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Genetic characteristics of infectious bursal disease viruses from four continents

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Abstract

Following the initial discovery of very virulent infectious bursal disease virus (vvIBDV) strains in Europe, these viruses spread to many parts of the world. In this study, we examined the phylogenetic relationship of never-before-published IBDV from 18 countries on four continents. All the samples were collected between 1997 and 2005 and were reported to be from broiler flocks experiencing higher than expected mortality which is often associated with acute very virulent infectious bursal disease. A total of 113 samples were imported into the U.S. and viral genetic material was used to determine the nucleotide sequence of the VP2 gene hypervariable region. Although all the samples were reported to be associated clinically with high mortality, genetic analysis suggests that some were not vvIBDV strains. Two viruses from South Africa were genetically similar to U.S. variant viruses. A majority (71/113) of the viruses examined had the amino acid Alanine at position 222 and sixty-seven of these suspect vvIBDV also had amino acids I242, I256, I294 and S299 which are highly conserved among vvIBDV strains. Phylogenetic analysis placed putative vvIBDV strains from many different countries and geographic regions in a single clade with some minor non-significant branching.

Keywords: Infectious bursal disease virus; Infectious bursal disease; Birnavirus; BDV; vvIBDV; genetic evaluation; Variant virus; Classic virus; Very virulent virus

Introduction

The first report of acute infectious bursal disease virus (vvIBDV) was in 1989 (Stuart, 1989). Since that time vvIBDV strains have been reported in many parts of the world (Van Den Berg, 2000). Molecular epidemiology indicates that all known vvIBDV strains are from a common ancestor (Brown et al., 1994; Eterradossi et al., 1997; Hon et al., 2006; Rudd et al., 2002; Van Den Berg, 2000; Yamaguchi et al., 1997). Comparisons of the variable VP2 (vVP2) nucleotide and amino acid sequences suggest that these viruses are clonal (Levin et al., 1999).

The quality that defines a vvIBDV strain is primarily the ability to cause high mortality in susceptible chickens. Since in vivo studies are expensive, time consuming and sometimes not possible, genetic characteristics that define the vvIBDV phylogenetic group have been explored (Jackwood and Sommer-Wagner, 2006; Mardassi et al., 2004; Rudd et al., 2002). The amino acids A222, I256 and I294 were reported to be unique to all known vvIBDV strains (Banda and Villegas, 2004; Hoque et al., 2001). In addition, the amino acids I242, I256, I294 and S299 were reported to be highly conserved among vvIBDV strains (Rudd et al., 2002). A study on vvIBDV strains from Indonesia however, indicates that A222 is not a unique characteristic of these viruses since some vvIBDV strains in that study had an A222S substitution mutation (Parede et al., 2003). A virus from Malaysia was reported to have the A222, I242, I256, I294 and S299 genetic characteristics of vvIBDV strains but it only produced 10% mortality in susceptible chickens (Hoque et al., 2001). That virus had a substitution mutation from G to S at position 254 and A to E at 270.

The conserved amino acids at positions A222, I242, I256, I294 and S299 do not control virulence of vvIBDV strains. This is supported by reverse genetic studies that concluded that VP2 is not the only protein responsible for the very virulent phenotype (Boot et al., 2000). Recent studies suggest that the

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increased virulence of vvIBDV strains may be due to a genome segment B that it gained through reassortment from an unidentified reservoir (Hon et al., 2006; Le Nouen et al., 2006). Together, these studies indicate that the virulence of vvIBDV strains is probably due to multiple genetic events.

