GENETIC CHARACTERIZATION OF POLISH INFECTIOUS BURSAL DISEASE VIRUS STRAINS

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Five Polish infectious bursal disease viruses (IBDVs) isolated from two epidemics on the turn of 70/80s (early IBDV) and in the 90s (recent IBDV) were characterized by nucleotide sequencing of VP2 variable domain (vVP2). They were compared to the Faragher 52/70 (F52/70) reference strain of European classical serotype 1 IBDV and to the 89/163 (antigenically typical) and 91/168 (antigenically atypical) French very virulent (vv) IBDV isolates. All Polish viruses, irrespectively of the isolation date, are different from the reference strain F52/70. The early Polish strains exhibit several amino acid changes that make them markedly different from recent vvIBDV. The recent Polish strains are closely related to other European vvIBDV. The 93/35 virus has the same amino acid sequence as the French atypical 91/168. Phylogenetic analysis suggests that the Polish isolates belong to two genetically distinct clusters of IBDV: the recent are significantly related to vvIBDV and the older form cluster unrelated to other groups of IBDV.

Key words: infectious bursal disease virus, nucleotide sequences, epidemiology.

Infectious bursal disease virus (IBDV) is a member of the genus Avibivirus within the family Birnaviridae (24). Due to its immunosuppressive effect, even in subclinical infections, and high rate of mortality in acute infections, infectious bursal disease (IBD) is of major importance to the poultry industry. Since the first report in the USA, the disease became widespread all over the world (5). In Europe, it appeared for the first time in the middle of 1960s. Until the 80s, the strains of the virus were of low virulence, they caused low mortality, and were satisfactorily controlled by vaccination but at the end of the 1980s vaccination failures were observed and a very severe form of the disease caused by very virulent IBDV (vvIBDV) was described first in Europe (4, 11, 23, 26) and later in the early 1990s in Japan (14, 21). In the USA, it
was demonstrated that the new isolates had been affected by an antigenic drift against which classical IBD virus (IBDV) vaccines were not satisfactorily protective (13, 25). During the 63rd General Session of the Office International des Epizooties (OIE, Paris, 15-19 May 1995), it was estimated that IBD has a considerable socio-economic importance at the international level, as the disease is present in more than 95% of Member Countries (7).

The major immunogenic component of IBDV is the viral capsid protein VP2, encoded by segment A, one of the two segments of IBDV genome which consists of double stranded RNA (12, 25). VP2 amino acid (aa) positions 206 to 353 are recognized as an important neutralizing antigenic site (1). This part is named “VP2 variable domain” (vVP2) because most of the aa changes between antigenically different IBDVs are clustered in this area. This region includes two major sets of hydrophilic aa, termed peak A and B, from aa positions 210 to 225 and 312 to 324, respectively, with smaller hydrophilic peaks present in between (2). So far, the four aa changes in vVP2 at positions 222A, 256I, 294I, and 299S have been shown to be present in all European-like vvIBDVs and to be differentiated from the classical viruses (3, 10, 27, 28). Although there is no experimental evidence that these aa changes play a role in the increased virulence of vvIBDVs, they have been generally considered as a putative marker for vvIBDV-related strains.

In Poland, the first real epidemic of IBD occurred at the end of the seventies (15). Despite of the wide spread of IBDV, the clinical form of the disease was rarely observed and the field strains isolated in 1978 and 1980 from broiler chickens exhibited a low pathogenicity for SPF chickens (16, 17). However, these strains caused economic losses due to impaired growth and acquired immunodeficiency (18).

The first cases of the acute form of IBD were diagnosed at the end of 1991. During the next year, the disease spread rapidly throughout the whole country and affected broilers and laying pullets flocks with mortality rates up to 50% and 70%, respectively (19). Pathogenic characterization of the IBDVs isolated in 1991, 1992 and 1993 confirmed their high virulence, as mortality rates in experimentally infected 4-week-old SPF chickens ranged from 40 to 100% (20).

In previous studies of Polish IBDV strains with the panel of monoclonal antibodies (Mabs) it was shown that they all had antigenic characterization different from the F52/70 reference strain. The early Polish strains isolated between 1978 and 1980 were also different from vvIBDVs. The recent Polish IBDV strains isolated after 1990 were similar to the French antigenically typical or atypical vvIBDV. Antigenically typical strains reacted with all studied Mabs but Mab 3 and Mab 4, and additionally antigenically atypical did not react with Mab 8 (6).

The aim of the present study was to characterize genetically the Polish IBDV isolates obtained from field outbreaks between 197 and 1981 and after 1990 and to compare them with other IBDV strains. The genetic relationships between these viruses were investigated by using reverse transcription, amplification and direct sequencing of a genome fragment encoding vVP2.

