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# Molecular characteristics of VP2 gene from wild-type infectious bursal disease viruses (IBDVs) in Saudi Arabia

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## *Abstract*

Infectious bursal disease (IBD) is caused by a virus related to the family Birnaviridae. The virus is a worldwide pathogen and causes immunosuppression and great losses in broiler flocks. In this study, molecular characterization of two Saudi Arabian IBDVs (IBD01/14/SA and IBD02/14/SA) was carried out by reverse transcriptase polymerase chain reaction (RT-PCR) and partial sequence of hypervariable region of VP2 gene. Nucleotide sequences of 740-bp PCR product of the Saudi Arabian isolates were compared with the corresponding nucleotide sequence of 56 reported IBDVs representing different pathotypes from different continents. Phylogenetic analysis showed that the Saudi Arabian isolates were split from the tree to form a distinguished cluster with the Canadian IBDVs and 586/USA field viruses. Deduced amino sequences from both strains were found to share the amino acid substitutions of the standard very virulent (vv) strains. The Saudi Arabian strains showed unique amino acid substitutions 254N and 359K and exhibited a modified antigenic profile. The deduced amino acid sequence and antigenic profile of both wild-type Saudi Arabian strains showed high similarity to the IBDI+ vaccines; hence, the Saudi Arabian strains might be more useful for protecting chickens against overt clinical signs and mortalities.

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**Keywords:** classical, IBDV, phylogenetic analysis, Saudi Arabia, sequencing, variant

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## Introduction

Gumboro disease (GD) is an immunosuppressive disease of chickens, even in subclinical infection, that infects lymphocytes in the bursa of Fabricius. Infectious bursal disease virus (IBDV) is the etiological agent of GD. Two distinct serotypes of IBDV are known to exist. Serotype 1 virus causes clinical disease in young chickens (3-6 weeks old), whereas serotype 2 is non-pathogenic (Lasher & Shane, 1994). The disease was first reported in Gumboro, Delaware in 1962. It was successfully controlled using either live and/or inactivated vaccines for many years. Some emerged IBDVs that could not be controlled by serotype 1 classic vaccines were called "variants" (Jackwood & Saif, 1985). This may be due to mutations in the neutralizing epitopes of their VP2, which help them to escape from the immunity stimulated by classical IBDVs (Giambrone & Closser, 1990 and Vakharia et al., 1994). Consequently, infections caused by serotype 1 IBDVs result in a variety of clinical pictures according to their pathotype and virulence; field IBDVs are often identified as classic, variant or very virulent viruses (van den Berg, 2000). IBDVs have been found in a naturally wide range of pathogenicity varied from low pathogenic or attenuated to the very virulent viruses (Sharma et al., 2000). The common impact of all IBDVs is the potential to cause immunosuppression that may result in great losses for broiler and layer industries. The pathognomonic lesions and damages to the bursa of Fabricius are lymphocytic depletion, inflammation, hemorrhage as well as decrease in bursa-body weight ratio (Boot et al., 2000; Le Nouen et al., 2006; Toro et al., 2009). The immune suppression caused by IBDV may be unnoticed until opportunistic bacteria cause diseases like gangrenous dermatitis (Rosenberger et al., 1975) or flock vaccination failure (Giambrone et al., 1977). Although vaccination is routinely used for disease prevention, GD continues to be one of the most important poultry diseases and the virus can still strike chicken flocks even they are vaccinated (Jackwood and Sommer-Wagner, 2007).

IBDV is a member of the genus Avibirnavirus of the family Birnaviridae (Etteradossi and Saif, 2008). IBDV is a non-enveloped bi-segment ds-RNA genome which consists of segments A and B. The large segment A encodes three structural virion proteins: VP2, VP3, and VP4, as well as a non-structural protein, VP5. The small segment B encodes the structural protein VP1. The VP2 protein contains the most important neutralizing antigenic sites and is known as the major host protective immunogen of IBDV. Moreover, most of the amino acid (AA) changes between antigenically different IBDVs are clustered in the hypervariable region of VP2 (Morgan et al., 1988; Levin et al., 1999; Lombardo et al., 2000; Wu et al., 2007). Recently, genomic sequencing and phylogenetic groups based on VP2 have been developed to define the very virulent infectious bursal disease virus (vvIBDVs) (Jackwood and Sommer-Wagner, 2007; Wu et al., 2007; Jackwood and Sommer-Wagner, 2011; Jenberie et al., 2014).

