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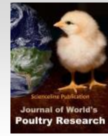

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Serological and Molecular Detection of Local Infectious Bronchitis Virus in Vaccinated Broiler Chickens in Diyala Governorate, Iraq

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ABSTRACT

The infectious bronchitis virus (IBV) is one of the most important *Coronaviridae* viruses, infecting the upper respiratory tract of chickens and leading to considerable losses in the poultry industry across the globe. Many outbreaks have recently occurred among IBV-vaccinated chicken farms in the Diyala Governorate of Iraq resulting in significant economic losses. As a result, the purpose of the present study was to investigate whether IBV can be a source of infection spread in IBV-vaccinated commercial broiler flock farms in Diyala Governorate. In this regard, ELISA was used as a serological test and RT-PCR as a molecular detection technique. Serum samples were collected from chickens suspected of IBV at 16 and 23 days of age. The results showed a significant increase of IgG antibodies in such serum samples at days 16 and 23 of age indicating the infections of the broilers with IBV. However, at the age of 2-3 weeks, the samples of kidney, liver, trachea, and lungs were collected from clinically and sub-clinically infected flocks, and also postmortem samples were sampled from all farms. Two sets of previously reported primers were created for this purpose in the S1-protein gene region. According to the findings of the present investigation, IBV was found in 83% of samples. Finally, despite immunization with IB4/91, IBV was prevalent in broiler chicken farms in the study area confirmed by serology and molecular biology tests. This finding indicates the possibility of genetic difference between the locally discovered IBV and the administered IBV vaccine. A study on the production of local vaccines can be useful in controlling IBV infections.

Keywords: ELISA, Infectious bronchitis viruses, RT-PCR

INTRODUCTION

Infectious bronchitis (IB) is one of the most common contagious respiratory disorders in poultry that affects chickens of all types, ages, sexes, and breeds. The *Coronavirinae* subfamily of the *Coronaviridae* family includes the infectious bronchitis virus. The IBV genome is a single-stranded, positive-sense RNA virus from the gammacoronavirus genus (Cavanagh and Gelb, 2008).

The first infectious bronchitis virus produced by the Mass serotype was discovered in North Dakota, USA, and spread around the world since then (Schalk and Hawn, 1931). Over the years and decades, hundreds of additional IBV genome variations emerged as a result of genetic instability during the propagation or development of the virus (Jordan, 2017). Although chickens are the most common natural hosts for IBV infection, other avian species, such as pigeons, geese, peafowl, pheasants, and ducks can also disseminate IBV albeit clinical indications appear to be limited (Liu et al., 2005; Awad et al., 2014). Infectious bronchitis virus infection in poultry has a short incubation period (24-48 hours) and spreads horizontally by aerosol (coughing and sneezing) and contaminates chicken litter, feed, drinking water, equipment, or other fomites, infecting the poultry flocks in a short time (Chhabra et al., 2015).

Although there has never been a record of IBV transmission via vertical circulation within the embryo, the virus can be transferred from infected hens by contaminating the shell of hatching eggs through shedding from the alimentary tract or the oviduct (Boltz et al., 2004; Saif et al., 2008). Hens of all ages are vulnerable to the IBV, which can lead to great economic losses, although very young chicks have demonstrated more significant respiratory distress, as well as higher morbidity and death,

as compared to older chickens (Britton and Cavanagh, 2007; Cavanagh and Gelb, 2008).

Initially, it was thought that all IB viruses belonged to a single prototype known as Massachusetts (Mass), which had been detected in commercial chicken flocks (Cavanagh and Naqi, 2003). Several IBV strains have been discovered and isolated from layer and broiler farms in Iraq, Iran, and Egypt, and the virus is considered a dominant strain (Liu et al., 2006; Mahmood et al., 2011; Kahya et al., 2013). Many outbreaks of clinically recognized IBV infections have been observed in chicken farms in the Diyala Governorate of Iraq despite the vaccination program of the private sector to suppress the disease.

The poultry business in Diyala Governorate suffers from economic losses as a result of these epidemics. Accordingly, the goal of this study was to examine the IBV molecular assay in selected broiler chicken flocks in the Diyala governorate using RT-PCR and show the status of disease outbreaks in vaccinated farms.

MATERIALS AND METHODS

Ethical approval

The Scientific Ethical Committee of the College of Veterinary Medicine, University of Diyala, Iraq, approved this study (Approval no: Vet Medicine (134); September 2020, K, A, T, and K).

