AVIAN DISEASES 51:597–600, 2007

*Research Note—*

# Molecular and Phenotypic Characterization of Infectious Bursal Disease Virus Isolates

T. V. Dormitorio,[A] J. J. Giambrone,[AC] K. Guo,[A] and D. J. Jackwood[B]

[A]Department of Poultry Science, Auburn University, Auburn, AL 36849
[B]Food Animal Health Research Program, Ohio State University, Wooster, OH 44691

Received 15 August 2006; Accepted 19 December 2006

SUMMARY. Two infectious bursal disease viruses (IBDVs 1174 and V1) were isolated from IBDV-vaccinated broiler flocks in California and Georgia. These flocks had a history of subclinical immunosuppression. These isolates are commonly used in IBDV progeny challenge studies at Auburn, AL, as well as vaccine manufacturer's vaccine efficacy studies, because they come from populated poultry-producing states, and are requested by poultry veterinarians from those states. Nested polymerase chain reaction (PCR) generated viral genome products for sequencing. A 491-bp segment from the VP2 gene, covering the hypervariable region, from each isolate was analyzed and compared with previously sequenced isolates. Sequence analysis showed that they were more closely related to the Delaware (Del) E antigenic variant than they are to the Animal Health Plant Inspection Service (APHIS) standard, both at the nucleotide level (96%, 97%) and at the amino acid level (94%, 97%). Both isolates had the glutamine to lysine shift in amino acid 249 which has been reported to be critical in binding the virus neutralizing Mab B69. Phenotypic studies showed that both isolates produced rapid atrophy of the bursae and weight loss, without the edematous bursal phase, in 2-wk-old commercial broilers having antibody against IBDV. A progeny challenge study showed both isolates produced more atrophy of the bursae (less percentage of protection) than the Del E isolate. Molecular and phenotypic data of these important IBDV isolates help in the improved detection and control of this continually changing and important viral pathogen of chickens.

RESUMEN. *Nota de Investigación*—Caracterización molecular y fenotípica de aislamientos del virus de la enfermedad infecciosa de la bolsa.

Se aislaron dos virus de la enfermedad infecciosa de la bolsa (1174 y V1) de parvadas de pollos de engorde vacunadas contra el virus de la enfermedad infecciosa de la bolsa provenientes de los estados de California y Georgia en los Estados Unidos. Las parvadas tenían una historia de inmunosupresión subclínica. Estos aislamientos son comúnmente utilizados en estudios de desafío de progenie en Auburn, Alabama, así como en estudios de eficacia realizados por los fabricantes de vacunas, esto debido a que los aislamientos provienen de estados con alta población avícola y son requeridos por los veterinarios avícolas de esos estados. Para secuenciación, se generaron productos de genoma viral mediante una prueba anidada de reacción en cadena por la polimerasa. De cada uno de los aislamientos se analizó un segmento de 491 pares de bases del gen de la proteína viral 2, que cubre la región hipervariable del virus y se compararon con aislamientos previamente secuenciados. El análisis de la secuencia mostró que tanto a nivel de nucleótidos (96%, 97%) como a nivel de aminoácidos (94%, 97%), los aislamientos estaban mas cercanamente relacionados con la variante antigénica Delaware E que con la cepa estándar del servicio de inspección de salud animal. Ambos aislamientos presentaron el cambio del aminoácido glutamina por lisina en la posición 249, que ha sido reportado como crítico en la unión del virus al anticuerpo monoclonal neutralizante B69. Estudios fenotípicos demostraron que ambos aislamientos producen atrofia rápida de la bolsa y pérdida de peso en pollos de engorde comerciales de dos semanas de edad con anticuerpos maternales contra el virus de la enfermedad infecciosa de la bolsa, sin presentar la fase edematosa de la bolsa. Un estudio de desafío de progenie demostró que ambos aislamientos producen mayor atrofia de la bolsa (menor protección) que el aislamiento variante Delaware E. La información molecular y fenotípica de estos importantes aislamientos ayuda a mejorar la detección y el control de este importante patógeno viral de las aves que cambia constantemente.

