ELTE Ph.D. School
Evolutionary genetics, evolutionary ecology, conservation biology program
Program Leader: Eörs Szathmáry, MHAS

Genetic diversity of infectious bursal disease virus strains and the molecular basis for change in virulence

PhD Thesis

Tamás Mató

Prepared under the guidance of Béla Lomniczi
at the Veterinary Medical Research Institute of the Hungarian Academy of Sciences

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Introduction

Infectious bursal disease (IBD) first described in the USA in 1962 is one of the most important, highly contagious viral disease of young chickens all over the world. It causes disease only in chickens, but other bird species can also be infected as it was shown by the detection of antibodies to IBDV. Replication of virus in the immature B-lymphocytes of *bursa Fabricii* is followed by a viraemic phase, when clinical signs and mortality is observed. Outcome of an infection is influenced by several factors. One of the most critical ones is the age of animals in correlation with the developmental stage of *bursa Fabricii*. Serious immunosuppression as a result of B-lympocyte depletion from bursa follicules, usually without any clinical signs, develops in chickens infected younger than 3 weeks of age. Affected chickens are more susceptible to several viral, bacterial and parasitic diseases and their immune response to vaccination is also suppressed. IBD with clinical signs is mostly observed in 3-6 weeks old chickens, when both the bursa and the complement system are matured and the physiological atrophy of bursa has not begun yet. Morbidity, after the rapid onset of disease, can reach 100% depending on the pathogenicity of the strain. Mortality can be as high as 5-25% in broilers and 30-70% in more susceptible layer pullets.

Since the end of ’60-s the disease had been well controlled by vaccination using attenuated IBD virus strains prepared from field strains by serial passage in embryonated hens eggs and chicken embryo fibroblast cell culture. New strains, able to cause disease in face of maternal immunity induced by vaccinal strains emerged in 1985 in the US and in 1987 in Europe. Losses caused by the American strains were mainly due to immunosuppression, while the European highly virulent IBDV strains were characterized by high mortality in susceptible flocks. IBDV strains are classified into three large groups: (i) so-called *classical virulent strains* (the earliest described ones and those which are genetically, serologically and pathologically similar to them), (ii) so-called *variant strains* (antigenically different from classical virulent strains, usually causing immunosuppression without clinical signs of IBD) and (iii) so-called *very virulent strains* (with significantly increased mortality without marked antigenic difference from classical virulent strains).

The etiological agent: IBD virus (IBDV) is a member of the genus *Avibirnavirus* within the family *Birnaviridae*. These viruses are characterized by bisegmented double-stranded RNA genome encapsidated into single-shelled capsid. The VP2 stuctural protein (consisting the

outer surface of the capsid) and also VP3, VP4 and VP5 proteins are coded by the bigger segment ("A"), while the viral polymerase (VP1) is encoded by the smaller genome segment ("B"). Amino acid changes among IBDV strains are concentrated on the *hypervariable region* of VP2 protein (between amino acids 206.-350.) Conformation-dependent epitopes recognized by neutralizing antibodies are located in this region; therefore it has an important role in the anti-viral immune response. Studies using monoclonal antibodies revealed significant structural changes in these epitopes in case of variant strains. No changes like this could be detected in case of very virulent strains, but most probably this region is involved in the increased virulence.

One of the methods used for attenuation of IBDV strains is the adaptation to non-lymphoid cell cultures (further referred to as cell culture adaptation). In this respect, VP2 plays the most important role on segment "A" at the following amino acid positions: i) histidine in position 253. (increases the virus titre), ii) asparagine at position 279. (probably supports intensive virus replication in tissue culture, but a virus can be cell-culture adapted without this marker) and iii) threonine at position 284. (this allows entrance of the virus into non-lymphoid cells). No changes related to cell culture adaptation were found in VP3 and VP4 proteins. VP1 - encoded by segment "B" determines the ability of the virus to replicate in cells of other species (like VERO, which is a green monkey kidney cell line) and the production of infectious virions in the cells.

Contradicting results were obtained regarding the role of the different viral proteins in virulence VP2 alone or VP2 and also VP1 were reported to have significant influence on the virulence by different authors. Moderate effect of VP3 on virulence was detected, too. The genetic basis for virulence can not however be clearly established on the basis of our current knowledge; this could be influenced by several factors.