In Saudi Arabia (SA), GD-related problems may be severe, as the number of submissions to our

Veterinary Teaching Hospital Laboratory has been increasing. These flocks characteristically had a history of health and production problems with or without apparent clinical signs or they had concurrent infections such as chronic respiratory disease, pneumonia, enteritis, and coccidiosis even they were vaccinated. Therefore, the use of a proper vaccine strain is important for effective protection, and the differentiation and identification of circulating local IBDV isolates are critical for the selection of an appropriate vaccine strain.

This study aimed to expand the epidemiological understanding of IBDVs in Saudi Arabia and determine the presence of variant strains in Saudi Arabia. In addition, molecular relatedness of the circulating local field isolates with different IBDV vaccine strains was determined for selection of appropriate vaccine.

## Materials and Methods

**Samples:** Five hundred, 4- to 5-week-old broiler chickens were examined, representing 10 poultry flocks from geographically separated poultry farms located in the Eastern region of Saudi Arabia, from January to September 2014. The birds showed mortality ranging from 15 to 18% and one or more of the following signs: depression, weakness, whitish diarrhea and vent picking. The birds were subjected to routine postmortem examination at the Avian Clinic, Veterinary Teaching Hospital (VTH), King Faisal University. Bursa of Fabricius, spleen, and kidneys were collected aseptically, processed and stored at -80°C until used.

**RNA extraction:** Total RNA was extracted from 140 µl of tissue homogenate using QIAamp Viral RNA Extraction Kit® (QIAGEN, USA). After lysis of the specimens, the mixture was transferred to a spin column according to the manufacturer's protocol. The extracted RNAs were eluted in 60-µl elution buffer and stored at -80°C until used.

**RT-PCR for detection of VP2 gene:** A set of primers were used for detection and sequencing of the VP2 gene of the DMSO denaturated IBDV RNA (Ojkic et al., 2007). Forward primer 5-GCC CAG AGT CTA CAC CAT AAC-3 and Reverse primer 5-CCC GGA TTA TGT CTT TGA AG-3 (Ojkic et al., 2007) were used to amplify 740 bp from the hypervariable region of VP2. The PCR reaction (25 µl) contained 6 µl of RNase free water, 5 µl of 5X Qiagen one-step RT-PCR buffer, 5 µl of Q buffer, 1 µl of dNTPs mix, 1 µl (50 pmol) of each primer and 1 µl of the enzyme mix. The optimized cycling condition was as follows: 1 cycle at 50°C for 30 min (RT reaction), followed by one cycle at 95°C for 15 min (for RT inactivation and hot start activation), then 35 cycles of denaturation (at 94°C for 30 sec), annealing (at 60°C for 30 sec) and elongation (at 72°C for 45 sec). The RT-PCR products were separated on 1% agarose gel and visualized by 0.5 µg/ml of ethidium bromide staining under UV light.

**Sequencing and construction of phylogenetic tree:** Approximately 740-bp PCR specific band of VP2 gene

was excised from the agarose gel, purified using Montage DNA Gel Extraction Kit® (Millipore, USA) and sequenced in an automated ABI 3730 DNA sequencer (Applied Biosystems, USA). The obtained sequences were aligned by the ClustalW method and compared with IBDV sequences available in Genbank by the BLAST web tool of Genbank. A phylogenetic tree was constructed using MEGA version 5.20 software. The obtained sequences were submitted to Genbank under accession numbers: IBD01/14/SA (KU640387) and IBD02/14/SA (KU640388).

**Antigenic Index:** Probable antigenic sites of deduced amino acid sequence were calculated considering surface probability using the Protean™ software (Dnastar® Inc., Madison, WI) based on formulations described by Jameson and Wolf (1988).

