

Molecular Survey and Phylogenic Analysis of Infectious Bronchitis Virus (IBV) Circulating among Chicken Flocks in Riyadh Province, Saudi Arabia

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Abstract: Infectious bronchitis is a disease of an economical importance of chickens. As result of emerging of new variant strains, the prevention and control of the disease is difficult. The study is the first to be conducted in Saudi Arabia. Results showed that there is a wide-spread of IBV amongst chicken farms in the Riyadh Province. Using RT-PCR, viral RNA was detected in 35 (42.68%) out of 82 poultry farm houses from 42 farms investigated in this study. The highest viral detection (55.55%) was recorded in Shaqra governate while the lowest (20%) was in the Northern governates (AL-Zolfi, AL-Dwadmi and Hremla). Detection of IBV in vaccinated chickens was evident that some of the vaccines used in the field did not provide the adequate protection against the disease. This investigation showed that viral detection was successfully done in 29 (35.36%) out of 82 farm houses from 42 farms using the conventional method of sample collection while the detection rate dropped to 21.95% (18 farms) when Flinders Technology Associates (FTA) card were used. Interestingly enough, viral detection was found positive in 6 samples using the FTA card but was negative using the conventional method. For further identification and phylogeny, the amplified PCR products of 2 isolates were sequenced and analyzed for phylogenetic analysis and compared with other avialable IBV strains from the GenBank. First isolate was designed as IBV/CHICKEN/KSA/101/2010)with accession No. HQ916668 was found to share high nucleotide sequence identity (98.9%) for Nucleocapsid (N) protein genes with IBV/CH/LDL/011 sequence; a strain from China which may be revertant attenuated vaccine strains that have arisen as a result of the widespread use of IBV vaccines in the local poultry population in China. The second isolate was designed as IBV/CHICKENS/KSA/102/2010 with accession number HQ916669 was found to share a high degree of identity (99.7 and 99.5%) with the IBV/INDIA/TN/92/03 and IBV/HB/CH isolated in India and China, respectively. The phylogenetic analysis for the isolates were found highly diverse from the same sequences of IBV/H120 strain that commonly used as commercial vaccine for prevention of IBV in poultry flocks in KSA.

Key words: Prevention, vaccines, detecion, chicken, disease

INTRODUCTION

Infectious bronchitis is one of the most economically significant diseases of intensive poultry production. The disease firstly reported in 1931 and defined as a highly contagious upper respiratory disease (Schalk and Hawn, 1931). The name of the disease refers to its most frequent clinical manifestation, although, it can infect many other epithelial cells including kidney, genital organs and many part of the alimentary tract (Cavanagh, 2005). Infectious bronchitis virus is the etiological agent of this condition and fall in genus *Coronavirus* of family Coronaviridae

contains a single stranded positive sense RNA of 27.6 kb (Murphy *et al.*, 1999). The genome consists of four structural proteins, namely a spike glycoprotein (S) an integral membrane glycoprotein (M), a nucleocapsid protein (N) and a small membrane envelope protein (E) (Cavanagh, 1981; Sutou *et al.*, 1988). More than fifty serotypes of IBV have been identified and new variants continue to emerge despite the use of different live attenuated and/or killed IBV (Ignjatovic *et al.*, 1997). It is very important to identify the field strains of IBV in circulation in order to select suitable vaccine strains for use in different geographical regions (Liu *et al.*, 2006).

Traditional methods for the identification of IBV serotypes include hemagglutination-inhibition and virus neutralization tests both of these procedures are labor-intensive and time consuming. Since the early 1990s, Reverse Transcription Polymerase Chain Reaction (RT-PCR) technology has been used successfully to N protein located in the capsid of the virion is involved in RNA replication and carries group-specific antigenic determinates (Krapez *et al.*, 2009). Nucleotide sequencing of *N* and *S1* genes fragment is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories. Comparison and analysis of sequences of unknown field isolates and variant with reference strains for establishing potential relatedness are significant advantages of sequencing (De Wit, 2000; OIE, 2008; Zulperi *et al.*, 2009). RT-PCR have been applied either directly to RNA extracted from clinical samples or to eluted RNA from FTA cards. Flinders Technology Associates cards (FTA) are cotton-based cellulose membrane containing lyophilized chemicals that lyses many types of bacteria and viruses, several recent publications report the use of FTA® cards as an convenient, adequate, reliable and safe method for storing, transporting and extracting nucleic acids from inactivated infectious organisms with no interference in molecular reactions (Ndunguru *et al.*, 2005; Moscoso *et al.*, 2005; Purvis *et al.*, 2006; Rogers and Burgoyne, 2000). In Saudi Arabia, there is very few case reports, there are little research work on the disease in the country. Nucleotide identity between isolates from Saudia Arabia, Iran and European isolates was 95% as reported (Cavanagh *et al.*, 2005). Two isolates from Iran and one from Saudi Arabia isolated in 2000 had been characterized as being of the 793/B serotype by the

