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RESTRICION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF JORDANIAN FIELD STRAINS OF INFECTIOUS BURSAL DISEASE VIRUS

(With One Table and 2 Figures)

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تقنية RFLP لدراسة العترات (المعزولات) الاردنية الحقلية لفيروس مرض غدة فايبريشيا المعدي (الجمبورو)

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استخدمت تقنية RT-PCR/RFLP لدراسة الصفات الاوليه للفيروسات الحقلية الاردنية المسببه لمرض التهاب غدة فايبريشيا الفيروسي. فحصت عينات الغدد (Bursas) والتي جمعت من ثمانية عشر حقلا مصابا او من الدجاج المربى منزلياً والذي ظهرت عليه الأعراض النموذجية للمرض وتميز باحداث نسب نفوق عاليه تراوحت بين ٢٠-٦٠ %. امكن مضاعفة السيطرة الموجبه الداخلية (IC) من ستة عينات فقط، كانت أربعة من العينات السته موجبة لاختبار RT/PCR والذي ضاعف منطقة 743-bp في جين VP2 للفيروس. تم تحديد ثلاثة اشكال من RT-PCR/RFLP بعد استخدام الانزيمات المحددة BstNI، MboI، SspI. تبين أن العتره الفيروسية رقم عشره كان لها شكل مطابق للفيروس الشرق اوسطي شديد الضراوة ، ويمكن أن تكون ضمن المجموعة الجزيئية السادسة. اما العترتان الخامسة و الثانية عشر يكون تصنيفهما ضمن المجموعة الجزيئية الثالثة. هذه الفيروسات لها اشكال RFLP مشابه للعترة الامريكية التقليدية 2512 والتي تستخدم على نطاق واسع كعترة لقاح اضافة الى انها موجبه SspI. ان شكل RFLP الذي تكون للعترة الثانية لم يكن متناسقا مع الاشكال المتوفره للمجاميع الجزيئية ولا يمكن تصنيف هذا الفيروس باستخدام طرق التشخيص الموصوفة. ان تشخيص العترة العاشرة لفيروس غدة فايبريشيا والتي لها شكل RFLP مطابقا لما يمكن ملاحظته غالبا لعترة فيروس مرض التهاب غدة فيبريشى الشديد الأمراضية ذا شان عظيم ويستحق العنايه بدراسات اخرى لغرض توصيفه كاملا للوقوف على مدى انتشاره في الاردن.

SUMMARY

Reverse transcriptase-polymerase chain reaction/ restriction fragment length polymorphism (RT-PCR/RFLP) techniques were used for the preliminary characterization of Jordanian field strains of Infectious

Bursal Disease Viruses (IBDVs). Bursal samples from 18 field outbreaks in commercial and backyard chicken flocks with typical signs of IBD and high mortality (20%-60%) rate were examined, however the internal positive control could be amplified from only 6 samples. Four of these 6 samples were positive by RT-PCR that amplified the 743-bp region of the VP2 gene of IBDV. Following digestion with *Bst*NI, *Mbo*I, and *Ssp*I restriction enzymes, three different RT-PCR/RFLP profiles were detected. Strain number 10 has RFLP profiles consistent with those of the Middle Eastern vvIBDV and can be designated to molecular group 6. Strains numbered 5 and 12 can be classified into molecular group 3. These viruses have RFLP profiles similar to the USA classic strain (2512) which is widely in use as a vaccine strain and is also *Ssp*I positive. The RFLP profile generated for strain number 2 was not consistent with the available profiles for the molecular groupings and using the described assays, this virus was not able to be classified. The detection of strain number 2 may requires further nucleotide and amino acid sequence analysis to completely characterize this strain. The identification of an IBDV strain number 10 with an RELP profile consistent with those most frequently observed for vvIBDV strains is of major concern and demands further research attention to determine its prevalence within Jordan

Key words: *Infectious Bursal Disease Virus, RFLP, VP2, vvIBDV, Chicken, Jordan.*

INTRODUCTION

Infectious bursal disease virus (IBDV) of the *Birnaviridae* family is a double-stranded RNA virus with bisegmented genome (Muller, *et al.,* 1979). It is the causative agent of an acute, highly contagious, and immunosuppressive disease in young chickens known as infectious bursal disease "IBD" (Saif, 1991). Of the two distinct viral serotypes, only serotype 1 viruses are able to cause the disease in poultry (Jackwood, *et al.,* 1985; Ismail, *et al.,* 1988). At least six antigenic subtypes of serotype I IBD viruses have been identified by *in-vitro* cross-neutralization (VN) assay (Jackwood and Saif, 1987). The commonly known variant viruses are in one of these antigenic subtypes and classic viruses are in another antigenic subtype. In the mid eighties very virulent IBDV (vvIBD) was recognized from acute cases of IBD in Europe (Chettle, *et al.,* 1989). Since then, vvIBDV has been isolated in many countries in Europe, Asia, the Middle East, and Latin America

