Virology 420 (2011) 98–105

Contents lists available at SciVerse ScienceDirect

Virology

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

Identification and pathogenicity of a natural reassortant between a very virulent serotype 1 infectious bursal disease virus (IBDV) and a serotype 2 IBDV

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ARTICLE INFO

Article history:
Received 27 May 2011
Returned to author for revision 8 August 2011
Accepted 29 August 2011
Available online 28 September 2011

Keywords:
Infectious bursal disease
Infectious bursal disease virus
Birnavirus
Reassortant
Serotypes 1 and 2
Pathogenicity

ABSTRACT

Infectious bursal disease virus (IBDV) causes an economically important, immunosuppressive disease in chickens. There are two serotypes of the virus that contain a bi-segmented double-stranded RNA genome. In December 2008, the first very virulent (vv)IBDV was identified in California, USA and in 2009 we isolated reassortant viruses in two different locations. Genome segment A of these reassortants was typical of vvIBDV serotype 1 but genome segment B was most similar to IBDV serotype 2. The CA-K785 reassortant caused 20% mortality in chickens but no morbidity or mortality in commercial turkey poults despite being infectious. There have been previous reports of natural reassortants between vvIBDV and other serotype 1 strains, but a natural reassortant between IBDV serotypes 1 and 2 has not been described. The apparent reassorting of California vvIBDV with an endemic serotype 2 virus indicates a common host and suggests vvIBDV may have entered California earlier than originally thought.

Introduction

Two serotypes of infectious bursal disease virus (IBDV) have been identified. The serotype 1 viruses typically infect chickens and cause immune suppression in that species (Cosgrove, 1962). Serotype 2 viruses infect several avian species including turkeys but no disease has been attributed to the viruses belonging to this serotype (Chettle et al., 1985; McFerran et al., 1980; McNulty et al., 1979). The IBDV are members of the family *Birnaviridae*, genus *Avibirnavirus*. They have a bi-segmented double-stranded RNA genome. Genome segment A encodes a polyprotein that is self-cleaved by the viral encoded protease VP4 to yield this protease and the structural proteins pVP2 and VP3 (Lejal et al., 2000). The pVP2 protein is further processed to yield VP2 a major virion capsid protein. A non-structural protein designated VP5 is encoded by an overlapping reading frame in this genome segment (Mundt et al., 1995). Genome segment B encodes the RNA directed-RNA polymerase designated VP1 which complexes with VP3 to initiate capsid formation (Lombardo et al., 1999).

A wide range of IBDV serotype 1 pathotypes exist in nature. They have been generally classified into sub-clinical (scIBDV), classic virulent (cvIBDV) and very virulent (vvIBDV) groups (van den Berg et al., 2004). The very virulent form of IBDV was first identified in Belgium during the early 1980s and causes an immunosuppressive disease characterized by high mortality (Chettle et al., 1989; van den Berg et al., 1991). Phylogenetic analysis indicates the vvIBDV have spread to nearly all poultry producing countries in the world including the United States (Eterradossi et al., 1999; Stoute et al., 2009; van den Berg et al., 2000). Phylogenetic studies indicate that all known vvIBDV are closely related (van den Berg, 2000) and molecular studies on the vvIBDV genome segment B indicate it is phylogenetically distinct from all other IBDV strains (Eterradossi et al., 1999; Islam et al., 2001).

The phylogenetic origins and population studies on vvIBDV suggest that the expansion of a single virus was initiated by an event where an endemic IBDV segment A was reassorted with a genome segment B of unknown origin (Hon et al., 2006). The worldwide spread of this vvIBDV confirms that the conserved co-evolution of both genome segments has been favored by the selection for this phenotype (Islam et al., 2001; Le Nouen et al., 2005; Le Nouen et al., 2006). In one study, co-evolution of the two genome segments was observed in approximately 70% of the IBDV isolates examined (Le Nouen et al., 2006). Although co-evolution of the genome segments is conserved, reassortant vvIBDV where segment A is typical of vvIBDV and segment B is from a serotype 1 non-vvIBDV have been reported to occur naturally in regions where these viruses are well established

Abbreviations: IBD, infectious bursal disease; IBDV, infectious bursal disease virus; RT-PCR, reverse transcriptase-polymerase chain reaction; TNE, buffer, 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM ethylene diamine tetra acetic acid; UPGMA, unweighted pair group with arithmetic mean; NJ, Neighbor-Joining; vv, very virulent.

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doi:10.1016/j.virol.2011.08.023

(Gao et al., 2007; Le Nouen et al., 2006; Wei et al., 2008). Two naturally occurring reassortant IBDV reported in China were unique compared to other natural reassortants of this virus because their genome segment A was from an attenuated vaccine strain and genome segment B was from a vvIBDV strain (Wei et al., 2006, 2008). This communication describes the molecular characteristics and pathogenicity of two reassortant IBDV isolates in the U.S. that contain a vvIBDV genome segment A and a serotype 2 genome segment B. This is the first report of a natural reassortant between IBDV serotypes 1 and 2. The phylogenetic origin, population dynamics and potential host for both serotypes in a country where vvIBDV has only recently been identified are discussed.

Results

Molecular characterization of CA-K785 and CA-D495

The RT-PCR amplified genome segment A from CA-K785 and CA-D495 was sequenced. The full-length CA-K785 (JF907702) and CA-D495 (JF907703) genome segment A nucleotide sequences were a 98.6% (3156 bp/3200 bp) match to each other. Of the 44 substitution mutations observed between the two viruses all but 3 were silent. The amino acid sequences across the highly variable (hv)VP2 region of these viruses were identical. Except for one synonymous base change, the hvVP2 sequence regions of genome segment A from CA-K785 and CA-D495 were a 99.8% (742 bp/743 bp) match to the ranch A (rA, GQ221682) and ranch B (rB, GQ221683) vvIBDV strains from California (Jackwood et al., 2009). When the full-length CA-K785 and CA-D495 segment A nucleotide sequences were compared to full-length segment A of the type strain vvIBDV, UK661 (NC004178) the viruses were a 98.2% and 97.9% match, respectively. When compared to the Cu-1 (AF362747) cvIBDV type strain the matches were 95.6% and 95.3%, respectively. Furthermore, the CA-K785 and CA-D495 had the A222, I242, I256, I294 and S299 amino acids typically observed in vvIBDV strains.

