Republic of Iraq
Ministry of Higher Education
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قال الله تعالى (و لقد اتينا داوود و سليمان علما و
قالا الحمدلله الذي فضلنا على كثير من عباده
المؤمنين)

Clinical and molecular detection of infectious bronchitis virus in Basrah 2021

By

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الاهداء

الى من شجعني على المثابرة طوال عمري ، الى الرجل الابرز في حياتي
(والدي العزيز)
الى من بها اعلو ، و عليها ارتكز ، الى القلب المعطاء
(والدتي الحبيبة)
الى من بذلوا جهدا في مساعدتي و كانوا خير سند
(اخواني و اخواتي)
الى اسرتي الى اصدقائي و زملائي .....
الى كل من ساهم و لو بحرف في حياتي الدراسية .....
الى كل هؤلاء : اهدي هذا العمل، الذي أسال الله تعالى ان يتقبله خالصا ....
و شكرا خاص لدكتور و مشرف البحث وليد المياحي الذي اتعب معنا على اعطاء هذة معلومات الثمينة

INTRODUCTION

Infectious bronchitis (IB) is an acute and highly contagious viral disease of chicken caused by the infectious bronchitis virus (IBV). This disease is prevalent throughout the world and affects the performance of both meat-type and egg-laying birds, thereby causing severe economic loss within the poultry industry. **IB** can affect chickens of all ages and result in respiratory disease, nephritis, proventriculitis, and decrease in egg production and egg quality **[Cavanagh ,2007 & Han et al.,2011].** Furthermore, this disease is often complicated by secondary bacterial infections that cause increased mortality. It may be the most economically important viral respiratory tract disease of chickens in countries and regions where there is no highly pathogenic avian influenza virus or velogenic (highly pathogenic) Newcastle disease virus. (Cavanagh ,.(2005 infectious bronchitis caused by a coronavirus, it is a positive sense-single stranded RNA genome virus **(Ababneh et al., 2012)** of the family coronaviridae of the Nidovirales. The infectious bronchitis disease virus placed in group 3 coronaviruses. It is a positive-stranded RNA virus, with a genome of about 27 kb containing 5' and 3' untranslated regions **(UTRs)** with a poly (A) **tail(BOURSNELL ET) AL., 1987** There are many serotypes of the IBV and new serotypes are still being discovered. For example, in the 1991 there was an increase in the outbreak and incidence of Infectious bronchitis in the U.K. as reported by **(Gough et al., 1992)**, which was attributed to a new more virulent strain **(Gough et al., 1996).** The infectious bronchitis spread quickly because of these there is possibility of all the flock becoming infected in a short time. Mortalities in chicks may reach 30- 60% (organicvet.co.uk), but production loss as a result of infectious bronchitis infection are said to be greater than those of mortalities **(Cavanaugh and Nagi,1997).**

Aims of the study :

1_ Diagnosis of IBV clinically

2_ Molecular detection of IBV

3_ Study the prvention programm of the fields .

IBV in Iraq

IB still causes serious problems in the Iraqi poultry industry due to the inability of vaccines to provide cross-protection between different genotypes. Due to the limited network of poultry diagnostic laboratories in Iraq, differential diagnosis is can only be done based on clinical signs and gross lesions. The characterization of IBV has raised additional problems in terms of both epidemiology and control. Although IBV on the poultry farms in Iraq (with H120 and 4/91 strains) is presently controlled by inactivated and live attenuated vaccines, outbreaks of IB have nevertheless been observed on broiler farms **(Al-Dabhawe *et al., 2013 ; Mahmood et al.,2011)*** . *First study concerning with IBV in Iraq , was by Azab et al., who diagnosed Infectious* bronchitis in 2- to 5-week-old broiler chickens in Iraq. The disease was characterized by respiratory symptoms and high mortality. Their isolate AM 88, was obtained from the trachea and lungs of infected birds. Those isolate produced curling, dwarfing and about 10% deaths in infected chick embryos after 3 passages, where physico-chemical characteristics of the isolate revealed that it did not agglutinate avian or ovine erythrocytes (1989).