(Nunoya, *et al.*, 1992; Lin, *et al.*, 1993; Cao, *et al.*,1998; Ture, *et al.*, 1998: Di Fabio, *et al.*,1999; Eterradossi, *et al.*, 1999; Abdel-Alim, *et al.*, 2003; Banda, *et al.*, 2003). High levels of maternally derived antibodies can protect chicks against wild type virus challenge but also depends on the IBDV strain used in the parent flock vaccination program and the circulating wild type viruses (Al-Natour, *et al.*, 2004). These vvIBDV strains are able to break through high levels of maternally derived antibodies in commercial flocks causing high mortality rates up to 60%-100% (Eterradossi, *et al.*, 1992).

Reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) have been used to identify the restriction enzyme markers *Ssp*I, *Bst*NI and *Mbo*I within the VP2 hyper variable region. These restriction markers were used to place IBDV strains into 6 broad molecular groups (Jackwood and Sommer, 1997; Jackwood and Sommer1998; Jackwood and Sommer, 1999). Jackwood and Sommer (1997) reported that the RT-PCR/RFLP analysis was an improvement over the RT-PCR-restriction enzyme (RE) assay because it examines a large portion of the VP2 gene (743 bp) for the presence of multiple restriction sites of *Bst*NI and *Mbo*I enzymes.

The *Ssp*I enzyme site in a 743-bp RT-PCR fragment of VP2 has been found to be present in many viruses designated with a vvIBDV phenotype (Lin, *et al.*, 1993; Ture, *et al.*, 1998; Jackwood and Sommer, 1999). However some workers detected this RE site in viruses other than vvIBDV (Cao *et al.*, 1998; Banda *et al.*, 2001; Hoque *et al.*, 2001). Typically, the vvIBDV strains exhibit RELP patters characteristic of molecular group 6 with the presence of the *Ssp*I site and correlating description of severe clinical disease with high mortality rates (Abdel-Alim *et al.*, 2003).

Ture, *et al.*, (1998), reported the use of three restriction enzymes (*Bst*NI, *Mbo*I, and *Ssp*I) in the RT-PCR/RFLP assay to characterize and compare five vvIBDVs from Turkey, Holland, and Taiwan with U.S. serotype 1 classic (STC) and variants (Maryland, Indiana) and serotype 2 (Ohio) viruses. When digested with *Bst*NI the RFLP profiles of the five vvIBDV isolates were different from the U.S. STC strain but were similar to the variant viruses (Maryland, Indiana). When digested with *Mbo*I the RFLP profiles for the isolates from Holland and Turkey were similar to the STC strains but were different from the variant viruses. When the two Taiwanese isolates were digested with *Mbo*I the RFLP profiles of these isolates were different from the Holland and Turkey strains, and the U.S. classic and variant viruses.

In Jordan disease due to IBD virus is widespread in the poultry industry (Anon, 2000). Severe outbreaks of IBDV with high rates of mortality (20-60%) in vaccinated flocks have been noted (Al-Natour unpublished observation). In light of the current situation in Jordan, the objective of this study was to undertake molecular typing of Jordanian IBDV field strains that caused severe outbreaks in broiler, layer and backyard flocks using RT-PCR/RFLP.

MATERIALS and METHODS

Bursal samples and RNA preparation:

Five bursal samples from each of the 18 field outbreaks in commercially reared broiler flocks (2-6 weeks of age), one layer flock (6 weeks of age) and one-backyard flock (8 weeks of age) with typical IBD signs were examined. The IBD virus was inactivated in a solution of phenol: chloroform: isoamyl alcohol (25:24:1 [v/v]) and shipped to the United States, (Jackwood, *et al.,* 1996). The samples were then rinsed in 10 volumes of TNE buffer (10 mM Tris-HCL, pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid), homogenized in TNE buffer, chloroform extracted and proteinase K (Sigma Chemical Co., St. Louis, MO) digested at a final concentration of 1.0 mg/ml, in 0.5% (w/v) of sodium duodecyl sulfate (Sigma). RNA was extracted by separation in acid phenol, pH 4.3 (Amresco, Solon, OH) followed by chloroform:isoamyl alcohol (24:1), and then ethanol precipitated from the aqueous phase. The RNA was resuspended in 100µl of 90% dimethyl sulfoxide (DMSO) as described by Jackwood and Sommer, (1997).

