to the serotype differences (12).

In our region, there is a high incidence of disease and is usually characterized by high morbidity and mortality. In addition, there is no report about the genotypes and the molecular detection of IBV in Iraq. Therefore, the aim of our study was to detect the IBVs by reverse transcriptase – polymerase chain reaction (RT-PCR) technique. PCR is fast and sensitive detection technique in comparison with the other traditional detection methods (13). Samples were collected from poultry farms to detect the viruses by two rounds of PCR run (single amplification by RT-PCR to detect IB viruses in general and nested-PCR to identify the virus serotype).

MATERIALS AND METHODS

Chicken flocks: history and samples

Eight commercial broiler (Cornish Cross) flocks, located in the east (Shat Al Arab), west (Zubair) and north (Qurna) of Basrah city/ south of Iraq were collected during the period from September 2014 to June 2015 and examined. Shat Al Arab and Al Qurna regions were included three farms each, while in Al Zubair region, two farms were included. The age of birds in all flocks was 2–6 weeks. Each flock affected with respiratory disease as suspected IBV cases. Broiler flocks were showing moderate to severe signs of respiratory disease, a decrease in feed intake, and deaths (20% to 30%). Post-mortem examination revealed lesions of inflamed trachea, cheesy exudates and swelling of the kidneys. All birds in the farms were vaccinated against IBV during the first week after hatching, according to the supervisor of each farm. All flocks were contacted through specialist veterinarians and all owners were very pleased to participate in this study.

Five to ten diseased birds per farm were killed, and tissues of the trachea and kidneys were collected as organ-specific pools of birds after necropsy. All samples were placed in a sterile tube containing phosphate buffer saline (PBS) and they were moved to the microbiology lab at the college of Veterinary Medicine/ University of Basrah in cold conditions for analysis. The samples were homogenized in sterile PBS to prepare pooled samples; 20% (w/v) homogenates. They were then centrifuged at 5,000 x g for 20 min; and the supernatants were then collected to extract viral RNA.

Viral RNA extraction

Two hundred micro liters of supernatants were used to extract viral RNA using a QIAamp viral RNA mini kit (Qiagen) following the manufacturer's manual. Viral RNA was stored at −70◦C until analysis by RT-PCR**.**

Single gene amplification by RT-PCR

The PCR reaction was performed using universal primers XCE2- and XCE2+, which are specific to all IBV. Table (1) shows the primer sequences which were used in the reaction. The RT-PCR produces a 466-bp fragment (figure 1) specific to all IBV positive samples and does not differentiate between different strains. One-step RT-PCR was performed to amplify the target RNA (AccuPower RocketScript RT-PCR PreMix kit, Bioneer, South Korea), according to the manufacturer's protocol. The RT-PCR was carried out in a DNA thermocycler. Reverse transcriptase was carried out for 1 RT cycle at 42°C for 60 min, followed by initial denaturation at 94°C for 5 min, then 35 PCR cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension cycle at 72°C for 7 min (14).

Table (1): Oligonucleotide (primer) sequences used in the RT-PCR and nested PCR determine IBV and IBV serotypes (14)

Primer	Sequence (5'-3')	Amplication fragment (bp)
XCE2+	5'-CACTGGTAATTTTTCAGATGG-3'	466
XCE2-	5'-CCTCTATAAACACCCTTGCA-3'	
XCE3-	5'-CAGATTGCTTACAACCACC-3'	
DCE1+	5'-TTCCAATTATATCAAACCAGC-3'	217
MCE1+	5'-AATACTACTTTTACGTTACAC-3'	295
BCE1+	5'-AGTAGTTTTGTGTATAAACCA-3'	154

