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# Investigations on bovine viral diarrhea virus and some of the disease forms caused by the infection

**PhD thesis**

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# 1. Introduction, aims

Bovine viral diarrhoea virus (BVDV) is a worldwide pathogen causing severe economic losses in cattle industry. The consequence of the infection may range from inapparent or subclinical infection through acute and severe enteric disease to the highly fatal mucosal disease (MD) complex characterized by profuse enteritis and typical mucosal lesions. In the epizootiology of the disease the most important role is played by cattle persistently infected with non virulent (noncytopathic) BVDV (ncpBVDV), because these animals serve as natural reservoirs for the virus. Persistent infection develops when ncpBVDV is transmitted transplacentally during the first 4 months of foetal development. The disease causes direct losses (cull and mortality) but besides these the birth rate of the herd is significantly reduced and the secondary infections by other pathogenes due to the immunosuppressive character of the virus will increase the losses as well.

The pathogen belongs to the *Pestivirus* genus of the *Flaviviridae* family, and is present all around the world. The BVDV strains belong to two virus species, currently called BVDV-1 and BVDV-2. The BVDV-2 was endemic on the American continent originally, but in the last 10-15 years it spread to other continents, including some parts of Europe. In Hungary only the BVDV-1 is present, BVDV-2 has not yet been found.

The strains are able to infect the fetus transplacentally. If the infection happens before the self-recognition of the immune system, immunotolerance will be the developed, and the calf is borne infected and carries the virus all along its lifetime, hence persistently infected (PI) calves will be present in the farm. The PI animals remain seronegative, therefore the identification of these calves is not possible using traditional serological methods, Hence, they can’t be easily eliminated from the stock and other animals can be infected in the same group through direct contact with the PI animal or it's excretions. The other consequence of the persisting virus infection is that the fatal mucosal disease may develop in these animals, if a genetic chang occures (via mutation or recombination). During our investigations we met with MD in several cases, sometimes causing mass mortality.

Since in Hungary till 2014 no serological survey has been performed to help the estimation of the BVDV infection rate, we planned a serological investigation involving several large scale cattle farms throughout Hungary. An ELISA kitt, used to detect antibodies produced against the BVDV was applied to identify the infected animals, since these antibodies persist longer in the animal then the virus, so we were able to gather more reliable data on the prevalence of

the virus. Besides collecting epidemiological data, our intention was also to help the voluntary BVDV eradication on the farms. In order to chose the proper method of the successful eradication it is necessary to know the current prevalence of the pathogen. We also intended to know if there were any farms among the investigated ones, which are free of the infection since these farms would to serve as source of BVDV free cattle to replace those which have to be removed and eliminated from other herds because they are seropositiv or PI animals. In herds with high infection rate it is very probable that one or more PI animals are present which ensures the continuous circulation of the BVDV within the stock.

We also wanted to investigate the recombination rate of the BVDV genome and its consequences, utilizing a software framework comprised with new statistical methods. The possibility of recombination has great significance, since live-attenuated vaccines were used in Hungary, and they were proven to have the tendency to create long or even persistent infection in some calves when pregnant cows were vaccinated. Hence vaccine strains were able to participate in simultaneous infections with field BVDV strains and this simultaneous infection is the prerequisite of the viral genetic recombination. In our research, the aim of the investigations were to detect new recombinants of the virus and to discover recombination events among BVDV and other *Pestivirus* species, and also to identify the recombination rate of the virus.

Further aim of the investigations was to study the clinical symptoms, pathological lesions and aetiology of the bovine neonatal pancytopenia (BNP) appearing between 2009 and 2011 in Hungary. The disease occured in herds, where the cows were well immunized against BVDV. At the time of the investigations, the connection between the immunization and the BNP was not identified.

The BVDV infection takes place as inapparent infection in several cases, however regularly causes clinical symptoms and rarely death as well. The severity of the disease is primarily influenced by the previously mentioned *in vitro* cytopathic ability of the virus strain. The cp biotype of BVDV is causing diarrhea in healthy, non-pregnant cattle, whereas ncpBVDV often causes merly inapparent infection and seroconversion, probably mild immune suppression in the same animal.