RT/PCR-RFLP:

The procedure was based on that described by (Jackwood and Nielsen, 1997; Jackwood and Sommer, 1997). Two µl of viral RNA was amplified using the GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular System, Inc., Branchburg, NJ) according to the instructions of the manufacturer. The RNA was denatured at 95°C for 5 minutes then reverse transcribed at 42°C for 1 hr. Products were amplified under the following conditions; 95°C for 5 min, 52°C for 1.0 min, and 72°C for 1.0 min. The 700-5' and 700-3' primers were used to amplify a 743-bp fragment of the VP2 gene from 710 bp to 1444 bp (Jackwood and Nielsen, 1997). The ssRNA internal control (IC) reagent (Smiley, *et al.,* 1999) that amplifies the 900 bp fragment was added. The PCR product was digested with the restriction enzymes *Bst*NI, *Mbo*I and *Ssp*I (Lin, *et al.,* 1993) and a 2.5% MetaPhor agarose gel (FMC Bio-Products,

Rockland, ME) with SYBER ™ green I nucleic acid gel stain (Molecular Probes, Inc., Eugene, OR) was used to detect the restriction fragments. A100-bp DNA size marker was included on each agarose gel (Jackwood and Sommer, 1997).

RESULTS

RT-PCR:

Four (2/16 broiler, 1/1 layer and 1/1 backyard flocks) out of the 6 bursal samples in which the internal positive control could be amplified were determined to have IBDV RNA as evidenced by amplification of a 743-bp region of the VP2 gene by the RT-PCR. The positive samples were obtained from flock number 2, 5, 10 and 12 as shown in Fig. 1. Digestion of the RT-PCR products of these viruses with three restriction enzymes *Bst*NI, *Mbo*I or *Ssp*I showed different RFLP profiles as shown in Fig. 2 and summarized in Table 1.

Digestion of the RT-PCR products with *Mbo*I restriction enzyme:

When using *Mbo*I enzyme for digestion of the RT-PCR products of these isolates, three different RFLP profiles were detected. The first profile was detected in the sample obtained from flock number 2 (flock number will be used to identify the virus) that generated three fragments with sizes consistent with a pattern of 170 bp, 229 bp, and 362 bp (Fig. 2 Lane 6). The second profile was seen in two samples (numbers 5 and 12) that generated two fragments with sizes consistent with a pattern of 229 bp and 403 bp (Fig. 2 Lane 7 and 12). The third profile was detected in sample number 10 that generated two fragments with sizes of 229 bp and 362 bp (Fig. 2 Lane 8).

Digestion of the RT-PCR products with BstNI restriction enzyme:

After digestion of the RT-PCR products of these viruses with *Bst*NI enzyme two viruses (numbers 2 and 10) had identical RFLP profiles that generated three fragments with sizes consistent with a pattern of 119 bp, 172 bp, and 424 bp. (Fig. 2 lanes 1 and 3). The RFLP profile of viruses (numbers 5 and 12) were identical and each yielded four fragments sizes consistent with a pattern of 119 bp, 139 bp, 154 bp and 172 bp (Fig. 2 lane 2 and 4).

Digestion of the RT-PCR products with *Ssp*I restriction enzyme:

All the detected viruses (numbers 2, 5, 10, and 12) were *Ssp*I enzyme positive and each yielded two fragments approximately 273 bp and 470 bp in size (Fig. 2 lanes 11-14 and Table 1).

Table 1: RT-PCR/RFLP results for the Jordanian infectious bursal disease virus strains[A].

Flock no.	Principal breed	RT-PCR results	*Ssp*I[B]	*Bst*NI enzyme	*Mbo*I enzyme
1	Broiler	Negative	-		
2	Broiler	Positive	+	119 bp, 172 bp, 424 bp	170 bp, 229 bp, 362bp
5	Broiler	Positive	+	119 bp, 139bp, 154bp, 172 bp	229 bp, 403bp
10	Backyard	Positive	+	119 bp, 172 bp, 424 bp	229 bp, 362 bp
12	Layer	Positive	+	119 bp, 139 bp, 154 bp, 172 bp	229 bp, 403 bp
15	Broiler	Negative	-		

[A] Values are the length in base pair of the restriction fragments. [B]Presence of *Ssp*I restriction site. RT-PCR = reverse transcriptase- polymerase chain reaction. RFLP = restriction fragment length polymorphism. All flocks were vaccinated once or twice with an intermediate plus IBDV vaccine in this area according to the manufacturer's recommendations except flock number 2 and 10.