Our objective was to get better knowledge on the hypervariable region of VP2, as this region is very important in the identification of IBDV strains and has important role in the virulence and cell-culture adaptation of IBDV strains.

Objectives

The aims of my work presented in the dissertation were:

i.) to investigate the genetic diversity of IBD viruses,

ii.) to categorize field isolates and vaccinal strains on the basis of genetic analysis;

iii.) by revealing the genetic differences among the groups obtained, analyse their impact on the immunogenic properties, virulence and adaptation to non-lymphoid cell culture.

Although this work is mostly based on my molecular investigations, other tests -i.e., *in vivo* pathogenicity tests, testing of adaptation to non-lymphoid cell culture *in vitro*- were also applied to provide help in the interpretation of the results.

The aims of my work are summarized in the following points:

1) Development of molecular diagnostic methods fro IBD viruses:

   a) Development of a nested RT-PCR system that amplifies the hypervariable region of VP2, which is suitable for detection of IBDV from organ samples.

   b) Development of a reliable molecular diagnostic for the differentiation of strains belonging to separate pathotypes of IBDV (i.e. vaccines and virulent strains, and among the latter ones *classical virulent*, *very virulent* and *variant* strains).

2) Investigation of the genetic diversity of IBD viruses:

   a) Genetic characterization of IBDV geno-groups (focusing on Hungarian isolates and concentrating on the hypervariable region of VP2),

   b) revealing their phylogenetic relationships,

   c) investigating the genetic basis of pathogenicity by comparing strains with different pathogenicity (including the checking the pathogenicity of representative strains *in vivo*),

   d) investigating the genetic basis of adaptation by genetic analysis of strains adapted to non-lymphoid cell cultures and others which are able to grow in lymphoid cells only.

Methods

Purification of viral RNA and reverse transcription

Viral RNA was purified directly from the bursa homogenate of infected chickens or from the reconstituted vaccine (or cell-culture supernatant). Samples were submitted to proteinase K digestion which was followed by purification with TRI-reagent according to the manufacturer's instructions. Reverse transcription was performed using random hexamer primers and M-MLV RT-enzyme.

Polimerase chain reaction (PCR)

A nested PCR system was developed using previously published primers (detailed description of the method is published in the following articles: *Domanska et al (2004)* and *Ivan et al (2005))*. Sequences of the primers used, which amplify a 474 bp long fragment of VP2 encompassing the hypervariable region are as follows: for external PCR P1: 5' TCACCGTCCTCAGCTTAC 3' and P2: 5' TCAGGATTTGGGATCAGC 3', for internal PCR P2.3: 5' CCCAGAGTCTACACCATA 3' and P5.3: 5' TCCTGTTGCCACTCTTTC 3'.

Analysis using restriction endonucleases (RFLP)

The nested PCR products were digested with the following enzymes to differentiate strains belonging to different groups: *Bse* DI; *Bsp* MI, *Ssp* I and *Eco* 91I. Restriction fragment patterns were analysed in correlation to the 50 bp DNA ladder.

Sequence analysis

Nucleotide sequence of the purified PCR product was determined. The sequence was aligned with sequences obtained from the GenBank or from our previous tests, using the ClustalW method option of the BioEdit sequence alignment editor program. Homology of sequences was calculated by the Kimura's two-parameter method (TREECON for Windows program) phylogenetic tree was generated by Neighbour-joining method.

Isolation and propagation of viruses

Only strains, which were planned to be involved in other tests or were proved to be important for establishment of an IBDV strain collection were isolated and propagated. Most of the IBDV field strains (*subclinical*, *variant* or unknown type) were propagated in 3-6 weeks old SPF chickens. Birds were infected with the organ homogenate *per os*, the *bursa Fabricii* of each chicken was harvested on the 3rd or 4th day post-infection. Then a 10% homogenate of the organ was prepared in PBS supplemented with antibiotics. *Very virulent strains* were

propagated in 9-10 days old SPF embryonated hens' eggs after inoculation onto the chorio-allantoic membrane. Allantoic fluids were harvested and used for further studies.