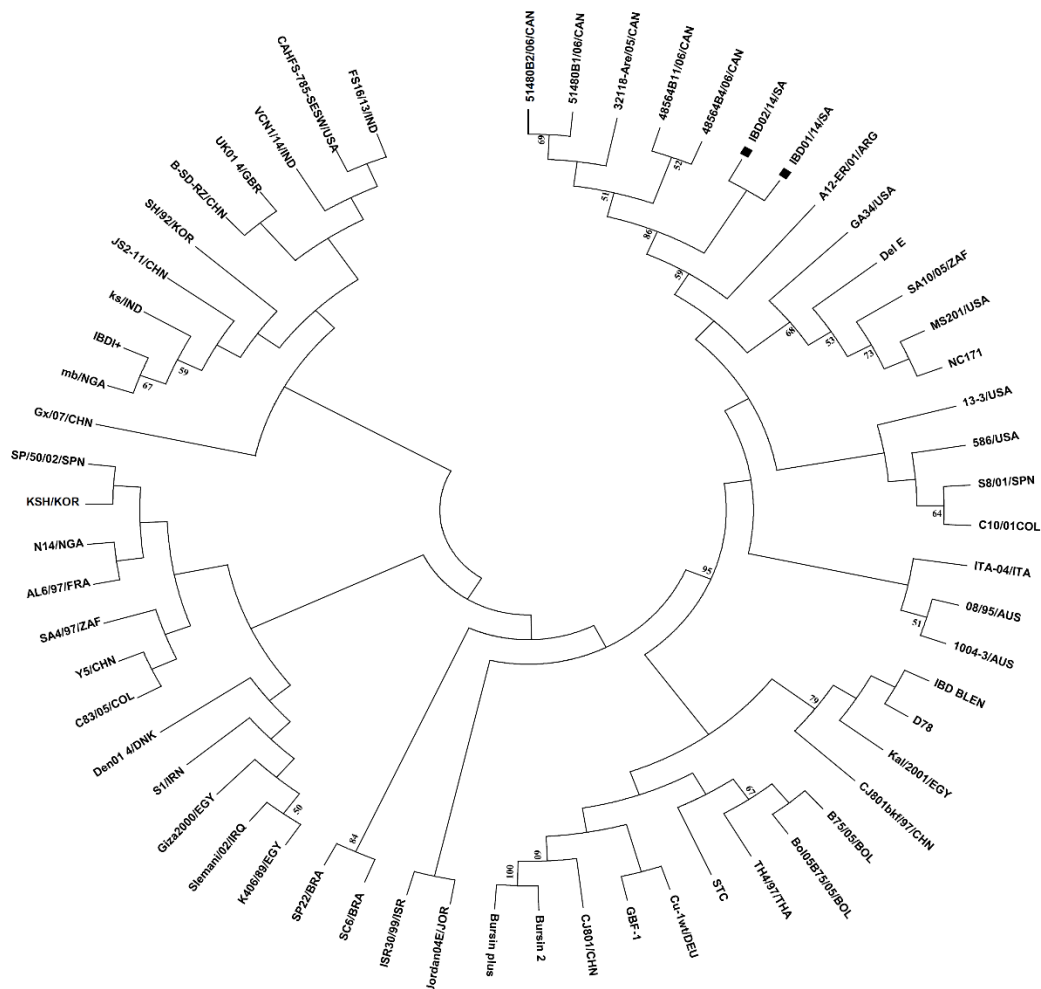
### Results

**Clinical examination:** Clinical examination and necropsy of the dead birds were carried out. The macroscopic lesions observed in bursa of diseases or sacrificed birds were enlarged edematous or hemorrhagic bursa with yellowish gelatinous exudate. In addition, hemorrhagic strikes on pectoral and/or

thigh muscles as well as enlarged spleen were observed in some birds.

**Molecular characterization of IBDV field isolates:** The 740 bp of VP2 gene was efficiently amplified from two farms out of the ten tested farms in the Eastern region of Saudi Arabia. The amplified region corresponded to the hypervariable region in VP2. The predicted nucleotide sequence was compared with other IBDV VP2 genes published in Genbank.