virus neutralization test (Cavanagh, 2005). The general oligonucleotides XCE-1 and XCE2+ (Capua *et al.*, 1999; Cavanagh *et al.*, 1999) corresponding to nucleotides 709-1166 in the *S1* gene of the 793/B serotype were used to amplify part of *S1* of the Iranian and Saudi Arabia isolates. Thus, the Iranian and Saudi Arabia isolates were clearly of the 793/B type by sequence as well as by serotype (Cavanagh *et al.*, 2005). IBV 793/B type detected in swabs collected in 1997 and 1998 from Saudi Arabia, Japan, Sweden, Denmark, Poland, Italy, France and Argentina (Roussan *et al.*, 2008; Asasi *et al.*, 2003). The aims of this research are to performing the first constructed molecular research study on the presence of IB in Saudi Arabia through a survey study for direct detection of IBV RNA in tracheal swabs collected from commercial chicken farms in Riyadh governorate using frozen PBS and/or FTA Cards Methods. To perform further strain identification and phylogeny, the nucleotide sequences of the amplified *N* gene PCR products were determined and comparative analysis was performed to estimate the phylogenetic relationship of the virus.

MATERIALS AND METHODS

Study area: Samples were collected from 82 poultry house representing forty three poultry farms (16.6% of total broilers and layers poultry farms) in Riyadh Province. Samples details from different localities are summarized in Table 1.

Samples collection and preparation: Samples (tracheal and cloacal swabs) were collected between October 2009 and May 2010. Five birds from each poultry house (total number of samples were 930) were randomly

Table 1: Distribution of the collected samples according to localities and farm type

Locality	Farms distribution		Total	Investigated farms		No. of samples
	B	L		B	L	
Al-Kharj	35	26	61	4	4	220
Al-Majmua	39	3	42	7	1	155
Shaqra	21	1	22	5	-	60
Thadeg	29	2	31	3	1	75
Wadi Al-Dwaser	8	1	9	1	1	-
Al-Zolfi	-	2	2	-	2	35
Al-Aflaj	9	-	9	2	1	45
Al-Dwadmi	4	3	7	-	1	30
Al-Riyadh	3	3	6	-	1	60
Thorma	4	9	13	-	2	65
Al-Mozahmia	14	3	17	3	-	80
Al-Goaeaia	7	3	10	2	-	65
Hremla	9	3	12	1	1	40
Affif	9	-	9	-	-	-
Al-Oiaena	7	-	7	-	-	-
Al-Ghat	2	-	2	-	-	-
Total	200	59	259	28	15	930