Using the real-time RT-PCR assay and probe specific for vvIBDV segment A sequences, both the CA-K785 and CA-D495 samples tested positive as expected (data not shown). When the probe specific for genome segment A of non-vvIBDV strains was used, the assays tested negative. Thus, classic and variant serotype 1 IBDV were not detected in the samples containing these viruses. The real-time RT-PCR assay conducted using primers specific for the hvVP2 encoding region in segment A of serotype 2 IBDV gave negative results when the CA-K785 and CA-D495 samples were tested. Thus, no known serotype 2 strains were detected in these bursa samples.

Genome segment B of vvIBDV is highly conserved and required for their high mortality phenotype (Le Nouen et al., 2006). Sequence characterization of this genome segment was necessary to confirm a molecular identification of vvIBDV. The full-length genome segment B nucleotide sequences of CA-K785 (JF907705) and CA-D495 (JF907704) were a 98.2% (2730 bp/2780 bp) match with each other. When they were compared to the partial segment B sequences of the California vvIBDV strains rA (GQ221684) and rB (GQ221685) they were an 88.1% (468 bp/531 bp) match.

Genome segment B sequence comparison and phylogenetic analysis

The genome segment B nucleotide sequences of the CA-K785 and CA-D495 viruses were not typical of vvIBDV, including the rA and rB California vvIBDV. Thus, we expanded the sequence comparison to include genome segment B sequences from vvIBDV and non-vvIBDV isolated in other parts of the world. Although we sequenced the full-length genome segment B from CA-K785 and CA-D495 our phylogenetic analysis was constrained by the sequences available in GenBank which included nucleotides 312 to 855. The viruses and their GenBank accession numbers were listed by Le Nouen et al. (2006). Segment B sequences used in the analysis included those from seven vaccine strains, thirty-eight wild-type strains representing scIBDV, cvIBDV and vvIBDV, three naturally reassorted strains from Venezuela and two serotype 2 strains from Europe. The genome segment B sequence of the vvIBDV isolate NIE/98/009/t (AY099457) from turkeys (Owoade et al., 2004), the U.S. serotype 2 OH strain (U30819) and four vaccines strains reportedly used in local California chicken flocks [D78 (AJ878654), Bursine (AJ878659), Bursine 2 (AJ878655) and Variant E (AJ878676)] were also included. The NJ and UPGMA phylogenetic analysis yielded identical results so only the NJ tree is shown (Fig. 1). The analysis indicated that the segment B nucleotide sequences of CA-K785 and CA-D495 were most closely related to the serotype 2 strain OH (Fig. 1). The nucleotide sequence identity across the 312–855 bp region of genome segment B was 94.5% (514 bp/544 bp) when the California reassortants were compared to the European serotype 2 viruses TY89 and 23–82. Compared to the serotype 2 OH strain from the U.S., there was a 97.8% (532 bp/544 bp) nucleotide sequence match with CA-K785 and CA-D495 across this 312–855 bp region. When we compared the full-length CA-K785 and CA-D495 genome segment B nucleotide sequences to the full-length OH (U30819) segment B sequence there was a 95.1% (2643 bp/2780 bp) and 96.0% (2669 bp/2780 bp) match, respectively. Although some regions were more conserved than others, the nucleotide mutations were generally spread across the entire sequence suggesting a recombination event had not occurred.

The OH serotype 2 strain was isolated in 1981 from turkeys in Ohio (Jackwood et al., 1982a). Since this is a relatively old strain of IBDV from a different species, more contemporary IBDV strains from commercial chicken flocks in California were examined to determine if their genome segment B sequences matched the CA-K785 and CA-D495 viruses. Partial genome segment B sequences (312–855 bp region) of the 15 non-vvIBDV strains were determined in this study (GenBank Accession Numbers HQ441144–HQ441158). The nucleotide B segment sequences of CA-K785 and CA-D495 were not as closely related to these contemporary California IBDV strains (<94% nucleotide sequence match) compared to the OH serotype 2 virus that had a 97.69% match across the same region. The genome segment B phylogenetic analysis using both NJ (Fig. 2) and UPGMA algorithms placed the CA-K785 and CA-D495 on the same branch but they were distant from the 15 California non-vvIBDV and the rA and rB vvIBDV.

Pathogenicity of CA-K785 in chickens

The ability of the CA-K785 reassortant to cause disease in 4-week-old SPF Leghorn chicks was determined and compared to the rB vvIBDV, STC cvIBDV and Variant E scIBDV. At 2 days following challenge all birds in the CA-K785, rB and STC groups became depressed. They had typical signs of clinical infectious bursal disease that included ruffled feathers, diarrhea, pasty vents and a reluctance to move. No morbidity was observed in the birds challenged with Variant E or in the non-challenged controls. On day 3 following challenge 2/10 birds in the CA-K785 group died and 6/10 birds in the rB group died. By day 4 the remaining birds in the rB challenged group had died. No additional mortality was observed in the CA-K785 challenged birds and no mortality was observed in the STC, Variant E or control groups throughout the remainder of the experiment.

