Virology 409 (2011) 33–37

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Amino acids contributing to antigenic drift in the infectious bursal disease Birnavirus (IBDV)

Daral J. Jackwood *, Susan E. Sommer-Wagner

Food Animal Health Research Program, Department of Veterinary Preventive Medicine, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA

ARTICLE INFO

Article history:
Received 8 June 2010
Returned to author for revision 13 July 2010
Accepted 27 September 2010
Available online 20 October 2010

Keywords:
Infectious bursal disease virus
Birnavirus
Infectious Bursal Disease
Antigenic drift
Point mutation

ABSTRACT

We examined the effect of amino acids 222 and 254 on antigenicity of the variant Del-E strain of infectious bursal disease virus (IBDV). Using molecular epidemiology, we identified a virus designated as Del-E-222 that was identical to Del-E except for alanine at position 222. A second virus was generated using reverse genetics of the Del-E backbone to create Del-E-254 that contained an asparagine at amino acid 254. The Del-E-222 and Del-E-254 viruses were tested for their ability to escape neutralizing immunity provided by parenteral vaccination. The bursas from birds vaccinated with parental Del-E and challenged with Del-E-222 or Del-E-254 had macroscopic lesions typical of an IBDV infection, and their B-BW ratios were significantly smaller than the controls. Microscopic lesions included lymphocyte depletion and confirmed the ability of Del-E-222 and Del-E-254 to break through the immunity induced by the parental Del-E virus vaccination. Both mutations appear to be contributing to antigenic drift.

Introduction

Infectious bursal disease virus (IBDV) causes an immunosuppressive disease in young chickens by infecting lymphocytes in the bursa of Fabricius. Infectious bursal disease (IBD) has been recognized as a significant problem in chickens since 1962 (Cosgrove, 1962). Despite the use of vaccines to control IBDV, it continues, directly and indirectly via immune suppression, to cause economic losses in the poultry industry. The magnitude of these economic losses is difficult to appreciate because the immune suppression caused by IBDV often goes unnoticed until opportunistic pathogens cause diseases like gangrenous dermatitis (Rosenberger et al., 1975) or flock vaccination programs fail (Giambrone et al., 1977).

Antigenic drift in IBDV is thought to be responsible for the formation of antigenic variant strains of the virus. Two substitution mutations (D318G and E323D) in the second hydrophilic region of the hypervariable sequence in VP2 (hvVP2) were necessary for the escape of the variant Del-E IBDV strain from neutralizing monoclonal antibodies (Heine et al., 1991). Substitution mutations in the first hydrophilic region of hvVP2 were also observed in that study: D213N and P222T. These data indicated the two hydrophilic regions are in close physical approximation to each other and together form a major neutralizing epitope of the virus, a property of VP2 that was later confirmed using X-ray crystallography (Coulibaly et al., 2005). These amino acid mutations in the hydrophilic regions of hvVP2 were also necessary for the escape of the Del-E variant from neutralizing antibodies produced by vaccination using a classic IBDV strain (Heine et al., 1991). Further studies on specific amino acid mutations that affect the binding of neutralizing monoclonal antibodies have been conducted (Eterradossi et al., 1997; Vakharia et al., 1994). These studies demonstrated that the major neutralizing epitopes of IBDV are located in the hypervariable region of VP2 by mapping neutralizing monoclonal antibodies to specific regions of the protein. Studies using reverse genetics have shown that several amino acids in the projection (P) domains of VP2 are important for reactivity to neutralizing monoclonal antibodies (Letzel et al., 2007). In this study, amino acid substitutions in the P_{BC} and P_{HI} loops of VP2 were shown to influence the binding of viral neutralizing epitopes to monoclonal antibodies.

Although amino acids in hvVP2 were shown to be important for binding and the escape of IBDV from neutralizing monoclonal antibodies, the contribution of these amino acids to antigenic drift still needs to be examined *in vivo*. Our studies identified IBDV strains that were capable of breaking through maternal immunity generated using variant and classic IBDV vaccines (Jackwood et al., 2001). Sequence analysis of these viruses indicated that in some cases only one or two amino acids had been substituted (Jackwood and Sommer-Wagner, 2005). These molecular epidemiologic studies suggest IBDV has continued to mutate amino acids in hvVP2 that are contributing to antigenic drift in the virus. Our hypothesis is that a single point mutation can significantly contribute to antigenic drift in the Del-E variant IBDV and specific point mutations will allow the virus to

Abbreviations: IBD, infectious bursal disease; IBDV, infectious bursal disease virus; RT-PCR, reverse transcriptase- polymerase chain reaction; B-BW, bursa body weight; SPF, specific pathogen free; hvVP2, hypervariable sequence region of viral protein 2.