Key words: infectious bursal disease virus, nested reverse transcription–polymerase chain reaction, sequencing, VP2 hypervariable region, vaccination

Abbreviations: APHIS = Animal Health Plant Inspection Service; AU = Auburn University; Del = Delaware; DEPC = diethyl pyrocarbonate; EDTA = ethylenediaminetetraacetic acid; ELISA = enzyme-linked immunosorbent assay; ETOH = ethyl alcohol; HVR = hypervariable region; IBDV = infectious bursal disease virus; PCR = polymerase chain reaction; PI = postinfection; RFLP = restriction fragment length polymorphism; RT = reverse transcription; SPF = specific pathogen free; VP = viral protein

Infectious bursal disease viruses (IBDVs) cause a contagious and common immunosuppressive disease in young chickens by targeting developing B lymphocytes located within the bursa of Fabricius. Despite the use of vaccine viruses from different antigenic subtypes of serotype I, chickens can be infected with antigenic IBDV variants that can breakthrough maternal antibodies and cause subclinical immunosuppression.

Molecular assays can identify and characterize IBDVs to help understand the molecular basis of antigenic variation and pathogenicity. This information can be used to develop new and more effective detection methods, vaccines, and vaccine efficacy testing. The reverse transcription (RT), polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) assay can place viruses into genetically related groups called molecular groups (8). Using this assay, 49% of IBDVs detected from 16 countries outside the United States, had the same RFLP pattern with *Bst*NI and *Mbo*I enzymes and were placed in Group 6 (9). The RFLP pattern of the 1174, also known as L1 strain, was previously published and placed in Group 6, because its RFLP pattern was identical to Group 6 pattern, except for an extra *Mbo*I band at 112 bp (9). Its sequence was also similar to the Group 6 viruses. The RFLP pattern of V1 was

[C]Corresponding author. 201 Poultry Science Building, Auburn University, Auburn, AL 36849-5416. E-mail: giambjj@auburn.edu

also reported and did not to belong to any group. In a similar molecular identification survey, IBDV isolates were found in 69.2% of 78 broiler flocks from 10 states in the United States (15). Most of the isolates, 53.6%, were "molecular variants," while 19.2% were of the Delaware (Del) E type. Because of these studies, vaccine manufacturers as well as independent laboratories include an IBDV strain that belongs in Group 6 in their challenge studies when determining vaccine efficacy. Our Auburn studies also use at least one Group 6 virus in broiler progeny challenge studies (6). These studies are used by industry veterinarians to determine the efficacy of their pullet vaccination programs.

The structural protein VP2 is the most widely studied fragment of the IBDV genome. It is the major antigen that elicits a host-protective immune response and contains at least three independent epitopes responsible for induction of virus-neutralizing antibodies in chickens (1,2). The major neutralizing epitope is located within the 145 amino acid fragment known as the hypervariable region (HVR). In this site certain amino acid changes may cause antigenic variation (1,7). The sequence of the variable VP2 fragment of isolates 1174 and V1 was determined and compared with previous isolates. This was done to identify genetic properties that are consistent with antigenic type and pathogenicity. Genomic characterization was by RT, nested PCR, and direct sequencing of variable region of VP2. Phenotypic studies were done using antigenic testing in broiler progeny challenge trials.

## MATERIALS AND METHODS

**Experimental chickens.** Specific pathogen free (SPF) broilers from an Auburn University (AU) flock were used for virus propagation in molecular studies, and commercial broilers from flocks from Georgia and California were used for progeny challenge studies. Chickens were housed in modified Horsfall–Bauer isolation units maintained with filtered air under negative pressure. All were given feed and water *ad libitum* and reared according to AU's Institutional Animal Care and Use Committee standards.

**Virus propagation.** IBDV isolates, 1174 and V1, were from IBDV-vaccinated broilers in California and Georgia, respectively, which had a history of subclinical immunosuppressive infections. Viruses were propagated in 3-wk-old SPF broilers. Each bird was inoculated intranasally and subconjunctively with 50 µl of a virus suspension containing at least 10 chicken infectious doses per milliliter. At 3 days postinfection (PI), the birds were euthanatized and bursae removed. The bursae were placed in NET buffer (100 mM NaCl, 1 mM ethylene-diaminetetraacetic acid [EDTA], 10 mM Tris, pH 8.) and stored at −70 C until used.