The phylogenetic analysis of both Saudi Arabian strains exhibited the highest identity of 97 to 98.5% with the Canadian vvIBDV strains (Fig. 1). In addition, both viruses showed different degrees of identity with IBDVs isolated from geographically related countries. The average identity percentages were 96, 94.5, 81, 94 and 89% with IBDVs from Iraq, Israel, Iran, Jordan and Egypt, respectively. Moreover, the Saudi Arabian IBDVs and Canadian strains showed the highest identity (96.8%) with the 568/US IBDV field strain, while the average identity with STC (standard challenge like strain), Delaware E, Variant A (Variant IBDVs), D78 (classic vaccine), and IBDI+ (intermediate plus vaccine) were 92, 92, 90.5, 94 and 96.5%, respectively.



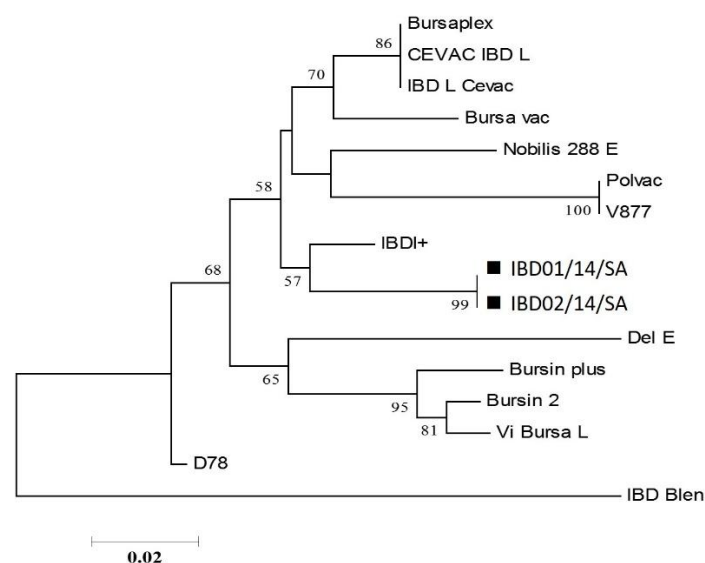
**Figure 1** Phylogenetic tree based on nucleotide sequences of VP2 of the Saudi Arabian IBDVs compared with many virulent, classical, variant and vaccine strains worldwide. Neighbour-joining tree was calculated from pairwise nucleotide differences of the VP2 gene. The length of the branches represents the distance between pairs.

The 255 amino acid sequences between positions 190 to 445 of both Saudi Arabian isolates were identical. Many amino acid substitutions were reported when compared with other classical, variant and vaccinal IBDV strains (Table 1). The amino acid at position 222 was serine (S), which is an important immunogenic site for IBDV. Both of the isolates had sequence characteristic of wild type virulent IBDVs. Both isolates had Asparagine (N) at position 254, also glutamine (Q) at position 253 and a serine-rich peptide SWSASGS at positions 326-332 next to the second hydrophilic region. Based on the amino acid sequence, the isolates were classified as classical IBDVs with similarity of 98.7, 97.6 and 96.4% to the field virulent 586/USA, 4854B406/CAN and 661/USA, respectively. The most interesting amino acid substitution was T (Threonine) substituted by K (Lysine) at position 359, a unique substitution in Saudi Arabian IBDVs and Canadian IBDVs. Lysine is a positively charged

