MOLECULAR DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS IN COMMERCIALLY REARED CHICKENS USING THE REVERSE TRANSCRIPTASE/ POLYMERASE CHAIN REACTION-RESTRICTION ENDONUCLEASE ASSAY

BY

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ABSTRACT

Twenty-four bursal samples collected from commercially reared chicken flocks suspected of having infectious bursal disease (IBD) were tested for the presence of infectious bursal disease virus (IBDV) using reverse transcription / polymerase chain reaction (RT/PCR) assay. Reverse transcription (RT) followed by PCR was used to amplify a portion of the genome (VP2 gene) of field IBD isolates and IBD vaccine virus (Bursa BLEN and IBD BLEN).

A single band of the PCR product of the expected size (800 base pairs in length), from the field IBDV isolates and IBD vaccine viruses, was visible on a polyacrylamide stained with ethidium bromide. The sequences of amplified VP2 gene of IBDV, isolated from commercially reared chickens was compared with those of vaccine viruses using restriction enzyme (RE) analysis. IBDV isolates (24 isolates) had RE profiles typical of classic vaccine viruses when using restriction enzymes, MboI, SmaI, StyI and BamHI.

These results demonstrated that there were no molecular differences in the region of VP2 gene among IBDV isolates compared with the classic vaccine viruses. IBD viruses responsible for outbreaks belong to classic strains and not variant strains..

In conclusion, this study demonstrated that the RT/PCR-RE assay can be used to diagnose IBDV in chickens and that IBDV strains existed in commercially reared chickens had VP2 gene sequences similar to those of classic IBD vaccine strains. IBD outbreaks were due to classic IBDV and were not due to variant strains. IBD outbreaks in commercially reared chickens might be due to other factors such as bad handling and improper administration of IBD vaccine or waning of maternal immunity.

INTRODUCTION

Infectious bursal disease virus (IBDV) is a member of family Birnaviridae (Lukert,1996). It responsible for a severe immunosuppressive disease in young chickens that causes significant losses to poultry industry worldwide (Cao et al, 1998). According to antigenic variation and virulence, IBDV can be divided into several groups: classical virulent strains, attenuated strains, antigenic variant strains and very virulent (VV) strains (Cao et al, 1998). Classical virulent strains cause bursal inflammation and severe lymphoid necrosis in infected chickens, resulting in immunodeficieny and moderate mortality. Attenuated strains have been adapted to

chicken embryo fibroblast (CEF) cells or other cell lines. These strains do not cause disease in chickens and therefore some of them are used as live vaccines. The very virulent strains can break through high level antibody and cause up to 60 - 100 % mortality (Brown et al, 1994). Chickens affected by the variant strains are characterized by severe atrophy of the bursa without showing inflammatory symptoms associated with infection by the classical strains (Sharma et al, 1989).

The virus genome consists of two segments of double-stranded RNA (dsRNA) (Brown, 1986). The smaller segment (segment B) (2.8 kb) encodes virus protein 1 (VP1). The larger segment (segment A) (3.2 kb) encodes VP2 , VP3 and VP4 (Hudson and Mckern , 1986). VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of IBDV that contains at least three independent epitopes responsible for the induction of neutralizing antibodies (Becht et al, 1988).

Sequences of the major host-protective immunogen VP2 indicate that they are conserved except a fragment designated as the hypervariable region (Yamagouchi et al, 1997). Studies showed that changes in the VP2 region account for antigenic or pathogenic variations among IBDV. These changes cause the viral isolate to lose an epitope and escape neutralization by a monoclonal antibody (Brown et al, 1994).

Antigenically variant strains were recognized by their ability to escape cross-neutralization by the antisera against classical strains (Jackwood and Saif , 1987).These antigenic subtypes of IBDV serotype 1 are probably responsible for the failure of maternal immunity and some vaccination programs of IBDV (Ismail and Saif, 1991). Therefore, diagnosis of serotype 1 subtypes is important when designing vaccination programs against IBDV infection.

