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The enhanced virulence of very virulent infectious bursal disease virus is partly determined by its B-segment

Brief Report

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Summary. There is a remarkable difference in virulence of infectious bursal disease virus (IBDV) strains ranging from sub-clinical infections for serotype 2 and cell culture adapted serotype 1 strains, to 100% mortality for very virulent serotype 1 strains in young SPF chickens. It is known that cell culture adaptation related attenuation is determined by distinct mutations in the hypervariable region of the VP2 outer capsid protein, encoded on the A-segment. Amino acid mutations in the hypervariable VP2 region however, offer no explanation for the difference in virulence of classical and very virulent serotype 1 strains. Here we show by *in vitro* and *in vivo* analysis of rescued segment reassorted IBDVs that virulence factors are not only located on the A-segment, but on the RNA Dependent RNA Polymerase (VP1) encoding B-segment as well. Insight into the virulence factors of very virulent IBDV will contribute to the improvement of live IBDV vaccines.

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Infectious bursal disease virus (IBDV), an avibirnavirus, is the causative agent of the highly contagious Gumboro disease that affects chickens (for review see [17]). Strict vaccination had kept IBDV associated loses in the European poultry industry under control until the appearance of IBDV strains with an enhanced virulence, namely, the very virulent (vv) IBDV strains [7]. Unlike antigenic variant strains found earlier in the United States, which have single amino acid changes in the hypervariable region of VP2 [14], the vvIBDV strains appeared to have the same antigenic structure as classical isolates [17]. Amino acid differences between viral proteins of vvIBDV and classical IBDV isolates were detected in all viral proteins, most of them in the hypervariable region of VP2 [6, 12]. Much attention has been given to differences in the proteins encoded by the A-segment, largely

because most of the neutralising epitopes occur in the hypervariable region (amino acids 224–314) of VP2. Rescue of recombinant IBDVs from chimeric A-segments revealed that amino acids within the hypervariable region are responsible for the cell culture adaptation [1, 5, 8, 11, 16]. A compilation of the adaptation associated mutations data narrowed it down to a specific combination of amino acids at position 253 and 284 of VP2.

A strict correlation has always been recognised between the cell culture adaptation and attenuation of serotype 1 IBDV strains. Although mutations in the A-segment are apparently the most important factor for (adaptation-related) attenuation, distinct nucleotide differences between the B-segment of classical and very virulent IBDV strains are present, which might also contribute to an enhanced virulence of the vvIBDV strains [1, 6, 18]. Comparison of the VP1/VPg sequences encoded by the B-segments of classical and virulent origin identified the presence of eight conserved amino acids differences [1]. To determine whether the B-segments of different origin (classical vs. very virulent) are associated with a different virulence phenotype, we rescued segment-reassorted IBDVs. These segment reassorted (chimeric) IBDV strains consist of a wild-type A-segment of either the cell culture-adapted CEF94 strain, or the very virulent D6948 strain, and the B-segment of CEF94 strain (designated as -ccB strains) or the very virulent D6948 strain (designated as -vvB strains). Furthermore, we used a chimeric D6948 A-segment in which the genome encoding the C-terminal part VP3 had been replaced by the corresponding part of the prototype (TY89) serotype 2 IBDV strain, designated as mDT-VP3C [2]. The exchange of the VP3 C-terminal encoding region gives rise to a partly attenuated vvIBDV strain. To be able to analyse the very virulent derivatives *in vitro* (cell culture), we also introduced two amino acid mutations (Q253H and A284T) in the VP2 part of the polyprotein, known to be both essential to and enough for cell culture adaptation of very virulent stains [16]. For the *in vivo* analysis (infection of young chickens) we used the viruses with the unmodified polyprotein, as cell culture adaptation also results in attenuation.