Determination of virus titre

Titre of cell culture adapted vaccines was determined on primary chicken-embryo fibroblast cell-culture in a 96-well tissue culture microplate; other strains were titrated in 9-10 days old embryonated SPF hens' eggs (inoculated onto the chorio-allantoic membrane) using standard methods.

Test method for adaptation to cell culture

Tests were conducted on primary chicken embryo fibroblast cell culture (CEF). Those strains which caused cytopathogenic effect in the cell culture were characterized as adapted.

Pathogenicity tests (in vivo)

Serology (virus neutralization)

Level of antibodies against IBDV was determined by micro-neutralization method in primary chicken embryo fibroblast cell culture against a cell-culture adapted classical IBDV strain.

Calculation of B:B index

B:B index (bursa weight:body weight index) was calculated as the quotient of bursa weight in milligrams and the body weight in grams (B:B ratio) correlated to the mean B:B ratio of non-treated control group.

Testing of vaccines

Pathogenicity test was conducted in 2 weeks old SPF chickens after per os immunization of different groups with the same amount of different vaccine viruses. Residual pathogenicity of the vaccine strains was evaluated on the basis of B:B index, histopathological lesions of the bursa and humoral immune response to vaccination.

Testing of sub-clinical strain

Two weeks old SPF chickens were infected *per os* by the bursa homogenate obtained during propagation of the virus in SPF chicks. Pathogenesis was evaluated on the basis of B:B index (only from the 7^{th} day post-infection) and the macroscopic and microscopic lesions observed.

Testing of South-African variant strain

Three days old broiler chickens with maternally derived antibodies against classical (antigenically) IBDV strain were infected *per os* with the isolate to be characterized or the referent *variant* Delaware E strain or *classical* W2512 strain. All strains were applied in the same titre. Take of virus and pathogenesis was followed by RT-PCR, B:B index and histology during a 21-day-long observation period.

Discussion

Investigation of pathogenicity, antigenicity and molecular genetic characteristics of infectious bursal disease virus strains (IBDV) led to the following results:

- Reverse transcription followed by nested PCR, which were found suitable for diagnosis and to follow the dissemination of IBD viruses in the body was developed *(Iván et al. 2005*).
- A restriction enzyme based method for rapid pre-screening and differentiation of IBDV strains was also developed.
- The first Hungarian IBDV strain (1975) causing clinical signs was characterized by nucleotide sequence analysis. It was concluded, that all European "classical virulent" strains were most probably the descendants of one strain, which became wide-spread in the continent.
- Strains isolated from sub-clinical cases in Hungary during the period of '77-81 -along with early polish IBDV strains- were also characterized and shown to constitute a separate, previously unidentified group. These strains differed antigenically from other known IBDV groups. These so-called sub-clinical strains, which could be the first IBD viruses in Europe, were later replaced from this continent by more virulent IBDV strains (*Domanska et al, 2004*). These sub-clinical strains are still present in Argentina and Brazil and also can be found among recent Canadian, Russian and US isolates on the basis of nucleotide sequences obtained from the GenBank. The relative large genetic distance within the sub-clinical group (11%) refer to the ancient origin of this group. These sub-clinical strains are not able to grow in non-lymphoid cell-culture, nevertheless they showed low pathogenicity in chicken. Attenuation of IBDV strains was usually reached by adaptation to non-lymphoid cell-culture. Our finding has shown, that the low pathogenic profile of IBDV strains can be reached without adaptation to non-lymphoid cell-cultures.
- "Very virulent" strains emerging in the late eighties were introduced to Hungary repeatedly and spread very quickly. This was verified by the phylogenetic examination of field samples from the period of 1995-2008.
- Occurrence of "variant" strains in South-Africa was demonstrated for the first time. This finding was supported by serological and pathological data. Three independent variant IBDV introductions were observed and one of them became endemic. The possible source of introductions was also fidentified. The South-African and Canadian strains seem to be

antigenically different from US variant strains, because of the amino acid change to alanine at position 222., which is characteristic to South-African and Canadian variants.

- Forty-eight vaccines of twenty-seven producers were characterized; their relationship to each other was tested. Vaccines tested formed eight separate groups.