Identification of the genetic elements that control the very virulent phenotype is not a requirement for tracking the dispersion and evolution of vvIBDV strains. Regions of the genome that do not control virulence and antigenicity but are unique to vvIBDV may be better for tracking viruses since selection pressures on these sequences would be minimal. Others have suggested that since the genetic basis for the very virulent phenotype has not been conclusively identified, genetic changes in VP2 should be considered to be evolutionary rather than virulence markers (Van Den Berg et al., 2004). The hypervariable sequence region of VP2 (vVP2) has been used for most molecular epidemiology and phylogenic studies. Although mutation rates are higher, this part of the genome also contains relatively conserved sequence regions unique to vvIBDV strains (Hoque et al., 2001; Jackwood and Sommer-Wagner, 2006; Parede et al., 2003; Rudd et al., 2002). Tracking IBDV using the vVP2 genetic region that is subject to frequent mutations allows greater discrimination between genomes that are considered to be closely related or clonal (Levin et al., 1999).

The goal of our study was to identify IBDV strains from diverse geographical regions that are associated with high mortality in chicken flocks. To achieve this goal, regions in the vVP2 nucleotide sequence that are unique to variant, classic and very virulent IBDV strains were used. The variable sequence regions in VP2 were also used to examine the short-term molecular evolution of those strains identified as vvIBDV.

Results

All 113 genomic dsRNA samples were submitted for testing because they were from broiler chicken flocks experiencing higher than expected mortality and clinical signs sometimes associated with vvIBDV infection. Because we are prohibited from importing live virus, it was not possible to confirm the pathogenicity of the viruses. Thus, we had to rely on molecular characteristics that are consistent with the vvIBDV phenotype. Not all of the samples contained these molecular criteria.

Nucleotide sequence analysis

The nucleotide sequence of the vVP2 region from nucleotide positions 737 to 1479 was determined for each virus in this study. The nucleotide sequences of these viruses were submitted to GenBank as a set; their accession numbers begin with DQ916164 and end with DQ916276. The predicted amino acid sequences that contained A222, I242, I256, I294 and S299 were observed in 68 samples (Table 1). One sample from Bolivia (05 B62) and two from South Africa (05 SA8 and 05 SA10) had A222 but had substitution mutations at one or more of the 242, 256, 294 and 299 positions. The most common amino acid at position 222 was Alanine with 71 viruses having that result. Proline at 222 was observed in 22 viruses, the next largest group. Serine was observed in 10 viruses, Threonine in 8 viruses and Glutamine was identified in 2 viruses.

Typical classic virus amino acid sequences that included P222, V242, V256, I294 and N299 were observed in 21 viruses (Table 1). One putative classic virus from Venezuela (VEN 34) had a substitution from N to D at position 299. Viruses with S222 varied in their amino acid sequences. Seven were similar to Lukert classic strain viruses and three S222 viruses from Singapore had amino acids more typical of vvIBDV; I242, I256, I294 and S299. Eight viruses that were found to have T222 and two that had Q222 also had amino acid substitutions that have been consistently observed in variant viruses.

Phylogenetic analysis

All 113 viral sequences were included in the phylogenetic analysis. Phylogenetic analysis of the amino acid sequences was conducted using the NJ, UPGMA and ME methods. An NJ analysis using the nucleotide sequences gave nearly identical results (data not shown). The results obtained using the UPGMA and ME methods on amino acid sequences were essentially the same as that shown for the NJ method in Figs. 1 and 2. Fig. 1 contains viruses with genetic characteristics consistent with the vvIBDV phenotype. They included all viruses with I242, I256, I294 and S299 plus viruses with A222 regardless of the amino acids at other key positions. Fig. 2 contains viruses with genetic characteristics that were consistent with non-vvIBDV strains. Reference IBDV strains including known variant viruses (Del E and T1), classic viruses (Cu1, STC, and Bursine), and vvIBDV strains (BD 3/99, UK661 and OKYM) were used for comparison in both phylogenetic trees. In addition to these reference strains, we included the El Salvadore01 ES1, El Salvador01 ES2, Spain97 SP11, Bolivia05 B62, South Africa05 SA10 and South Africa05 SA8 viruses in both trees because of their unique amino acid sequences.