Co-transfecting of the cDNA into QM5 cells resulted in rescue titers that were about 10^2 to 10^3 lower when the D6948 derived very virulent B-segment was used, in stead of the CEF94 derived cell culture adapted B-segment (Table 1). Factors located on the B-segment of a cell culture-adapted isolate apparently contributed to the enhanced cell culture replication. To prove that indeed the replication of the segment- reassorted (chimeric) viruses was reduced, and that the differences is not an artefact of our rescue system, we analysed the replication in single step growth curves of either the rescued unmodified cell culture-adapted strain (rCEF94-ccB) or its B-segment reassorted counterpart (rCEF94-vvB), and with the cell culture adapted variant of the vvIBDV strain (rD6948^{HT}-vvB) or its B-segment reassorted counterpart (rD6948^{HT}-ccB). We inoculated a near confluent monolayer of QM5 cells with 10^6 to 10^7 $TCID_{50}$ of each virus as described before [3]. After thorough washing and addition of fresh medium, we removed samples of the supernatant at regular time points, and amount of virus ($TCID_{50}$) was determined in each sample (Fig. 1). The segment reassorted cell culture adapted strain which contains the B-segment of the vvIBDV strain (rCEF94-vvB) showed a clear reduction in

Table 1. Origin and phenotype of rescued IBDV viruses

Virus	A-segment plasmid	B-segment plasmid	Growth on eggs[a]	Growth on QM5 cells[b]
rCEF94-vvB	pHB-36W	pHB-55	+	1.7 (0.4)
rCFE94-ccB	pHB-36W	pHB-34Z	+	4.8 (0.1)
rD6948^{HT}-vvB	pDA-60(HT)	pHB-55	+	1.1 (1.0)
rD6948^{HT}-ccB	pDA-60(HT)	pHB-34Z	+	3.5 (0.3)
rD6948-vvB	pDA-60	pHB-55	+	–
rD6948-ccB	pDA-60	pHB-34Z	+	–
mDT-VP3C-vvB	pHB60-s2VP3C3	pHB-55	+	–
mDT-VP3C-ccB	pHB60-s2VP3C3	pHB-34Z	+	–

[a]Five 9-days-old embryonated SPF eggs were inoculated with 0.1 ml of the transfection supernatant via the choirlois allointric membrane. The embryo's were examined for IBDV specific clinical signs at 7 days post inoculation

[b]The mean $TCID_{50}$ titer was determined (QM5 cells) of three independent transfections (standard deviation in parenthesis). Dash means that no infectious centers were present in the analysis of the supernatants of all three independent transfections