Seven days following challenge, birds that survived were examined for macroscopic and microscopic lesions (Table 1). At necropsy, macroscopic lesions were observed in the bursa of all birds that survived the challenge in groups 1–4 but not in the group 5 controls. The bursas from birds in groups challenged with CA-K785 and STC were similar in appearance. They included large edematous bursas that were yellowish in color and had striations on the serosal surfaces. The macroscopic lesions in the rB challenge birds were typical of a vvIBDV and included large bursas with varying degrees of hemorrhage and mottled colored kidneys. Some of the rB challenged birds

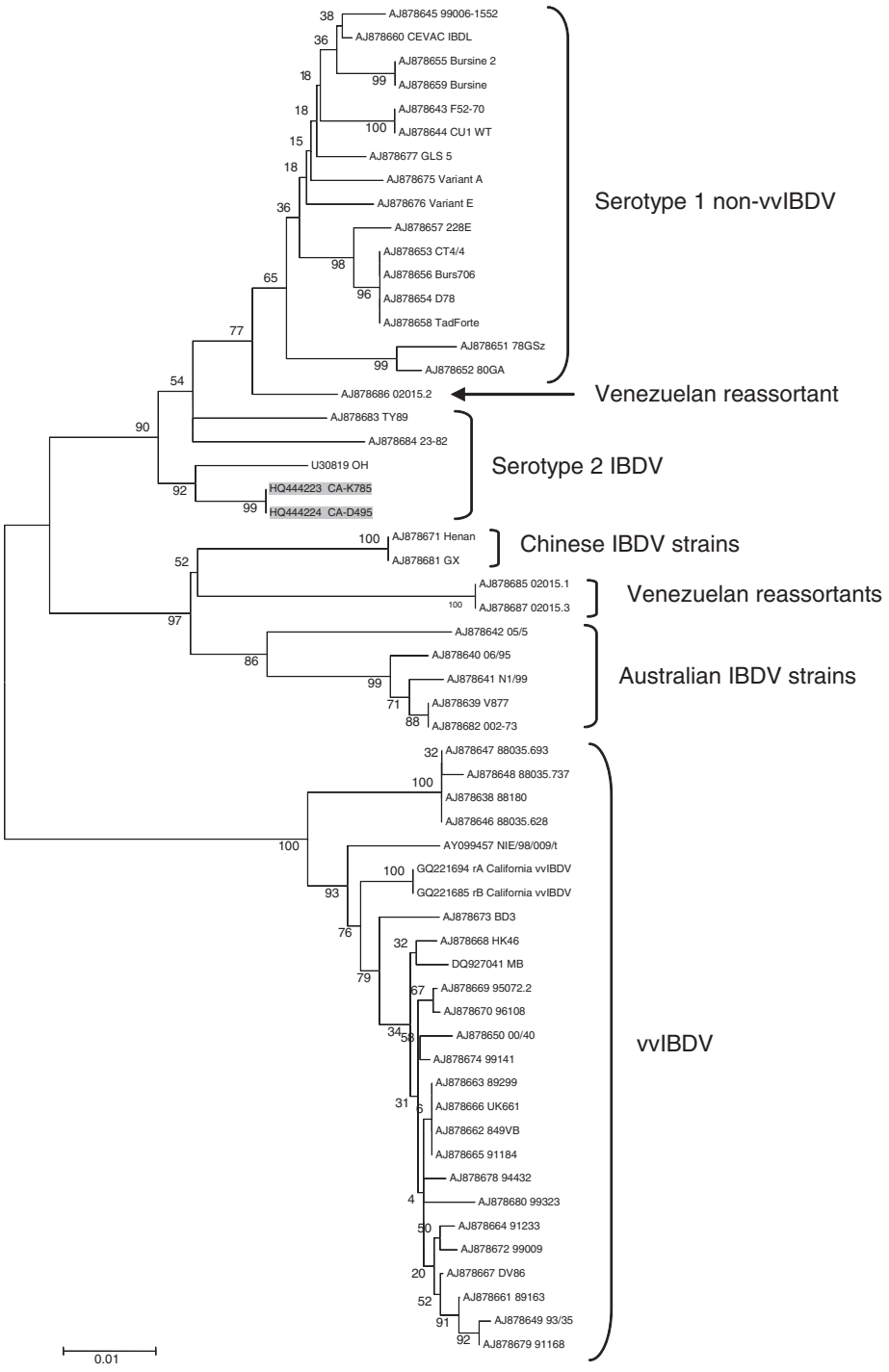

Fig. 1. Phylogenetic analysis of genome segment B from reassortant viruses CA-K785 and CA-D495 with serotype 1 and 2 IBDV from around the world. The Neighbor Joining method using 1000 bootstrap replicates was used. GenBank accession numbers are followed by the common name of each virus.

also had ecchymotic hemorrhages on the mucosal surface at the proventriculus and gizzard junction. Birds challenged with the Variant E strain had small and friable bursas without discoloration or hemorrhage and macroscopic lesions in other organs were not observed. The mean bursa/body weight ratios of the birds in groups 1, 3 and 4 were significantly lower ($p<0.05$) when compared to the controls. Since death was accompanied by varying degrees of post-mortem dehydration, body weights of birds that succumbed to the infection in groups 1 and 2 were not reliable for the calculation of bursa/body weight ratios.

Microscopic lesions were observed in the bursa tissues of birds from groups 1–4 (Table 1). They included moderate to severe lymphocyte necrosis, atrophy of the follicles and follicular depletion of lymphocytes. Bursa tissues from birds challenged with CA-K785, rB and STC also had signs of inflammation and some congestion in the stroma. The most severe lesions were observed in the rB vvIBDV group followed by CA-K785, STC and then the Variant E group. No significant microscopic lesions were observed in the spleen, liver, kidney and proventriculus of birds challenged with CA-K785, STC and Variant E. However, birds that were challenged with rB had