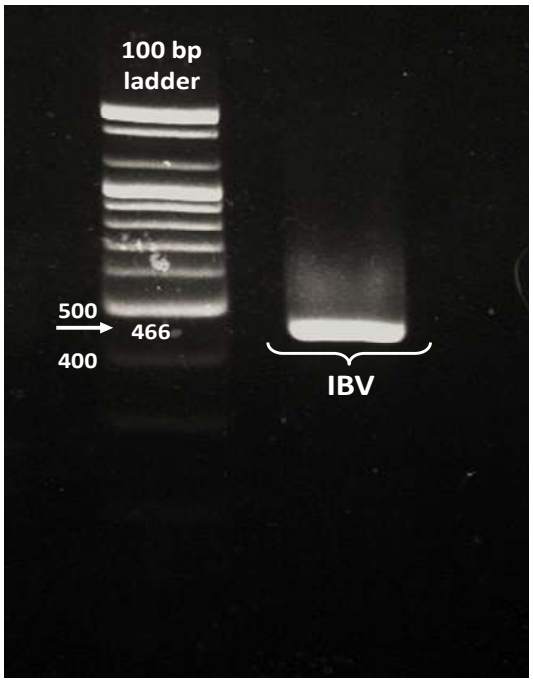

Figure (1): Results of RT-PCR in commercial broiler flocks affected by respiratory disease. The PCR products were separated using a 1.5% agarose gel pre-stained with ethidium bromide. Single band which represents the presence of IBV appeared clearly on the gel. A 100 bp ladder was also loaded on the gel to determine the size of the band.

Nested amplification by PCR

A type specific PCR was performed to identify the virus serotype. For this purpose, a type specific nested PCR was employed. Oligonucleotide primers including the reverse primers MCE1+, DCE1+ and BCE1+ respectively specific for a hypervariable region in the S1 gens of serotypes Massachusetts, D274 and 4/91, and the forward primer XCE3- which is common for the three serotypes were used (14-16).

Agarose gel electrophoresis

The PCR products were electrophoresed using 1.5% agarose gel and stained with ethidium bromide and visualized by UV transilluminator. A DNA ladder of 100 bp was also loaded on the gel to estimate the size of the PCR product**.**

RESULTS

Detection of IBV

Single RT-PCR based on using universal primers specific to all IBV with 466 bp was carried out on tracheal and kidney samples of eight commercial broiler flocks affected with respiratory disease (figure 1). The results showed that 7 out of 8 (87.5%) of the sampled flocks were positive to IBV by RT-PCR. Four out of 7 positive, flocks gave positive results with trachea or kidney samples while three flocks, gave positive results with kidney samples only. Infectious bronchitis virus was identified in samples from all geographic regions (Table 2).

Table (2): Positive results of RT-PCR according to geographical distribution and animal organ

Flocks	Sample	RT PCR Result
Zubair 1	Trachea	Negative
	Kidney	Negative
Zubair 2	Trachea	Positive
	Kidney	Positive
Shat al Arab 1	Trachea	Negative
	Kidney	Positive
Shat al Arab 2	Trachea	Positive
	Kidney	Positive
Shat al Arab 3	Trachea	Positive
	Kidney	Positive
Qurna 1	Trachea	Positive
	Kidney	Positive
Qurna 2	Trachea	Positive
	Kidney	Positive
Qurna 3	Trachea	Negative
	Kidney	Positive

Results showed that the disease was identified from all geographic areas in Basra city. In general, the prevalence of the virus was slightly higher in kidney samples in comparison with trachea.

IBV typing

Specific nested PCR was performed on RT-PCR positive flocks. Massachusetts serotype was the specific serotype of infectious bronchitis virus in broiler flocks of Basrah. The 295 bp fragment, including a portion of the S1 gene of the Massachusetts serotype was amplified by PCR (figure 2). The prevalence of Massachusetts serotypes of IBV was about 71.4% (5 farms) and in two farms, unknown IBV serotypes (table 3).

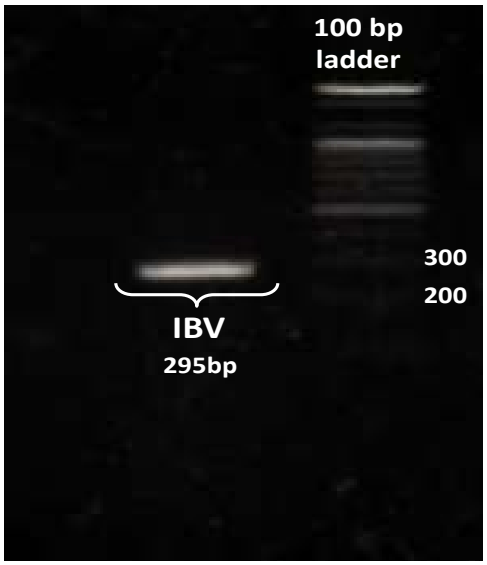

Figure (2): Nested PCR results in commercial broiler flocks affected by respiratory disease. The PCR products were separated using a 1.5% agarose gel pre-stained with ethidium bromide. Single band which represents the presence of Massachusetts serotype appeared clearly on the gel. A 100 bp ladder was also loaded on the gel to determine the size of the band.