In case of pregnant cows, the symptoms and gynecopathological signs are influenced not only by the biotype of the virus, but also the time of the infection. The foetus is highly susceptible to the infection of the more virulent cpBVDV. At the beginning of the pregnancy, embryonic death, embryo absorption and recurrent oestrus can be seen, in the later phase of thepregnancy abortion, and in the last trimester malformation of the foetus may develop. In this case bone

malformations (ankylosis, limb and skull abnormalities) and if the infection happened after the full development of the skeletal system, abnormalities in central nervous system may happen (hydrocephalus, cerebellar hypoplasia).

The ncpBVDV strains are less virulent, so they are lethal for the fetus only at the beginning of the pregnancy. At this time they cause absorption of the embio, embryonic death and recurrent oestrus. If the infection happens later (after the 40th day but before the development of the immuncompetent organs: bone marrow, thyme, spleen, etc) the developing immune system will accept the viral antigens as part of the body in the progress of the self-recognition phase and the presence of the virus will be tolerated. So, , the virus is continuously present in these animals and they excrete the infective virions into the environment all along their lifetime. These PI animals can not be immunized against the virus and turn to be seronegative in later examinations.

My investigations described in my PhD thesis could be grouped among three topics:

1.) Survey on the prevalence of the BVDV in Hungary.
2.) Investigation of the bovine neonatal pancytopenia (BNP) which is seen in newborn calves and is considered to be connected with BVDV infection.
3.) Examination of intramolecular recombination ability of the BVDV based on the deposited genomic sequences of different BVDV strains using computational biological methods.

**Survey on the prevalence of the BVDV in Hungary**

The herds were grouped according to disease prevalence: No. 1. Herds of this sub-set may be classified as clearly seropositive to BVDV. Total or nearly total seropositivity was exhibited.. No. 2. This cohort showed variable rates (10-85%) of seroprevalence throughout various herds and geographical regions. The presence of the virus however unquestionable in these stocks. No. 3. These herds display very low seropositivity (<10%): one sample was positive or questionable. This low prevalence may be interpreted as test error as it is within the ELISA error range.

No. 4. The results from these herds can be regarded as statistically invalid due to lack of data and/or low sample size lacking statistical validity. The results may be valid for the individual herd but are discounted from the study as inclusion would induce artificially seronegative skewed herd-level seroprevalence. No. 5. BVDV-free status in these herds is highly probable. High populations were sampled but no age data were recorded (except that the animals were older than 6 months), thus reducing the degree of certainty with which results can be

analyzed. No. 6. The animals sampled in this group were of sufficient age to have been exposed to the virus if it had been present in their environment for a clinically significant time period. In addition the sample sizes at these locations were large enough to provided statistical validity and thus be conclusively considered as ”BVDV-free”.

Considering BVDV as a production limiting disease (like Johne’s disease, neosporosis or enzootic bovine leukosis) losses can be described as direct production losses (reduced milk yield on dairy farms, reduced beef cattle slaughter value, abortion and reproductive losses), and treatment costs (veterinary and medication costs, increased labour demands). Therefore the most effective means of reducing the threat of BVDV is with and eradication program. BVDV surveillance, control and eradication measures in European countries are influential in shaping Hungary’s own BVDV strategy. Vaccination against the virus prevents foetal infection with questionable efficacy, since PI calves have been born into vaccinated herds. Researchers have shown that because 100% efficacy is required to prevent infection when a herd is exposed to the disease, vaccination does not reduce the prevalence or incidence of BVDV.

Our results suggest that the seropositivity rate on individual level is 43.4% (521 seropositive animals from 1200 sampled). If we compare this level to other countries, we find that it is higher then it had been in the Scandinavian countries prior launching the eradication campaign, but lower then it was in certain regions of Austria or Denmark.

If we calculate the seropositivity on herd level we find that 29.6% of the investigated farms (16 out of 54 farms) was not infected (Figure 3.). This result confutes the previously assumed nearly 100% seroprevalence. The ratio of seronegative farms is important, since these herds may serve as sources of animal replacement, because in case of an eradication program, only animals from BVD-free stocks can be introduced to farms where the program has started. Hence completely seronegative herds should be used as market seeds. The proprietors of these herds can demand higher premiums for their stock as sales come without risk of BVD propagation. The monetary value of the herd is enhanced by BVD-free status as various countries among Hungary’s trading partners screen for BVDV when import a stock. Seronegativity guarantees export to these countries -particularly valuable for farmers wishing to establish such trade links.