B: Broiler; L: Layer

Table 2: IBV *N* gene nucleotide sequences used in this study

Accession No.	Virus ID	Country	Serotype	References
AY790350	IBV/K210-02	S. Korea	Arkansas	Park <i>et al.</i> (2005)
DQ287910	IBV/CK/CH/LDL/98I	China		Unpublished
DQ352148	iBV/CK/CH/LDL/01I	China		Unpublished
EF602445	IBV/CK/CH/LDL/97I	China		Unpublished
HQ916668	IBV/Chicken/KSA/2010/101	KSA		Unpublished
GQ229232	IBV/3382/06	Taiwan		Chen <i>et al.</i> (2010)
FJ888351	IBV/H120	Netherlands	H120	Zhang <i>et al.</i> (2010)
AY514485	IBV/California 99	USA	California99	Mondal and Cardona (2004)
FN430414	IBV/ITA/90254/2005	USA		Unpublished
EU418975	IBV/Arkansas/DPI	USA		Ammayappan <i>et al.</i> (2009)
GQ504724	IBV/Massachusetts	USA		Unpublished
GU391029	IBV/Jin-13	China		Unpublished
AY363968	IBV/JP9758	Japan		Shieh <i>et al.</i> (2004)
GQ149079	IBV/ZJ981	China		Unpublished
AY121093	IBV/SAIBwj	China		Unpublished
M28566	IBV/Mass41	-		Bournnell <i>et al.</i> (1985)
EF602457	IBV/CK/CH/LSD/03I	China		Unpublished
EF185916	IBV/Ind/TN/92/03	India		Unpublished
HQ916669	IBV/Chickens/KSA/102/2010	KSA		Unpublished
DQ473615	IBV/HB/CH	China		Unpublished
DQ659366	IBV/Brasil/TII/1975	Brasil		Unpublished
M28565	IBV/Beaudette	-		Bournnell <i>et al.</i> (1985)
AY319651	IBV/BJ	China		Unpublished
EU362619	IBV/SD0611	China		Unpublished
EU362625	IBV/SD0708	China		Unpublished
HQ014604	IBV/Ma5	China		Unpublished
HM230752	IBV/CK/CH/SD09/002	China		Unpublished

selected and tracheal swabs were taken using sterile cotton-tipped swabs then soaked in 1 mL Phosphate Buffer Saline (PBS) (SIGMA®) pH 7.2 with antibiotic mixture prepared according to Burlison *et al.* (1997).

Another swabs from the same birds were imprinted over the FTA cards (Whatman® company) stored at room temperature till being used. As an alternative method for inactivation, transportation and preservation to be analyzed by Reverse-Transcriptase PCR (RT-PCR) and sequenced for the molecular characterization.

RNA extraction and cDNA synthesis: RNA extracted using Qiagen RNA Easy Protect Mini kit (QIAGEN®, Germany) according to manufacturing instructions. cDNA was synthesis in 20 µL reaction using transcription first strand cDNA Synthesis kit (Roche®, USA) according to the manufactures procedures.

Polymerase chain reaction: The primers were selected to ensure a wide detection range of IBVs according to Handberg *et al.* (1999) the universal oligonucleotide primer pairs: IBVN (+) [GAAGAAAACCCAGTCCCAGAT GCTTGG] and IBVN (-) [GTTGGAATAGTGCGCTTGCA ATACCG] manufactured by TIB-MOL Biol syntheslabor Gmb H Berlin, Germany.

Amplification was done in 50 µL reaction using the following PCR profile 95°C for 3 min for initial denaturation then 35 cycles of 95°C for 30 sec (denaturation) 60°C for 1 min (Annealing) and 68°C for

3 min (Extension). Then, final extension at 68°C for 7 min. The PCR products were analyzed on 1.0% agarose gel.

Sequence analysis: The amplified PCR complimentary to the *N* gene products were send for direct sequencing in King Faisal Specialist Hospital and Research Center. The sequences alignment and compared to those registered in GenBank (Table 2) using MEGALIGN Program in DNASTar Laser gene 8 Software package.

RESULTS AND DISCUSSION

Molecular detection of IBV using RT-PCR: Out of 82 tested poultry house in 43 poultry farms, 29 (35.36%) were shown to be positive for the detection of IBV nucleic acid using the ordinary method of sample preparation. On the other hand, only 18 (21.95%) house are shown to be positive using FTA Card Method (Table 3). Some clinical signs similar to those previously reported associated with IB infection were observed in 15.9% of poultry houses.

Phylogenetic analysis: Identification of homologies between nucleotide and amino acid sequences of the obtained Saudi IBV and other IBVs strains published on Gen-Bank was done using BLAST 2.0 and PSI-BLAST Search Programs (National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>), Clustal W multiple alignments of sequenced viruses and reference IBV *N* gene nucleotide sequences were carried

Table 3: Detection of IBV RNA from the whole Riyadh Province using by RT-PCR using Conventional (C) and FTA Card Preparation Method

Regions	No. of farm houses tested	C		FTA cards		Total positive farm houses	
		+ve	%	+ve	%	No.	%
Al-Kharj	15	8	53.33	6	40.00	8	53.33
Al-Majmaua	15	4	26.66	5	33.33	7	46.66
Shaqra	9	3	33.33	4	44.44	5	55.55
Thadeg	7	3	42.85	0	0.00	3	42.85
Northern region	10	2	20.00	1	10.00	2	20.00
Southern region (Makkah Rd)	18	7	38.88	2	11.11	8	44.44
Southern region	8	2	25.00	0	0.00	2	25.00
Total	82	29	35.36	18	21.95	35	42.68