Material and Methods

Viruses. Two early Polish isolates 78/GSz and 80/GA were collected during the first epidemic of IBD in Poland between 1978 and 1980. Three recent IBDV strains were isolated between 1990 and 2000 from chickens with clinical symptoms of acute
form of IBD. The reference IBDV strains included in this study were the Faragher 52/70 (F52/70) strain as reference for the classical European serotype 1 strains, the French 89/163 strain as reference for the very virulent (vv) IBDV, and the 91/168 strain as an atypical vvIBDV (8). The viruses (isolates and strains) were propagated in 4-to-6-week-old SPF chickens, with sampling of the infected bursae 4 d p.i. Bursal homogenates were prepared by blending the bursae w/v in phosphate buffered saline (PBS), then clarified v/v in Freon. All the viruses were stored at -70°C.

**Genome analysis strategy.** The genetic characterization of the Polish isolates was achieved by reverse transcription and amplification using chimeric oligonucleotide primers (coupling the sequence of M13 and 21M13 standard primers to IBDV-specific L2 and U2 sequences, respectively) according to Eterradossi et al. (10). The resulting primer pair generated a 604 base pair (bp)-long genome fragment, 516 bp of which were IBDV-specific and span the region encoding vVP2 (11). The RT-PCR products were purified with the Qiaquick Gel purification kits (Qiagen, Chatsworth, CA, USA). Sequencing of the purified RT-PCR products was performed using M13 and 21M13 specific primers with an automated 373 ABI DNA sequencer (Applied Biosystems Inc.).

**Phylogenetic analysis.** The phylogenetic tree was generated as follows: the nucleotide sequences (nt positions 762 to 1151 according to Bayliss et al. (2) were aligned using the Clustal W programme. A set of 500 aligned sequences was then generated by the bootstrap method (Seqboot programme). Genetic distances were calculated for each set of aligned sequence with the DNADIST programme, and the trees were calculated by the neighbor joining method (Neighbour programme) using the OH strain (serotype 2) as an extra group. Finally, the consensus tree for 22 representative isolates was generated using the Consense programme (all programmes by J. Felsenstein, Department of Genetics, University of Washington, Seattle, Washington, USA, 1993).

**Results**

The comparison of the 390 nt-long sequences that have been determined in all studied viruses revealed two groups with a high level of genetic identity. The first group included two early isolates (97.2 to 99.0% identity), the second group included three recent Polish isolates and the 89/163 and 91/168 vvIBDV strains (97.2 to 99.7% identity). Fig. 1 presents amino acid sequence of VP2 variable domain, positions 183 to 353 (according to Bayliss et al., 1990) in the studied IBDV strains. The early 78/GSz and 80/GA exhibited several amino acid changes, the first of these conserved changes was from proline to serine at aa position 222 (P 222→S). The other aa changes conserved in the early isolates were I 242→V, E 245→G, A 270→T, I 272→T, D 279→N, L 289→P, M 290→I, and I 296→F. There was a difference at the amino acid in position 254 between the 78/GSz (glucose) and 80/GA (aspartic acid) strains. All three recent Polish strains exhibited four amino acids: 222 A, 256 I, 294 I, and 299 S which differ vvIBDVs from F52/70 and have been found so far in all European – like vvIBDV. The 93/35 virus had the same amino acid sequence as the 91/168 French atypical vvIBDV. The changes rely on substitution of leucine in the place of glutamine in amino acid position 324.
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**Fig. 1.** Amino acid sequence of VP2 variable domain (aa positions 183 to 353 according to Bayliss *et al.*, 1990) in IBDV strains Faragher 52/70 (classical), 89 163 (French vvIBDV with typical antigenicity), 91 168 (French vvIBDV with atypical antigenicity) and Polish strains 78/GSz, 80/GA, 93/35, 94/48, and 00/40. Dots indicate aa positions where the sequence is identical to F52/70.
Fig. 2. Phylogenetic consensus tree of the studied IBDV strains.

The nucleotide (nt) sequences encoding VP2 variable domain in Polish strains (underlined) were compared with several very virulent, classical, variant or cell culture adapted viruses of various geographical origin. The OH strain was used as an extra group. Branch length has no special meaning. The figures at the forks are bootstrap values, i.e. they measure the likelihood that viruses which are to the right of that fork are indeed genetically related.
Fig. 2 presents the consensus phylogenetic tree constructed following analysis by the neighbor joining method of the previously aligned nucleotide sequences encoding VP2 variable domain of the studied IBDV strains: Polish strains (underlined) and several very virulent, classical, variant or cell culture adapted viruses of various geographical origin. The OH strain was used as an extra group. All serotype 1 strains appear genetically related (bootstrap 100%) however the Australian strain differs significantly (it branches out of other serotype 1 strains in 93% of the bootstrap generated trees). Polish early strains 80/GA and 78/GSz (100% bootstrap) form a separate genetic cluster (cluster i). There were four other remarkable genetic clusters with a bootstrap exceeding 75%, namely the US variant viruses (cluster ii), bootstrap 76.6%, the cell culture adapted viruses (cluster iii), bootstrap 99.2%, the classical viruses including the F52/70 isolate (cluster iv), bootstrap 99.8%, and the very virulent group (cluster v), bootstrap 88.2%, which included recent Polish strains 93/35, 94/48, 00/40 as well as French 89/163 and 91/168. The genetic relationships between early strains (cluster i) and clusters ii, iii, and iv are unclear. Significant subclustering was apparent within group v: the Polish 93/35 and French 91/168 antigenically atypical strains appeared genetically related (bootstrap value 87.8%), as could be expected since they differed only by one nucleotide over the sequenced region.