Design of the study

From September 2020 to June 2021, many outbreaks of clinically identified IBV occurred in broiler chicken farms in the privet sections of Diyala Governorate. Clinical signs included gasping, dyspnea, and nasal discharge in these broiler chickens. The mortality rates varied from 30% to 50%. The tracheal bifurcation was blocked with fibrinonecrotic cast in deceased chickens, as well as pericarditis and perihepatitis due to co-infections. There were also swollen kidneys with inflated tubules (Figure 1).

Sample collection

Blood samples

For blood samples, 2 ml of the blood was collected aseptically from the wing vein of the clinically infected chickens aged 16-23 days using disposable syringes (Terumo, Japan). All collected blood samples were allowed to remain for 1 hour for clotting at room temperature. In the next step, the serum was collected, placed in a sterile Eppendorf tube, labeled, and then frozen at -30°C for antibody evaluation against IBV by ELISA test.

Tissue samples

The samples were collected from kidney, liver, trachea, and lungs of infected chickens (5 samples from each farm). The postmortem samples of the mentioned tissues were also collected from each farm too (5 samples from each farm, Figure 1). These samples were processed later for molecular detection of the IBV.

ELISA test

The Zoetis IBV ELISA kit was used to track IBV antibody levels in infected flocks. All ELISA monitoring studies were conducted by Zoetis IBV Ab, United States using the synbiotic developed Profile 2.0 Windows computer program (ELISA – Synbiotics Corporation, United States). The ELISA kits were used according to the manufacturer's protocol using an automated microplate reader (ELx800, BIO-TEK Instruments Inc., USA). The antibody titer in each sample was quantified using the software provided by the manufacturer. The geometric mean titer for each group of serum samples was also calculated using the same software. Processing of serum samples for the ELISA test was followed according to the instruction manual of the above-mentioned ELISA kit manufacturer.

Molecular detection

The reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect IBV at the molecular level. Accordingly, postmortem tissue samples of the kidney, liver, trachea, and lung (n=10) from clinically and sub-clinically infected broiler chickens aged 2-3 weeks were collected as necropsy findings and were processed for RT-PCR (Figure 1).

Viral RNA was isolated from tissue samples of the kidney, liver, trachea, and lung by the Quick-RNATM Viral Kit (France) following the instruction provided by the manufacturer. In this technique, the first step was the conversion of RNA to cDNA, and the second was to amplify the DNA template using a customized program employing PrimeScriptTM RT reagent Kit intending to execute reverse transcription optimized for RNA to cDNA conversion (O'Connell, 2002).

The RT-PCR reaction volume was expected to be 25 µl (23.5 µl and 1.5 µl as template). All PCR components were vortexed together and centrifuged quickly to remove any remaining liquid from the tubes' sides. A new reaction was made in ice, gently mixed and vortexed for 4 seconds,

and then centrifuged shortly. A programmed thermal cycler was used to transfer the RT-PCR tubes (Eppendorf, USA, O'Connell, 2002).

Using the commercially available kit Maxime TM PCR PreMix Kit (i-Taq^{TM}), the following RT-PCR condition was used to amplify the IBV S1 gene in a single-tube experiment with a final reaction volume of 25µl. As previously stated by the company of Integrated DNA Technologies firm, Canada, the oligonucleotide primers were utilized in the detection of IBV (Jones et al., 2005). Two pairs of primers were used which included (SX1 and SX2) and (SX3 and SX4). The first PCR amplification (SX1 and SX2) and the second nested PCR (SX3 and SX4) were used to generate a copy DNA of 393 base paris region of S1 gene (Table 1).

Amplification was performed using a 35-cycles protocol that included denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes (O'Connell, 2002).

The amplicons were electrophoresed on a 1.5% (w/v) agarose gel in 1XTBE buffer, stained with Red safe Nucleic acid staining, and flanked by a 100 bp ladder as a molecular weight marker purchased from Intron (Korea) following the study conducted by Jones et al. (2005).

Statistical analysis

Statistical analysis was run in SPSS program version 24. The Chi-square test was chosen to indicate the differences between the samples. The significant differences were indicated by the Duncan test at the level of $p \leq 0.05$ (Steel and Torrie, 1980).