* Corresponding author. Fax: +1 330 263 3760.
E-mail address: Jackwood.2@osu.edu (D.J. Jackwood).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.09.030

circumvent immunity induced by variant IBDV vaccines. To test this hypothesis, we identified a Del-E strain with a single substitution mutation (T222A) in the first hydrophilic hvVP2 region (P_{BC}) and using reverse genetics generated another Del-E virus with a substitution mutation (S254N) in the P_{DE} region of hvVP2 that is between the two major hydrophilic projections P_{BC} and P_{HI}. These mutant viruses were then tested for their ability to cause disease in chickens with immunity generated using a Del-E vaccine strain.

Results

Identification and production of substitution mutations in Del-E

In a previous study, we identified an IBDV strain that had an hvVP2 sequence identical to Del-E except for a substitution at position 222 (Jackwood and Sommer-Wagner, 2005). To determine if this was the only amino acid mutation, we determined the complete nucleotide sequence of both genome segments from this virus. The nucleotide sequences of genome segments A and B confirmed that Del-E-222 had an identical amino acid sequence to Del-E in both genome segments (AF133904 and AF133905) except for an alanine (A) at position 222 in VP2 (Fig. 1). This virus was propagated in 9-day-old embryonated chicken eggs.

The Del-E-254 virus generated using site-directed mutagenesis was also identical to Del-E except for an asparagine (N) at position 254 in VP2 (Fig. 1). This virus also produced lesions typical of a Del-E infection when inoculated into 9-day-old embryonated chicken eggs (data not shown).

Vaccination/challenge experiments

Although the Del-E strain is pathogenic in SPF chickens, the virulence of Del-E-222 and Del-E-254 needed to be tested. Birds that were not vaccinated but were challenged with Del-E (Group 3), Del-E-222 (Group 5), or Del-E-254 (Group 7) had small friable bursas. Gross lesions in these bursas were typical for variant strains in the acute and chronic phase of the disease. The B-BW ratios calculated for these groups were significantly smaller ($p \leq 0.05$) than the controls in Group 1 (Table 3). Microscopic lesions in the bursa tissues consisted of moderate depletion of lymphocytes, variable loss of follicles, and reticuloendothelial cell proliferation with lymphocyte necrosis. The histopathologic lesion score for Del-E was 2, while a score of 3 was observed for both Del-E-222 and Del-E-254 (Table 3). These histopathologic lesion scores were subjective and may not represent increased virulence in the Del-E-222 and Del-E-254 viruses compared to the parental Del-E strain.

Antibodies to IBDV were not detected in serum samples collected from SPF birds at hatch and prior to vaccination on day 10 using the virus neutralization and ELISA procedures. Neutralizing antibodies were detected at challenge in birds vaccinated with the homologous 89/03 vaccine. These neutralizing antibody titers ranged from 200 to 800 with a geometric mean of 264 (Table 2). The ELISA titers in vaccinated birds prior to challenge ranged from 77 to 701 with a geometric mean of 125 (Table 2). At 7 days following challenge, relatively high virus-neutralizing and ELISA titers were observed in all the challenged groups (Table 2). No antibodies to IBDV were detected in the un-vaccinated/non-challenged birds in Group 1.

At 7 days following challenge, birds in Group 4 that were vaccinated with 89/03 and challenged with the homologous Del-E virus did not have signs of macroscopic or microscopic lesions in the bursa (Table 3). However, birds that had been vaccinated with 89/03 and then challenged with Del-E-222 or Del-E-254 had gross lesions in the bursa that were similar to the Group 3 un-vaccinated/Del-E challenged controls. In addition, their B-BW ratios were similar to those observed when these challenge viruses were used in un-vaccinated birds (Groups 5 and 7) (Table 3). Microscopic lesions were also similar to those seen in Groups 5 and 7 although they appeared to be slightly less severe and received histopathologic lesion scores of 2 (Table 3).