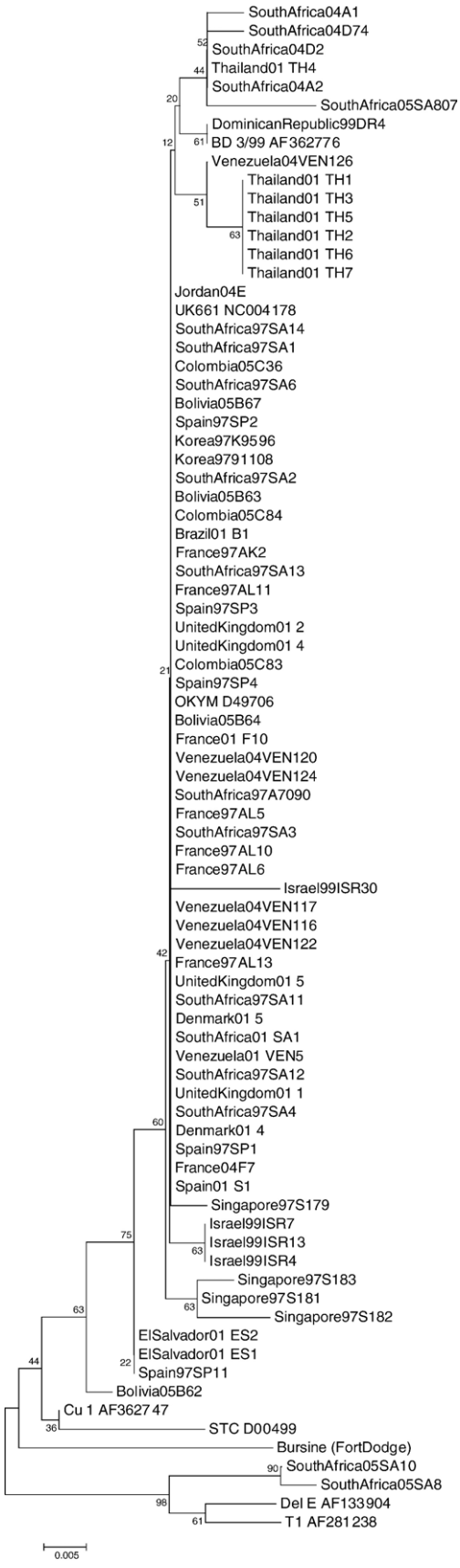
The variant viruses Del E and T1 group together in one clade and the classic viruses from the US and Europe were in a separate clade as expected. In both trees, the two viruses from South Africa (05SA8 and 05SA10) formed a separate branch but were related to the U.S. variant viruses. The 05SA8 virus had amino acids A222, V242, V256 and L294 while the 05SA10 virus had A222, I242, V256 and L294. Although many amino acids were common between these South African viruses and the U.S. variants, the K249 and I286 were unique to both groups and were not found on any of the classic or vvIBDV strains examined in this study. The only other virus that was A222 but did not have Isoleucine at all three positions (V242, V256 and I294) was from Bolivia 05B62. It was alone on a separate branch in both trees.

Viruses examined in the phylogenic analysis that contained the characteristic vvIBDV amino acids A222, I242, I256, I249 and S299 were placed together in a phylogenic clade that included the vvIBDV reference isolates UK661 BD 3/99 and OKYM (Fig. 1). The viruses in this largest clade were isolated between 1997 and 2005. They originated from Europe, South Africa, South America, Central America, the Middle East and

Table 1
Origin, year of isolation and key amino acids of IBDV strains examined in this study