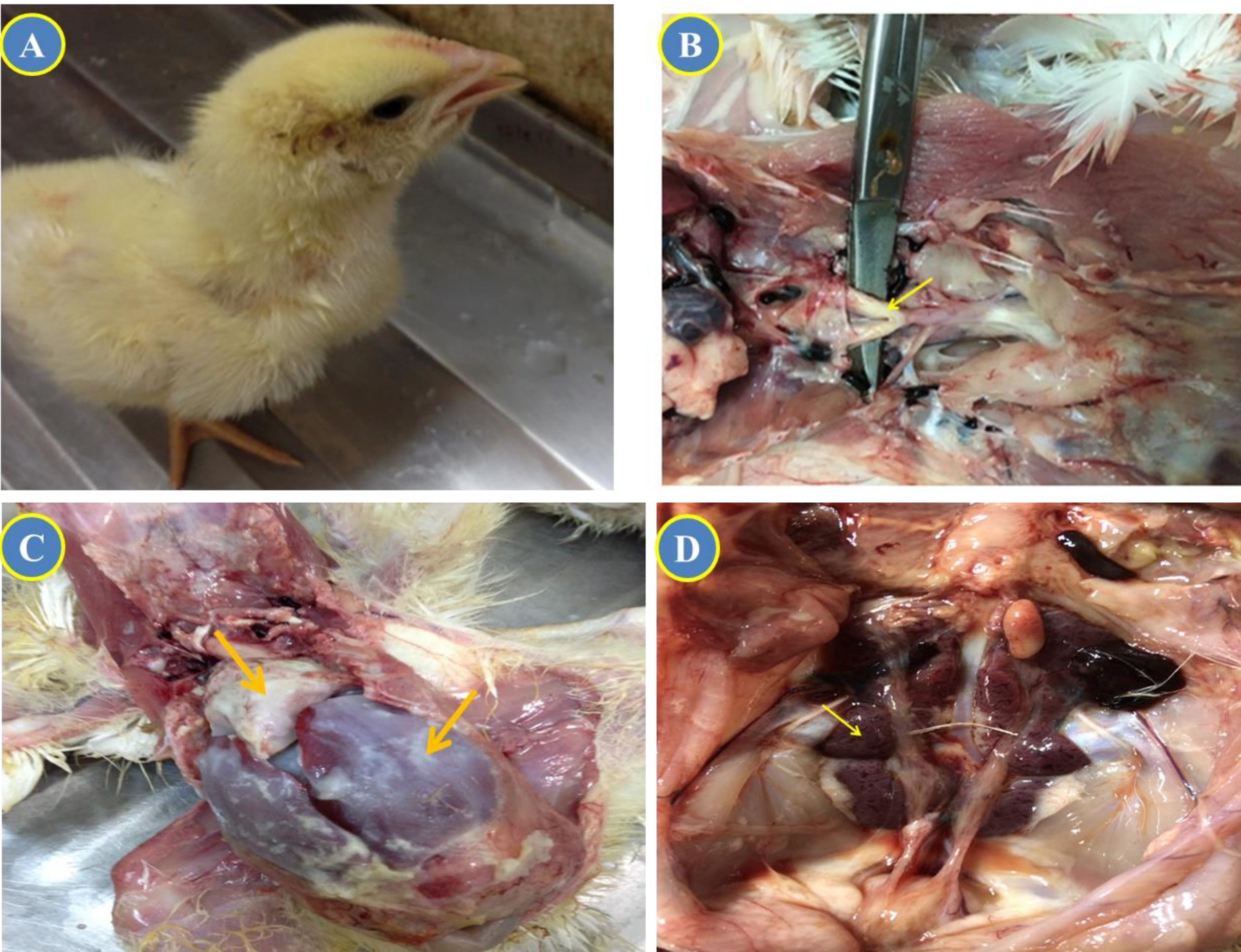

Figure 1. Broiler chickens infected with infectious bronchitis virus showed dyspnea with abdominal breathing (gasping, **A**), tracheal bifurcation showed completed obstruction with fibrinonecrotic cast (arrow, **B**), Pericarditis and perihepatitis (arrow) due to co-infections across with IBV (**C**), and swollen kidneys with distended tubules (**D**).

Table 1. Oligonucleotide primers used in the detection of IBV as previously described

Primer Name	Sequence(5′-3`)	Company	Product Size (bp)
SX1F SX2R	5'-TCCACCTCTATAAACACCYTTAC - 3′ 5'-TCCACCTCTATAAACACCYTTAC - 3′	Integrated DNA Technologies Canada).	393bp
SX3F SX4R	5'-TAATACTGGYAATTTTTCAGATGG - 3′ 5'-AATACAGATTGCTTACAACCACC - 3′	Integrated DNA Technologies Canada).	393bp

RESULTS

ELISA test results

ELISA was performed using serum diluent at a single dilution (1:50). All tests included both positive and negative controls. The ELISA test was used to detect specific antibodies to the IBV infection.

