Iraqi Journal of Veterinary Sciences

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Genotyping of avian infectious bronchitis virus in broiler farms in Duhok province, north of Iraq

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Article information

Article history:
Received February 28, 2021
Accepted May 20, 2021
Available online November 23, 2021

Keywords:
Broiler
Infectious bronchitis
Duhok
Iraq
Nested RT-PCR

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Abstract

Infectious bronchitis disease is becoming the most challenging disease in broiler fields in Iraq. This disease leads to massive economic loss every round of production. Three vaccine strains H120, Ma5, and 4/91 are used to combat the disease during the production period while outbreaks occur continuously. This study aimed to investigates the prevalence and circulation of Mass and 4/91 IBV strains in the broiler fields in Duhok province which is the first record in the area. Positive samples were obtained from suspected flocks to this disease during 2018-2020. A specific region of S1 was amplified using specific pairs of primers. Genotyping was performed by nested PCR using specified primers for detecting both Mass and 4/91 strains. In the results, all of the positive flocks were infected with nephropathogenic strain 793/B or 4/91. Two of the positive flocks had an infection with both Mass and 4/91. These results indicate that more than one strain circulates in the area as well as shows the weakness of vaccines used in broiler fields.

DOI: 10.33899/ijvs.2021.129635.1670,

Introduction

Avian Infectious bronchitis disease (IB) is one of the most challenging diseases in the poultry industry. Chicken is exposed to IBV through its entire lifespan (1). Massive economic loss is due to the fast and rises emergence of novel variants or serotypes in both broiler and layer flocks (2). Morbidity reaches 100% with a mortality of 20-30% (3) while secondary infection may increase flock mortality (4). Moreover, the upper respiratory tract, kidney, digestive tract, and ovary are the main infection sites of this virus. Clinical symptoms are difficulty in breathing, plaque in the trachea and/or bifurcation, ovary damage, kidney swelling, and sneezing (2). The main consequences are weight loss, drop in egg production, and mortality (1). Although no vertical transmission has been claimed (5), it is transmitted horizontally such as direct and indirect chicken contact and air-born route (contamination and poor field biosecurity). Although successful vaccination is considered one of the best methods for immunizing the birds from this disease, genotyping the field strain may determine the vaccine strains that should be applied (6). Genomic deletions, insertions, substitutions, point mutations, and RNA recombination of the S1 gene are associated with the emergence of new variants (7). The S1 is responsible for viral attachment to the host cell receptors. Additionally, there are three hypervariable regions in the structure of this gene. For these reasons, this gene has been the preferable region for genotyping this virus by many researchers (8). Different serotypes of newly evolved variants from chickens may cause partial or complete vaccination failure (5,9). In Iraq, the first report on this disease was made by (10). Later, the isolation and identification were performed for a specific isolate AM 88 by (11). However, the use of three inactivated and/or live-attenuated IBV vaccines H120, Ma, and 4/91 couldn't stop the massive economic loss in the Iraqi poultry industry due to continuous outbreaks of this disease (12). In the south and middle of Iraq, studies on this disease have been performed to genotype this infection agent (13). The new variant, Sul/01/09 (GQ281656) has

been detected in Sulaimani province, North of Iraq (14). However, In Duhok province, besides one study on virus detection (15), circulation of IBV strains in the broiler fields has not been documented yet. Therefore, this study aims to genotype the IB virus isolated from broiler fields in this area by Nested-PCR.

Materials and methods

Sample collection

A total of 180 trachea tissue samples (3 samples from each field) were obtained from 60 suspected broiler fields located in Duhok governorate, North of Iraq. The sample details were recorded during the collection. The tracheal tissue samples were transported in sterile tubes and kept on ice until RNA extraction.

RNA extraction

Total RNA was extracted within 24 hours from sample collection using QIAamp Viral RNA Mini Kit (Qiagen, Germany). A pool of three tracheas tissues was homogenized manually from each field. The RNA extraction was applied according to the procedure given by the manufacturer. NanoDrop 2000/2000 was used to measure RNA quality and quantity and saved at -20 ºC for further downstream applications.