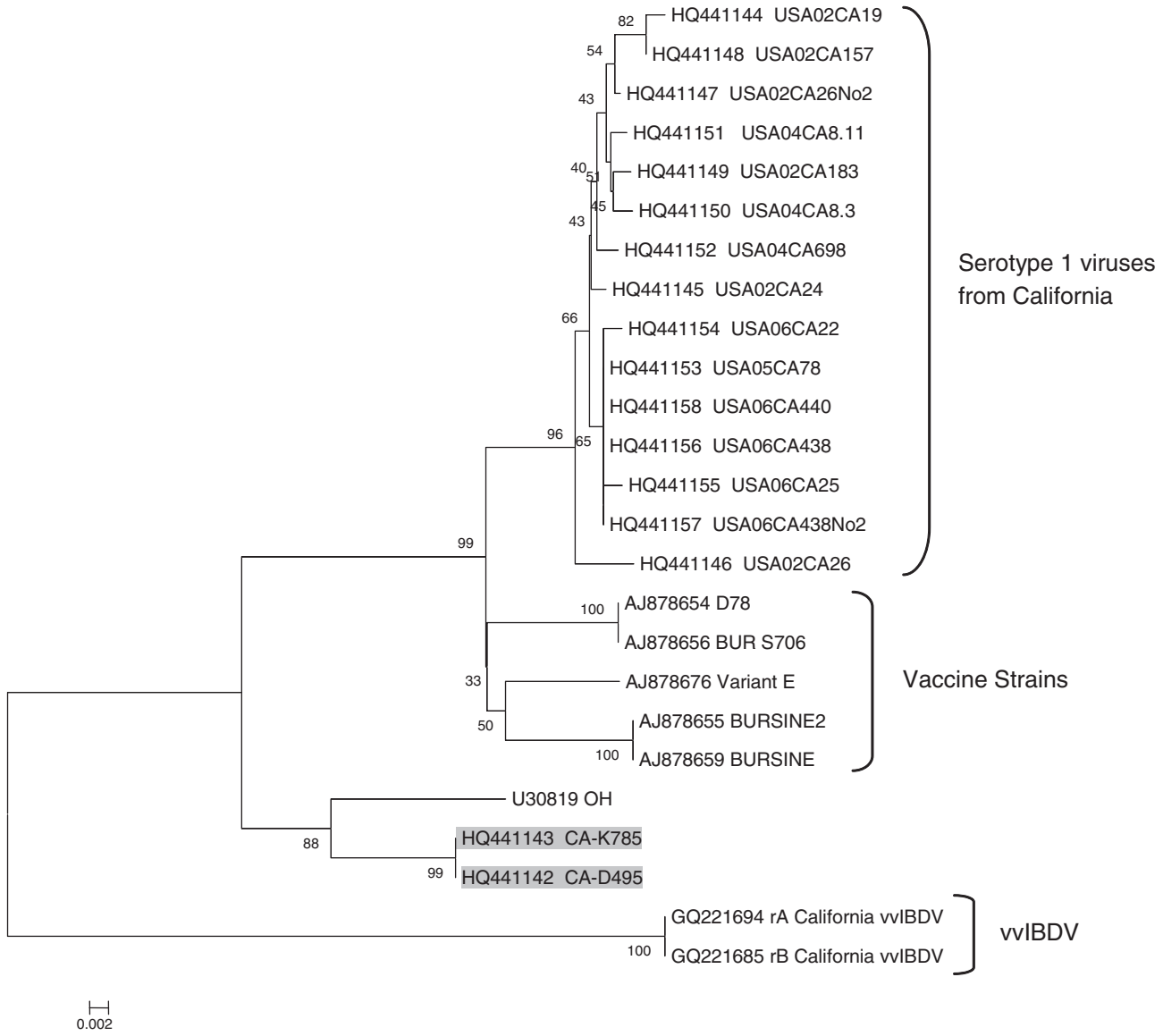

Fig. 2. Phylogenetic analysis of genome segment B from California IBDV isolates. The Neighbor Joining method using 1000 bootstrap replicates was used to compare the California reassortant viruses CA-K785 and CA-D495 with 15 non-vvIBDV strains, the rA and rB vvIBDV strains and the serotype 2 OH virus. GenBank accession numbers are followed by the common name of each virus.

Table 1
Pathology of CA-K785 in SPF chickens.

Group	IBDV challenge strain[a]	Mortality	Mean bursa/body wt. ± SD[b]	Histopathologic lesion scores[c]
1	CA-K785	2/10	2.44 ± 0.48^{A}	4,4,4,3,4,4,4,3,4,4
2	rB vvIBDV	10/10	ND	4,4,4,4,4,4,4,4,4,4
3	STC cvIBDV	0/10	2.56 ± 0.12^{A}	3,4,3,4,4,4,3,4,4,4
4	Variant E scIBDV	0/10	2.61 ± 0.30^{A}	3,3,2,2,3,3,3,2,3,3
5	Control	0/10	5.33 ± 0.79^{B}	0,0,1,0,0,0,0,0,0,1

Different superscripted letters indicate statistically significant differences among the group means ($p<0.05$). ND = not determined.

[a] Specific-pathogen-free chickens were challenged with IBDV at 4 weeks of age. Each bird received $10^{5.0}$ EID_{50} in 0.1 ml oral-nasally.

[b] The mean B-BW ratios ± standard deviations were calculated for birds that survived the challenge. Mean B-BW ratios = bursa wt. (g)/body wt. (g) × 1000.

[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 bird are reported.

lymphocyte lysis and depletion in their spleen, thymus and cecal tonsil tissues. Hemorrhages were observed in the isthmus of the proventriculus of some birds and varying degrees of lymphoid cell necrosis were observed in the liver and kidneys of most birds in this group.

The RT-PCR assays for IBDV were used to detect CA-K785, STC and Variant E in the bursa tissues collected at necropsy and from bursa of birds that succumbed to the infection. The bursa tissues collected at necropsy from each group were pooled and all except the control non-inoculated group were positive in the RT-PCR assays for genome segments A and B. Bursa tissues from birds that died during the experiment were pooled by group and the day of mortality. Each bursa pool from birds that died as a result of the infection was also positive in the RT-PCR assays for both genome segments. All the RT-PCR products were sequenced across the hvVP2 region of genome segment A and 722 bp region of genome segment B. The results confirmed the identity of each virus in their respective groups (data not shown).

Pathogenicity of CA-K785 in turkeys

The pathogenicity of the CA-K785 reassortant in 3-week-old turkey poults was determined and compared to the rB vvIBDV, and OH serotype 2 IBDV. Two weeks prior to challenge, a randomly selected group of 16 poults were bled and found to be negative for IBDV serotype 1 and 2 neutralizing antibodies. At 3 weeks of age on the day of challenge, a second randomly selected group of 10 poults were bled and also found to be sero-negative for neutralizing antibodies to both serotypes of IBDV.