The interpretation of ELISA results was done according to the manufacturer's instruction of the kit supplied information (ProFlok TM IBV, Zoetis, USA). The results of IBV titer represented a comparison between IBV antibody level of field serum samples and the positive samples of control sera. In case of the Coefficient of Variance (CV) is greater than 30%, it means a very early exposure of the flock to IBV or the flocks have a bimodal population or a non-uniform flock. According to the manufacturer guidelines of the kit, chickens with ELISA titers below the detectable level throughout the testing period were considered negative (titer < 600, OD = 0.529, and S/P ratio = 0.150). In all sampled fields, sera were collected for detection of the antibodies twice (days 16 and 23 of age).

The antibody titers of chickens aged 16 days in Baqoubah, Kanaan, Baladroze, Almoqdadia, Alkhalis, and Alwajehia groups were found to be 1044.2778 ± 249.95382, 761.4444 ± 182.60843, 2380.3889 ± 317.56748, 1190.8333 ± 200.12109 1373.4444 ± 183.23802 and 728.6111 ± 101.57859 respectively.

In comparison to the day 16, field samples had considerably greater levels of anti-IBV IgG ELISA titer on 23 days of age, the mean titers (11488.153 ± 2376.1111 for Baqubah, 14008.657 ± 3103.2312 for Kanan, 12527.342 ± 6164.3434 for Baladroze, 15709.5563 ± 3589.3356 for Almoqdadia, 11328.6732 ± 3436.5900 for Alkhalis, and 13744.6754 ± 1305.6578 for Alwajehia) showed significant differences among different farms ($p \leq 0.05$) and revealed that all flocks were considered infected according to the instructions of the manual kit of ELISA in six different regions of Diyala Governorate as showed in (Table 2 and figures 2 and 3).

Table 2. Titers of antibodies of IBV (n= 10) at different ages and different regions of Diyala Governorate, Iraq, in broiler chickens (Mean ± standard error)

Regions	16 days	23 days
Baqubah	1044.2778 ± 249.95382 $^{BC\ b}$	11488.153 ± 2376.1111 $^{C\ a}$
Kanaan	761.4444 ± 182.60843 $^{C\ b}$	14008.657 ± 3103.2312 $^{B\ a}$
Baladroze	2380.3889 ± 317.56748 $^{A\ b}$	12527.342 ± 6164.3434 $^{C\ a}$
Almoqdadia	1190.8333 ± 200.12109 $^{BC\ b}$	15709.5563 ± 3589.3356 $^{A\ a}$
Alkhalis	1373.4444 ± 183.23802 $^{B\ b}$	11328.6732 ± 3436.5900 $^{C\ a}$
Alwajehia	728.6111 ± 101.57859 $^{C\ b}$	13744.6754 ± 1305.6578 $^{B\ a}$

^{abc}: Different superscripts in a row means significant ($p \leq 0.05$). ^{ABC}: Different superscripts in a column mean significant ($p \leq 0.05$).