**IBDV RNA extraction.** Virus-infected frozen bursae were thawed, then homogenized with equal volume of RNA extraction buffer (10 mM Tris, 10 mM NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate, 0.1% diethyl pyrocarbonate [DEPC] in a Virtis homogenizer [Virtis Co. Inc., Gardiner, NY]). Samples were frozen at −70 C for 5 min, thawed then centrifuged at 2400 × *g* for 15 min. Supernatant was removed and digested with 100 µl of proteinase K at 37 C for 1 hr. An equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1) was added. Mixture was centrifuged at room temperature and 12,000 × *g* for 15 min. Supernatant containing RNA was aspirated and precipitated by adding 2.5 volumes of 100% ethyl alcohol (ETOH) and 1/10 volume of 3 M sodium acetate. After an overnight precipitation at −70 C, the mixture was centrifuged at 12,000 × *g* for 30 min. Supernatant was removed, the pellet washed with 70% cold ETOH, dried and suspended in 30 µl DEPC-treated water.

**RT-PCR and nested PCR primers.** Primers flanked the variable region of the VP2. These were selected from the sequence of the plus strand of segment A–cloned cDNA of standard Animal Health Plant Inspection Service (APHIS)-IBDV (11). The APHIS strain is an antigenic standard strain used in most IBDV challenge studies. Sequences of the outer set of primers were as follows: P1 (5′-TCACCGTCCTCAGCTTAC-3′; corresponds to positions 587–604) and P2 (5′-TCAGGATTTGGGATCAGC-3′; complementary to positions 1212–1229). Sequences of the inner set of primers were as follows: P3 (5′-GCCCAGAGTCTACACCATAACTGC-3′; corresponds to positions 703–726) and P4 (5′-GCGACCGTAACGACA-GATCC-3′; complementary to positions 1174–1193).

Reagents and protocols in the Perkin-Elmer's RT-PCR kit (Perkin-Elmer Co., Norwalk, CT) were used for RT, PCR, and nested PCR and were described previously (12). Nested PCR products were purified using Magic™ PCR Preps DNA purification kit (Promega Co., Madison,

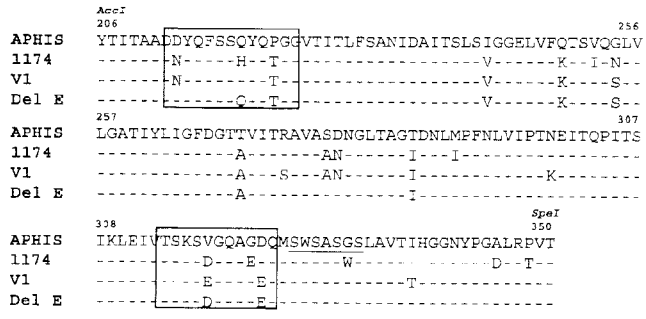


Fig. 1. Deduced amino acid sequences of the central *AccI–SpeI* (206–350) region of the VP2 gene of four IBDV isolates (APHIS, 1174 [GenBank accession no. AF303895]), V1 [GenBank accession no. AF303896], and Del E). The two hydrophilic regions are in boxes. Dashes indicate the same amino acid as that of APHIS. A heptapeptide conserved in virulent isolates only is underlined (7).