Identification of the IBDV serotype 1 subtypes is currently possible by using the virus neutralization assay (Jackwood and Saif , 1987). Before this assay is conducted, viruses must be isolated and propagated in vitro. These procedures are time consuming and it can be difficult to adapt some IBDV isolates to propagate in cell culture system. Therefore, a diagnostic assay is needed that can easily identify and differentiate the antigenic variants of serotype 1 IBDV. Reverse transcription (RT) followed by the polymerase chain reaction (PCR) has been used to detect IBDV infection among chickens (Wu et al. 1992 and Jackwood and Jackwood, 1994). Differentiation of IBDV strains has been accomplished followed by restriction endonuclease(RE) digestion of the PCR products (Jackwood and Nielsen, 1997). The reverse transcriptase/ polymerase chain reaction-restriction endonuclease (RT/PCR-RE) assay was used to differentiate IBDV subtypes that can not be differentiated by virus neutralization assay (Giambrone et al, 1994 and Jackwood and Jackwood 1994).

The focus of this study is to demonstrate the availability of using RT / PCR for detecting IBDV infection among commercially reared chickens and to determine the extent of genetic heterogeneity among field isolates of IBDV in comparison to classic IBDV vaccinal strains.

MATERIAL AND METHODS

1. Bursal samples:

Bursa tissues submitted for diagnosis were collected from commercially reared chicken flocks suspected of having IBDV infection. The samples were collected from January 1998 to October 1999. A total of 24 samples was collected from 24 different IBD outbreaks and different farms. Each sample contains five bursas from each outbreak . The ages of the flocks ranged from 28 to 42 days old.

2. Vaccine viruses:

Bursa Blen (batch No. 1308E1H and IBD Blen (Batch No. 0702E1H produced by Phylaxia Sanofi, Vet. Biol. Co. LTD were used as classical IBDV.

3. Reverse transcription / polymerase chain reaction (RT/PCR):

3.1. Preparation of samples for viral RNA extraction (Jackwood and Jackwood 1994):

a. Lyophilized vaccine viruses:

Each vaccine virus vial was reconstituted in 5.0 ml TNE buffer (10mM Tris-HCL (pH 8.0), 100 mM NaCl and 1mM ethylenediaminetetraacetic acid).The vaccine viruses are prepared for viral RNA extraction.

b. Bursal samples:

After freezing and thawing for three times, the bursae were homogenized in TNE buffer. The homogenates were centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatants were collected for viral RNA extraction .

3.2. Extraction of viral RNA (Jackwood et al., 1996):

A volume of each viral sample was extracted with an equal volume of chloroform and the aqueous layer was collected. Sodium dodecyl sulfate (Sigma Chemical Co. St. Louis, MO) and proteinase K (Sigma) were added to a final concentration of 0.5% and 1.0 mg/ml, respectively. Following incubation at 37^0C for 1 hour, the samples were extracted with an equal volume of acid phenol (pH 4.3) (Amersham Co, LTD) and then chloroform : isoamyl alcohol (24:1). The viral.RNA was precipitated with 2.5 volumes cold ethanol. The viral RNA was collected by centrifugation at 14,000 rpm for 20 minutes at 4^0C. The RNA pellet was suspended in 100 ul of 90 % dimethyl sulfoxide solution at 98^0C for 5 minutes.