Morbidity and mortality were not observed in the non-inoculated controls or in any of the turkeys inoculated with CA-K785, rB or OH viruses during the 7 days following inoculation. At necropsy on day 7 post-inoculation, all birds were examined for macroscopic lesions and none were observed in any organs including the bursa, spleen, liver, kidney, proventriculus and thymus.

The turkey bursa and spleen tissues were examined for microscopic lesions. There were no microscopic lesions in the spleens of any bird. Bursa tissues from the control group also appeared normal. Although there was some mild lymphocytic depletion and atrophy in 2 bursas, most bursa tissues from the CA-K785 inoculated turkeys had normal lymphocytic populations in the follicles (histopathologic lesion scores ranged from 0 to 1). However, a mild interfollicular edema and inflammation was observed in the bursa of most birds in this group. The turkeys inoculated with the rB vvIBDV had similar microscopic lesions in their bursa tissues. Turkeys inoculated with the OH serotype 2 strain had mild interfollicular edema and inflammation in their bursa tissues and most had a mild lymphocytic depletion and atrophy in the follicles. Histopathologic lesion scores for the bursa in this group ranged from 1 to 2. Two bursas had large pyogranulomatous foci characterized by a central area of necrosis that was surrounded by multinucleated giant cells often associated with a bacterial infection.

The serotype 1 genome segment A RT-PCR assay for IBDV was used to detect the CA-K785 reassortant and rB vvIBDV strains in the turkey bursa tissues collected at necropsy. Bursa tissues from these two groups were positive in this RT-PCR assay and nucleotide sequencing confirmed the identity of each virus. Bursa tissues from turkeys inoculated with the serotype 2 OH strain and non-inoculated controls were negative in the serotype 1 RT-PCR assay. The RT-PCR assay for the genome segment A of the serotype 2 OH strain was positive in the turkeys inoculated with that virus and nucleotide sequencing confirmed its identity. The serotype 2 RT-PCR assay was negative when turkey bursa tissues from the rB, CA-K785 and control non-inoculated groups were tested.

Discussion

This is the first description of natural reassorting between serotype 1 and 2 IBDV strains. The serotype of IBDV is defined by epitopes on the surface protein VP2 that is encoded by genome segment A (Letzel et al., 2007). The hvVP2 nucleotide sequences of CA-K785 and CA-D495 were typical of serotype 1 vvIBDV strains. Genetically the CA-K785 and CA-D495 reassortants were identical across this hvVP2 region. Since the hvVP2 sequence is highly variable among IBDV strains, it is likely that these two isolates are clonal (Le Nouen et al., 2005; Levin et al., 1999). Across the full-length of the genome segment A there were 44 substitution mutations (98.6% nucleotide match) between CA-K785 and CA-D495. Of these mutations all but 3 were silent. Thus, the polyproteins of CA-K785 and CA-D495 were a 99.7% match to each other and across the hvVP2 region they were a 99.86% match to other vvIBDV from California suggesting these viruses are closely related.

The genome segment B of vvIBDV is genetically distinct from all other strains of IBDV (Hon et al., 2006; Islam et al., 2001). When the nucleotide sequences of the full-length B genome segments from CA-K785 and CA-D495 were compared, they had 50 nucleotide differences (98.2% match). All were silent except 3 giving these viruses a 99.7% amino acid identity across the VP1 protein.

Previous phylogenetic studies on the nucleotide sequence of genome segment B indicated that results obtained on the 5′ two-thirds of this molecule were comparable to results observed for the entire segment B sequence (Le Nouen et al., 2005) and therefore, most GenBank sequences of the IBDV genome segment B include only this 5′ region (Le Nouen et al., 2006). When the genome segment B sequences of CA-K785 and CA-D495 were compared to this 5′ region of those sequences available in GenBank, they were not characteristic of vvIBDV, cvIBDV or scIBDV serotype 1 viruses. They were very closely related to the genome segment B of a U.S. serotype 2 IBDV designated OH. Compared to the full-length VP1 protein encoded by genome segment B from OH, the CA-K785 and CA-D495 viruses had a 98.5% and 98.6% amino acid sequence identity, respectively.

To rule out the possibility of a mixed infection of serotype 1 and 2 viruses, real-time RT-PCR assays were conducted. The assay for serotype 1 hvVP2 sequences employed specific serotype 1 primers 743-1 and 743-2 plus a probe that was specific for either the serotype 1 vvIBDV or serotype 1 non-vvIBDV. This assay only detected the serotype 1 vvIBDV genome segment A in the original CA-K785 and CA-D495 samples. A second set of primers (S2-F and S2-R) designed to amplify a region of genome segment A was specific for serotype 2 viruses and in our real-time RT-PCR assay did not amplify serotype 1 strains. No serotype 2 hvVP2 sequences were detected in the original bursa tissues containing CA-K785 and CA-D495 using this assay. The absence of a mixed serotype 1 and 2 virus infection was also demonstrated when bursa tissues from the CA-K785 infection experiments in chickens and turkeys were examined in the real-time RT-PCR assays. Only vvIBDV serotype 1 hvVP2 sequences were detected.