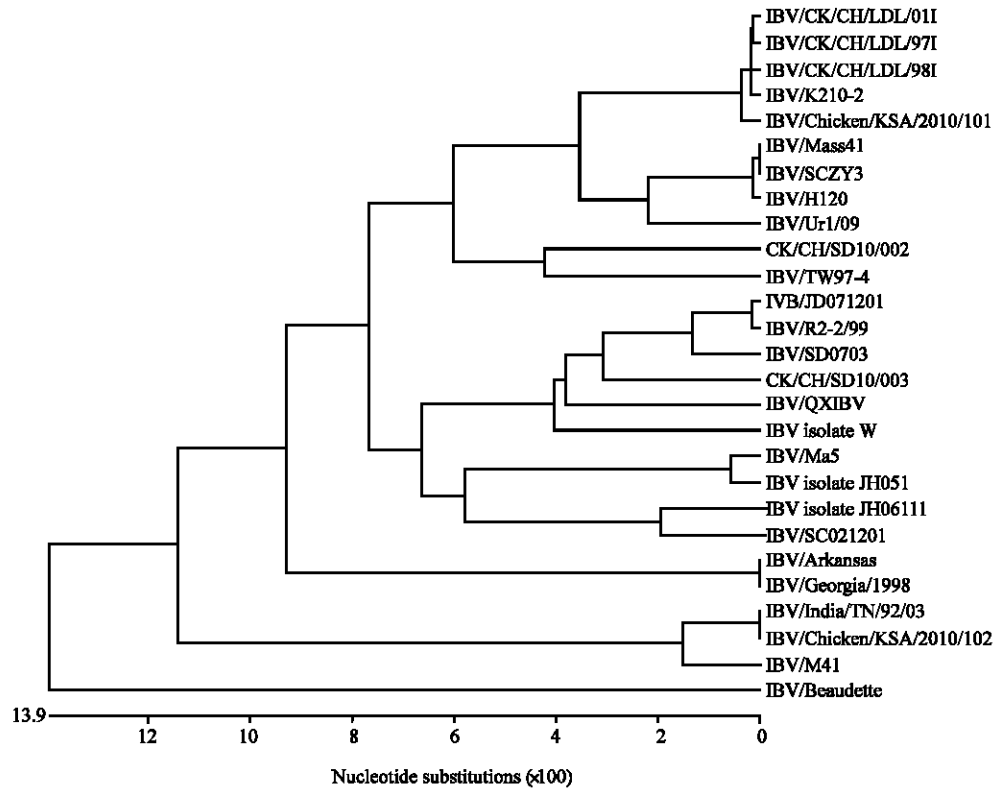


Fig. 1: Phylogenetic analysis of IBV strains IBV/Chickens/KSA/101/2010 and IBV/Chickens/KSA/102/2010

out. The results showed that multiple nucleotide substitutions were observed along the length of the studied *N* gene. The sequence identity matrix developed for the sequences and reference IBV *N* gene nucleotide sequences showed identity percentages 98.9% between IBV/Chickens/KSA/101/2010, IBV/CH/CK/LDL/011 and IBV/CH/CK/LDL/981. The identity percentage between IBV/Chickens/KSA/102/2010 and IBV/India/TN/92/03 was 99.7% while the identity percentage between the sequences was 89.5%. On other hand, the percentage of identity between the sequences (IBV/Chickens/KSA/101/2010 and IBV/Chickens/KSA/102/2010) and IBV/H120 were 94.1 and 91.6%, respectively. Phylogenetic analysis clustered the IBV/Chickens/KSA/101/2010 with four IBV isolated from China while IBV/Chickens/KSA/102/2010

clustered with IBV strain isolated from India and another one isolated from China (Fig. 1 and Table 2).