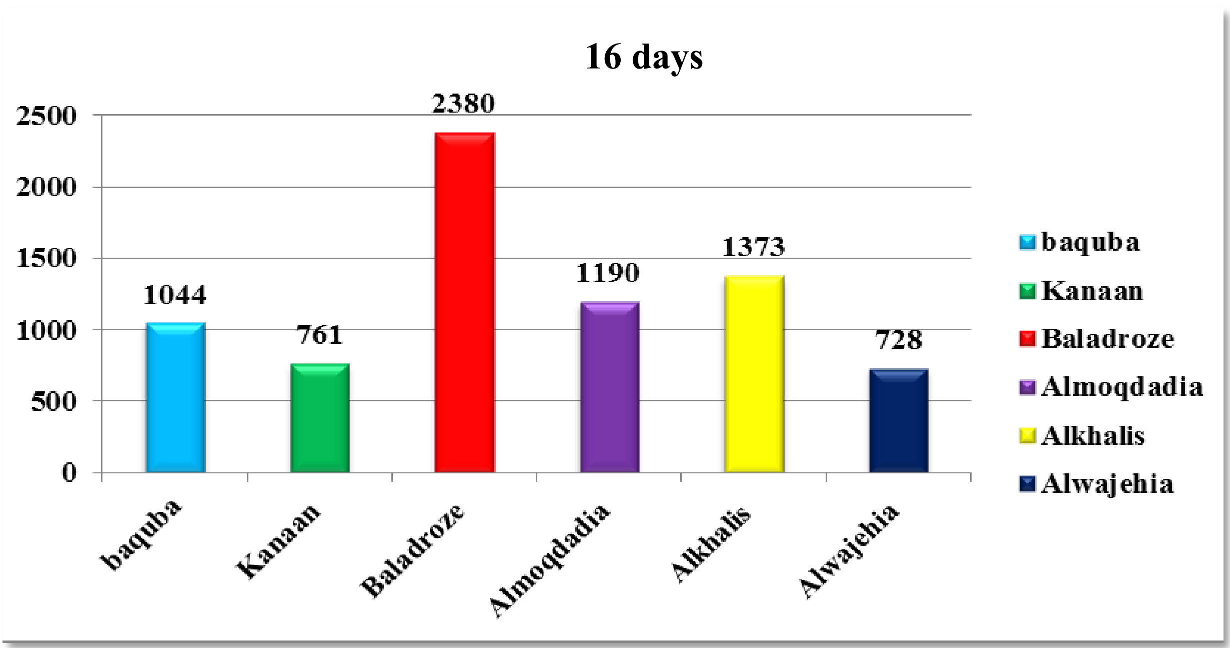

Figure 2. Anti-IBV rate by age groups among infected broiler chickens aged 16 days old in different regions of Diyala Governorate, Iraq

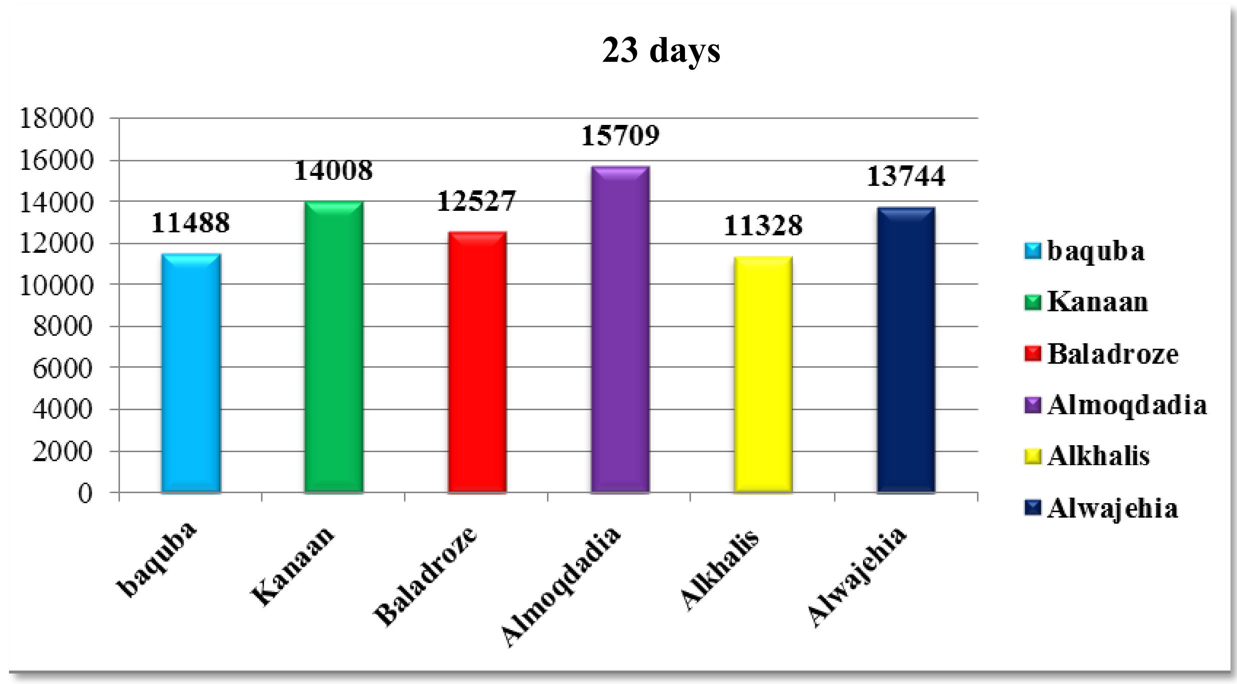

Figure 3. Anti-IBV rate by age group among infected broiler chickens aged 23 days old in different regions of Diyala Governorate, Iraq

Molecular detection of infectious bronchitis virus from tissue samples using RT- PCR

Kidney, liver, trachea, and lung were among the samples collected for molecular analysis. As the samples were subjected to RT-PCR, IBV was found in 25 out of the 30 samples (83%). A 393bp DNA band was produced in positive samples (Figures 4 and 5).