Another study which included isolation of (IBV) from broilers chicks; the virus isolation done by samples inoculation in chicken egg embryos via allontoic cavity route which showed sensitivity for virus isolation resulting in stunting and rounding of inoculated embryos, as well as thickening of chorioallantoic membrane (CAM). In the latter study, cultivation of the isolated virus was carried out in the yolk sac and embryo vein .The virus was grown in chicken kidney cells (CKC) cultures resulting in cytopathic effect (CPE) after 24 hour post inoculation including swelling and accumulation of cells which increased in 2nd& 3rd day post inoculation. Their diagnosis of isolated virus carried out by Agar gel precipitation test (AGPT) using reference positive antiserum and Serum neutralization test (SNT) using CKC tissue culture **(Al-Haseerchy and Al-Attar ,2007)** .

A molecular study was conducted to detect Infectious bronchitis virus in broilers chicken farms in Diwaniya province. Tracheal swab from 30 infected chicken flocks located in different areas of Diwaniya province were collected to make Rapid immunochromatography test for IBV by using Antigen Rapid IBV Test Kit , and tissue samples were collected from flocks which showed positive for rapid test to make rRT-PCR. Twenty eight (93.33%) flocks were positive for IBV by rapid immunochromatography test, while the molecular detection of IBV

showed that all flocks (100%) were positive for IBV(Abbas and Nafea ,2013).Until the 2013 , the molecular detection of IBV in middle and south of Iraq was performed by (Al-Dabhawe *et al) , who reported the prevalence of IBV broiler chicks in middle of Iraq with Real time PCR . In other hands, Diagnosis of Infectious Bronchitis virus in broiler chickens by Serological test and RT-PCR in Duhok region-Iraq was done by (Dyar et al.,2012) .*

Infectious bronchitis virus (IBV) was isolated from trachea and kidney tissues of eight broiler farms in Kurdistan region of North Iraq from 2008 to 2010, Sequence analysis and BLAST homology search in GenBank data base indicated that two of the farms were infected with the 4/91 strain, , one with an unidentified IBV and five were infected with Sul/01/09 (Mahmood *et al., 2011) . The genotype, QX IBV, was detected in Erbil City –Kurdistan Iraq by (Amin et al., 2012) .*

IBV in Middle East

In Israel, Mass was the only type detected for many years until the 793B type of IBV (Israel/793B/variant 1/96) was identified in 1996 (Meir *et al., 1998.)Two years later a new virus Israel/variant 2/98 was characterized (Callison et al., 2001). Other variant viruses have also been characterized in Israel including Israel/IS720/720/99 and Israel/IS720/885/00 both belonging to the IS720 type and IS/1494 (Meir et al., 2004.) The IS720 type virus was also identified in Iraq (Iraq/IS720/Sul/01/09) and Egypt (Egypt/IS720/Beni-Seuf/01) (Abdel-Moneim et al., 2006). Some of these genotypes in particular IS/885/00 and IS/1494/06, have become dominant in the majority of farms in the Middle East countries, causing respiratory and renal diseases (Awad et al., 2014)*

IS/1494/06 IBV genotype was isolated in Turkey as TR_IS1494_06_B12011 and TR_1494_06_7201 IBV (Serpil *et al., 2013) .Other viruses in Egypt include Mass (Egypt/Mass/F/03) and D274 (Egypt/D274/D/89) and a recent report found QX-like IBV in Zimbabwe (Toffan et al., 2011). In Iran, the 793B type was reported (Shapouri et al., 2004),also in Iran There was detection of six distinct phylogenetic groups (IS/1494/06 [Var2] like, 4/91-like, IS/720-like, QX-like, IR-1 and Mass-like) that were related to variants isolated in the region(Najafi et al.,2014). In addition, Mass was reported in Iran (Cavanagh et al., 2005). And in Iran, IRFIBV6 and IRFIBV9, TC07-1*

, the Iraq/Sul/01/09 strain belonging to the IS720 type was characterized (Mahmood *et al., 2011).*