3.3. Reverse transcription /polymerase chain reaction (RT/PCR) (Jackwood and Jackwood, 1997):

a. Reverse transcription:

2-ul volume of viral RNA was removed and used in RT/PCR (Pharmacia). The viral RNA was denaturated at 95 ^{0}C for 5 minutes and then used in the RT reaction. The reaction mixture was incubated at 42^0C for 1 hour and then at 95 ^{0}C for 5 minutes for inactivating reverse transcriptase.

b. Polymerase chain reaction (PCR):

PCR was conducted using 2.5 units Taq polymerase in a reaction buffer containing 50 mM KCL , 10 mM Tris-HCL (pH 9.0) and 200 uM each of dATP, dCTP, dGTP and dTTP. The sequences of the primer pair were as follows: primer 1, 5'-AGCGGCCGCAGCGATGAC-3' and primer 2, 5'-GGGATTCTGGGGCACCTG-3' (synthesized by DNA synthesizer , Pharmacia).The primers were homologous to the conserved regions at 5' and 3' termini of VP2 gene of IBDV. The primers were used at

a concentration of 1.0 uM , and MgCl2 was 2.5 mM .The PCR cycle was as follows: denaturation at 95 ^{0}C for 5 minutes, annealing at 52 ^{0}C for 1.5 minutes, and polymerization at 72^{0}C for 3 minutes. The cDNA was amplified for 30 cycles. Following the last cycle, a 7-minute polymerization step was done at 72 ^{0}C.

4. Restriction enzyme (RE) assay :

The procedure used to examine RT/PCR products by restriction enzyme analysis has been described by Jackwood and Nielsen (1997).The RT/PCR products (1ug) were digested with 10 units of each restriction enzymes (BamHI, Smal, Styl and Mbol) (pharmacia) at a temperature and incubation period recommended by the manufacturer. The restriction fragments were examined on a polyacrylamide gel followed by staining with ethidium bromide.

RESULTS

cDNA of IBDV was synthesized from IBDV RNA by reverese transcription (RT) using random oligonucleotides primer. The cDNA was amplified by PCR with the use of pair of synthetic oligonucleotides homologous to the conserved regions at the 5' and 3' termini of the VP2 gene of IBD. The RT / PCR products yielded a clear single band on
a polyacrylamide gel stained with ethidium bromide (Fig. 1). No differences in the length of the RT/PCR products were detected in IBDV isolated from commercially reared chickens and IBD vaccine viruses.

The size of this cDNA fragments corresponded to the 800 base pairs (bp) which was expected from the known sequence of IBDV (Fig. 1).

The amplified VP2 genes of both IBDV isolates and IBD vaccine viruses were not cut with any restriction enzyme used (BamHI, Smal, Styl and Mbol). Therefore, RT/PCR-restriction enzyme profiles of IBDV isolated from commercially reared chickens were typical to IBD vaccine viruses (Table, 1). Both isolates and vaccine strains gave a single band of size similar to the non-enzyme treated RT/PCR on a polyacrylamide gel stained with ethidium bromide.

DISCUSSION

In the present study , RNA of IBDV isolates, from commercially reared chickens suspected of having IBD, was detected using reverse transcription followed by PCR. A portion of the IBDV genome (VP2 gene) has been successfully amplified by PCR to yield a large amount of genomic product of expected molecular size in a very short period. IBDV is a double-stranded RNA virus. Reverse transcription is needed in the PCR for IBDV to synthesize a complementary DNA (cDNA) from RNA by a reverse transcriptase. Reverse transcriptase is an RNA-dependent DNA polymerase that uses single-stranded RNA as a template in the presence of a random primer to synthesize cDNA strand (Wu et al. 1992).

The presence of a single band of RT / PCR products of all IBDV isolates on a polyacrylamide gel indicated IBDV infections in commercially reared chickens. No differences in the size of band of RT / PCR product of IBDV isolates and IBD vaccine strains (Bursa BLEN and IBD BLEN) were detected on a polyacrylamide gel. These results indicated that there is no deletions or insertions within the region flanked by the used primers (Dormitorio et al. 1997).

The RT/PCR technique is a sensitive method for detecting IBDV. It was possible to detect IBDV in as little as two fragments of viral RNA from infected bursae by ethidium bromide staining after 30 cycles of PCR (Wu et al. 1992). The PCR eliminates the need to grow or isolate IBDV before amplification. It has also been shown that no purification steps are required to process impure bursal samples. RT/PCR is not time consuming since all procedures involved take about 6 hours to be completed (Davis and Boyle, 1990).