Discussion

Recent Polish isolates had the aa sequence of their VP2 variable domain as typical vvIBDV represented by the 89/163 French isolate containing four aa (222A, 256I, 294I, and 299S) different in comparison to classical virulent F52/70 (222P, 256V, 294L, and 299N). These changes have so far been demonstrated in most vvIBDVs, especially those of European origin. The phylogenetic analysis of the nucleotide sequences confirmed these relationships, as the three recent Polish IBDV strains appeared significantly genetically related to other vvIBDVs, with which they were associated in 95% of the bootstrap generated trees. Hence, the present study confirms previous pathotypic studies and demonstrates that vvIBDVs are now present in Poland. Interestingly, one of the recent Polish viruses (93/35) appeared similar to the French strain 91/168. These both isolates were found to share 99.8% identical nucleotide sequences, and the same deduced aa sequence (one aa change in VP2 hydrophilic peak B). Because these atypical French and Polish vvIBDV-related isolates have been isolated over a relatively short period of time (1991-1993), share a very high nucleotide identity although they originate from geographically distant areas, and have not so far been isolated elsewhere, it could be speculated that the same IBDV strains were exchanged between the two countries or that the outbreaks in the two countries might have a common source. However, the epidemiological links between the outbreaks in the two countries are not known, and a survey of the French vvIBDV isolates from 1989 to 1997 suggested that the 91/168 strain had caused only a sporadic outbreak in France (one farm) and did not replace the more typical 89/163-like vvIBDVs (9).

The aa sequence of two early Polish strains (78/GSz and 80/GA) revealed that these strains exhibit 9 to 10 aa changes as compared with the classical F52/70 strain, and 12 to 13 as compared with typical vvIBDVs.

The present results with early Polish isolates could give new insights into the epidemiology of the early outbreaks of IBD infection in Europe. The current idea of
IBD epidemiology in Europe is that strains antigenically and genetically similar to the classical virulent strains (i.e. F52/70-like) had been prevalent worldwide and they were replaced in Europe at the end of the eighties by the so called very virulent viruses (4, 26), which in turn seem to have now spread worldwide with the exception of North America and Australia (7, 21, 23). This theory stems from the antigenic and genetic studies first of a limited number of early pathogenic viruses which have been maintained as reference strains (e.g. the 002/73, STC, Cu1 and F52/70 strains), and second of a limited number of some IBD attenuated vaccine strains which have been developed from several early IBDV isolates (Lukert strain, D78). However, the extent to which early IBDVs were actually antigenically and genetically homogeneous is not known precisely because of a limited number of available early IBDV strains. Similarly, it is not known if other IBDV strains, possibly involved in subclinical infections, were present in Europe before the F52/70-like viruses were isolated. These points might be important to investigate, as the epidemiological origin of the IBDV strains that have recently emerged in Europe is still unknown. The present report showing that early Polish isolates share unclear genetic relationships with the classical IBDVs (bootstrap value ranging 26-52%) and the previous one concerning antigenic characterization of these isolates suggest that more diversity than currently admitted might have existed in Europe prior to the emergence of the vvIBDV. Similar strains described Palya et al. (22). The early Hungarian IBDV isolates collected in the late 70s and early 80s belonged to a genetic cluster significantly different from the “classical” viruses.

The first cases of acute form of IBD were recorded in 1970. Even though the early IBDV strains were isolated from 1978 to 1980, so much later than classical F52/70 strain, it could be suggested that early IBDV strains were present in Poland before the F52/70-like viruses. The early viruses might have been replaced in the mid-seventies to the late eighties by F52/70-like viruses and process of this replacement might have lasted for some time when both types of the viruses coexisted. Similar phenomenon was noted at the end of 1980s when the classical viruses were replaced by the vvIBDV’s. On the other hand, it might be possible that the early and F52/70-like viruses stem independently from one common ancestor. They might expand putting pressure one on another thus easier acquiring new characteristics (antigenicity and virulence). Such effect might be also reason for raising very virulent form of IBDV. However, such hypothesis should be confirmed by testing more of early viruses, which unfortunately may not be available unless fixed infected material is investigated.

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References