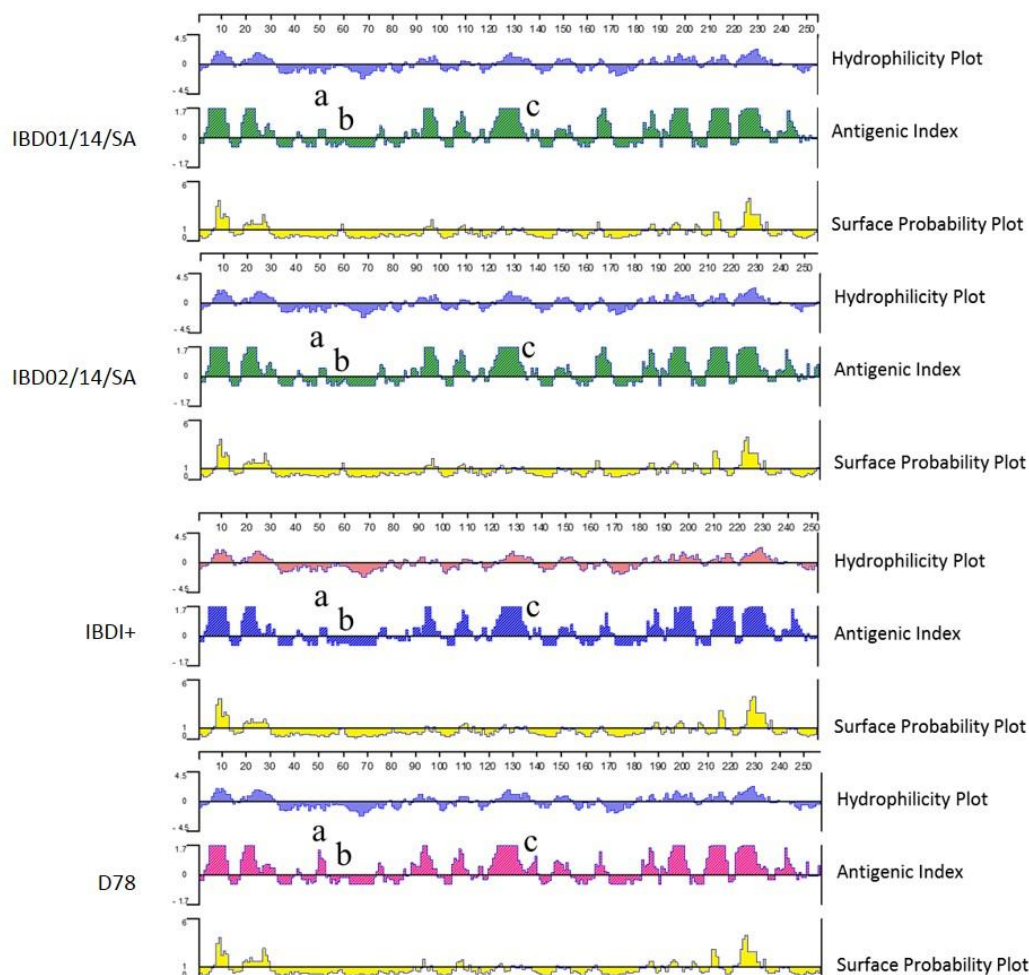
hydrophilic amino acid while threonine is a non-charged hydrogen bonding hydrophilic amino acid that alters the protein folding and antigenic index at this site (Fig. 3). According to the phylogenetic analysis to determine the phylogenetic related vaccine, the amino acid sequences of the Saudi Arabian isolates were compared with the corresponding sequences of commonly used vaccines (Fig. 2). It was found that the more phylogenetic related vaccines were IBDI+ (intermediate plus vaccine) and D78 vaccine strains with the similarity of 97.2 and 96.1%, respectively. The antigenic sites within the amino acid sequence of the Saudi Arabian isolates and the closely related vaccines (Fig. 3) showed that the antigenic profile of the IBDI+ vaccine was almost identical to the Saudi Arabian IBDV isolates. Three discrepancies were observed between the Saudi Arabian isolates and the D78 vaccine strain at positions 242, 330 and 353.