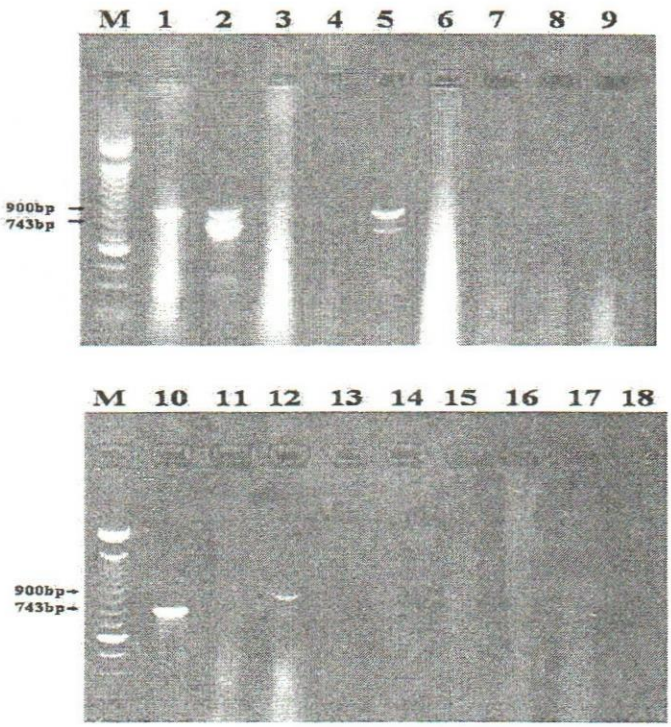

Fig. 1: RT/PCR products of the Jordanian field IBDV isolates. The PCR products were separated into a 2.5% agarose gel, and a 743-bp fragment was observed in the positive lanes. The IC (900 bp) reaction products are shown. The 100-bp ladder (Gibco, BRL Gaithersburg, MD) was used as a molecular weight marker (M).

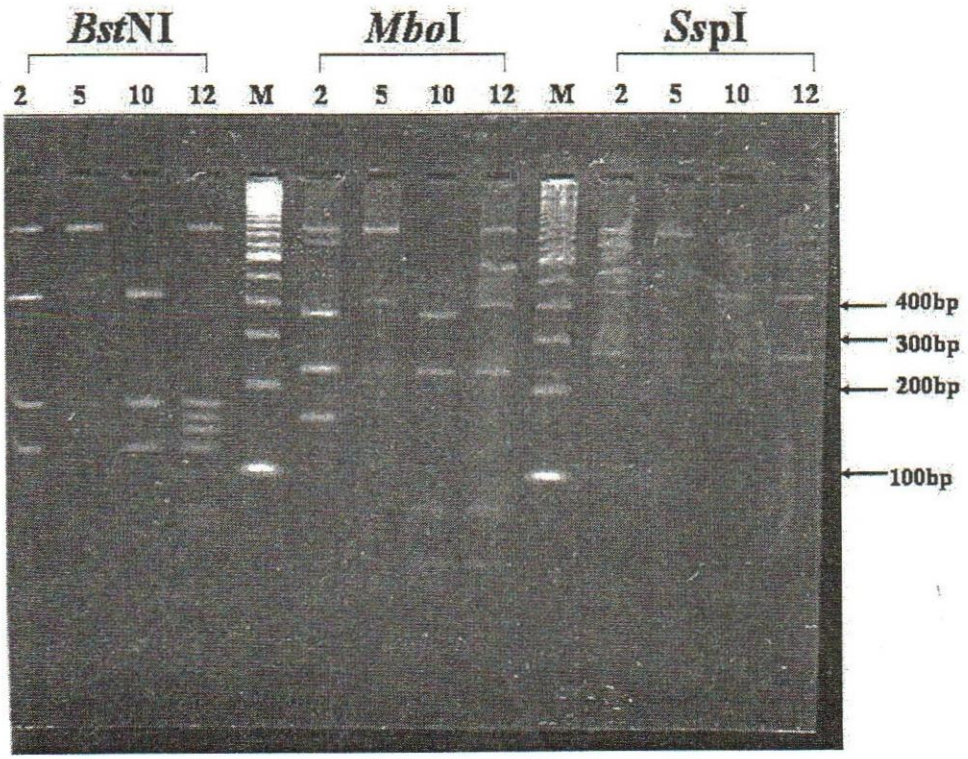

Fig. 2: RFLPs observed when *Mbo*I, *Bst*NI and *SsP*I enzymes were used. Lanes designated M contain the 100-bp ladder molecular weight marker.