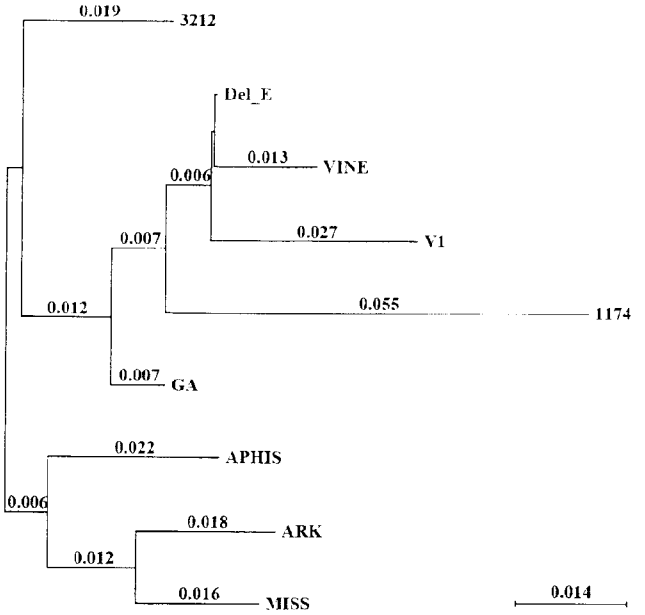


Fig. 2. Phylogenetic analysis of the amino acid sequences of the variable fragment of VP2 gene region of 1175, V1, and other published IBDV isolates using the Vector NTI® Software. The tree was generated with Mega2 program by neighbor-joining method with 1000 bootstrap replicates. Dendrogram was drawn using PHYLO_WIN (5). The higher the distance number when added up indicates the farther the two isolates are from each other.

Table 1. Percent base and amino acid identities for the 435-bp hypervariable fragment of various IBDV isolates.[A]

| | 1174 | V1 | Del E | APHIS |
|---|---|---|---|---|
| 1174 | | 94 | 96 | 93 |
| V1 | 91 | | 97 | 94 |
| Del E | 94 | 97 | | 96 |
| APHIS | 90 | 91 | 94 | |

[A]Percent nucleotide identity is in upper triangle; percent amino acid identity is in lower triangle.

WI). Nested PCR was used because sufficient RNA could be produce from one bursa.

**Sequencing and sequence analysis.** Purified 491-bp PCR products were used as templates for automated sequencing by ABI-373A (Perkin-Elmer Co.). Primers (P3 and P4) and templates were sent to Scott-Ritchey Research Center (College of Veterinary Medicine, AU) for sequencing.

Base sequence print-outs generated by the ABI-373A machine were entered into the Vector NTI® software (Invitrogen, Carlsbad, CA) for analysis such as amino acid transcription, percent identity, and amino acid sequence multiple alignment with other previously sequenced strains. The same software was used for phylogenetic analyses. The tree was generated with Mega2 program by neighbor-joining method with 1000 bootstrap replicates. Dendrogram was drawn using PHYLO_WIN (5).

**Progeny challenge studies.** This was done using 2-wk-old broilers from commercially vaccinated pullet flocks according to a previous publication (6). The six flocks, from which the broilers were obtained, received multiple live standard and killed standard and variant (Del E) viruses as pullets. Three flocks were from one company and the other three from a different company. The companies used different vaccination programs. No attempt was made to compare the vaccine programs to avoid commercialism. For each flock, broilers were placed in four challenged groups (20 chickens per group). One group was not challenged, one received the APHIS, one Del E, one 1174, and one V1 at the same dosages ($10^{3.5}$ chicken infective doses) by eye and nose drop. At 1 wk PI, all birds were killed and bursal to body weight ratios determined. The mean and standard deviation were calculated for all groups using the SAS software package (SAS Institute, Inc., Cary, NC). A multiple comparison test was used to compare percent protection scores of challenged groups, and bursa weight and body weight *vs.* control group. All bursa with bursal to body weight ratios 2 standard deviations below the nonchallenged control groups were considered atrophied (not protected). A percent protection score was determined based on the percentage of birds within a flock that were protected. Percent protection scores below 30 were considered poor and correlate with poor performance in the field (6). At day of age, 20 chicks per flock were sacrificed and blood was taken for maternal antibody determination using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Affinitech Ltd., Bentonville, AR).

## RESULTS AND DISCUSSION

A 491-bp product was generated from a single virus-infected bursa, using the simplified RNA extraction procedure and nested PCR. It was used as template for automated sequencing. Fig. 1 shows amino acid sequences of the hypervariable region of the VP2 gene of four isolates. The boxed hydrophilic regions are important in binding of neutralizing monoclonal antibodies and are presumed to be a main part of the neutralizing domain (7). In residue 222, located within the first hydrophilic region, there is a change from proline to threonine on both 1174 and V1 isolates. This amino acid is threonine, serine, or glutamine in the U.S. variants, and alanine in very virulent IBDVs in United Kingdom, Dutch, and Japanese isolates (3,4).