The results of RT/PCR-RE analysis revealed that the amplified Vp2 genes of both IBDV isolates and vaccine viruses were not cut by any enzyme used. These results indicated that there were no restriction site(s) in the amplified VP2 genes for any enzyme used. The RT/PCR-RE profiles of IBDV isolates were typical to classsic IBD vaccine strains. These results indicated that IBDV isolated from commercially reared chickens belong to classic strains and there were no differences in the nucleotides sequences of VP2 gene of IBDV isolates compared with with IBDV vaccinal strains. These results were confirmed by those described by Jackwood and Nielsen (1997 and Jackwood and Sommer (1998).

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Table (1): Results of RT/PCR-RE analysis of IBDV isolates and vaccine strains.

Restriction enzymes	IBDV isolates	IBD vaccine strains
MboI	Negative*	Negative
BamHI	Negative	Negative
SmaI	Negative	Negative
StyI	Negative	Negative

* Negative results mean absence of restriction site in the RT/PCR products (VP2 gene).

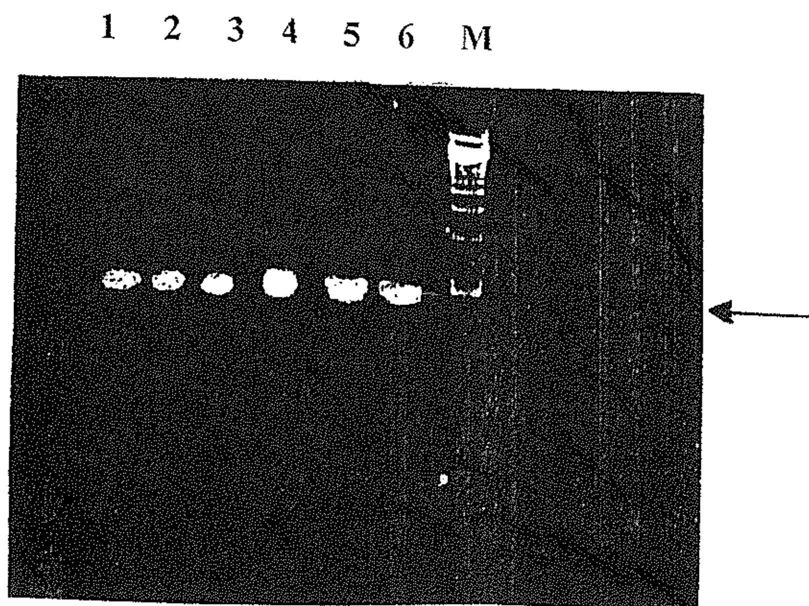

Fig. 1 Ethidium bromide staining of amplified 800 base pair cDNA from infectious bursal disease virus (IBDV) after 30 cycles of polymerase chain reaction with a set of primers in a polyacrylamide gel electrophoresis. The amplified cDNA was 800 base pairs in length (arrow). Lane 1 = Bursa BLEN vaccine , lane 2 = IBD BLEN vaccine , lanes 3, 4, 5 and 6 = IBDV isolates from commercially reared chicken flocks suspected of having IBD. Lane M = molecular weight marker (100 base pairs DNA ladder).

الملخص العربى

استبيان فيروس البرسا المعدى فى دجاج التسويق

سامى عبد السلام خليل و هانى اللقانى

تم فحص اثنين واربعون عينة اخذت من الدجاج المربى لغرض التسويق بحثا عن الصابة بفـــيروس التهاب البرسا المعدى باستخدام تفاعل ترانس كربتاز / بلوميراز العكسى وقد اوضحت النتائج انه ليوجـد اختلاف فى التركيب الجزيئى للجين VP2 بين مختلف العترات للفيروس المسبب لمرض البرسا المعدى.