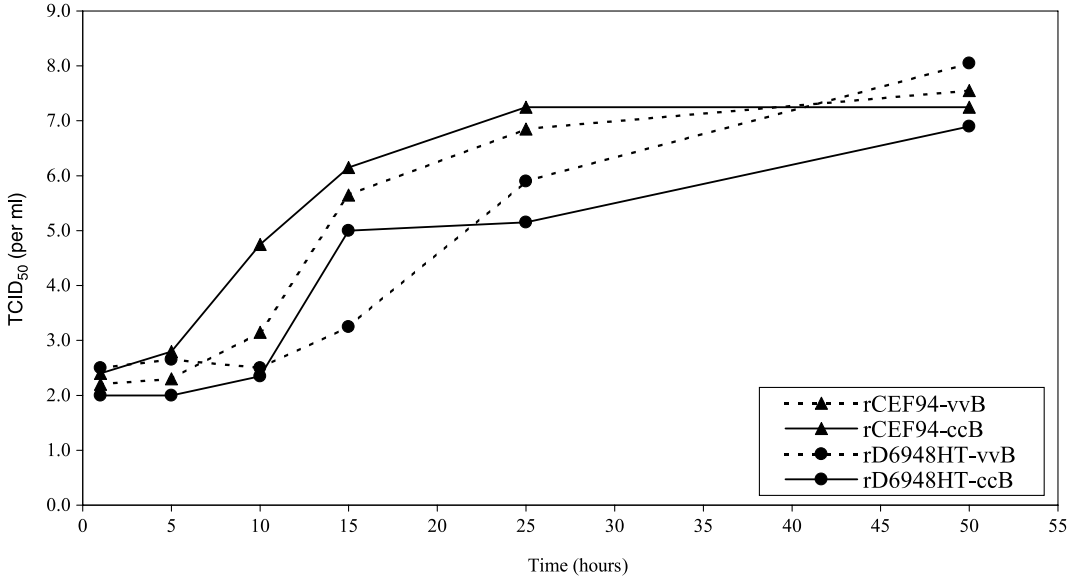

Fig. 1. Single step growth curves of the rIBDV that contain either the B-segment of the cell culture adapted CEF94 isolate (rCEF94-ccB and rD6948^{HT}-ccB), or the B-segment of the very virulent D6948 isolate (rCEF94-vvB and rD6948^{HT}-vvB). QM5 cells were infected with rIBDV (m.o.i. = 3; T = 0 h), and at different time points post infection (T = 5, 10, 15, 25, and 50 h), samples were taken from the supernatant and the IBDV titer ($TCID_{50}$ per ml) was determined

replication, as no progeny virus was detectable at 10 h post infection (pi), whereas in the case of non-segment reassorted cell culture adapted strains progeny virus is already released by that time (Fig. 1, and [3]). The same phenomenon was found

for rD6948^{HT}-vvB virus, for which we found almost no progeny virus at 15 h pi (titer of 10^3), while the segment reassorted counterpart already had a titer of 10^3 (Fig. 1).

To evaluate the *in vivo* virulence of the segment reassorted IBDV strains we orally inoculated 7-day-old layer type chicks (SPF chickens, Spafas) with a 50 embryo lethal dose 50% (ELD_{50}) of non-cell culture adapted IBDV. Chickens are highly susceptible to (virulent) IBDV during the first 6 weeks of their lives, but mortality is rather low if they are infected during the first two weeks of life [17]. To analyse the virulence of the segment reassortant rIBDV and to be able to follow the recovery after infection we chose to use 7-days old SPF chickens. Groups of 35 chicks were housed separately in isolators and observed during the first 7 days pi for mortality and IBDV specific morbidity (Fig. 2). At 1, 2, and 3 weeks pi we removed 5 aselect birds from each group, weighed them, and took a blood sample from each of them. The birds were subsequently euthanised and the bursa of Fabricius was isolated, weighed and stored in formalin. We observed that as early as 7 days pi, all examined chicken sera contained an IBDV neutralising antibody titer of $\geq 5\log_2$, irrespective of the viruses used (data not shown). Young chicks with an IBDV neutralising antibody titer of $5\log_2$ (active immune responds) are considered to be protected against a subsequent infection with (vv) IBDV. The VN-titers rose considerably during the following two weeks, but again no differences were found between the mean values of each group. Compared to the mock infected group, all the birds in the experimental groups with the exception of the group that was infected with mDT-VP3C-ccB showed a significant ($P < 0.05$) decrease in weight gain over the three weeks (Table 2).