The Mass and 793B types were the most common IBV types reported in India since 1991 (Elankumaran *et al.,1999). Around the year 2000, including India/PDRC/Pune/9/99. Variant IBVs have been recognized in Israel since at least the mid-1990s (Cook et al., 1996; Callison, et al., 2001) on the basis of both virus neutralization tests and molecular techniques, and protection studies have shown that Mass vaccines provide inadequate protection against some of these novel variants. In Jordan, the use of RT-PCR enabled European IBV variants D274 and 4/ 91 to be detected (Roussan et al., 2008), but since the primers used were designed to detect only these specific variants, it is possible that others are present in that country. Similar methods have been used to identify 4/91 in Iran (Seyfi Abad et al., 2004) .*

Materials and Methods :

Sample collections :

The samples were collected from infected broilers in Qarmat Ali district from 3 farms with clinical signs related to IB as gasping , nasal discharge , Face swelling . About 40 samples included kidney and trachea were taken from such farms and subjected to laboratory test .

1- Homogenization

Tracheal and kidney samples which were collected from dead or scarified suspected dead bird . The samples were taken into sterile pestle and homogenized with the help of sterile scissors and mortar and added phosphate buffered saline (PBS) (20%) to make a suspension and the suspension were clarified by speed (1000 rpm for 10 minutes) centrifugation. The suspension was treated with penicillin (1000 IU/ml of suspension) and a broad spectrum antibiotic Gentamycin (500 µm per ml).

2- RNA extraction

1. Transter 150 µl, Homogenized fluid, cell-free fluid or virus Infection tissue or cell in the 1.5ml microcenterfuge tube.

Note: If sample Volume is less than 150 µl, sample should be adjusted to 150 µl with DEPC treated water.

2.Add 250 µl of Lysis buffer

Note: if the sample Volume is larger than 150 µl ,increase the amount of Lysis buffer(e.g a 300 µl sample will requine 500 µl Lysis buffer) and if the Lysis buffer become solid, include in 80°C for 10min.

3.Mix by vortexing for 15sec .

4.Incubate at room temperature (15_25°C)for 10min.

5.Add 350 µl of Binding buffer, and completely mix well by gently vortexing

Note: If the sample Volume is more than 150 µl, increase the amount of Binding buffer (e. g.. a300 µl sample will require 700 µl of Binding buffer) This step is conductive efficient passage of cell lysates through a column and to increase binding onto column resins and important for effective deproteinization.

6.place a spin column in a provided 2ml collection tube.

7. Load lysates on the column and centrifuge at 13,000rpm for 1min

Note : The maximum Volume of the column reservolrs 800 µl. for sample Volume of more than 800 µl, samply load and spin again. if the solution has not completely passed through the membrane ,centrifuge again at higher speed until all of the solution passed through.

8. Discard solution in collection tube and place the column back in the same 2ml collection tube.

9. Add 500 µl of washing buffer A to column and centrifuge for 1min at 13,000rpm.

10. Discard solution in collection tube and place the spin column back in the same 2ml collection tube.

11. Add 500 µl of washing buffer B to the column and centrifuge for 1min at 13,000rpm.

12. Discard solution in collection tube and place the spin column back in the same 2ml collection tube. centrifuge for 1min at 13,000rpm.

Note: it is important to dry the membrane since residual ethanol may interfers wirh downstream reactions.

13. Place the column in a RNase-free 1.5ml microcenterfuge tube (not provided),and add 30-60?l of Eluion buffer directly onto the membrane.

14. Incubation at RT for 1min, and then centrifuge for 1min at 13,000rpm.

15. Use 2-5 µl of eluted solution for PCR or RT-PCR .

One step qPCR

Table 1. General Thermocycler Program.