Proline is an amino acid that introduces a bend in polypeptide chain and, therefore, this change can disrupt epitope conformation and consequently virus evasion of neutralization. A shift in the antigenicity was predicted by the lack of the Mab B69 epitope on field strains of IBDV (14). It was shown that amino acid 249 is critical in binding the virus neutralizing B69, which variants failed to bind because of a glutamine to lysine substitution (16). Both 1174 and V1 had the glutamine to lysine shift. The serine rich heptapeptide, SWSASGS, has been hypothesized to be involved in virulence and shown to be conserved in all virulent isolates (3). Isolate 1174 had tryptophan instead of glycine in this motif; however, this change cannot be correlated with virulence in this present study. Furthermore, it has been reported that VP2 is not the sole determinant of IBDV virulence, and that VP1 (RNA polymerase protein) may also play an important role (17).

Table 1 and Fig. 2 showed the relatedness of the 1174 and V1 to other IBDV strains including two of the most commonly used IBDV isolates (Del E and APHIS) in challenge and vaccine efficacy studies. In addition, APHIS and Del E (13) are used in many commercial inactivated vaccines. The two isolates are more closely related to Del E than they are to APHIS or to each other. Both isolates especially V1, which was *Spe*I negative (data not shown) just like other vaccine or attenuated strains, may be vaccine derived. This possibility is based on the fact that the Del E strain is included in some live vaccines and the virus may be shed by the birds, undergo mutation, and evade neutralization. Furthermore, it has been reported that with the discovery of reassortant viruses in nature, there is an additional risk of using live IBDV vaccines. These vaccine strains could act as genetic donors for genome reassortment. A Chinese IBDV reassortant, ZJ2000, has been identified, whose genomic segment A is derived from an attenuated strain, and whose segment B is derived from a very virulent strain (17).

Phenotypic studies showed that 1174 and V1 are similar to other antigenic variants such as Del E, in that they cause rapid atrophy of

Table 2. Bursa and body weights of commercial broilers at 1 wk postinfection with Del E, 1174, and V1 isolates *vs.* uninoculated control group.

| | Del E | | 1174 | | V1 | | Control | |
|---|---|---|---|---|---|---|---|---|
| Flocks | Body weight (kg) | Bursa weight (g) | Body weight (kg) | Bursa weight (g) | Body weight (kg) | Bursa weight (g) | Body weight (kg) | Bursa weight (g) |
| 1 | 798 | 1.227* | 687** | 1.008* | 616** | 1.000* | 865 | 1.675 |
| 2 | 858 | 1.021* | 703** | 0.901* | 798** | 0.899* | 903 | 1.416 |
| 3 | 809 | 1.018* | 698** | 0.910* | 653** | 0.759* | 806 | 1.391 |
| 4 | 795 | 0.802* | 721** | 0.649* | 709** | 0.631* | 804 | 1.566 |
| 5 | 801 | 1.035* | 691** | 0.798* | 714** | 0.695* | 784 | 1.455 |
| 6 | 785 | 1.019* | 725** | 0.689* | 683** | 0.628* | 790 | 1.385 |

*Bursa weights are significantly lower than control group within the same flock at $P < 0.05$.

**Body weights are significantly lower than control group within the same flock at $P < 0.05$.

Table 3. IBDV serology and challenge data for two-wk-old broiler progeny.

| Flocks | % Protection scores[A] | | | GMT titer[B] |
|---|---|---|---|---|
| | Del E | 1174 | V1 | |
| 1 | 55 | 42* | 35* | 4930 |
| 2 | 46 | 33 | 40 | 2784 |
| 3 | 62 | 41* | 28* | 3830 |
| 4 | 40 | 28* | 25* | 2209 |
| 5 | 63 | 40* | 48* | 3290 |
| 6 | 58 | 31* | 27* | 3705 |

[A]Percentage of birds within a flock that were protected (bursa not atrophied).

[B]Day old geometric mean antibody titers against IBDV determined by ELISA.

*Statistically lower than the Del E challenged group within the same row ($P \leq 0.05$).

the bursae without the inflammatory phase (Table 2). However, in contrast to other variants (14), these two viruses produced decreased weight gain in progeny challenge studies (Table 2). Table 3 also showed that the 1174 and V1 variants produced more atrophy (less percent protection) in commercial broilers, than did the Del E isolate, indicating that the 1174 and V1 differ antigenically from the Del E virus. Both isolates caused significant atrophy of the bursae in birds with maternal immunity against IBDV. Maternal immunity was measured by ELISA on serum of day-old progeny of breeders that have been vaccinated with live and killed vaccines containing Del E strain. As shown in Table 3, all flocks had moderate antibody titers for IBDV, and yet a significantly high percentage of birds had atrophied bursa which is indicative of poor protection against IBDV infection. A previous progeny challenge study (6) showed little correlation between day-old ELISA titers and resistance to variant IBDVs.