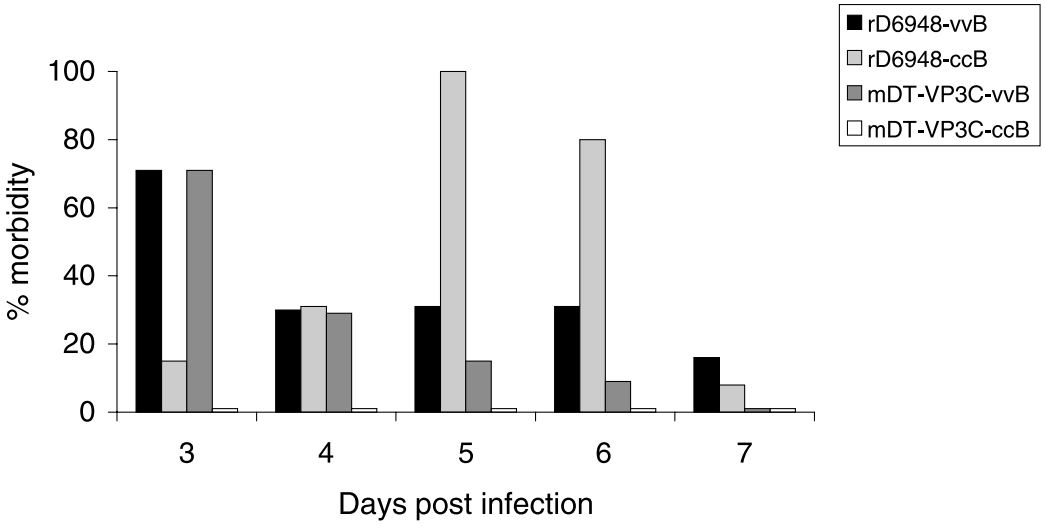

Fig. 2. Total morbidity in the groups of chickens that received the indicated rIBDVs was determined at each indicated day, and expressed as percentage of total number of birds present in each group that particular day. Dead chicks were removed daily from the isolators. No mortality nor morbidity was observed on days other than indicated in this graph

Table 2. Body and bursa weight, and bursa damage after infection with rIBDV

Virus	Mean Weight[a] (g) at weeks post infection			Mean BBW Ratio[a] (*1000) at weeks post infection			HBLS[a,c] at weeks post infection			Mortality[d]
	1	2	3	1	2	3	1	2	3	
PBS	132 (7)	228 (11)	343 (35)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	3.9 (1.5)	6.7 (2.1)	6.2 (1.4)	0% (0)
rD6948-vvB	120 (8)	212 (35)	294 (16)	5.0 (0.0)	4.8 (0.0)	4.6 (0.5)	1.0 (0.3)	1.1 (0.2)	0.9 (0.2)	3% (1)
rD6948-ccB	97 (9)	187 (17)	298 (57)	5.0 (0.0)	5.0 (0.0)	4.4 (0.5)	1.5 (1.0)	1.0 (0.2)	0.9 (0.2)	11% (4)
mDT-VP3C-vvB	117 (7)	200 (14)	312 (28)	5.0 (0.0)	4.8 (0.0)	5.0 (0.0)	1.0 (0.1)	1.1 (0.4)	1.3 (0.3)	3% (1)
mDT-VP3C-ccB	122 (8)	207 (23)	345 (45)[b]	3.0 (0.7)	2.8 (0.5)	1.8 (0.5)	1.0 (0.1)	2.2 (1.0)	3.1 (0.4)	0% (0)

[a]The given mean values are based upon the values of 5 chickens (standard deviation)

[b]Pairwise comparison (Fisher LSD method) of the log-transformed overall weight data showed that only the mDT-VP3c-ccB infected group did not differ significantly ($P < 0.05$) from the Mock infected (PBS) group

[c]The mean (standard deviation) of the Histopathologic Bursa Lesion Score (HBLS) of three different section of each H&E stained bursa was determined using the following scale:

0 = absence of damage
1 = necrosis of isolated follicles
2 = moderate general depletion of lymphocytes or severe depletion limited to a few follicles
3 = severe depletion of lymphocytes in more than 50% of follicles
4 = remains of follicular contours showing a few lymphocytes with hyperplasia of related tissues, cysts, thickened and folded epithelium
5 = loss of the entire follicular structure with associated fibroblast

[d]Relative cumulative mortality is given for each group (n = 35), and absolute numbers of dead chicks are given in parenthesis. Mortality was only found on day 3 pi, except in the group receiving rD6948-ccB, where 1 chick died at day 3 pi, 1 chick at day 4 pi, and 2 chicks at day 5 pi