Comparative viral loads following challenge

A real-time RT-PCR assay was used to determine the relative viral loads in bursa tissue following challenge. This assay was originally developed to differentiate very virulent IBDV from endemic IBDV in the United States (Jackwood et al., 2009). The efficiency reported for the assay in detecting endemic IBDV that includes the variant Del-E strain was 99.96% ($R^2 = 0.999$). The mean cycle threshold (Ct) values were determined for 4 replicates of each sample (Table 3). No significant differences ($p > 0.05$) were observed between the mean Ct values for Groups 5, 6, 7, and 8. The mean Ct value of the vaccinated group was subtracted from the mean Ct value of the non-vaccinated group to achieve a ΔCt. The ΔCt for the Del-E-222 challenged Groups 5 and 6 was −0.25. An identical ΔCt was observed for the Del-E-254 challenged Groups 7 and 8. These ΔCt values were less than one standard deviation observed for the individual Ct values and indicate no difference in viral load between the vaccinated and unvaccinated groups.

Discussion

Antigenic drift in the Birnavirus IBDV has been attributed to substitution mutations in the hypervariable sequence region of the capsid protein VP2 (Heine et al., 1991). Much is known about the mutations that occur in the hvVP2 region (Van Den Berg et al., 2000) and the three-dimensional structure of the VP2 viral protein (Coulibaly et al., 2005). Studies have also been conducted on the specific substitutions needed to significantly alter the antigenic property of the virus (Letzel et al., 2007; Eterradossi et al., 1997, 1998; Vakharia et al., 1994). These studies used neutralizing monoclonal antibodies to assess antigenic changes that were the result of substitution mutations in VP2. To our knowledge, this is the first report where the antigenic property of IBDV was assessed *in vivo* following single substitution mutations in the hvVP2 region.

Variant Del-E viruses with single point mutations at amino acids T222A and S254N in VP2 were identified and tested for antigenic drift in SPF layer chickens. These mutations were selected because they were observed in viruses that caused disease in commercial chicken flocks with immunity to variant IBDV strains (Jackwood and Sommer-Wagner, 2005). Since these substitution mutations were based on sequence analysis of viruses causing disease in the field and not on escape from neutralizing monoclonal antibodies, it is likely that they are pragmatically contributing to antigenic drift in these viruses. The 222 and 254 amino acids are located at the tips of the loop structures in VP2. The T222A mutation is located in the first hydrophilic projection loop P_{BC}, and substitution mutation S254N is located in Loop P_{DE}, which is between the two major hydrophilic projections

```
            210        220        230        240        250        260        270        280
Del-E         AADNYQFSSQ YQTGGVTITL FSANIDAITS LSVGGELVFK TSVQSLVLGA TIYLIGFDGT AVITRAVAAN NGLTAGIDNL
Del-E-222     .......... ..A....... .......... .......... .......... .......... .......... ..........
Del-E-254     .......... .......... .......... .......... ....N..... .......... .......... ..........
89/03_Vaccine .......... .......... .......... .......... ...H...... .......... .......... ....T.....
```

Fig. 1. Partial amino acid sequences of the hvVP2 region showing substitution mutations of viruses Del-E-222 and Del-E-254 compared to the parent Del-E (AF133904) strain. The genome segment B sequences of all four viruses are identical to the Del-E virus (AF133905).

(Coulibaly et al., 2005). Although mutations in the two major hydrophilic projections (Loops P_{BC} and P_{HI}) have been extensively studied, few studies on antigenicity have examined substitutions in the minor hydrophilic loop P_{DE} (Vakharia et al., 1994). Our molecular epidemiology data suggest loop P_{DE} may be important in the antigenic drift of variant IBDV strains because mutations at position 254 were frequently observed in viruses that could break through maternal immunity to IBDV (Jackwood and Sommer-Wagner, 2005; Jackwood et al., 1997).

In the present study, the data indicate the T222A and S254N point mutations created a measurable antigenic change in Del-E that was detected in this controlled vaccination/challenge study. The Del-E vaccine 89/03 was administered according to the manufacturer's instructions and induced neutralizing and ELISA antibody titers to the virus. At the time of challenge, these titers were relatively low, but it has been shown that humoral and cellular immunity are important in protection against IBDV infections (Rautenschlein et al., 2002). We did not measure the cellular immune response to the 89/03 vaccine, but it is clear that the immunity induced by this vaccine protected the SPF chickens from disease following challenge with an antigenically homologous and virulent Del-E virus. Immunity to the 89/03 vaccine also appears to have appreciably affected infection since we were unable to detect RNA from the homologous Del-E challenge virus in the bursa of vaccinated birds. We were able to detect the Del-E-222 and Del-E-254 following challenge of 89/03 vaccinated birds using this real-time RT-PCR assay. The relative quantities of each of these challenge viruses were determined between the non-treated group (unvaccinated) and treated group (vaccinated) using a comparative Ct method. The ΔCt values indicated that immunity induced by vaccination with the Del-E 89/03 virus did not significantly alter the quantity of Del-E-222 and Del-E-254 challenge viruses detected in the bursa.