The CA-K785 reassortant was from a commercial layer chicken flock and CA-D495 originated from a backyard flock of mixed breed poultry which was approximately 64 km from the commercial layer flock. For these viruses to exist in nature, a common host and cell tropism must exist for vvIBDV and serotype 2 IBDV. Antibodies to serotype 2 viruses have been found in commercial chicken flocks but there are no reports of these viruses being isolated from chickens (Jackwood et al., 1985; Jackwood and Saif, 1983). Furthermore, it has been reported that serotype 2 viruses do not replicate in chicken lymphoid cells but they have been isolated from the bursa tissue and the respiratory tract of turkeys (Chettle et al., 1985; Jackwood et al., 1982b; McNulty et al., 1979; Nieper and Muller, 1996). The pathogenicity of vvIBDV for other poultry and avian species has not been thoroughly investigated but vvIBDV strains have been isolated from turkeys (Owoade et al., 2004; Razmyar and Peighambari, 2009), migratory aquatic fowl (Jeon et al., 2008), and several other sedentary avian species (Jeon et al., 2008; Kasanga et al., 2008). The cell tropism of vvIBDV in these species has not been reported. The higher virulence of the vvIBDV compared to cvIBDV and scIBDV may allow them to infect a wider variety of avian species and thus, increase the likelihood that vvIBDV strains will encounter serotype 2 viruses. Serologic studies have shown that serotype 2 viruses are commonly found in California turkeys (Chin et al., 1984) and are more common than serotype 1 viruses in free-living wild avian species (Ogawa et al., 1998). It is possible that the backyard flock where CA-D495 originated provided a unique opportunity for reassorting of the vvIBDV and serotype 2 genomes.

Studies on serotype 1/serotype 2 reassortant viruses generated using reverse genetics indicate that viruses with a serotype 1 genome segment A and serotype 2 genome segment B can infect chicken lymphocytes in vivo but reassortants with a serotype 2 segment A and serotype 1 segment B did not (Zierenberg et al., 2004). Although it might be possible for a serotype 1 non-vvIBDV (cvIBDV or scIBDV) reassortant with a serotype 2 genome segment B to exist naturally, the 15 serotype 1 non-vvIBDV strains we identified in commercial and backyard California

chicken flocks between the years 2002 and 2006 all had genome segment B sequences that were not related to serotype 2 viruses (Fig. 2). Furthermore, other studies on the molecular epidemiology of IBDV genome segments A and B have not found naturally occurring serotype 1 cvIBDV and scIBDV strains with a serotype 2 genome segment B (Hon et al., 2006; Le Nouen et al., 2006) . Together, these data suggest that the genome segment B in the CA-K785 and CA-D495 viruses came directly from a serotype 2 virus and not from a serotype 1 strain endemic to California.

The vvIBDV typically cause very high mortality in SPF chickens (van den Berg et al., 2004). In our previous studies with the rA and rB vvIBDV from California, these viruses caused 90–100% mortality in 4-week-old SPF chicks (Jackwood et al., 2009). We again observed 100% mortality in the 4-week-old SPF chicks inoculated with rB vvIBDV in this study. Comparatively, the CA-K785 reassortant only caused 20% mortality in these 4-week-old SPF chicks. This is similar to the reduced virulence seen with a vvIBDV reassortant from Venezuela (Le Nouen et al., 2006). When the same dose of rB and CA-K785 was administered to 3-week-old turkey poults, we did not observe any morbidity or mortality. Although these turkeys were obtained from a commercial source, they were free of antibodies to both serotypes of IBDV prior to inoculation. We also did not observed macroscopic lesions in organ tissues at 7 days post-inoculation and only minor histopathologic lesions were observed in the bursa tissues of these turkeys. The data suggest that the rB vvIBDV and the CA-K785 reassorted virus are not pathogenic in turkey poults. Historically, the serotype 1 and 2 IBDV have not caused disease in turkeys (Giambrone et al., 1978; Jackwood et al., 1982a). Although recent isolates of vvIBDV have not been examined using a controlled challenge experiment in turkeys, vvIBDV has been isolated from 6 to 8 week old commercial turkey flocks with unexpectedly high mortality (Owoade et al., 2004). Our data demonstrate that, the rB vvIBDV which is highly pathogenic in chickens, is not pathogenic in turkeys. Furthermore, the vvIBDV reassortant virus CA-K785 with a serotype 2 genome segment B was also not pathogenic in turkeys. These viruses infected the turkeys in our study as evidenced by positive RT-PCR assays and thus turkeys could act as an asymptomatic reservoir for IBDV. Since the virulence of vvIBDV varied in different genetic lines of chickens (van den Berg et al., 2004), it is possible that the vvIBDV and viruses like the CA-K785 reassortant could be pathogenic in different genetic lines of domestic and wild turkeys. The presence of serotype 1 genome segment A and serotype 2 genome segment B reassortant viruses in California also suggests a serotype 2 genome segment A and serotype 1 genome segment B reassortant may emerge. Although the serotype 2 segment A/serotype 1 segment B virus created using reverse genetics was unable to replicate in chickens, it was not tested in turkeys (Zierenberg et al., 2004). The pathogenicity of such a reassortant for turkeys would need to be examined but reassorting of genome segments among IBDV serotypes might eventually lead to a strain that is pathogenic in a species other than the chicken.

The first samples containing vvIBDV were submitted to the CAHFS Laboratory System in December of 2008. Samples containing the CA-K785 reassortant virus were submitted 5 months later in May of 2009. If the first detection of vvIBDV in December 2008 coincided with the initial introduction of vvIBDV in the U.S., it seems unlikely that reassortants would be detected 5 months later because studies have shown that there is a strong co-evolution of vvIBDV genome segments A and B (Le Nouen et al., 2006). Studies on the time of emergence of the most recent common ancestor (TMRCA) suggest that the vvIBDV emerged in the 1980s following the reassorting of an IBDV genome segment A, which emerged approximately 20 years earlier, with a genome segment B from an unidentified reservoir (Hon et al., 2006). These studies suggest that the California vvIBDV may not have had sufficient time to reassort with a serotype 2 IBDV in California.

It is possible that vvIBDV were introduced in California much earlier than their initial detection. Our preliminary data indicate that maternal immunity and competition with endemic viruses may reduce the mortality caused by vvIBDV in the U.S. layer and broiler chicken flocks (Jackwood, 2011). Without routine surveys for vvIBDV in these commercial poultry flocks, it is possible that the disease went unrecognized until the right combination of vvIBDV, environment and a fully susceptible host resulted in an acute high mortality disease. The presence of reassortant vvIBDV in commercial layer and backyard chicken flocks in California supports the theory that vvIBDV strains have been in this region longer than the initial December 2008 isolation would suggest.