**Table 1** Comparison of amino acid sequences of VP2 hypervariable region of Saudi Arabian isolates and reference IBDVs

AA Site	Infectious Bursal Disease Virus Strains										
	Conesus	IBD01/14/SA	IBD02/14/SA	B4/06/CAN	Del E	IBDI +	586/USA	04E/JOR	STC	661/UK	Nobilis 228E
222	S (Ser.)				T			-	A	A	
242	I			V	V		V				V
249	Q (Gln.)				K (Lys.)						
254	G	N	N	N	S		N				
256	I			V (Val.)	V		V				
270	T				A			A	A	A (Ala.)	
272	I			T (Thr.)			T				
279	N							D (Asp.)	D	D	
286	T				I (Ile.)						
289	L	P (Pro.)	P	P			P				
290	M (Met.)			I			I				
294	L (Leu.)							I	I	I	
296	I			F			F				
299	S			N	N		N (Asn.)				N
318	G (Gly)				D						
321	A						D				
323	D				E						
329	A										V
359	T	K	K	K							-



**Figure 2** Phylogenetic tree based on deduced amino acid sequences of VP2 of the Saudi Arabian IBDV isolates compared with commercially available vaccine strains. Bootstrap values are shown only on main branches for clarity.



**Figure 3** Identification of potential antigenic sites within the amino acid sequence of hypervariable region of VP2 from Saudi Arabian field isolates, D78 and IBDI+ vaccines. Letters a, b and c show differences in the antigenic profile.

### Discussion

IBD is a highly contagious immune suppressive disease affecting mainly chickens. In spite of the extensive use of live and/or inactivated vaccines to control the disease in chickens, IBD is still one of the major problems facing poultry industry and many outbreaks reported in different parts of the world (Hon et al., 2006). In the present study, the first molecular characterization of IBDVs in Saudi Arabia was demonstrated. RT-PCR was used for rapid detection of the virus in field samples combined with genomic sequence of VP2, which was found to be a more reliable method for characterization to overcome the RFLP limitations and inadequacy. VP2 is the major immunogenic determinant; the amino acids between positions 206 to 350 represent a major neutralizing domain and hypervariable region (Jackwood et al., 2008 and Xu et al., 2011).

The samples were collected from ten geographically separated broiler flocks. The signs and obvious gross lesions found were suggestive to IBD (Lukert and Saif, 2003). RT-PCR was successfully generated and the 740 bp was amplified using the specific primers of IBDVs (Ojkic et al., 2007). The nucleotide sequence analysis and alignment showed the identity with several published IBDV strains. The phylogenetic analysis based on the nucleotide sequences of VP2 hypervariable region revealed that

the Saudi Arabian strains showed 99.5% identity. The phylogenetic tree was divided into distinct lineages (Fig. 1). The Saudi Arabian IBDVs and Canadian IBDVs were grouped within the same lineage, including the classical field viruses 586/USA (Ojkic, et al., 2007) and A12-ER/01 (Vera, et al., 2015).

The antigenic variations of IBDVs usually due to amino acid substitutions in the hypervariable region in two main hydrophilic peaks are located between amino acids residues 212-223 and 314-324 (Vakharia et al., 1994). The Saudi Arabian isolates have Serine (S) at position 222, which is a characteristic for classical or variant viruses (Dormitorio et al., 1997; Eterradosi et al., 1998; Jackwood et al., 1997; Letzel et al., 2007). The amino acid residues at positions 222A, 256I, 294I and 299S play an important role in the virulence (Sreedevi and Jackwood, 2007; Jackwood et al., 2008; Kasanja et al., 2007). Both Saudi Arabian IBDVs had serine as a residue at 222, which is a hydrophilic amino acid that alters the genetic profile at this site, and shared the same substitutions with B4/06/CAN, 586/USA and IBDI+. The amino acid substitution L294I that are isomer for each, therefore, this substitution did not modify the Jameson and Wolf antigenic profile (Fig. 3). Dormitorio et al. (1997) mentioned that all standard strains contained Glutamine (249Q) and Glycine (254G). The Saudi Arabian IBDVs, B4/06/CAN IBDVs and 586/USA showed a substitution of G254N. The residues 253Q and 284A in the Saudi Arabian isolates