The variant IBDV including Del-E typically produces a bursa lesion score in the range of 2-3 in susceptible SPF chicks (Perozo et al., 2009). Although the bursa lesion score is a subjective measure, the strength of the challenge for all three viruses (Del-E, Del-E-222, and Del-E-254) appeared to be similar. Although this may be considered a weak challenge compared to classic and very virulent IBDV strains, too strong a challenge could overwhelm the immunity produced by the vaccine and thus produce false positive data. The fact that a weak challenge was still capable of breaking through the vaccine induced immunity is strong evidence that the amino acid mutations at positions 222 and 254 in VP2 were contributing to antigenic drift.

The results support our hypothesis that a single point mutation can significantly contribute to antigenic drift in IBDV. Although the 222 and 254 amino acid positions in hvVP2 appear to be important for antigenic drift in Del-E variant viruses, it is possible that substituting different amino acids at these sites would not have significantly altered antigenicity. Furthermore, monoclonal antibody studies suggest the 222 and 254 amino acid sites are not the only two that may be contributing to antigenic drift (Eterradossi et al., 1997; Letzel et al., 2007; Vakharia et al., 1994). Nevertheless, these studies contribute to our understanding of the amino acids that cause antigenic drift *in vivo* and the ability of IBDV to evade immunologic control efforts used in the poultry industry.

The S→N mutation at 254 was produced using reverse genetics in this study, and although we have observed many Del-E variant strains of IBDV with this mutation, all have substitutions in at least one other site in hvVP2 (Jackwood and Sommer-Wagner, 2005). Thus, we were unable to study the contribution of the 254 site to antigenicity using a naturally occurring mutant of the Del-E variant virus. The T→A mutation at amino acid 222 has only been observed in a few variant strains of IBDV, but alanine at this position is a common substitution in the very virulent (vv) IBDV strains. Finding this single point mutation in a naturally occurring Del-E variant is important not only because it can significantly alter antigenicity but also because it may represent a new class of variant viruses that appear to be breaking through maternal immunity to the Del-E type variant vaccines. Epidemiological studies are needed to determine if the A222 mutant virus is becoming widespread and replacing Del-E strains in the United States. The spread of this virus may be affected by classic virus vaccines. Classic vaccines have been shown to be efficacious against vvIBDV strains, which are A222 viruses (Van Den Berg and Meulemans, 1991), but other amino acid differences in the hvVP2 region of the variant A222 viruses may circumvent the utility of these classic vaccines. Studies to determine if a classic virus vaccine would protect against the A222 variant viruses are needed.

Materials and methods

Viruses

The variant Del-E strain was used for the production of full-length clones and reverse genetic experiments. The Del-E vaccine strain-designated 89/03 (Intervet, Schering-Plough Animal Health, Millsboro, DE) has an identical amino acid sequence to the variant Del-E except for two mutations in VP2 that contribute to attenuation and allow replication in cell culture; histidine at position 253 and threonine at position 284 (Brandt et al., 2001; Mundt, 1999). These mutations do not appear to affect antigenicity as 89/03 is widely used in the poultry industry to successfully vaccinate against the virulent Del-E strain (Intervet, Schering-Plough Animal Health) (Rosenberger, 1986). The BGM-70 cell line is used routinely in our laboratory to isolate and propagate IBDV strains (Dahling and Wright, 1986). The 89/03 vaccine strain of Del-E was propagated in BGM-70 cells.

The Del-E-222 virus was identified using molecular epidemiology and had an identical sequence in both genome segments to Del-E (AF133904 and AF133905) except for the T→A mutation at position 222 in genome segment A (Fig. 1) (Jackwood and Sommer-Wagner, 2005). This virus was propagated in 9-day-old embryonated chicken eggs inoculated via the chorioallantoic membrane using standard procedures (Jackwood et al., 2009). Lesions typical of a Del-E infection were observed in the embryos 7 days following inoculation.