Country (year) (isolate)	Key amino acids				
	222	242	256	294	299
Argentina05 A17	S	V	V	L	N
Argentina05 A24	P	V	V	I	N
Argentina05 A25	S	V	V	L	S
Bolivia05 B51	P	V	V	I	N
Bolivia05 B56	P	V	V	I	N
Bolivia05 B59	P	V	V	I	N
Bolivia05 B60	P	V	V	I	N
Bolivia05 B61	Q	V	V	L	N
Bolivia05 B62	A	V	V	I	N
Bolivia05 B63	A	I	I	I	S
Bolivia05 B64	A	I	I	I	S
Bolivia05 B66	P	V	V	I	N
Bolivia05 B67	A	I	I	I	S
Bolivia05 B68	Q	V	V	L	N
Bolivia05 B75	P	V	V	I	N
Bolivia05 B81	P	V	V	I	N
Brazil01 B1	A	I	I	I	S
Colombia01 C10	P	V	V	I	N
Colombia01 C5	S	V	V	L	S
Colombia01 C6	T	V	V	L	N
Colombia01 C9	T	V	V	L	N
Colombia04 C81	T	V	V	L	N
Colombia05 C36	A	I	I	I	S
Colombia05 C83	A	I	I	I	S
Colombia05 C84	A	I	I	I	S
Denmark01 4	A	I	I	I	S
Denmark01 5	A	I	I	I	S
Dominican Republic99 DR4	A	I	I	I	S
ElSalvador01 ES1	A	I	I	I	N
ElSalvador01 ES2	A	I	I	I	N
France97 AL13	A	I	I	I	S
France01 F10	A	I	I	I	S
France04 F7	A	I	I	I	S
France97 AK2	A	I	I	I	S
France97 AL10	A	I	I	I	S
France97 AL11	A	I	I	I	S
France97 AL5	A	I	I	I	S
France97 AL6	A	I	I	I	S
Israel99 ISR13	A	I	I	I	S
Israel99 ISR30	A	I	I	I	S
Israel99 ISR4	A	I	I	I	S
Israel99 ISR7	A	I	I	I	S
Jordan04 E	A	I	I	I	S
Korea97 91108	A	I	I	I	S
Korea97 K9596	A	I	I	I	S
Mexico01 M	P	V	V	I	N
Mexico04 M101	T	V	V	L	N
Mexico04 M83	T	V	V	L	N
Mexico04 M84	T	V	V	L	N
Mexico04 M92	T	V	V	L	N
Mexico04 M95	T	V	V	L	N
Singapore97 S179	A	I	I	I	S
Singapore97 S181	S	I	I	I	S
Singapore97 S182	S	I	I	I	S
Singapore97 S183	S	I	I	I	S
South Africa01 SA1	A	I	I	I	S
South Africa04 A1	A	I	I	I	S
South Africa04 A2	A	I	I	I	S
South Africa04 D2	A	I	I	I	S
South Africa04 D74	A	I	I	I	S
South Africa05 SA10	A	I	V	L	N
South Africa05 SA8	A	V	V	L	N
South Africa05 SA807	A	I	I	I	S
South Africa97 A7090	A	I	I	I	S
South Africa97 SA1	A	I	I	I	S
South Africa97 SA11	A	I	I	I	S
South Africa97 SA12	A	I	I	I	S
South Africa97 SA13	A	I	I	I	S
South Africa97 SA14	A	I	I	I	S
South Africa97 SA2	A	I	I	I	S
South Africa97 SA3	A	I	I	I	S
South Africa97 SA4	A	I	I	I	S
South Africa97 SA6	A	I	I	I	S
Spain01 S1	A	I	I	I	S
Spain01 S8	P	V	V	I	N
Spain01 S9	P	V	V	I	N
Spain97 SP1	A	I	I	I	S
Spain97 SP11	A	I	I	I	N
Spain97 SP2	A	I	I	I	S
Spain97 SP3	A	I	I	I	S
Spain97 SP4	A	I	I	I	S
Thailand01 TH1	A	I	I	I	S
Thailand01 TH2	A	I	I	I	S
Thailand01 TH3	A	I	I	I	S
Thailand01 TH4	A	I	I	I	S
Thailand01 TH5	A	I	I	I	S
Thailand01 TH6	A	I	I	I	S
Thailand01 TH7	A	I	I	I	S
Thailand97 TH4	P	V	V	I	N
United Kingdom01 1	A	I	I	I	S
United Kingdom01 2	A	I	I	I	S
United Kingdom01 4	A	I	I	I	S
United Kingdom01 5	A	I	I	I	S
Venezuela01 VEN1	P	V	V	I	N
Venezuela01 VEN10	S	V	V	I	N
Venezuela01 VEN2	P	V	V	I	N
Venezuela01 VEN3	P	V	V	I	N
Venezuela01 VEN4	P	V	V	I	N
Venezuela01 VEN5	A	I	I	I	S
Venezuela01 VEN6	P	V	V	I	N
Venezuela01 VEN7	P	V	V	I	N
Venezuela01 VEN8	S	V	V	I	N
Venezuela04 VEN 116	A	I	I	I	S
Venezuela04 VEN 117	A	I	I	I	S
Venezuela04 VEN 120	A	I	I	I	S
Venezuela04 VEN 121	P	V	V	I	N
Venezuela04 VEN 122	A	I	I	I	S
Venezuela04 VEN 123	P	V	V	I	N
Venezuela04 VEN 124	A	I	I	I	S
Venezuela04 VEN 126	A	I	I	I	S
Venezuela05 VEN 32	S	V	V	I	N
Venezuela05 VEN 33	S	V	V	I	N
Venezuela05 VEN 34	P	V	V	I	D