Furthermore there are quite a few positive herds (11.1%), where the seropositivity rate is extremely low (1 sample is positive or questionable from 15-20), which either means that the test result could be false, and it has to be repeated (especially if the OD value were near to the positivity margin) or the virus is freshly introduced into the stock. These herds were needed to

be rechecked, however this was not possible since the sample collection was not organized by us. In these herds identification and selection of PI animals may be carried out (by PCR from peripheral lymphocytes or by Ag ELISA from ear notches), and their removal may result in successful eradication of BVD from the herd.

If the seropositivity rate is very high within a herd, then eradication by the selection method is not possible. In this case the only solution is the total replacement of the stock BVDV free animals, or the calves should be separated from the cows as soon as possible, tested for their PI status (from ear notches) and if negative, raised on a separate farm. The heifers raised in this new, separated stock should not be mixed to the cows. This is possible if a farm has facilities at more than one location.

Farms with multiple premises were tested and results showed different rates of seropositivity and seronegativity. This later should serve as breeding stocks. Using the offsprings from these herds will help the farm to get rid of BVDV completely, without having to pay higher prices for guaranteed BVD-free stock. Such farms must also prevent exposure to disease by only purchasing from other BVDV-free herds and not allowing their animals to mix with those of undefined or BVDV-positive herd status.

BVD-free herds must prioritize protection of their status. This requires the design of disease prevention protocols, ideally including breeding, animal purchase and stock replacement, herd and operator hygiene policies.

**Investigation of the bovine neonatal pancytopenia (BNP)**

The symptoms were seen on young, 1-4 weeks old calves.:multiple haemorrhages were dominant. As the sign of the reduced blod clotting, long lasting bleedings were observed after every skin damage (injection, insect bytes), and spontaneous bleedings (haematuria, epistaxis, bleeding of the gums) was also seen. After developing the clinical signs, the calves soon died, mostly within 2-7 days. The lethality of the syndrome was very high, nearly every calf which developed sympthoms was lost.

At the necropsy large haemorrhages were found throughout the body, subcutaneously, also under mucosal membranes or in the wall of the intestines and parenchymatous organs. Body cavities contained reddish exudates. Histopathology examinations revealed multifocal hemorrhages at the same areas. The bone marrow was severly aplastic. Also at the microscopic analysis of the organs lymphoid depletion of the spleen, extensive bleeding and vascular damage in the wall of the reticulum, bleeding in the lungs, fibrin excretion in the

alveoli and microtrombi as the signs of disseminated intravascular coagulopathy (DIC) in several small arteries.

The PCR-based examinations intended to identify the presence of different viral agents (herpes virus, circo virus, BVDV) turned to be all negative. The bacterium cultivations from spleen samples of the calves were also negative.

As for explanation for the BNP syndrome several hypothesis were stated, however neither of them proved to be feasible for long time. Recently many assume the role of the major immunhysto-compatibility complex (MHC) proteins, originating from the cattle cell lines used for producing the vaccines in the background. According to this hypothesis, a specific vaccine used to minimalise the losses caused by the BVDV infection (Pregsure, Pizer Inc.) this vaccine contains an extremely effective adjuvant. This adjuvant can be capable to enhance the immunogenity not only the inactivated virus particles but the remnants of the allogenic MHC molecules as well. Hence, the vaccinated cows will generate antibodies also against these MHC epitops, of course, only in case, if these are incompatible with the cow MHC-s. These antibodies will be present in the colostrum and in the case if the specific calf inherited MHC-s similar to the contaminant in the vaccine, the mentioned antibodies will cause cytotoxic reaction in the newborn animal. This reaction damages the bone marrow at first palace, thus causes pancytopeny as a consequence. This theory also serves as explanation to the fact that the disease is nearly exclusively seen in herds with premium level keeping environment where the cows are vaccinated multiple times during pregnancy and the consume of the colostrum by the calves is provided. These conditions were met in case of the Hungarian cases as well.