The Del-E-254 virus was generated using reverse genetics and had an identical amino acid sequence to Del-E except for a substitution mutation S→N at amino acid position 254. Following the rescue of this virus and propagation in 4-week-old SPF chickens, the titer was determined by inoculating the chorioallantoic membrane of 9-day-old embryonated chicken eggs (Jackwood et al., 2009).

Reverse genetics

The full-length genome segments A and B from Del-E were prepared using RT-PCR (Mundt and Vakharia, 1996). The forward primers contained the T7 promoter region and an *Eco*RI sequence (Table 1). The full-length products were ligated into pCR-XL-TOPO (Invitrogen Corp., Carlsbad, CA), and the products were used to transform DH5α *Escherichia coli*. Transformed bacteria were grown on LB agar containing 50 μg/ml kanamycin.

The GeneTailor site-directed mutagenesis system (Invitrogen Corp.) and mutation primers (Table 1) were used to create a mutation at amino acid 254 in genome segment A. The mutant full-length cDNA of genome segment A and the full-length genome segment B cDNA were sequenced (University of Wisconsin biotechnology Center DNA Sequence Facility, Madison, WI) and analyzed using our standard procedures (Jackwood et al., 2008). These cDNAs were excised from the plasmids using *Eco*RI and used in a transcription reaction that employed the T7 promoter (Invitrogen Corp.). The segment A and B RNA transcripts were combined and prepared for transfection into BGM-70 cells (Dahling and Wright, 1986) using the Lipofectamine 2000 reagent (Invitrogen Corp.). Briefly, the transcripts were added to 50 μl of Opti-MEM I medium (Invitrogen Corp.) and in a separate tube

Table 1
Primers used in site-directed mutagenesis and to amplify genome segments A and B.

Primers[a]	Primer Sequences 5′–3′
S254N site-directed mutant—forward	GTTCAAAACAAGCGTCCAAAaCCTTGTACTGG
S254N site-directed mutant—reverse	TGGACGCTTGTTTTGAACACGAGCTCTC
Full-length segment A—forward	AGA**GAATTC***TAATACGACTCACTATAGG*ATACGATGGGTCTGAC
Full-length segment A—reverse	AGGGGACCCGCGAACGGATCC
Full-length segment B—forward	AGA**GAATTC***TAATACGACTCACTATAGG*ATACGATGGGTCTGACC
Full-length segment B—reverse	GGGGGCCCCCGCAGGCG

[a] The overlapping sequences of the site-directed mutagenesis primers are underlined, and the mutation is shown in lower case. For the full-length primers, the *Eco*RI site is in bold, the T7 promoter sequences are in italics, and the IBDV-specific sequences are underlined. The IBDV-specific sequences of the full-length primers were similar to those previously published (Jackwood et al., 2008), and the forward full-length primers were modified according to Mundt and Vakharia (1996).

Table 2
Antibody titers to IBDV in SPF chickens [a].

Group	Treatment vaccine/challenge[b]	Virus neutralization[c]	ELISA[d]
1	Control	<100	<50
2	89/03/None	264	125
3	None/Del-E	3763	7769
4	89/03/Del-E	4081	8428
5	None/Del-E-222	4579	7852
6	89/03/Del-E-222	5571	6046
7	None/Del-E-254	8163	6646
8	89/03/Del-E-254	4688	3878

[a] Birds were vaccinated at 10 days of age and challenged at 21 days of age.
[b] Serum samples were collected prior to challenge on day 21 in Groups 1 and 2 and at 7 days post-challenge in Groups 3, 4, 5, 6, 7 and 8.
[c] Virus neutralization titers are the geometric means of 5 samples.
[d] The IBD-XR ELISA (IDEXX, Corp.) was used to assay the sera. Each serum sample was tested in duplicate. Each titer represents the geometric mean of 5 samples.

the 2 µl of Lipofectamine 2000 reagent was added to 50 µl Opti-MEM I medium. After 5 min at room temperature, the mixtures were combined and incubation at room temperature continued for 20 min. Following the addition of the Lipofectamine/RNA transcript mixture to the BGM-70 cells, they were incubated at 37 C for 48 h. The transfected cells were then frozen and thawed 3 times before being used to inoculate 4-week-old SPF chickens via the oral/nasal route. At day 4 post-inoculation, the birds were euthanized and bursa tissues were collected. These tissues were examined for the presence of virus using our standard RT-PCR amplification of the hvVP2 region and nucleotide sequencing procedures (Jackwood et al., 2008). To identify the desired S254N substitution and confirm that no other mutations had occurred, the full-length genome segment A and the full-length genome segment B were amplified using RT-PCR and sequenced (University of Wisconsin biotechnology Center DNA Sequence Facility, Madison, WI) (Jackwood et al., 2008).