Asia. Although not significant based on bootstrap values, related sub-branches of the vvIBDV clade were observed. One contained viruses from South Africa, Venezuela and Thailand. Other sub-branches of the tree that were phylogenically related to the vvIBDV strains included one with three 1999 viruses from Israel, another with 1997 isolates from Singapore and a third with one 1997 virus from Spain and two 2001 El Salvador viruses. Both phylogenetic trees placed these two viruses from



El Salvador (ES1 and ES2) and one from Spain (SP11) in a clade that appeared to be genetically divergent from the main vvIBDV clade. The viruses had the typical A222, I242, I256 and I294 of vvIBDV but they had a substitution mutation at 299 from S to N.

The non-vvIBDV strains separated into two major groups; one characterized by variant viruses and the other by classic viruses (Fig. 2). The branch containing the variant viruses Del-E and T1 also contained the two viruses from South Africa (05 SA10 and 05 SA8) seen in Fig. 1. A second branch related to these variant strains contained viruses from Venezuela and Columbia with K249 and I286. The Spain01 S8 and Spain01 S9 viruses were also on this branch suggesting they were related to variant IBDV strains. The branch containing classic viruses Cu-1, STC and Bursine also contained viruses from Bolivia, Thailand, Mexico, Argentina and Colombia.

Discussion

The goals of this study were to assess the genetic variability among relatively recent isolates of IBDV associated with high mortality in chicken flocks and to track short-term evolution of the viral dsRNA from vvIBDV strains. Samples were collected from a wide geographic region that included 18 countries on four continents. All the samples were collected between 1997 and 2005.

Although the viruses examined were from flocks experiencing higher than expected mortality, we did not assume they were vvIBDV strains. Currently, the only acceptable criterion for identification of the vvIBDV phenotype is mortality rate in SPF chickens (Ignjatovic et al., 2004; Van Den Berg et al., 2004). In the study by Ignjatovic and coworkers (Ignjatovic et al., 2004), an IBDV isolate associated with high mortality in the field was determined not to be a vvIBDV strain using a combination of molecular assays and pathogenicity studies. Based on molecular characteristics, an IBDV isolate from Malaysia was predicted to have the very virulent phenotype, but this isolate only caused 10% mortality in susceptible chickens (Hoque et al., 2001). It was reported that vvIBDV induced mortality in SPF chickens could not be anticipated on the basis of antigenicity and genetic lineage (Van Den Berg et al., 2004). These scientists also noted that the use of different genetic lines of SPF chicks for vvIBDV pathogenicity studies may contribute to variability in the mortality results. The genetic and antigenic identification of vvIBDV strains that cause lower than expected mortality may suggest that these viruses are evolving to less virulent phenotypes. Thus, reliable identification of the vvIBDV phenotype may ultimately require a combination of field observations, pathogenicity studies in susceptible chicks and molecular based assays.