were found to be responsible for pathogenicity and unique to highly virulent IBDVs (Brandt et al., 2001). These results suggest that these isolates are field vvIBDVs (Van Loon et al., 2002). Moreover, the virulence determinant serine rich repeat <sup>326</sup>SWSASGS<sup>332</sup> strengthens our suggestion. The amino acid residue 359K is a unique substitution in Saudi Arabian and Canadian isolates. Lysine (K) is a strong positively charged hydrophilic amino acid; and according to the data provided by antigenic index, this substitution modifies the Jameson and Wolf antigenic profile (Fig. 3), making them antigenically different from other classic IBDVs. The phylogenetic analysis and potential antigenic sites within the 255 amino acids of VP2 of the Saudi Arabian isolates and the commonly used vaccines were compared by calculating the antigenic index (Jameson and Wolf, 1988). The Saudi Arabian isolates were significantly different from commonly used vaccines (D78, Nobilis 228E) in the antigenic profile, while they exhibited high similarity to the IBDI+ vaccine.

In conclusion, two IBDVs were successfully characterized. The genotyping and genomic sequence analysis indicated that both Saudi Arabian isolates were related to the classical very virulent viruses. The isolates showed a unique amino acid sequence and high identity when compared with the Canadian and 586/USA IBDVs, making them different from other classical strains. According to the phylogenetic analysis and the antigenic index, the Saudi Arabian isolates were found to be more related to IBDI+ vaccine and could be effectively controlled using this type of vaccine.

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## บทคัดย่อ

### ลักษณะทางโมเลกุลของยีน VP2 ของเชื้อไวรัส infectious bursal disease viruses (IBDVs) ในประเทศซาอุดีอาระเบีย

มามูด โมฮาเหม็ด<sup>1,3</sup> มามูด อีสมา<sup>1,4</sup> อิบราฮิม อัล ซาบั<sup>2,5</sup>  
 अबดุลrahمان อัล อัลคาร์<sup>1</sup>

โรค Infectious bursal disease (IBD) มีสาเหตุจากเชื้อไวรัสในแฟมิลี Birnaviridae เชื้อไวรัสชนิดนี้ทำให้เกิดการกดภูมิคุ้มกัน และเกิดโรคในฝูงไก่กระเทย การศึกษาครั้งนี้ได้ศึกษาคุณลักษณะทางโมเลกุลของเชื้อไวรัส IBDVs ในประเทศซาอุดีอาระเบีย จำนวน 2 ตัวอย่าง (IBD01/14/SA และ IBD02/14/SA) โดยวิธี RT-PCR และถอดรหัสพันธุกรรมบางส่วนของบริเวณที่มีความหลากหลายสูง (hypervariable region) บนยีน VP2 จากนั้นได้เปรียบเทียบสายพันธุกรรมความยาว 740 bp ของเชื้อไวรัสจากซาอุดีอาระเบีย และเชื้อไวรัส IBDVs ที่มีความรุนแรงหรือแหล่งที่มาแตกต่างกัน จำนวน 56 เชื้อ ผลการวิเคราะห์ทาง Phylogenetic analysis พบว่า เชื้อไวรัสจากซาอุดีอาระเบีย จัดอยู่ในกลุ่มที่แยกออกจาก IBDVs ในแคนาดาและอเมริกา และพบการลดลงของกรดอะมิโน (deduced amino sequences) ในเชื้อไวรัสทั้งสองตัวอย่าง ซึ่งเหมือนกับเชื้อไวรัสที่มีความรุนแรงสูง (vv strain) นอกจากนี้ยังพบเปลี่ยนกรดอะมิโนที่ตำแหน่ง 254N and 359K ซึ่งเกี่ยวข้องกับการเปลี่ยนแปลงแอนติเจน (antigenic profile) ผลการศึกษานี้แสดงให้เห็นว่าเชื้อไวรัส IBDV ในประเทศซาอุดีอาระเบียและเชื้อไวรัสที่เป็นวัคซีน มีความใกล้เคียงกันในแง่ของกรดอะมิโนที่ลดลง และการเปลี่ยนแปลงแอนติเจน ดังนั้นเชื้อไวรัสดังกล่าวสามารถนำมาใช้ในการป้องกันโรคในไก่ได้ในอนาคต

**คำสำคัญ:** IBDV phylogenetic analysis ซาอุดีอาระเบีย ถอดรหัสพันธุกรรม

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