Vaccination/challenge experiments

These experiments were conducted to examine if a measurable antigenic drift had occurred as a result of a single mutation in VP2. Ten-day-old SPF chickens were randomly assigned to eight groups containing 5 birds each; Group 1, non-vaccinated, non-challenged control; Group 2, 89/03 vaccinated, non-challenged control; Group 3, non-vaccinated, Del-E challenged; Group 4, 89/03 vaccinated, Del-E challenged; Group 5, non-vaccinated, Del-E-222 challenged; Group 6, 89/03 vaccinated, Del-E-222 challenged; Group 7, non-vaccinated, Del-E-254 challenged; Group 8, 89/03 vaccinated, Del-E-254 challenged (Tables 2 and 3).

Vaccination

A live-attenuated Del-E vaccine designated 89/03 (Intervet, USA) was used to vaccinate 10-day-old SPF chickens in Groups 2, 4, 6, and 8 (Table 3). Each bird was given a single dose/bird orally according to the manufacturer's instructions (Intervet, USA).

Challenge

Birds in Groups 3 and 4 were inoculated with the Del-E virus (Table 3). Birds in Groups 5 and 6 were challenged with the Del-E-222 virus and birds in Groups 7 and 8 were challenged with the Del-E-254 virus (Table 3). The challenge viruses were administered orally at a $10^{3.0}$ 50% mean egg infectious dose (EID_{50})/bird. At 7 days following challenge, all birds were euthanized and examined for macroscopic and microscopic signs of disease. Body and bursa weights were recorded and bursa/body weight (B/BW) ratios were calculated.

Histopathology

Bursa tissues were collected at necropsy and placed in 10% neutral phosphate-buffered formalin. The fixed tissues were sectioned at 4 µm and stained using hematoxylin and eosin. They were examined using light microscopy and graded based on the extent of lymphocyte necrosis, follicular depletion, and atrophy. Scores of 0 to 4 were used to indicate relative degree of severity, a score of "0"indicated absence of lesions, and scores 1 to 4 were for <25%, 25–50%, 50–75%, and >75% of the follicles affected, respectively.

Quantitative real-time RT-PCR

To determine viral load in the bursa tissue of IBDV challenged chicks, a relative real-time RT-PCR assay was conducted (Crossley et al., 2009). Following challenge, 0.1 g of each bursa from a treatment group was pooled, homogenized in 1.0 ml TNE buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM ethylene diamine tetra acetic acid) and an 800 µl volume was extracted to obtain RNA using a standard Trizol procedure (Invitrogen, Corp. Carlsbad, CA). The RNA extracted from each tissue pool was placed in a 50 µl volume of 90% DMSO in sterile water, and 1.0 µl was used for each real-time RT-PCR assay. A 743 bp region of the hypervariable sequence region of VP2 (hvVP2) was amplified using primers 743-1: 5′-GCCCAGAGTCTACACCAT-3′ and 743-2: 5′-CCCGGATTATGTCTTTGA-3′. The amplified products were identified using a TaqMan probe designed for the challenge virus (VIC-5′-CTGTTCTCAGCCAACATTGATGC-3′) (Applied Biosystems, Custom Oligo Synthesis Service, Foster City, CA). A real-time RT-PCR assay kit (AgPath-ID, Ambion, Inc., Austin, TX) was used. The RT was at 48 °C for 10 min followed by a 95 °C denaturation for 10 min. Forty cycles of PCR were conducted in a two-step reaction at 95 °C for 15 s and 58 °C for 90 s. Total reaction volumes were 25 µl, and the assays were conducted in a Roche LightCycler 480 instrument (Roche Diagnostics Corp., Indianapolis, IN). Relative quantities of challenge virus in the bursa tissues were determined using a comparative Ct method. Four extractions of each sample were tested in the assay and the ΔCt was determined by subtracting the mean Ct of the non-treated (unvaccinated control) samples from the mean Ct of the treated (vaccinated) samples for a given challenge virus.