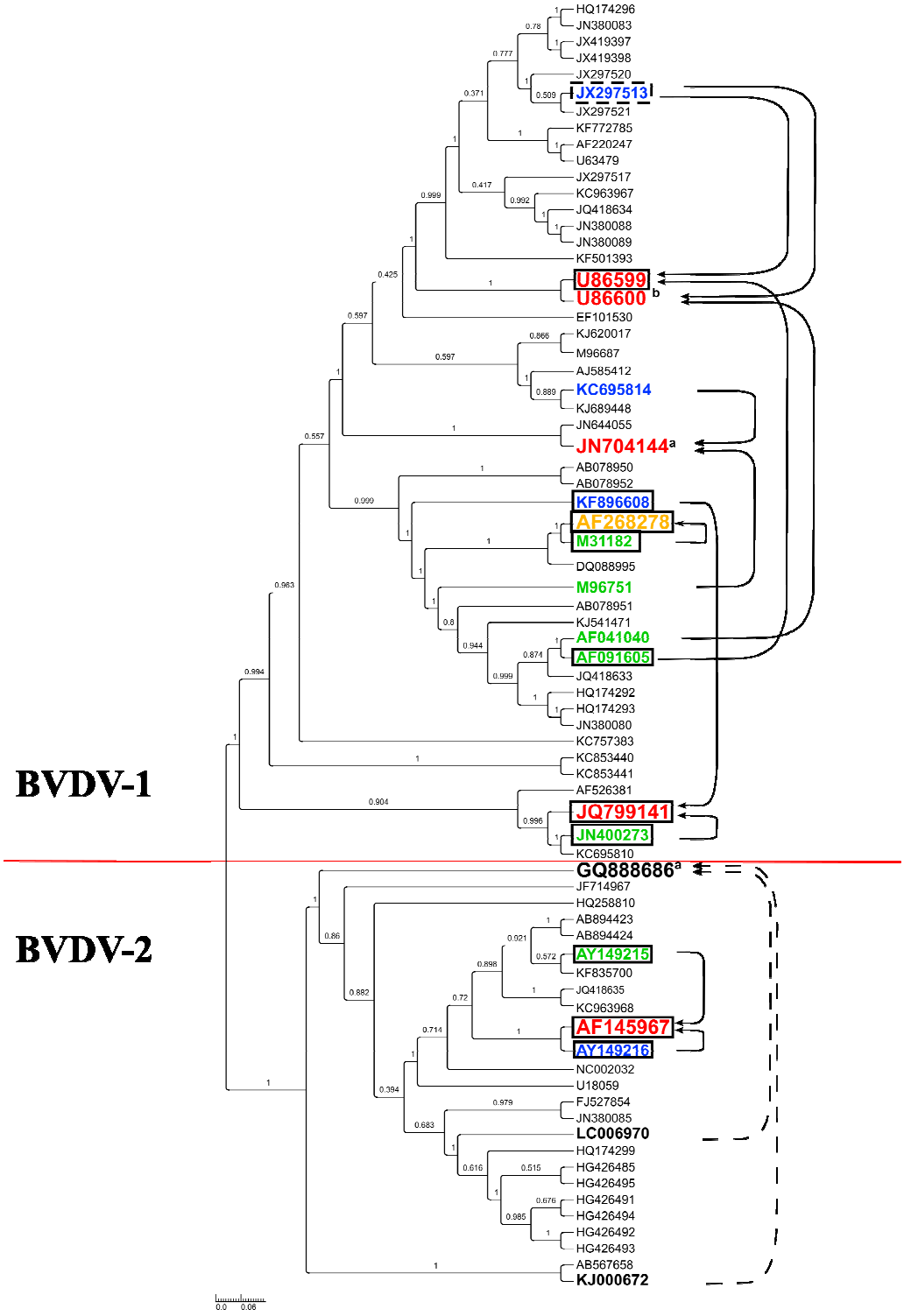
In our cases the symptoms were absolute characteristic to the one mentioned in the literatures. Since at the time of the examinations there were no acceptable explanation for the syndrome we extended the search for causative agent into several directions especially the exclusion of the probable role of the BVDV in the cases. According to the results, non of the cases contained sign of BVDV infection. Also, the PCR-based tests were not able to identify further viral agents from the samples and no bacterium was found, too.