Infectious bronchitis is an acute, highly contagious viral infection of chickens of all ages worldwide with adverse effects on growth and egg quality and egg production in the laying birds. It was surprising that a huge country such as Saudi Arabia with a large poultry industry has little or in fact no structured published scientific research on IB disease. Results of this study showed that 42.68% of the chickens tested were positive for IBV. This is quite high and indicates the wide spread of IBV in the Riyadh Province. However, vaccine strains may interfere with the obtained findings; virus detection accompanied with clinical signs was observed in 15.9% of tested farms. This may be due to the outdoor survival of

the virus (Ahmed *et al.*, 2007). This result is comparable to that obtained from Pakistan, 57.3% in commercial poultry farms (Ahmed *et al.*, 2007). In United Kingdom, 85% among commercial broiler flocks in the East and North of England were positive (Cavanagh *et al.*, 1999). The 72% of tested flock were IBV positive in Iran in an epidemiological study carried out by Shoushtari *et al.* (2008). However, low detection rates were also reported such as in China (16.6%) (Zhu *et al.*, 2007).

The prevalence of IBV among different localities varied from 20-55.55%. The highest percentage was reported in Shaqra and the lowest was in Northern region (Al-Zolfi, Al-Dwadmi and Hremla). This may be due the persistence of IBV infection, intermittent shedding of IBV and risk of flock to flock transmission via contamination of personnel or equipment (Cook *et al.*, 1987). The high prevalence of IBV even in vaccinated poultry flocks may be due to inappropriate vaccination, vaccination strain or rapid emergence of new strains (Smati *et al.*, 2002; Sharawi and Zidan, 2011) as well as environmental factors such as heat, mycotoxins and others that can induce significant stress on birds have the potential to make chickens immune-suppressed thereby increasing the chances of viral infections (Neldon-Ortiz and Qureshi, 1991; Qureshi and Hagler, 1992).

The FTA card ability to inactivate several avian pathogens has been studied previously (Perozo *et al.*, 2006; Purvis *et al.*, 2006). The results showed a lower detection of IBV in FTA card (21.95%) might be due to the presence of non-specific inhibitors of the Taq DNA polymerase in FTA card which could not be completely removed by RNA Extraction Method (Sharawi and Zidan, 2011) or some detrimental effects of FTA inactivation on the viral RNA as previously reported for other chemical inactivated samples (Masuda *et al.*, 1999). Lower sensitivity of the RT-PCR identification of IBV in stored FTA cards was reported to be possible after 43 days was explained as a consequence of RNA denaturation (Rogers and Burgoyne, 2000; Moscoso *et al.*, 2005).

Phylogenetic analysis from the 2 pools of samples collected from diseased birds showed that both IBVs sequences detected by this study were genetically diverse. The obtained nucleotide sequences IBV/CHICKENS/KSA/101/2010 shared high nucleotide sequence identity (98.9%) for Nucleocapsid (N) protein genes with IBV/CH/LDL/01I sequence a strain from China which may be revertant attenuated vaccine strains that have arisen as a result of the widespread use of IBV vaccines in the local poultry population in China (Zhang *et al.*, 2005). The another nucleotide sequence IBV/CHICKENS/KSA/102/2010 sharing a high degree of identity (99.7 and 99.5%) with the IBV/INDIA/TN/92/03

and IBV/HB/CH, respectively. Results of the phylogenetic analysis for the IBVs circulating were highly diverse from the same sequences of IBV/H120 strain that commonly used as commercial vaccine for prevention of IBV in poultry flocks in KSA.

The detected IBV strains in this study were identical to most IBV strains isolated in different parts of China. It is possible that several species of birds mostly waders are known to migrate between parts of Africa and their breeding grounds in Siberia through communal stopover grounds in the African Rift Valley, the Middle East and central Asia where they are likely to come into contact with birds from the far East and that may be explained the source of the identified IBV in current research. Anyhow in the same aforementioned results; the possibility of widespread dissemination of IBV by wild birds such as wild migratory ducks. In addition, IBV has been isolated from domestic peafowl (*Pavo cristatus*), partridge (*Alectoris* sp.), guinea fowl (*Numida meleagris*), teal (*Anas* sp.) (Liu *et al.*, 2005), racing pigeons (Barr *et al.*, 1988) Turkeys, pigeons and parrots (Sharawi and Zidan, 2011).

CONCLUSION

The present study proves the prevalence of IBV variants in Saudi Arabia poultry. The phylogenetic analysis of the 2 IBV in this study make the ability of the existing used vaccines to contain the field strains questionable and a raise the demand for a new vaccines a national control preventive strategy to minimize the economic losses results from IBV infection in different types of chickens.

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