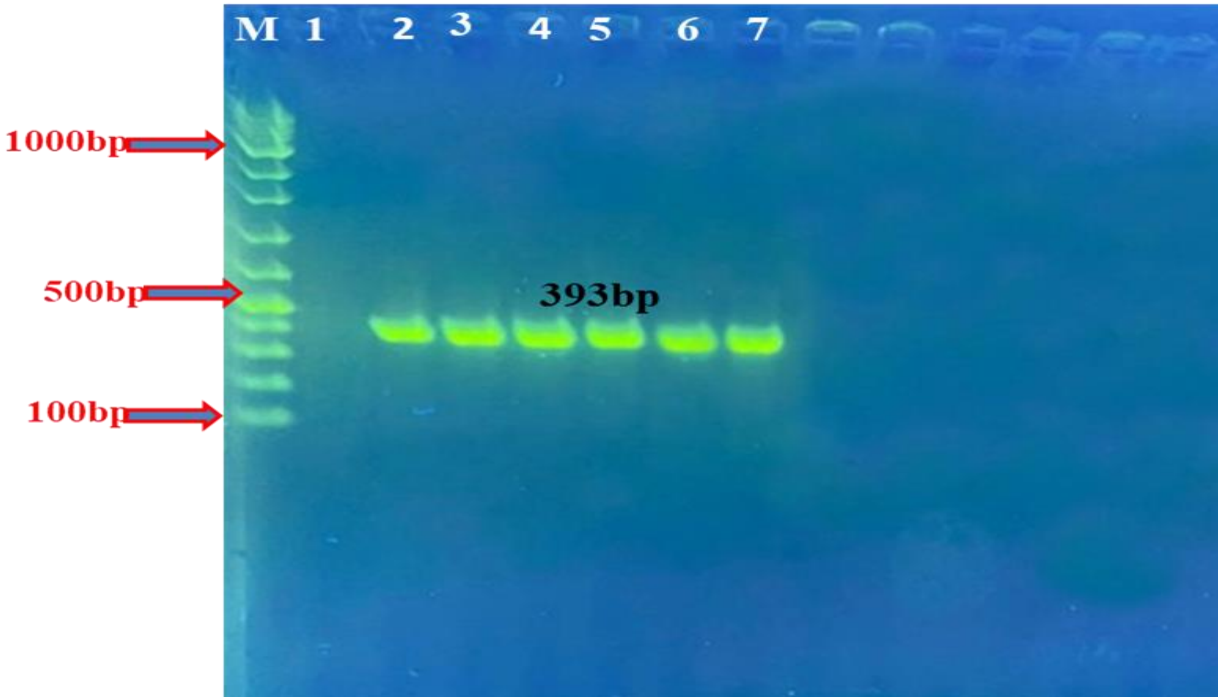

Figure 4. Amplicons of RT-PCR technique for detection infectious bronchitis virus utilizing specific primer pairs. The S1 region of IBV is detected using a pair of primers (SX1 and SX2, SX3 and SX4), which results in a 393-bp PCR product. Electrophoresis was performed on a 1.5% agarose gel. Lane 1 (NTC): Non-template control, Lane 2: Positive control, Lane 3-5: Positive samples, M: 100 bp DNA molecular weight marker.