Vaccination against BVDV can not be avoided in the infected herds in the future because of the possible economic losses that the virus is capable to cause. Considering the economic importance of the disease there is still available reliable BVDV vaccine that is licensed in Hungary, but the multiple vaccination of the pregnant cows should be avoided. The immunopathogeniy background of the disease emerges the possibility that using an extremely

effective adjuvant material in vaccines can cause syndromes similar to BNP in case of other species or vaccines.

**Examination of intramolecular recombination ability of the BVDV based on the genomic sequences stored BVDV strains using computational biology methods**

Recombination breakpoint p-distribution plot analysis of the genomes detected four hot-spots (A: 0.5 kb, B: 1 kb, C:7.5 kb, D:12 kb) on the BVDV-1 genome, which correlate with the detected recombinations rather well (Fig. 3A). Recombination can be identified more exactly when only the recombinant and the two parents are compared, but when all 62 BVDV-1 genomes were fed into the program used for hot spot analysis (RDP 4.46 BETA), the minor differences in the localization of the breakpoints and the different length of the genomes resulted in relatively longer sequences; they may vary with a few hundred bases in the case of the different recombinants. Two similar hot spots are also detected in the BVDV-2 genome, but their location is slightly different compared to that of BVDV-1, they are around 0.15 kb and 11.7 kb .




Phylogenetic tree constructed using sequences selected from the 112 BVDV-1 and 2 full genome sequences deposited in the GenBank. Only one of the genomes was used if higher than 98% identity was indicated between two sequences, therefore only 72 representative genomes are on the tree. Red: recombinants, Blue: major parent, Green: minor parent. Orange: artificial chimaera strain, used as internal control. Recombinants and parents identified by our survey are framed. [a] [b].

The most prominent BVDV recombination events detected after complete sequence alignment of all BVDV sequences. Some of the events were identified earlier by others [ [a] [b]], but our investigations resulted different parental genomes in some cases (See details in text). Double line separates BVDV-1 and BVDV-2 recombinations. ([c]Our examinations did not support this recombination event, however it is placed to the figure and in the table, since we refer to it further in the text.

| Position in recombinant sequence | | Recombinant sequence | Parental sequences | | Significance values found by the different detection methods* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Start | End | | Minor | Major | RDP | GENE-CONV | Bootscan | Maxchi | Chim-aera | SiSscan | LARD | 3Seq |
| 7780 | 11923 | JQ799141 | JN400273 | KF896608 | $1.73*10^{-136}$ | $1.02*10^{-145}$ | $9.71*10^{-156}$ | $1.49*10^{-45}$ | $5.62*10^{-35}$ | $2.29*10^{-69}$ | $1.44*10^{-217}$ | $1.12*10^{-80}$ |
| 7348 | 8500 | U86599 | AF091605 | JX297513 | $8.57*10^{-27}$ | $3.71*10^{-05}$ | $8.00*10^{-39}$ | $5.97*10^{-13}$ | $3.91*10^{-15}$ | $2.83*10^{-25}$ | $1.29*10^{-09}$ | NS |
| 332 | 1004 | AF268278 | Unknown | M31182 | $2.38*10^{-159}$ | $5.98*10^{-113}$ | $1.80*10^{-147}$ | $4.41*10^{-34}$ | $1.14*10^{-29}$ | $2.76*10^{-26}$ | $7.96*10^{-314}$ | $2.61*10^{-165}$ |
| 2939 | 4536 | JN704144 [a] | M96751 | KC695814 | $6.22*10^{-177}$ | $4.38*10^{-164}$ | $2.23*10^{-87}$ | $5.23*10^{-45}$ | $7.23*10^{-43}$ | $1.72*10^{-68}$ | $1.60*10^{-152}$ | $1.28*10^{-230}$ |
| 8783 | 12046 | JN704144 [a] | M96751 | KC695814 | $4.90*10^{-147}$ | $2.73*10^{-143}$ | $1.01*10^{-146}$ | $1.59*10^{-58}$ | $1.93*10^{-58}$ | $3.08*10^{-89}$ | $1.24*10^{-221}$ | $1.55*10^{-166}$ |
| 5976 | 6575 | U86600 [b] | AF041040 | JX297513 | $5.52*10^{-7}$ | $1.35*10^{-05}$ | $7.89*10^{-34}$ | $1.680*10^{-15}$ | $7.99*10^{-15}$ | $9.89*10^{-11}$ | $2.52*10^{-62}$ | NS |
| 11490 | 11679 | AF145967 | AY149215 | AY149216 | $7.74*10^{-19}$ | $4.40*10^{-18}$ | $3.05*10^{-20}$ | $1.74*10^{-05}$ | $1.26*10^{-04}$ | $4.24*10^{-07}$ | $8.36*10^{-19}$ | $9.39*10^{-08}$ |
| 3586 | 7357 | GQ888686 [a, c] | Unknown | AY149215 | $1.31*10^{-14}$ | NS | $4.04*10^{-14}$ | $8.5*10^{-14}$ | $2.77*10^{-14}$ | $1.24*10^{-22}$ | $6.46*10^{-12}$ | $7.57*10^{-08}$ |