Important features of vaccine groups

Vaccine group	No. of vaccines in the group	Important amino acid positions				Adaptation to non-lymphoid cell-culture	Pathogenic Group
		222	253	279	284		
D78	14	Pro	His	Asn	Thr	adapted	intermediate
P2	11	Pro	His	Asn	Thr	adapted	Mild
228E	2	Ser	Gln	Asn	Ala	non-adapted*	intermediate plus
STC	2	Pro	Gln	Asp	Ala	non-adapted*	intermediate plus
W2512	5	Pro	Gln	Asp	Ala	non-adapted	intermediate plus
MB	6	Ala	Gln	Asn	Ala	non-adapted*	intermediate plus
Lukert	3	Ser	Gln	Asp[$]	Thr	non-adapted*	Mild
V877	4	Pro	Gln	Gly	Ala	non-adapted	intermediate plus

[$] 278. Ala→Ser change can have an influence on 279. Asp

*After inoculation of non-lymphoid cell-culture with the vaccine, the following changes could be observed, which led to the ability of virus to replicate and induce cytopathic effect: 253. Gln→His and 284. Ala→Thr.

Groups according to pathogenicity:

- **Mild/highly attenuated**: mild bursal lesions, slow development of immune response.
- **Intermediate/moderately attenuated:** moderate bursal lesions, slow development of immune response.
- **Intermediate plus/slightly attenuated:** more serious bursal lesions, rapid immune response.

- Groups possessing markers of cell-culture adaptation (H^{253} and T^{284}) were found to be adapted. Asparagine at position 258 was also required for growing in non-lymphoid cells in the case of Lukert group.

-Asparagine in position 279 is probably a determinant of low pathogenicity as it is characteristic to most of the vaccine strains and all of sub-clinical strains showing low pathogeneticity, too. This amino acid residue is not enough for cell-culture adaptation as it was shown by the 228E vaccine group.

- Serological difference was observed in case of the following groups from the ones containing Pro at 222.: (i) 228E and Lukert groups (222. Ser) and (ii) MB group (222 Ala). This result verified the key function of amino acids at position 222 in determining antigenicity.

-Ability of cell-culture adapted strains to loose one of the markers of cell-culture adaptation (253. His to Gln change) accompanied by increasing in pathogenicity was verified by passage some of these strains in lymphoid cells (in chickens or in embryonated hens' eggs). Change in the reverse direction (253. Gln to His) could also be reached by passage the virus in non-lymphoid cells. These results verified the important role of His at position 253 in the adaptation to non-lymphoid cell-culture and determination of pathogenicity.

Articles related to the thesis

Ivan J, **Velhner M**, **Ursu K**, **German P**, **Mato T**, **Dren CN**, **Meszaros J**. (2005). Delayed vaccine virus replication in chickens vaccinated subcutaneously with an immune complex infectious bursal disease vaccine: quantification of vaccine virus by real-time polymerase chain reaction. Can J Vet Res. Apr; 69(2):135-42.

K. Domanska, T. Mato, G. Rivallan, K. Smietanka, Z. Minta, C. de Boisseson, D. Toquin, B. Lomniczi, V. Palya, N. Eterradossi (2004). Antigenic and genetic diversity of early European isolates of Infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of Infectious bursal disease virus revisited? Archives of Virology 149(3): 465-80. *(megosztott elsőszerzős cikk)*

Congress presentations:

T. Mato., B. Lomniczi, V. Palya (2004) Molecular characterisation of Hungarian field isolates and vaccinal infectious bursal disease virus strains World's Poultry Congress & Exhibition Istambul Turkey Fulltext CD

V. Palya T. Mato. (2004) Molecular grouping of current live infectious bursal disease vaccines. COST 839 Agriculture & Biotechnology, Immunosuppressive viral diseases of poultry, Final meeting, Barcelona

T. Mató, V. Palya and B. Lomniczi (2001) Molecular Characterisation of Hungarian field isolates and vaccinal infectious bursal disease virus strains. II International Symposium of Infectious Bursal Disease and Chicken Infectious Anaemia, Rauischholzhausen Germany Proceedings p172