Serology

Sera from blood samples collected at necropsy were examined for antibodies to IBDV using the IBD-xr ELISA kit (IDEXX, Inc. Westbrook, ME) according to the manufacturer's instructions. The sera were also tested in an *in vitro* virus neutralization assay previously described (Dybing and Jackwood, 1998). The assay was conducted in BGM-70 cells grown in 96-well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). The 89/03 vaccine virus was used as antigen. Sera were diluted twofold and tested against 100 median tissue culture infectious doses ($TCID_{50}$) of virus. Neutralizing antibody

Table 3
Mean bursa body weight (B-BW) ratios and the bursal histopathology scores for SPF chickens following challenge [a].

Group	89/03 Vaccinated	Del-E challenge	Del -E-222 challenge	Del-E-254 challenge	Mean bursa/body weight ± SD[b]	Histopathologic lesion score[c]	Mean Ct ± SD[d]
1	−	−	−	−	5.58 ± 1.16[e]	1,0,0,0,0	Neg
2	+	−	−	−	5.49 ± 0.91[e]	0,0,0,1,0	Neg
3	−	+	−	−	2.72 ± 0.78[f]	2,2,3,2,2	31.75 ± 0.97
4	+	+	−	−	4.82 ± 0.92[e]	0,1,0,0,1	Neg
5	−	−	+	−	1.82 ± 0.59[f]	2,3,3,4,3	31.75 ± 1.71
6	+	−	+	−	1.90 ± 0.48[f]	2,2,2,2,3	32.00 ± 1.41
7	−	−	−	+	1.62 ± 0.24[f]	2,3,4,2,3	31.25 ± 0.50
8	+	−	−	+	2.14 ± 0.31[f]	2,3,3,2,2	31.50 ± 0.58

[a] Specific-pathogen-free chickens were vaccinated with the Del-E attenuated strain 89/03 at 10 days of age. Each bird received $10^{3.0}$ EID_{50} in 0.1 ml orally. At 21 days of age (11 days post-vaccination), birds were challenged with Del-E, Del-E-222, or Del-E-254. Each bird challenged received $10^{3.0}$ EID_{50} in 0.1 ml orally.
[b] The mean B-BW ratios ± standard deviations for 5 samples were calculated. Mean B-BW ratios = bursa wt. (g)/body wt (g) × 1000. Different superscript letters indicate statistically significant differences among the group means ($p \leq 0.05$).
[c] The severity of the microscopic lesions was graded based on the extent of the lymphocyte necrosis, follicular depletion, and atrophy. Scores of 0 to 4 were used to indicate relative degree of severity, a score of '0'indicated absence of lesions, and scores 1 to 4 were for <25%, 25 to 50%, 50 to 75%, and >75% of follicles affected, respectively. Individual scores for each of the five birds examined are reported.
[d] The mean cycle threshold (Ct) values ± standard deviation were used to determine relative vial load. Four replicates of each sample were tested in the real-time RT-PCR assay. Neg = real-time RT-PCR was negative.

titers were reported as the geometric mean of the last dilution that neutralized 50% of the replicates.

Statistical analysis

The B/BW ratios were compared by ANOVA and Fisher's least squares test for differences among groups.

Acknowledgments

This work was funded in part by funds appropriated to the Ohio Agricultural Research and Development Center and by funds from the US Poultry and Egg Association, Tucker, GA. The authors thank Dr. S. G. Krakowka, Veterinary Biosciences Department, College of Veterinary Medicine, The Ohio State University, for histopathologic analysis of bursa tissue samples.