* Threshold for significance: $p<0.005$; NS: not significant

In our study, the program selected those deposited sequences as parent sequences, which were nearest to the probable parents. This does not mean that the recombinants originated really from these parents, since the real donors were most probably not sequenced and deposited in the GenBank. Therefore the respective genomic regions of the parents and the derived recombinants are not identical nucleotide by nucleotide only very closely related on the exchanged genomic regions.

For to test and verify the robustness of the used mathematical methods, we used AF268278 (BVDV-1a) recombinant strain as control. Only one of its parents (M31182) could be detected, because the recombinant virus is an artificial chimera BVDV-1 strain, produced by replacing the BVDV $N_{pro}$ gene with a human hepatitis C genomic segment. This partial replacement caused an "unknown" result of the search in case of the minor parent, since hepatitis C genomes were not included into the alignment as possible parents. At the same time this finding proves the robustness of the applied test methods. The programs confirmed the results of Weber et al. (2015) by revealing a possible double cross-recombinant (JN704144), which strain was demonstrated in China from a field case. The major parent is the Av69 Vedevac strain also sequenced in China, the minor parent is a field strain (SD1). Besides being an example of a presumably rare double recombination event, this strain clearly demonstrates the risks of applying traditional attenuated live vaccines to reduce economical losses.

Another recombinant, JQ799141, is also a Chinese strain as well as its parent strains JN400273 and KF896608. It is interesting that the recombinant itself was isolated from a yak, while one of the parents, JN400273, was isolated from swine. The strain must also circulate in cattle, otherwise the recombination could not occur; but the species variety among the two parents and the recombinant (cattle, swine, yak) is remarkable from the epizootiological point of view. Previous authors publishing results on recombination events did not report on recombinations between different viral species of different hosts (border disease virus of sheep, bovine viral diarrhea virus of cattle and classical swine fever virus of pigs) within the *Pestivirus* genus.

U86599 is also interesting for more than one reason. A very similar strain U86600 was previously identified (Jones and Weber, 2004) as a recombinant using SimPlot analysis. The major parent was the same in our investigations as well (JX297513), but the minor parent was different (AF091605 instead of AF041040), though very closely related to the one identified as minor parent in the previous study. Besides finding another minor parent, the more sophisticated programs used in our study found a longer recombination region and stronger p

values in U86599, which proves that this strain was a recombinant, and U86600 is a derivate of U86599 different in less than 1% of the nucleotides, which may be the consequence of point mutations. Another interesting feature of this strain is, that it is the least supported recombination in our study the p values were not as strong as in the case of the other chimeras, what is more, one of the tests did not reveal recombination in this case, the p value was below threshold for the 3Seq method. We decided to introduce this event to prove that results of the tests must be treated with consideration; comparison of the results of the different methods and visual re-evaluation of the graphs is always necessary, as it was suggested previously. The major parent (Neill et al., 2015) was demonstrated in the USA, the closest minor parent in the UK. Since close relatives of the real parents were not sequenced in this case, the p value is relatively low. It is unique in the length of the total genome (15.521 kb) which is the consequence of three repetitions in its genome within the NS2-3 region, but these are most probably consequences of duplication of a 3263 nt long sequence of a partially overlapping part of three genes (partial NS2, NS2-3, NS3) within the genome of the strain itself, since the identity of the repetitive sequences is 98–100%. This strain also draws the attention of the users to the risk of false positive results since this strain was detected as recombinant, though the repetitive regions are most probably of self origin. But the program doesn't compare the genomes to themselves, therefore repetitions will be detected as recombinations with the closest relative of the investigated strain, since the duplicated genomic region shows the highest identity to the closest relative.