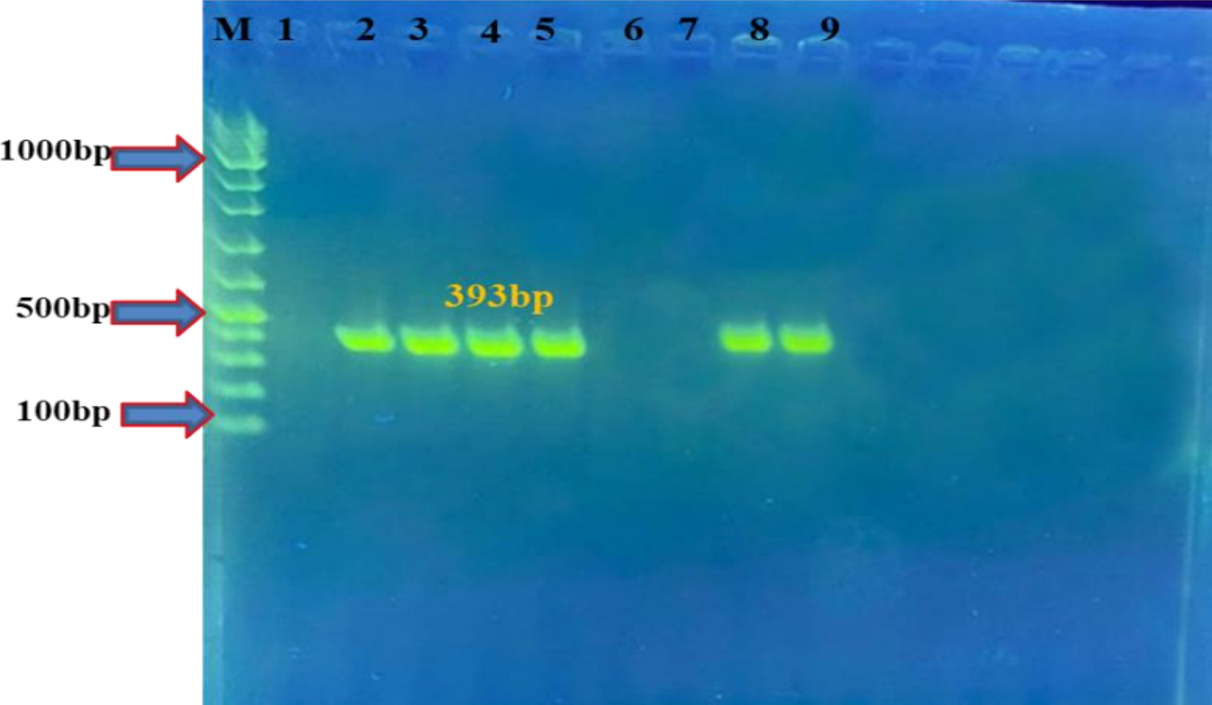

Figure 5. Amplicons of RT-PCR technique for detection infectious bronchitis virus utilizing specific primer pairs. The S1 region of IBV is detected using a pair of primers (SX1 and SX2, SX3, and SX4), which results in a 393-bp PCR product. Electrophoresis was performed on a 1.5 agarose gel. Lane 1 (NTC): Non-template control, Lane 2: Positive control, Lanes 3, 4, 5, 8, 9: Positive samples (IBV), Lane 6, 7: Negative samples. M: 100 bp DNA molecular weight marker.

DISCUSSION

Infectious bronchitis virus is a highly infectious viral disease that affects chicken respiratory and urogenital systems (Cavanagh and Naqi, 2003). The virus has also tissue tropism to kidneys as well as the respiratory and reproductive systems. The IBV is reported all over the world, and it has various varieties with different genomes (Cavanagh, 2007).

Anti-IBV-specific IgG antibodies were identified using commercially available tests (ProFLOK1IBV PLUS ELISA antibody test kit, Synbiotics Zoetis, USA). Based on the OD values, anti-IBV antibody titers were estimated from samples taken from 6 groups (18 chickens in each group) from different farms in Diyala Governorate. The present study showed a high elevation of anti-IBV IgG antibody levels in these flocks (Table 2).

Since the immune system is not well-formed in young chickens, they are susceptible to numerous types of infections (bacterial and viral causes) during the first few days of life (Grindstaff et al., 2003).

In the first eight days following infection, death rates in these groups were low. However, the death rate began to rise after this period. It is possible that once the maternally produced IgG is catabolized, the chicks become more vulnerable (De Wit et al., 2011).

Despite the fact that all flocks were vaccinated (Nobilis® IB4/91vaccine, Netherlands), all six groups of broiler chickens in the current study showed a high level of IgG antibody at 23 days of age. This could be attributed to infection with IBV according to the manufacturer instructions of ELISA kit (Intervet Company, Netherlands) in six different regions of Diyala Governorate. All the investigated flocks were immunized using various vaccination techniques.

Depending on the technique of immunization used by breeders, the vaccine was given through an ocular route, coarse spray, or drinking water but the IBV infection occurred regardless of the vaccination programs. These findings matched those of Bhuiyan et al. (2021), who found that IBV is an acute multiple-system infection in commercial chicken farms because of multiple serotypes that do not cross-protect and so the vaccination cannot cover all environmental serotypes.