Stage	# of Cycles	Program in Standard or Fast Mode
1. Reverse Transcription	1	≥37°C for 15 minutes
2. RT inactivation/Hot-start activation	1	95°C for 10 minutes
3. 3-Step qPCR:		
a. Denature	40	95°C for 10 seconds
b. Anneal/Collect Data		60°C for 30 seconds
c. Extend		72°C for 30 seconds
4. Dissociation	1	60-95°C

The following instructions describe the preparation of 20µl or 50µl reactions . Prepare TaqR 1-Step RT-qPCR Reaction Mix as a single batch that includes the common components, such as TaqR 1-Step RT-qPCR Master Mix, CXR dye, nuclease-free water and RT Mix. Divide the batch into individual volumes then add the remaining components (Table 2).Determine the number of control and experimental reactions in the assay. Make a sufficient volume of reaction mix to provide for ≥3 replicates of each reaction, plus a 10% volume excess. Prepare new reaction mix for each experiment.

1. Thaw the components of the GoTaqR 1-Step RT-qPCR System, the RNA

templates and the primer pair on ice, at room temperature or at 37°C.

2. Visually inspect to ensure that thawing is complete. Immediately mix each thawed component solution thoroughly. Swirl using a vortex mixer at **low speed to minimize aeration. Keep the thawed reagents on ice.** Protect all reagents that include GoTaqR qPCR Master Mix from light.

3. Combine the component volumes in a nonstick sterile tube, on ice (Table 2). After each addition, mix the combinations gently and thoroughly, by drawing up and down in the pipette tip. If using a vortex mixer, use low Speed

Table 2. GoTaq® 1-Step RT-qPCR Reaction Mix.

Component	Volume per 20µl Reaction	Volume per 50µl Reaction	Final Concentration in Reaction
GoTaq® qPCR Master Mix, 2X	10µl	25µl	1X
Forward Primer, 10X	2µl	5µl	50–300nM
Reverse Primer, 10X	2µl	5µl	50–300nM
GoScript™ RT Mix for 1-Step RT-qPCR, 50X **or** Nuclease-Free Water for Minus-RT control	0.4µl	1.0µl	1X
RNA Template (500fg–100ng) (or Nuclease-free Water for No-Template Control)	4µl	10µl	variable
Optional: Supplemental $MgCl_2$, 25mM*	__µl	__µl	≥2mM
Optional: Supplemental CXR Reference Dye, 30µM**	__µl	__µl	≥33nM**
Nuclease-Free Water	**to 20µl**	**to 50µl**	–

*Add to supplement the $MgCl_2$ provided in Master Mix

Guidelines for addition of CXR Reference Dye (30µM) to the reaction mix to achieve a final concentration of 0.5µM:

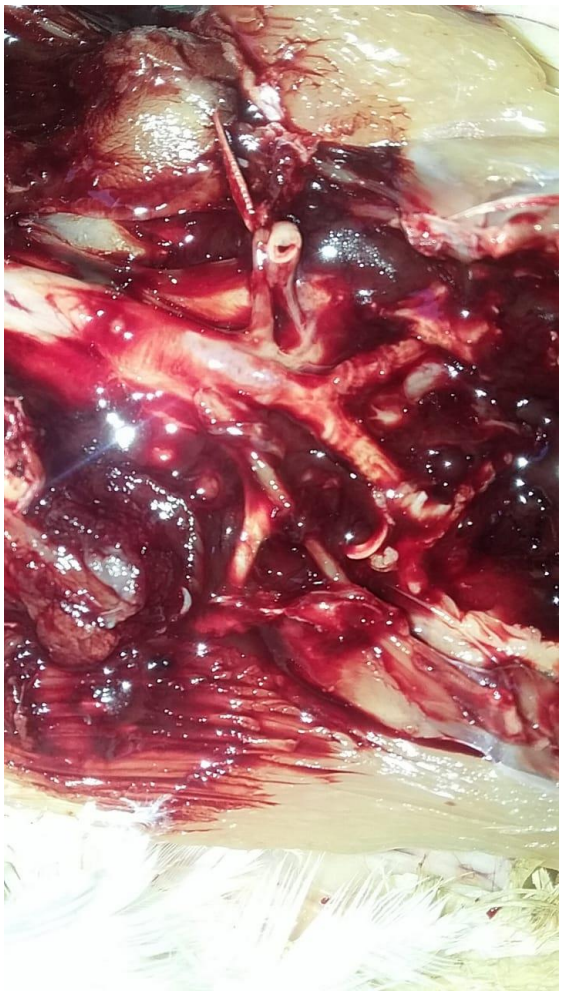
31µl per 100-reaction batch for 20µl reactions, **or**
78µl per 100-reaction batch for 50µl reactions.