Fig. 1. Phylogenic analysis was conducted on viruses with genetic characteristics consistent with vvIBDV strains. The Neighbor-joining analysis was used for amino acid sequences located in vVP2 between 210 and 369. Numbers at the nodes indicate the bootstrap confidence values calculated using 1000 bootstrapping replicates. Included for comparison are vvIBDV strains OKYM, UK661 and BD 3/99; classic IBDV strains Cu 1, STC and Bursine; and variant IBDV strains Del E and T1.

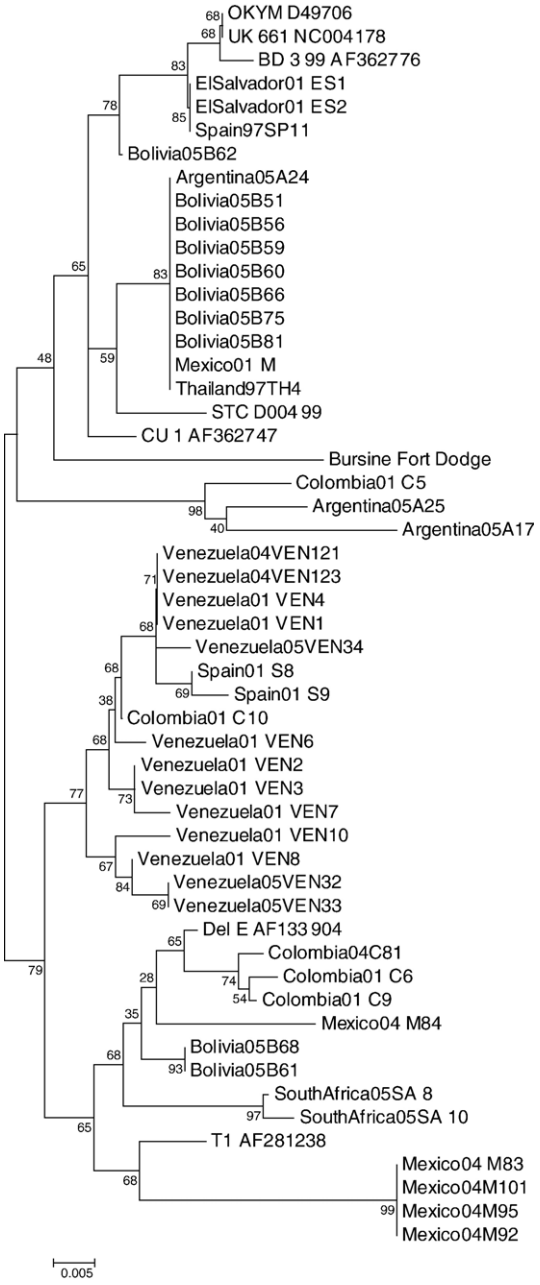


Fig. 2. Phylogenic analysis was conducted on viruses with genetic characteristics consistent with non-vvIBDV strains. The Neighbor-joining analysis was used for amino acid sequences located in vVP2 between 210 and 369. Numbers at the nodes indicate the bootstrap confidence values calculated using 1000 bootstrapping replicates. Included for comparison are vvIBDV strains OKYM, UK661 and BD 3/99; classic IBDV strains Cu 1, STC and Bursine; and variant IBDV strains Del E and T1.

Determining the actual virulence of the viruses in our study was not possible since importation of all samples to our laboratory required treatment with phenol and chloroform (Import Permit #44226). Thus, we relied on several genetic characteristics consistent with the very virulent phenotype of IBDV to identify those viruses that could be vvIBDV strains (Banda and Villegas, 2004; Hoque et al., 2001; Mardassi et al., 2004; Rudd et al., 2002). Furthermore, we examined the viruses for genetic characteristics typically found in classic and variant IBDV strains in the U.S. (Jackwood and Sommer-Wagner, 2005). Genetic sequencing to discriminate between classic IBDV and exotic vvIBDV strains was reported to be of value (Ignjatovic et al., 2004). Others have reliably used molecular characteristics to distinguish vvIBDV from non-vvIBDV strains (Mardassi et al., 2004; Owoade et al., 2004).