References

Brandt, M., Yao, K., Liu, M., Heckert, R.A., Vakharia, V.N., 2001. Molecular determinants of virulence, cell trophism, and pathogenic phenotype of infectious bursal disease virus. J. Virol. 75, 11974–11982.
Cosgrove, A.S., 1962. An apparently new disease of chickens—Avian Nephrosis. Avian Dis. 6, 385–389.
Coulibaly, F., Chevalier, C., Gutsche, I., Pous, J., Navaza, J., Bressanelli, S., Delmas, B., Rey, F.A., 2005. The Birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell 120, 761–772.
Crossley, B.M., Jackwood, D.J., Woods, L., Kinde, H.H.S.K., 2009. Bench validation of a high throughput real-time RT PCR assay to detect very virulent infectious bursal disease virus. American Association of Veterinary Laboratory Diagnosticians Conf Abstr #52.
Dahling, D.R., Wright, B.A., 1986. Opitmization of the BGM cell line culture and viral assay procedures for monitoring viruses in the environment. Appl. Envrionmental Microbiol. 51, 790–812.
Dybing, J.K., Jackwood, D.J., 1998. Antigenic and immunogenic properties of baculovirus-expressed infectious bursal disease viral proteins. Avian Dis. 42, 80–91.
Eterradossi, N., Toquin, D., Rivallan, G., Guittet, M., 1997. Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. Arch. Virol. 142, 255–270.
Eterradossi, N., Arnauld, C., Toquin, D., Rivallan, G., 1998. Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. Arch. Virol. 143, 1627–1636.
Giambrone, J.J., Eidson, C.E., Kleven, S.H., 1977. Effect of infectious bursal disease on the response of chickens to *Mycoplasma synoviae*, Newcastle disease virus, and infectious bronchitis virus. Am. J. Vet. Res. 251–253.
Heine, H.G., Haritou, M., Failla, P., Fahey, K., Azad, A.A., 1991. Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strains. J. Gen. Virol. 72, 1835–1843.
Jackwood, D.J., Sommer-Wagner, S.E., 2005. Molecular epidemiology of infectious bursal disease viruses: distribution and genetic analysis of newly emerging viruses in the United States. Avian Dis. 49, 220–226.
Jackwood, D.J., Jackwood, R.J., Sommer, S.E., 1997. Identification and comparison of point mutations associated in classic and variant infectious bursal disease viruses. Virus Res. 49, 131–137.
Jackwood, D.J., Sommer, S.E., Knoblich, H.V., 2001. Amino Acid comparison of infectious bursal disease viruses placed in the same or different molecular groups by RT/PCR-RFLP. Avian Dis. 45, 330–339.
Jackwood, D.J., Sreedevi, B., LeFever, L.J., Sommer-Wagner, S.E., 2008. Studies on naturally occurring infectious bursal disease viruses suggest that a single amino acid substitution at position 253 in VP2 increases pathogenicity. Virology 377, 110–116.
Jackwood, D.J., Sommer-Wagner, S.E., Stoute, S.T., Woolcock, P.R., Crossley, B.M., Hietala, S.K., Charlton, B.R., 2009. Characteristics of a very virulent infectious bursal disease virus from California. Avian Dis. 53, 592–600.
Letzel, T., Coulibaly, F., Rey, F.A., Delmas, B., Jagt, E., van Loon, A., Mundt, E., 2007. Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. J. Virol. 81, 12827–12835.
Mundt, E., 1999. Tissue culture infectivity of different strains of infectious bursal disease virus is determined by distinct amino acids in VP2. J. Gen. Virol. 80, 2067–2076.
Mundt, E., Vakharia, V.N., 1996. Synthetic transcripts of double-stranded Birnavirus genome are infectious. Proc. Natl Acad. Sci. USA 93, 11131–11136.
Perozo, F., Villegas, P., Cruz, J., Pritchard, N., 2009. Efficacy of single dose recombinant Herpesvirus of Turkey infectious bursal disease virus (IBDV) vaccination against a variant IBDV Strain. Avian Dis. 53, 624–628.
Rautenschlein, S., Yeh, H.-Y., Njenga, M.K., Sharma, J.M., 2002. Role of intrabursal T cells in infectious bursal disease virus (IBDV) infection: T cells promote viral clearance but delay follicular recovery. Arch. Virol. 147, 285–304.
Rosenberger, J.K., 1986. Update on Delmarva respiratory complex and use of variant IBDV vaccines. Proc.Poultry Health Meeting, Ocean City, MD.
Rosenberger, J.K., Klopp, S., Eckroade, R.J., Krauss, W.C., 1975. The role of the infectious bursal agent and several avian adenoviruses in the hemorrhagic–aplastic–anemia syndrome and gangrenous dermatitis. Avian Dis. 19, 717–729.
Vakharia, V.N., He, J., Ahamed, B., Snyder, D.B., 1994. Molecular basis of antigenic variation in infectious bursal disease virus. Virus Res. 31, 265–273.
Van Den Berg, T.P., Meulemans, G., 1991. Acute infectious bursal disease in poultry: protection afforded by maternally derived antibodies and interference with live vaccination. Avian Pathol. 20, 409–421.
Van Den Berg, T.P., Enterradossi, N., Toquin, D., Meulemans, G., 2000. Infectious bursal disease (Gumboro disease). Rev. Sci. Tech. Off. Int. Epiz. 19, 527–543.