4. Carefully distribute the reaction volumes to the wells of the reaction plate on ice, using caution to avoid cross contamination.
5. Cover the wells with an optical plate seal.
6. Centrifuge the plates for up to 1 minute at room temperature to collect the reaction volumes and eliminate residual air bubbles from the well contents.
7. Keep the reactions chilled on ice or in a cold block whenever possible.
Protect the reactions from light. Keep the plate on ice during transfer to the instrument.
8. Place the reaction plate into the prepared instrument (prewarmed and
programmed). Start the run immediately.

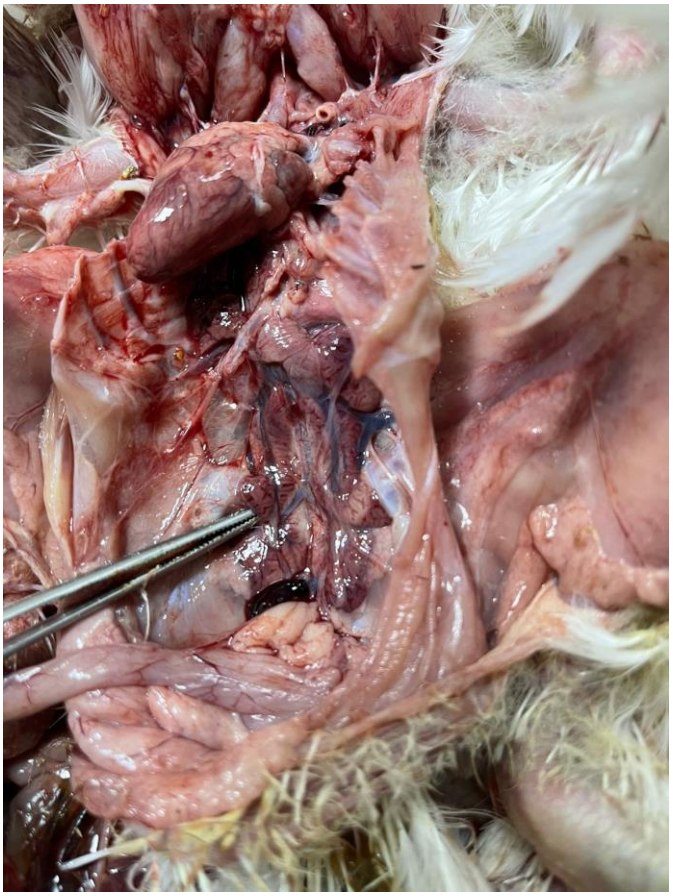
Results :

Clinical finding :

The clinical results included gasping , coughing , nasal ocular discharge , rales while the postmortem lesions , trachritis , caseous plug in trachea bifurcation , kidney swelling , urate depisit , and enteritis .



Caseous plug in tracheal bifurcation enlargement



Nephritis , Kidney

Assay Information

Assay Name	Assay Version	Lot Number	Expiration Date	Assay Status	Assay Type
IBV One Step RT-PCR 5'-UTR 45	NA			Valid	Research

* indicates that a particular field is entered using a barcode scanner

Sample Results

Site ID	Sample ID	Assay Result	Warning/ Error Code	Sample Type	Notes
A1	1	Positive		SPEC	
A2	2	Positive		SPEC	
A3	3	Positive		SPEC	
A4	4	Positive		SPEC	
A5	5	Negative		SPEC	
A6	6	Positive		SPEC	
A7	7	Positive		SPEC	
A8	9	Negative		SPEC	
A9	10	Positive		SPEC	
A10	11	Negative		SPEC	
A11	12	Negative		SPEC	
A12	13	Positive		SPEC	