The Bursa-Body Weight (BBW) ratio was determined for each euthanised chicken, and the average BBW ratio for each group was determined for each time point (Table 2). Apart from the mock infected group of chickens, all groups showed a large reduction in the BBW ratio at one week pi, indicating a severe damage of the bursa of Fabricius due to the rIBDV infection. The BBW ratio of chickens infected with rIBDV remained very low during the two following weeks, with the exception of the mDT-VP3C-ccB, infected group, which demonstrated a distinct gain in the BBW ratio (Table 2). The increase of the BBW ratio after infection with mDT-VP3C-ccB reflects the recovery of the damaged bursa by infiltration of new B-lymphoid cells. This B-lymphoid cell infiltration was confirmed by analysis of the Histopathologic Bursa Lesions of the recovered bursas at the different times pi (HBLS, Table 2). A clear increase in the B-lymphoid cell population was found for segment reassorted mDT-VP3C-ccB at 3 weeks pi, whereas both the BBW ratio and the HBLS bursa of chickens infected with non-segment reassorted counterpart (mDT-VP3C-vvB) did not change over time.

We anticipated that the *in vivo* analysis would reveal the virulence of the segment reassorted vvIBDV strain (rD6948-ccB) to be less than its wild-type counterpart (rD6948-vvB). To our surprise we found that the cumulative mortality induced by the segment reassorted strain was much higher (31%) then the cumulative mortality (9%) of its wild-type counterpart (Fig. 2). The difference in absolute mortality rates was mainly attributable to the differences at days 5 and 6 pi, while the mortality rates were almost equal at day 3 and 4 pi. The difference in the morbidity pattern of these two groups, was even more extreme. Morbidity was the highest (71%) at 3 days pi for the wild-type rD6948-vvB and subsequently decreased (Fig. 2), as opposed to a morbidity of only 15% at 3 days pi among the segment reassorted rD6948-ccB. which increased to 100% at day 5. The difference in mortality and morbidity patterns suggests that the replication rate of the segment reassortant rD6948-ccB is indeed reduced, as the peak of morbidity and mortality was not found at 3 days pi, as normally found with wild-type IBDV strains [7], but delayed to 5 days pi. This delayed replication rate results however in an enhanced virulence phenotype in our experimental set-up of 7-day-old SPF chickens.

The morbidity and mortality pattern of the partly attenuated, recombinant IBDV strain (mDT-VP3C-vvB) mimics the pattern of the wild-type vvIBDV strain (mD6948-vvB), as both morbidity and mortality is the highest at day 3 pi (Fig. 2). The segment reassorted counterpart of this attenuated strain showed neither mortality nor morbidity (Fig. 2). The further reduction in virulence of this chimeric and reassorted IBDV strain is also clear from the gain in bursa-body-weight ratio, and from the reduction in bursa damage (Table 2) during the 3-week pi period.

Segment reassortant viruses of highly segmented dsRNA viruses, like rotavirus (11 segments), and Reovirus (10 segments) are well described, and are even found during natural infections (for review see [13]). Only one report describes a single segment reassorted rotavirus concerning the RDRP encoding segment 1, but no change of phenotype has been reported for that virus [9]. Point mutations in the

RDRP of the attenuated poliovirus Sabin-1 strain have also been observed to have an additional attenuating effect on the virulence phenotype [4, 10, 15].

In this report we show that factors contributing to the enhanced virulence of very virulent IBDV strains are not only located on the A-segment, but also on the B-segment. The single protein encoded by the B-segment has, at least, two functions, i.e., RNA dependent RNA polymerase (VP1), and Viral Protein genome-linked (VPg). Amino acid mutations in this protein might only affect one of these two functions. Whether the difference in virulence between the B-segment of wild-type and cell culture adapted origin is solely due to a single amino acid change, or a combination of different amino acid changes, or whether silent nucleotide substitutions have an influence on virulence, awaits further studies involving site-directed mutagenesis.

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