The failure of vaccination programs to protect chickens against IBV is due to the inability of chickens to mount a sufficient immune response following inoculation (Bosha and Nongo, 2012). The possibility of long-term immunity, the selection of most virulent serotypes, and the scheduling of applications according to flocks requiring revaccination are all connected with appropriate IBV vaccines. Farm owners blame the ineffectiveness of vaccines to immunize their flocks. More than half of vaccination failures in vaccinated flocks were found to be related to poor vaccine application. Furthermore, the increased risk of vaccine delivery, as well as vaccine maintenance and storage quality, are critical factors for vaccine failure leading to IBV outbreaks in immunized farms (Ganguly et al., 2010; Bosha and Nongo, 2012; Boelm, 2018).

The Nobilis® IB 4 / 91 vaccine (Intervet Company, Boxmeer-Holland) was given to all broiler farms in this investigation although it did not appear to provide complete protection against IBV. This might be due to vaccination failures or the existence of genetic changes that allowed the circulating virus (emerging virus) to evade antibodies generated against the IBV vaccine strain. Furthermore, according to Cavanagh and Naqi (2003), regular vaccination with many IBV strains has been associated with many environmental risk factors that have different influences on a successful vaccination.

The findings of this study also contradicted those of Bhuiyan et al. (2021), who found that IBV exists as multiple serotypes that do not cross-protect, making it extremely difficult to control since there are only a few types of IBV vaccines available for prevention. These findings were also in line with those of Mahmood et al. (2011), and Seger et al. (2016), who expressed that the characterization of IBV has resulted in new issues in terms of epidemiology and control. The IB infections with various varieties in genomes have been discovered around the world in recent decades (Cavanagh, 2007).

Detection of infectious bronchitis virus from tissue samples using RT-PCR

The infectious bronchitis virus's S (spike) gene-encoded protein was used to connect up with the host receptor. The immunization status of the chicken industry may be connected to the biological variety of the viral S gene (Alazawy, 2013; Fraga et al., 2013; Umar et al., 2016). S1 gene sequencing has been an alternative method for distinguishing vaccination from field viruses in recent years (Jones et al., 2011). This (S1) region appears to be preserved in each geographically separated virus, making it useful for viral genotyping. It is the most likely serotype-specific determinant of IBV and contains an antigenic epitope that is serologically significant for IBV serotyping (Mahmood et al., 2011). Due to their sensitivity

and short reaction time, molecular assays are now the most widely used method for IBV detection.

Aside from viral RNA detection, they enable genetic characterization of discovered strains, correct design and evaluation of vaccination programs, and assessment of the existence of specific field strains (Legnardi et al., 2020).

By RT-PCR, 25 out of 30 samples (83%) were positive for IBV. A 393bp DNA band was produced in positive samples. In the current study, there was a high rate of detection (83%), compared to a study by Setiawaty et al. (2019), who found that 26 of 47 (55.3%) samples had a positive result for the S1 gene of IBV using RT-PCR. The detection percentage of IBV by RT-PCR in the current study was close to the findings of Seger et al. (2016) in Iraq, who reported positive samples of IBV by RT-PCR in broiler chickens. The AlNajaf governorate has the highest prevalence of IBV in broiler chickens at 10/20 (50%), and the Al-Muthana governorate has the lowest rate at 4/20 (20%).

CONCLUSION

Infectious bronchitis virus infection was endemic in commercial broiler farms of Diyala Governorate regardless of the control program using commercial vaccines. It seems the maternal antibodies and vaccination did not protect chickens against IBV infection, which might indicate the virus circulation in industrial farms in the study area. Therefore, it is recommended to conduct more molecular studies for local detection of the viruses and the preparation of IBV vaccine from local isolates.

DECLARATIONS

Acknowledgments

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Authors' contribution

Karrar Awni Jasim, Amer Khazaal Al-Azawy, and Karim Sadun Al-Ajeeli proposed the hypothesis, designed the study, and conducted the serological and molecular works. Karrar Awni Jasim and Talib J.Kadhim collected samples from poultry farms. All authors contributed to manuscript preparation and approved the final manuscript.

Ethical considerations

Ethical issues (including plagiarism, consent to publication, misconduct, data fabrication and/or forgery, double publication and/or submission and replication). Exactly done by all authors.

Competing interests

The authors declare that they have no competing interests.

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