Reverse transcription

Reverse transcription was applied for cDNA synthesis using SuPrimeScript RT Premix Kit (GeNet Bio, South Korea). Briefly, about 500 ng/µl to 1 µg/µl of total RNA and 100 pmol of Random Hexamers were mixed with 10 µl of ready-to-use RT mixture (2X) and adjusted to 20 µl reaction with nuclease-free water. The cycling program was performed in GeneAmp PCR System 9700 (Applied Biosystems, Singapore). The sample was incubated at 25ºC for 5 minutes then heated for 60 minutes at 50ºC and finally, the reaction was stopped at 70ºC for 10 minutes.

Partial S1 gene amplification

A region 464 bp located on the Hypervariable region 3 (HVR-3) of S1 gene of IBV was amplified by conventional PCR using HS Prime Taq Premix kit (GeNet Bio, South Korea) in GeneAmp PCR System 9700 (Applied Biosystems, Singapore). PCR reaction was made in a 20 µl reaction volume containing 10 µl HS Prime Taq Premix (2X), 2 µl cDNA, 1 µl of each reverse and forward primers 10 pmol (Table 1) and reaction volume was adjusted to the desired volume using nuclease-free water. The cycling program was as follows; initial denaturation 94ºC for 5 minutes followed by 35 cycles of denaturation 94ºC for 30 seconds, annealing 50ºC for 30 seconds, extension 72ºC for 35 seconds and final extension 72ºC for 2 minutes then cooled at 4ºC. Results were confirmed using a 1.5% agarose gel.

Amplified DNA (464 bp) from the first PCR round was used in Nested-PCR to detect specific amplicons of 154 bp and 295 bp for 4/91 and Mass serotypes, respectively. A total of 20 µl reaction was prepared to contain 10 µl of HS Prime Taq Premix (2X), 2 µl of the first-round PCR product, 1 µl of forward primers BCE1+, MCE1+ Specific for 4/91 and Mass serotype, respectively and 1 µl of reverse primer XCE3- (a universal primer for IBV) (Table 1), and volume was adjusted by adding nuclease-free water. Amplification was carried out in Ependrof AG Mastercycler (Hamburg, Germany) according to the following conditions; 1 cycle for Initial denaturation 94ºC for 5 minutes followed by 35 cycles of denaturation 94ºC for 30 seconds annealing 50ºC for 35 seconds extension 72ºC for 1 minute and final extension 72ºC for 8 minutes then cooled at 4ºC. Results were visualized by 2% agarose gel.

Table 1: Primer sequences were used in both partial and nested-PCR reactions

Oligonucleotide	Sequences	Band Size (bp)	Specificity
XCE1+	5'-CACTGGTAATTTTTCAGATGG-3'	464 bp	Universal
XCE2-	5'-CTCTATAAACACCCTTACA-3'		
MCE1+	5'-AATACTACTTTTACGTTACAC-3'	295 bp	Massachusetts
BCE1+	5'-AGTAGTTTTGTGTATAAACCA-3'	154 bp	793/B (4/91)
XCE3-	5'-CAGATTGCTTACAACCACC-3'	-	Universal

Results

All the collected samples were having symptoms related to IBV such as gasping, nasal discharge, sneezing, difficulty in breathing. According to the information that was taken on the day of sample collection, five of the positive flocks were vaccinated with 4/91 and/or Ma5, two of them had Mass types of vaccine, however, the vaccination history for one of them was unknown. On the day of sample collection, all of these samples were having a mortality rate of around 1-2% except in one field which was 8% (Table 2).

The viral extraction kit was efficient in isolating RNA from collected tissue samples with high quality and quantity. Primers (XCE1+ and XCE2-) were able to amplify 464 bp of IBV S1 gene from eight collected samples (Figure 1).