Using all 112 genomes in our study and a threshold limit of p=0.005 ($5\times10^{-3}$) we did not identify this strain as recombinant. When parameters were changed and merely 72 representative genomes were analysed (see phylogenetic tree in Fig. 1) the program found this recombinant, but the supporting p values were relatively weak (Table 1). We did not consider this strain as a recombinant.

It is interesting that relatively less recombinations were found in the case of BVDV-2, though the number of the deposited sequences was nearly as high as in the case of BVDV-1. Furthermore, most BVDV-2 strains were demonstrated and sequenced in North America (USA and Canada), which means that these virus variants coexisted in the same restricted geographical region, which increases the possibility of simultaneous infections. It may prove the relative stability of BVDV-2 compared to BVDV-1, which is also indicated by the lower genetic variance, and fewer putative subgroups in this viral species (Frey et al., 1996). Besides the recombinant described by Weber et al. mentioned above and not approved as recombinant by us, the only recombinant detected in our study was AF145967 introduced in

Fig. 2. The recombinant was described by Ridpath et al. (2006), but they detected a recombination different from ours. They identified not a recombination but an insertion within the NS3 gene of the genome, which is signed by a smaller drop of identity percentage in the graph in our investigation and was not confirmed as a recombination event by our results. We have detected a much more prominent recombination closer to the 3' end within the NS5b region, and it is interesting that the minor parent from which this 195 base long part is derived was identified by the program as AY149215 BVDV-2. Searching further, after cutting out this 195 bases, the BLAST algorithm search identified the origin of this partial sequence as a BVDV-1 strain which was deposited with accession number KF896612 by Gao et al. in the GenBank in 2013. It seems that the minor parent recognized by our program was a recombinant itself with major parent AY149216 and minor parent KF896612. This minor parent was not identified originally by RDP because partial sequences were not among the investigated genomes. The length of the recombined genomic part is 195 nt, the identity to KF896612 partial sequence was 100%. So considering this event, the existence of interspecies recombinants is also supported by the identification method used in our study, though only between the two BVDV species infecting the same host (cattle).

Our results prove the relatively high frequency of recombination in the evolution of BVDV. Though reassortment is known to occur frequently in case of viruses with segmented genomes (i.e. influenzaviruses, bluetongue virus), it is not so in virus families with unsegmented genomes. In the case of BVDV the special pathomechanism, the occurrence of persistently infected animals in the endemic countries may help the intergenomic recombinations. Also the value of utilisation of multiple statistical methods in the identification of such events is further supported. Also it is necessery to mention that using multiple methods for identifying recombination is important, since this way one can ballance between the strenght and weaknesses of each different detection methods, and the final result can be more reliable than using only one method.

# 2. Own publications

Kővágó, C., Hornyák, Á., Kékesi, V., Rusvai, M.,: Demonstration of homologous recombination events in the evolution of bovine viral diarrhoea virus with in silico investigations, Acta Vet Hung, 2016, Közlésre elfogadva.

Kővágó, C., Balka, G., Mándoki, M., Abonyi, T. and Rusvai, M.: **A borjak újszülöttkori pancytopeniája néven ismert kórkép és annak hazai előfordulása**, Magy. Áo. Lapja **135**(5): 259-266. 2013

Kővágó, C., Forgách, P., Szabára, Á., Mándoki, M., Hornyák, Á., Duignan, C., Gere, E. P. and Rusvai, M.: **Seroprevalence of Bovine Viral Diarrhoea Virus in Hungary - Situation before Launching an Eradication Campaign**, Acta Vet Hung **63**(2): 255-263. 2015