Studies have indicated that IBDVs in molecular Group 6 as determined by PCR-RFLP are widespread (10,15). Therefore, vaccine manufacturers include these viruses in their vaccine efficacy trials. It is therefore important to continuously characterize IBDVs as they emerge in the field so that molecular and phenotypic data can be used in field strain identification, vaccination programs, designing effective vaccines, etc. Studies of this nature are necessary as this RNA virus changes continuously due to vaccine pressure.

## REFERENCES

1. Azad, A. A., M. N. Jagadish, M. A. Brown, and P. J. Hudson. Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. Virology 161:145–152. 1987.

2. Becht, H., H. Muller, and H. K. Muller. Comparative studies on structural and antigenic properties of two serotypes of infectious bursal virus. J. Gen. Virol. 69:631–640. 1988.

3. Brown, M. D., P. Green, and M. A. Skinner. VP2 sequences of recent European 'very virulent' isolates of infectious bursal disease virus are closely related to each other but are distinct from those of 'classical' strains. J. Gen. Virol. 75:675–680. 1994.

4. Dormitorio, T. V., J. J. Giambrone, and L. W. Duck. Sequence comparisons of the variable VP2 region of eight infectious bursal disease virus isolates. Avian Dis. 41:36–44. 1997.

5. Galtier, N., M. Gouy, and C. Gautier. SEAVIEW and PHYLO_-WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biosci. 12(6):543–548. 1996.

6. Giambrone, J. J., T. V. Dormitorio, T. Brown, and K. Takeshita. Monitoring of the immune status of broiler breeders against infectious bursal disease virus using progeny challenge and serological data. J. Appl. Poult. Res. 8:362–367. 1999.

7. Heine, H. G., M. Haritou, P. Failla, K. Fahey, and A. A. Azad. 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. 22:1835–1843. 1991.

8. Jackwood, D. J. Molecular identification of infectious bursal disease virus strains. Vineland Update. No. 62. November. 1998.

9. Jackwood, D. J., and S. E. Sommer. Genetic heterogeneity in the VP2 gene of IBDVs detected in commercially reared chickens. Avian Dis. 42:321–339. 1998.

10. Jackwood, D. J., and S. E. Sommer. Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease virus from outside the U.S. Avian Dis. 43:310–314. 1999.

11. Kibenge, F. S. B., D. J. Jackwood, and C. C. Mercado. Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. J. Gen. Virol. 71:569–577. 1990.

12. Liu, X., J. J. Giambrone, and T. V. Dormitorio. Simplified Sample Processing combined with a sensitive nested PCR assay for detection of infectious bursal disease in the bursa of Fabricius. Avian Dis. 42:480–485. 1998.

13. Rosenberger, J. K., S. S. Cloud, and A. Metz. Use of infectious bursal disease virus variant vaccine in broilers and broiler breeders. In: Proc. 36th Western Poultry Disease Conference, Davis, CA. pp. 105–106. 1987.

14. Snyder, D. B., D. P. Lana, P. K. Savage, F. S. Yancey, S. A. Mengel, and W. W. Marquardt. Differentiation of infectious bursal disease viruses directly from infected tissue with neutralizing monoclonal antibodies: evidence of a major antigenic shift in recent field isolates. Avian Dis. 32:535–539. 1986.

15. Takeshita, K. Practical use of molecular infectious bursal disease virus identification. Vineland Update. No. 62. November. 1998.

16. Vakharia, V. N., J. He, B. Ahmad, and D. B. Snyder. Molecular basis of antigenic variation in infectious bursal disease virus. Virus Res. 31:265–273. 1994.

17. Wei, Y., J. Li, J. Zheng, H. Xu, L. Li, and L. Yu. Genetic reassortment of infectious bursal disease virus in nature. J. Biochem. Biophy. Res. Comm. 350:277–287. 2006.