To accomplish our goal we focused on the vVP2 region of the viral genome. This variable sequence region was selected because it has the highest mutation frequency compared to other regions of the IBDV genome and can be used to uniquely identify variant, classic and very virulent IBDV strains. To track short-term evolution of the virus, genetic characteristics that change frequently will provide more valuable information than relatively constant sequence regions of the genome (Levin et al., 1999).

All the viral genomes examined in our study were reported to be from chicken flocks experiencing higher than expected mortality. However, not all of the 113 viral genomes examined had characteristics consistent with the vvIBDV phenotype. The sequence analysis demonstrated that some viruses had genetic characteristics consistent with variant and classic strains. Fourteen viruses from Central and South America were considered to have vVP2 amino acid sequences typical of variant IBDV. A total of 22 viruses had P222 and all had vVP2 amino acid sequences typical of classic IBDV strains. Two viruses from Spain (01 S8 and 01 S9) had P222, K249 and I286 but were on a branch with viruses from South America that were otherwise more related to U.S. variant viruses than they were to the Cu-1 and STC classic viruses. We previously reported the presence of IBDV in France and Spain that were genetically similar to U.S. variant strains (Jackwood et al., 2006).

The presence of higher than expected mortality in these broiler chicken flocks was not always associated with detection of the very virulent genotype of IBDV. There may be several reasons for this including other infectious agents, IBDV induced immune suppression, poor management practices, inaccurate disease records or the presence of multiple IBDV strains. The 1999 high mortality disease outbreak in Australia was not due to a vvIBDV strain but rather, a native classic IBDV strain was detected (Ignjatovic et al., 2004). The authors suggested that low protective antibody levels combined with poor husbandry could have elevated the mortality in that outbreak.

Two viruses from South Africa with A222 were related to the U.S. variant viruses. This was unexpected because most U.S. variant viruses have Threonine at position 222. Although Australian variant viruses have been reported to have A222, these viruses were not related to U.S. variants (Sapats and Ignjatovic, 2000). The K249, I286 and D318 amino acids are unique to U.S. variant viruses. All three were observed in the South African viruses. These data support the need for further studies on IBDV from South Africa to conclusively determine if these viruses are antigenically similar to U.S. variant viruses.

We identified 74/113 viruses with genetic characteristics consistent with the vvIBDV phenotype. All but one virus from Bolivia were in a single phylogenic clade. The Serine at position

299, thought to be conserved among vvIBDV, was not observed in three putative vvIBDV strains. The El Salvador ES1 and ES2 viruses and the Spain SP11 virus had N299 and phylogenetic analysis placed them on a separate branch in the vvIBDV clade. Previous studies reported vvIBDV strains containing N299 (Eterradossi et al., 1999) and non-vvIBDV strains with S299 (Rudd et al., 2002). We also observed S299 in the non-vvIBDV strains from Argentina (A25) and Colombia (C5). Three viruses from Singapore had Serine at position 222 and were on a separate branch of the vvIBDV clade. Although rare, previous studies reported vvIBDV strains from Indonesia that had Serine at position 222 (Parede et al., 2003; Rudd et al., 2002).

The virus isolated from Bolivia in 2005 (B62) was not part of the vvIBDV clade or the classic virus clade. Bootstrap values are not confidence limits but they are a measure of statistical validity of the tree branches; the higher the bootstrap value the greater the internal reliability of the branching. The bootstrap values in Figs. 1 and 2 indicated the differences between the B62 virus and vvIBDV strains are significant. B62 appears to be a unique IBDV strain but it may be more closely related to classic strains based on lower bootstrap values with those viruses.