In the second round of PCR (nested PCR), 154 bp was amplified in all positive samples indicating that all the fields were infected with 793/B (4/91) strain of IBV. Also, 295 bp and 154 bp were detectable in two samples (samples 2 and 6) which means that the fields were having co-infection with both IBV strains (Mass and 4/91) (Figure 2).

Table 2: Information of the positive samples to IB from broiler field in Duhok governorate

Location	Age (D)	Mortality (%)	Vaccination
Duhok*	29	1%	4/91+Ma5
Sumel	23	< 1%	4/91
Amedi	12	1%	4/91
Bardarash	24	< 1%	4/91+Ma5
Duhok*	8	< 1%	4/91
Duhok*	34	1%	Ma5
Duhok*	13	8%	H120
Duhok*	15	2%	Unknown

*Center of Duhok province (Duhok subdistrict). 4/91 and H120 at first day, Ma5 at 8 days.

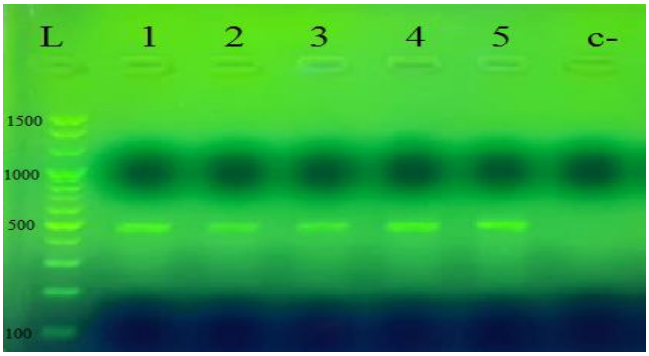

Figure 1: RT PCR for partial S1 gene of IBV from some of the collected samples in broiler fields. L: DNA Ladder (100+ bp); Lane 1-5 positive samples to IBV (464 bp); Lane c-: Negative control.

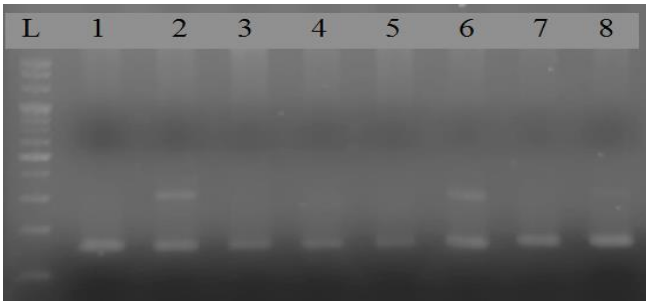

Figure 2: Nested PCR for partial S1 gene of Mass (295 bp) and 793/B (4/91) (154 bp) of IBV in the positive samples collected from broiler fields. Lane L: DNA ladder (100+ bp), Lane 1-8 positive samples to IBV.

Discussion

Infectious bronchitis disease is considered one of the most spread diseases among broiler fields in Iraq. This leads to massive economic losses in this sector. Protection from this disease is mainly dependent on vaccinating the birds with different strains from different origins in every production cycle (12,15). However, the outbreaks still rising which may indicate the weakness of these vaccines in making enough protection. According to Jahantigh *et al.* (5) vaccination against IBV may fail in case of entering a new strain in a specific geographical region. Mahmood *et al.* (14) linked the low efficiency of vaccines used for IBV in Sulaimani province, North of Iraq to the presence of new strain Sul/01/09 (Genebank accession number: GQ281656).

Although continuous IBV cases are detectable in most of the broiler farms in Duhok province, there have been no reports on circulating IBV strains or genotyping of this virus in the area. RT-PCR and Nested PCR have been widely used in this concern due to their efficiency in the identification and genotyping of the isolated IBV and other infectious agents in the broiler and other animals (16-19). Therefore, this study was aimed to genotype the collected positive samples to IBV in the broiler fields in Duhok province, in Iraq. Positive results of PCR are an indication of widely spread of this disease among poultry farms in this area. Due to its sensitivity, the nested PCR technique is widely used to differentiate the obtained amplicons (20). Consequently, in this study, the published primers were able to amplify specified amplicons from 793/B (4/91) and Massachusetts types. Besides the usages of the 4/91 vaccine, the results indicated that all of the collected samples were infected with nephropathogenic strain with the detectable amplicon size that has been targeted with the used primers. Infection of broiler with the same vaccine strain may be due to administration of a vaccine to the IBV-infected chickens with IBV. An additional reason may be due to recombination between vaccine strains with field isolates shortly after vaccination or the use of multiple vaccine strains in the fields (21).