Number of Sites: 12

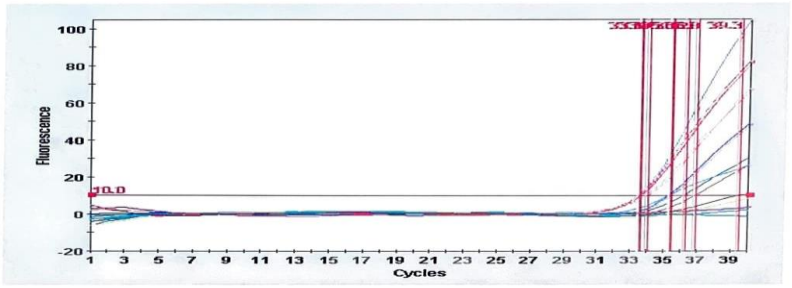
Valued Smart Cycler Dx Customer 14/10/2021 13:01:09

Run Report

IBV

Site ID	Sample ID	Channel Result	Ct	EndPt
A1	1	POS	35.3	49
A2	2	POS	33.4	105
A3	3	POS	33.9	68
A4	4	POS	36.2	30
A5	5	NEG	0	8
A6	6	POS	36.8	26
A7	7	POS	33.5	83
A8	9	NEG	0	2
A9	10	POS	35.3	26
A10	11	NEG	0	-1
A11	12	NEG	0	3
A12	13	POS	39.3	11

Run Date: 14/10/2021 09:21:08



Site Legend

Site		Assay	Sample ID
A1		IBV One Step RT -PCR 5'-UTR 45	1
A2		IBV One Step RT -PCR 5'-UTR 45	2
A3		IBV One Step RT -PCR 5'-UTR 45	3
A4		IBV One Step RT -PCR 5'-UTR 45	4
A5		IBV One Step RT -PCR 5'-UTR 45	5
A6		IBV One Step RT -PCR 5'-UTR 45	6
A7		IBV One Step RT -PCR 5'-UTR 45	7
A8		IBV One Step	9

Data Type	Line Type
Primary Curve	

Channel	Symbol
FAM	None

Site Legend

Site		Assay	Sample ID
A8		RT-PCR 5'-UTR 45	
A9		IBV One Step RT -PCR 5'-UTR 45	10
A10		IBV One Step RT -PCR 5'-UTR 45	11
A11		IBV One Step RT -PCR 5'-UTR 45	12
A12		IBV One Step RT -PCR 5'-UTR 45	13

Discussion :

Viral respiratory diseases are common causes of economic losses in poultry industry (Dekich .,1998). IBV causes reduction of growth rate and production, high rate of; mortality , prevention and treatment costs (Cavanagh .,2007). Rapid detection of causative viruses can play an important role in controlling of these viruses (31). IB viruses are the viruses that frequently affect the respiratory tract of chickens. All IBV types can be isolated from upper respiratory tract, with highest concentration found in the trachea during 3 to 5 days post-infection. After this period, virus titer fall quickly in the second week, below the detection levels (De Wit, 2000).the present study showed presence of IBV in samples that collected from suspected farms with real time PCR .this results were I line with that of (callison et al., 2006 , Jackwood et al.,2009) who diagnosed IB by using UTR gene detecting real time PCR .The studied area characterized by intensive broiler farms and random rearing without veterinary supervision . In the other hand the histopathological changes were excessive vacuolation mostly in sub capsular renal tubules , aggregation of inflammatory cells in sub capsular renal tubules and show hemorrhage and inflammatory cell in mucosal and sub mucosal layers of trachea and there is discharge in tracheal lumen .the present results were similar to (Gola et al., 2017) who mentioned trachea of affected birds on histopathological examination revealed deciliation, desquamation of epithelial cells and serous exudation. Trachea of some birds showed haemorrhage. Kidney revealed tubular necrosis, hyaline degeneration, desquamation of epithelial cells, congestion, infiltration of inflammatory cell and deposition of urate crystal surrounded by inflammatory cell .

Conclusions and Recommendations

- Our study have shown that qpcr very sensitive technequie to detect IBV in the field and there are clinucal signs and postmortem lesions which pathognomonic to IB in broulers
- 2.sequence may be need to detect circulating strains in the field in yhe future study

References

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