Table (3): Stereotypes of IBV in Basrah city

Flocks	Serotypes
Zubair 2	Unknown IBV serotypes
Shat al Arab 1	Massachusetts
Shat al Arab 2	Massachusetts
Shat al Arab 3	Massachusetts
Qurna 1	Massachusetts
Qurna 2	Massachusetts
Qurna 3	Unknown IBV serotypes

The results showed that the dominant serotype of IBV in Basrah city was the Massachusetts. Unknown serotypes were in some chicken flocks**.**

DISCUSSION

In Basrah, poultry producers seek assistance for disease diagnosis through a limited network of poultry diagnostic laboratories. In general, the diagnosis of IBV infection is usually based on clinical signs and gross lesions and, likewise, other respiratory agents are excluded as the cause of the respiratory disease, based on clinical signs and gross lesions only. The clinical signs of the of respiratory diseases are generally similar, therefore, sensitive and specific detection and identification techniques are required. The most common methods to diagnose IB are based on virus isolation in embryonated hen's eggs, followed by immunological detection of the isolated viruses. This procedure is time consuming and quite expensive as it requires specific polyclonal or monoclonal antibodies for virus detection. The single RT-PCR based on S1 gene developed by Keeler (17) greatly improved the speed of the serotyping and diagnosis of IBV. In this study, we optimized PCR techniques to detect IBV which had been shown to be very efficient for the detection of IBV and for the identification of IBV types that also detected by others (18,19).

In the present study, 7 out of 8 (87.5%) broiler flocks were positive to IBV by RT-PCR confirming that infectious bronchitis is one of the main viral respiratory diseases of broilers in different geographic regions of Basrah, south of Iraq. Our study undoubtedly demonstrates that there is a relatively high prevalence of IB disease in commercial broiler flocks in Basrah. Poorbaghi *et al*. (20) found that IBV was detected in 72% of broilers suffering from respiratory signs. The failure of PCR to detect the IBV in samples taken from diseased broilers would be attributed to the chicken infected with another agent causes respiratory signs. Kidney samples are better than tracheal sample in term of the detection of IBV. The current virus strain has more affinity to replicate in kidney tissues, or the kidney tissues are softer than tracheal tissues, therefor the virus can be released more easily during sample homogenates.

This study was conducted to monitor the existence of serotypes of IBV which have been reported, in part, from the countries around Iraq. In commercial broilers in Basrah, five broiler flocks out of seven positive flocks to IBV were infected with Massachusetts serotype and the other two flocks were unknown IBV serotypes. The reason of the presence of unknown IBV serotype may be due to some variations and genetic changes that have occurred recently. Molecular analysis of IBV has shown that genetic variants of this virus can emerge, mainly as a result of a few alterations (insertions or deletions) or mutations in nucleotide sequences of the S1 gene which eventually might change the

amino acid sequence. The genetic variability in the S1 subunit of the envelope spike glycoprotein gene represents an adaptive mechanism of the virus to immune selective pressures associated with intensive IBV vaccination and other management practices (21,22).

Interestingly, a study had reported the identification and genotyping of IBV isolate in Kurdistan region of Iraq in symptomatic vaccinated broiler and found poor efficacy of vaccines used in this region, since vaccinated broilers were symptomatic and found positive by PCR. IBV was determined by RT-PCR in vaccinated broiler as results of studies conducted by (23, 24), and they suggested that flocks were naturally exposed to new variant strains of IBV, which is why the vaccines were not protected. This could be applied to the results of RT-PCR in vaccinated broilers in this study, where 7 flocks examined by RT-PCR were found positive to IBV although they were vaccinated. So virus isolated could be mutant of vaccine strain, or broiler flocks may be infected simultaneously with several type of IBV (18).

Future work suggests the isolation of IBV using the embryonated chicken eggs. In addition, virus serotyping through gene sequencing is so important to determine the current serotype in our region. This will have a fundamental role to adopt a suitable vaccination program, using the common field serotypes as vaccines, to protect against IBV caused disease.

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