In two fields, the presence or coexistence of both strains Mass and 4/91 was observed. This may be to the same reasons which were mentioned above by Cavanagh (21). This phenomenon was detected by Nouri (22) when they found both Mass and 793/B (4/91) strains in 33 tested flocks. These authors claimed that there is no or poor cross-protection between these two vaccines against IBV. Infection with 4/91 in Duhok province is almost the same as that rate recorded in Sulaimani, Basra, Thi-Qar, and Muthana governorates (14,23). Additionally, infection with Mass types was the same as the prevalence rate in mentioned provinces. Finally, the results showed that the virus can affect birds of all ages and this was concluded by Cavanagh and Al-Jameel and Al-Mahmood (1,24).

Conclusion

In our study genotyping of IBV by Nested PCR was investigated for the first time in Duhok province, north of Iraq. The results of this study indicate the presence of more than one strain of IBV in the broiler fields. Additionally, this technique was successful in amplifying and genotyping the targeted region of the S1 gene of IBV. Complete infection with 793/B (4/91) type of collected samples may indicate the poor vaccination programs against IBV in broiler fields. Co-infection with both strains may be due to low cross-protection among circulating and vaccine strains of IBV. We highly recommend further studies on sequence study of isolated IBV from broiler fields as well as efficient biosecurity and vaccination program against this virus in this area.

Acknowledgment

Special thanks to the Animal Production Department, College of Agricultural Engineering Sciences, University of Duhok for their continuous support. Great thanks and appreciation to Mr. Merkhan Mahdi Mustafa, New vet private veterinary clinic for his priceless help throughout the project.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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النمط الجيني لفايروس التهاب الشعب الهوائية المعدية للطيور في حقول الدجاج اللاحم في محافظة دهوك، شمال العراق

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الخلاصة

أصبح مرض التهاب الشعب الهوائية المعدي أكثر الأمراض تحديا في حقول الدجاج اللاحم في العراق. يؤدي هذا المرض إلى خسائر اقتصادية فادحة في كل وجبة من وجبات الإنتاج. بالرغم من أستخدم ثلاث عترات مختلفة من اللقاح أج ١٢٠، م ي ٥ و ٩١/٤ لمكافحة المرض خلال فترة الإنتاج يبقى المرض متفشي بشكل دائم. الهدف هذه الدراسة هي التحقق من انتشار وتداول العترات ٩١/٤ وماس لفايروس التهاب الشعب الهوائية المعدية في حقول الدجاج اللاحم في محافظة دهوك وتعد هذه الدراسة الأولى في المنطقة للنمط الجيني لهذا الفايروس. تم الحصول على العينات الإيجابية من القطعان المشتبه بها لهذا المرض خلال فترة ٢٠١٨-٢٠٢٠. وتم تضاعف منطقة معينة من جين *S1* باستخدام أزواج محددة من البادئ. ومن ثم إجراء التنميط الجيني بواسطة تفاعل السلسة المتبلمرة المعشعش باستخدام بادئات محددة للكشف عن سلالات ٩١/٤ وماس. ومن خلال النتائج تبين بأن جميع القطعان الإيجابية كانت مصابة بالسلالة الكلوية الممرضة ٩١/٤ أو ٧٩٣/ب. وكذلك اثنان من القطعان الإيجابية كانا مصابين بكل من ٩١/٤ وماس. تشير هذه النتائج إلى وجود أكثر من سلالة واحدة منتشرة في المنطقة وكذلك تظهر ضعف اللقاحات المستخدمة في حقول الدجاج اللاحم.