In conclusion, high mortality in chicken flocks may not always be associated with vvIBDV. It could be the result of other factors including sequelae in a non-vvIBDV infected immune-compromised host. Short-term evolution of vvIBDV, although not significant based on bootstrap values, appears to be responsible for mutations in vVP2. The truncated vVP2 sequences examined in this study are expected to have substitution mutation rates that do not follow a molecular clock (Hon et al., 2006). Selection pressures from the host's immune system and competition with other viruses probably account for this observation. These selection pressures may be different in different regions of the world. Although the molecular basis for the very virulent phenotype has not been conclusively elucidated, vVP2 sequences are responsible for controlling antigenicity and tissue tropism of the virus. Amino acid changes in this region could affect these characteristics and in the future complicate control of vvIBDV strains.

Materials and methods

Viruses

During the years from 1997 to 2005, we received genetic material from suspect vvIBDV strains. Import regulations prohibit us from importing live virus. The viral genomic dsRNA, however, was imported into our laboratory under import permit #44226 from the U.S. Department of Agriculture Animal and Plant Health Inspection Service (Riverdale, Maryland, USA). Examined in this study were 113 samples from Argentina, Bolivia, Brazil, Venezuela, Colombia, El Salvador, Dominican Republic, Mexico, France, Spain, Denmark, United Kingdom, Israel, Jordan, South Africa, Singapore, Korea and Thailand. Nineteen of these viruses were previously described (Jackwood and Sommer-Wagner, 2006). All the samples were obtained from broiler chicken flocks with a reported history of higher than expected mortality.

Reverse transcription-PCR

Bursa tissue samples arrived at our laboratory after being treated in phenol/chloroform/isoamyl alcohol (25/24/1) for a minimum of 2 weeks (Jackwood et al., 1996). The tissues were washed three times in TNE buffer (10 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid), homogenized and then treated with proteinase K (Sigma Chemical Co., St. Louis, MO) using our standard procedures (Jackwood and Sommer, 1997). Following proteinase K treatment, the samples were extracted in acid phenol (pH 4.3) (AMRESCO, Solon, OH) and ethanol precipitated. The viral genome was suspended in 90% DMSO (Sigma Chemical Co.) and used for reverse transcription-PCR (RT-PCR).

The RT-PCR assay was conducted using a primer pair that amplifies a 743 bp region of vVP2. This region of the genome was selected because it encodes the most highly variable sequence of the virus and has numerous genetic markers for vvIBDV, classic and variant IBDV strains (Banda and Villegas, 2004; Hoque et al., 2001; Parede et al., 2003; Rudd et al., 2002). The forward primer used was 5′-GCCCAGAGTCTA-CACCAT-3′ and the reverse primer was 5′-CCCGGAT-TATGTCTTTGA-3′. The RT-PCR reactions were conducted using the GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ) according to the manufacturer's instructions. The RT incubation was at 42 °C for 60 min followed by the PCR incubations at 95 °C for 1.0 min, 53 °C for 1.5 min and 72 °C for 1.0 min. A 7.0 min extension at 72 °C was added at the end of the 35 PCR cycles.

Nucleotide sequence analysis

The RT-PCR products were purified using a Wizard SV Gel and PCR Clean-up System (Promega Corp. Madison, WI). They were sent to the University of Wisconsin Biotechnology Center DNA sequence Facility (Madison, WI) for nucleotide sequencing. The sequence results were downloaded using Chromas (Technelysium Pry Ltd., Queensland, Australia) and analyzed using Omiga software (Oxford Molecular, Campbell, CA, USA). Sequences were aligned using Clustal W and phylogenetic trees of the nucleotide and predicted amino acid sequences were conducted using MEGA version 3.1 (Kumar et al., 2004) and up to 1000 bootstrapping replicates. The neighbor joining (NJ), minimum evolution (ME) and unweighted pair-group methods using arithmetic averages (UPGMA) distance methods were used.

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