It is also possible that these reassorted viruses and vvIBDV were coincidentally introduced into California chicken flocks at about the same time. Compared to other vvIBDV, the hvVP2 sequences of the reassorted viruses CA-K785 and CA-D495 are phylogenetically most closely related to the rA and rB vvIBDV from California, suggesting that these viruses evolved in the same geographic location. That geographic location may have been California but it is possible that they co-evolved in another country and were introduced in the U.S. at approximately the same time.

In conclusion, the CA-K785 and CA-D495 isolates contain a serotype 1 vvIBDV genome segment A and a genome segment B from serotype 2 IBDV. The viruses were closely related genetically and the sequences of their full-length genome segments A and B indicated they were true reassortants and had not recombined to obtain only partial serotype 1 and 2 sequences. The CA-K785 isolate was pathogenic in 4 week-old SPF chickens but not in 3-week-old turkeys. Virulence of the CA-K785 reassortant for chickens was lower than typically seen for non-reassorted vvIBDV. The CA-K785 reassortant and rB vvIBDV were able to infect 3-week-old turkeys but they were not pathogenic in these birds. The data suggest that the reassorted viruses either entered the U.S. at about the same time vvIBDV strains were first detected in that same region of California or vvIBDV have been replicating and spreading among commercial and backyard poultry flocks in California longer than the initial isolation of these viruses would suggest. The data reaffirm that molecular identification of vvIBDV from disease outbreaks requires sequence analysis of both genome segments. If the U.S. is going to control or at least contain vvIBDV in California, routine surveys for these viruses in commercial and backyard poultry flocks will be essential.

Materials and methods

Viruses

The CA-K785 isolate was identified in commercially reared layer chickens submitted to the California Animal Health and Food Safety (CAHFS) Laboratory System in May 2009. The flock of origin was 4 weeks old and birds submitted to the laboratory were stunted with ruffled feathers, signs of dehydration and blood stained vents. The flock was vaccinated for Newcastle disease, avian infectious bronchitis and infectious bursal disease (IBD, Bursine live-attenuated vaccine) 4 days prior to being submitted to CAHFS.

The CA-D495 isolate was obtained from two female Araucana chickens submitted to the CAHFS Laboratory System after they died acutely in October 2009. These birds came from a backyard flock of 19 chickens consisting of several breeds and an undetermined number of pigeons. It was located about 64 km from the commercial layer flock that yielded CA-K785. Both birds were approximately 6 months old and had not been vaccinated for IBD. The chicks were purchased in April 2009 from a supplier located in California but geographically distant from any commercial poultry. The two birds had congested and edematous lungs, enlarged spleens and one bird had petechial hemorrhages on the serosal surfaces of the small intestine.

Macroscopically, the bursas of both birds were large and appeared normal.

Fifteen IBDV strains from commercial chicken farms in California were isolated between 2002 and 2006. A portion of their genome segment B was sequenced and compared to the CA-K785 and CA-D495 viruses.

Detection and identification of IBDV

Bursa tissues from commercial layer, broiler and backyard chicken flocks in California were submitted to The Food Animal Health Research Program at The Ohio Agricultural Research and Development Center, The Ohio State University. The seventeen bursa tissue samples which include the CA-K785 and CA-D495 viruses, were homogenized in TNE buffer (10 mM Tris–HCL, pH 8.0, 100 mM NaCl, 1 mM ethylene diamine tetra acetic acid) and examined for IBDV genomic RNA using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

The bursa homogenates were extracted using a standard Trizol procedure (Jackwood et al., 2008). Using a GenAmp RNA PCR kit (Applied Biosystems, Roche Molecular Systems, Inc., Branchburg, New Jersey) portions of genome segments A and B were amplified. A 743-bp segment of the highly variable region of VP2 (hvVP2) from nucleotides 737 to 1479 was amplified using primers 743-1 (5′-GCCCAGAGTCTACACCAT-3′) and 743-2 (5′-CCCGGATTATGTCTTTGA-3′) (Jackwood and Sommer-Wagner, 2005). These primers used for both RT and PCR are located in a conserved sequence region and will amplify this portion of genome segment A from all serotype 1 IBDV strains including vvIBDV and non-vvIBDV. They do not amplify genome segment A from serotype 2 viruses. The RT-PCR conditions and incubation times for amplifying hvVP2 of serotype 1 IBDV were previously described (Jackwood et al., 2008).

To amplify the hvVP2 region of serotype 2 IBDV, primers S2-F (5′-AAGTTGATGGCCACGTGCG-3′) and S2-R (5′-CTCGGATTATGTCCT-TAAAACC-3′) were used in a real-time RT-PCR assay that was similar to that previously described (Jackwood et al., 2008) except the annealing temperature was increased to 57 °C. These primers were used in both the RT and PCR conducted using a one-step SYBR Green RNA-to-C_T real-time RT-PCR kit (Applied Biosystems, Foster City, CA). They amplify a 778 bp region of the serotype 2 VP2 gene from nucleotide 686 to 1463. They did not amplify genome segment A of serotype 1 rB vvIBDV, STC cvIBDV and Variant E scIBDV. The viruses CA-K785 and CA-D495 were negative in this assay.

A portion of genome segment B was amplified using primers B-168AF (5′-CATAAAGCCTACAGCTGGAC-3′) and B-889R (5′-GTCCACT-TGATGACTTGAGG-3′) and reaction conditions previously described (Jackwood et al., 2009). The B-168A and B-889 primers were used in both the RT and PCR and amplify a 722 bp region of genome segment B starting at nucleotide 168 and ending at 889. These primers are located in a relatively conserved sequence region and under the conditions used in this study they amplify this portion of genome segment B from both serotypes of IBDV.