DISCUSSION

In this study the bursal samples were collected from 18 outbreaks that had clinical signs and lesions of IBD however only 6 generated the expected 900 bp band by RT-PCR corresponding to the internal positive control. These results indicate either RNA degradation during preparation and storage of the samples or inhibition of the RT-PCR reaction by contaminants in a large proportion of the samples. Of the 6 samples positive for the internal control 4 bursal samples were determined to contain IBDV as evidenced by positive RT-PCR reaction.

When the RT-PCR products of the four detected isolates of IBDV were digested with *Mbo*I and *Bst*NI restriction enzymes, three different RFLP profiles were generated. The RFLP profile of virus number 10 (backyard flock) yielded two fragments with sizes of 229 bp

and 362 bp when digested with *Mbo*I enzyme and three fragments with sizes of 119bp, 172bp and 424bp when digested with *Bst*NI enzyme. This RFLP profile resembles the RFLP profile of the previously published Dutch, Turkish and Egyptian vvIBDV (Ture, *et al.*, 1998; Eterradossi, *et al.*, 1999; Abdel-Alim, *et al.*, 2003). Viruses number 5 (Broiler flock) and number 12 (Layer flock) were similar. In both viruses the RFLP profile yielded two fragments of 229 bp and 403 bp in size when digested with *Mbo*I enzyme and four fragments of 119bp, 139bp, 154bp and 172bp after digestion with *Bst*NI enzyme. These two viruses with identical patterns are similar to the US (26th egg passage) 2512 strains and the US IBD BLIN^{TM} vaccine strain of the classic serotype 1 IBDV. (D.J. Jackwood personal communication). Virus number 2 (Broiler flock) generated RFLP profile with three fragment sizes of 119bp, 172bp and 424bp when digested with *Bst*NI enzyme which is similar to virus number 10, and when digested with the *Mbo*I enzyme the RFLP profile of this virus produced three fragments with sizes of 170bp, 229bp, and 362bp. The presence of an additional 170 bp band in the RFLP pattern does not resemble any previously published IBDV RFLP profiles (Jackwood and Nielsen, 1997; Jackwood and Sommer, 1998; Ture, *et al.*, 1998; Jackwood and Sommer, 1999; Abdel-Alim and Saif, 2001; Abdel-Alim, *et al.*, 2003).

Of the four viruses detected all were *Ssp*I positive. Most commonly vvIBDV strains have RFLP profiles characterized as molecular group 6 combined with the *Ssp*I marker. These features are present for virus number 10 and therefore are consistent with the most frequently identified RFLP profiles of vvIBDV strains. Conclusive identification of strain number 2 as vvIBDV will require further sequence analysis.

From this study, it is concluded that 4 Jordanian IIBDV RFLP profiles were identified. Strain number 10 has RFLP profiles consistent with those of the Middle Eastern vvIBDV and can be designated to molecular group 6. Strains numbered 5 and 12 can be classified into molecular group 3. These viruses have RFLP profiles similar to the US classic strain (2512) which is widely in use as a vaccine strain and is also *Ssp*I positive. The RFLP profile generated for strain number 2 was not consistent with the available profiles for the molecular groupings and using the described assays, this virus was not able to be classified. This strain could be addressed by further nucleotide and amino acid sequence analysis.

In the Middle East and Europe vvIBDV strains have been reported to break through high levels of maternal antibodies in commercial flocks, causing up to 60%–100% mortality rates in chickens and producing lesion typical of IBDV (Eterradossi, *et al.,* 1992). Therefore, the identification of an IBDV strain number 10 with an RFLP profile consistent with those most frequently observed for vvIBDV strains is a major concern and demands further research attention to determine its prevalence within Jordan.

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Running Head: RFLP analysis of Jordanian Field Strains of IBDV

Abbreviations: bp = base pair; DMSO = dimethyl sulfoxide; IBDV = infectious bursal disease virus; IC = internal control; RE = restriction enzyme; 2512 strain = (26^{th} egg passage) USA serotype 1 classic; TNE buffer = 10mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM. TNE = Tris-sodium chloride and EDTA buffer, IBD= infectious bursal disease, vvIBDV = very virulent infectious bursal disease virus, RFLP = restriction fragment length polymorphism, RT-PCR = reverse transcription-polymerase chain reaction, VN = virus neutralization.

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