A real-time RT-PCR assay was used to detect segment A genetic material of serotype 1 IBDV strains (Jackwood and Sommer-Wagner, 2011). Briefly, a 1.0 µl volume of RNA extracted from the samples containing the CA-K785 and CA-D495 isolates was used for the assay. Primers 743-1 and 743-2 were used to amplify the highly variable sequence in the VP2 gene from serotype 1 viruses and two Taq-Man probes (Applied Biosystems, Foster City, CA) were used to detect the RT-PCR products. One probe was specific for non-vvIBDV viruses that are endemic to California (VIC-5′-CAGCCAACATT-GATGC-3′) and the other probe was specific for vvIBDV (FAM-5′-CTCAGCTAATATCGATGC-3′). The reactions were conducted using a real-time RT-PCR assay kit (AgPath-ID, Ambion, Inc., Austin, TX). The RT was conducted at 48 °C for 10 min followed by a 95 °C denaturation for 10 min and then forty cycles of PCR at 95 °C for 15 s, 58 °C for 90 s and 72 °C for 90 s in a Roche LightCycler 480 instrument (Roche Diagnostics Corp., Indianapolis, IN). Control samples used in all real-time RT-PCR assays included the OH serotype 2 virus, rB vvIBDV, STC cvIBDV and Variant E scIBDV. Nucleic acid extracted from the bursa of non-infected SPF chicks was used as a negative control.

Nucleotide sequencing and analysis

Full-length genome segment A and B sequences were determined for the CA-K785 and CA-D495 viruses using primers and procedures previously described (Jackwood et al., 2008). The positive RT-PCR samples were prepared for sequence analysis using a Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI). The cleaned RT-PCR samples were sent to the University of Wisconsin, Biotechnology Center, DNA Sequence Laboratory (Madison, WI) for cycle sequencing. Nucleotide sequence results were downloaded using Chromas (Technelysium Pty. Ltd., Queensland, Australia). Sequence analysis and Clustal W alignments were conducted using Accelrys Gene v2.5 (Accelrys, San Diego, CA). Phylogenetic analysis was conducted using MEGA version 4 software (Tamura et al., 2007). Phylogenetic trees were prepared using the unweighted pair-group methods using arithmetic averages (UPGMA) and Neighbor-Joining (NJ) distance methods each with 1000 bootstrap replicates.

Pathogenicity in chickens and turkeys

The pathogenicity of the CA-K785 virus was examined in 4 week-old specific-pathogen-free (SPF) chickens (Charles River Laboratories, North Franklin, CT) and 3 week-old large white turkey poults from a local commercial hatchery that certified they were free of *Mycoplasma*, *Salmonella* and avian influenza. The birds were housed in Horsfall isolation units and given feed and water ad libitum.

The SPF chickens were allotted into 5 groups containing 10 birds each. Birds in each group except the control were inoculated oral-nasally with 10^5 mean egg infectious doses (EID_{50}) as previously described (Jackwood et al., 2009). The EID_{50} titers were determined in 9-day-old embryonated chicken eggs inoculated via the chorioallantoic membrane (CAM) route with a 0.1 ml volume of bursa homogenate (Jackwood et al., 2009). Birds in group 1 were inoculated with the CA-K785 virus, group 2 received the vvIBDV isolate designated ranch B (rB), group 3 were inoculated with the STC classic isolate, group 4 received the Variant E strain and group 5 were non-inoculated controls. The rB, STC and Variant E strains were used for comparison because they represent the three IBDV pathogenicity types: vvIBDV, cvIBDV and scIBDV, respectively (van den Berg et al., 2004). Birds that survived the challenge were euthanized 7 days after being inoculated and examined for gross lesions. Bursa and body weights were recorded and the tissues collected for histopathology included the bursa, spleen, liver, kidney, proventriculus, cecal tonsils and thymus.

Turkey poults were allotted into 4 groups of 8 birds each. At 2 and again at 3-weeks of age, prior to being inoculated, serum samples from randomly selected poults were collected and examined for antibodies to serotype 1 and 2 IBDV using a virus-neutralization (VN) assay in cell culture. BGM-70 cells grown in 96-well plates were used in a constant virus-varying antibody VN assay that employed the serotype 1 Lukert strain virus and serotype 2 OH virus as antigens (Dybing and Jackwood, 1998). The same inoculation procedure used for SPF chickens was used for the turkey poults. Each bird in the respective groups except the control was inoculated oral-nasally with 10^5 EID_{50} of virus. Turkeys in group 1 were inoculated with the CA-K785 virus, group 2 received the vvIBDV isolate rB, group 3 were inoculated with the serotype 2 OH isolate and group 4 were non-inoculated controls. The vvIBDV rB strain and serotype 2 OH strain were used for comparison because both vvIBDV and serotype 2 strains

have been reported in turkeys (Chin et al., 1984; McFerran et al., 1980; Owoade et al., 2004; Razmyar and Peighambari, 2009). Turkeys that survived the challenge were euthanized 7 days post-inoculation and examined for gross lesions. Bursa and body weights were recorded and the tissue collected for histopathology included the bursa, spleen, liver, kidney, proventriculus, cecal tonsils and thymus.

Bursa tissues from the chickens and turkeys were collected for molecular detection of the viruses using RT-PCR assays. Detection of serotype 1 IBDV strains CA-785, rB, STC and Variant E was conducted using the 743-1 and 743-2 primers. Detection of the OH strain was conducted using serotype 2 specific primers S2-F and S2-R.

Histopathology

Tissues collected at necropsy were fixed in 10% neutral buffered formalin, sectioned at 4 μm and stained using hematoxylin and eosin. They were examined by light microscopy and bursa tissues were 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 to 50%, 50 to 75% and >75% of the follicles affected, respectively.

Statistical analysis

Because IBDV infections in chickens cause atrophy of the bursa, the bursa and body weights measured at necropsy for chickens were used to calculate a bursa/body weight ratio (B/BW). These B/BW ratios were compared for statistical differences using the SAS: Proc GLM program.

Acknowledgments

We thank Dr. G. S. Krakowka, The Ohio State University, for histopathologic analysis of chicken and turkey tissues and Dr. John Hughes, The Ohio State University, for reviewing this manuscript. This study was supported in part by funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

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