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Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses*

Brief Report

N. Eterradossi[1], **C. Arnauld**[2], **D. Toquin**[1], and **G. Rivallan**[1]

[1]Unité de Virologie Immunologie et Parasitologie Aviaires et Cunicoles, Centre National d'Etudes Vétérinaires et Alimentaires (CNEVA), Ploufragan, France
[2]Unité de Biologie Moléculaire, CNEVA, Ploufragan, France

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Summary. Classical serotype 1 infectious bursal disease viruses (IBDV), but not very virulent (vv) isolates, react with neutralizing monoclonal antibody (NMab) 3 in virus neutralization tests or antigen-capture ELISA. Two other NMabs, 6 and 8, bind to both classical and most vv strains, but not to the atypical 94 432 and 91 168 vv strains, respectively. The basis for such reactivities was investigated by sequencing the genome region encoding the VP2 major immunogenic domain. In classical, variant, vaccine or vv IBDV strains, negative reactions with NMab3 were associated with changes in the Proline-Glycine pair at amino-acid (aa) positions 222–223 (hydrophilic peak A), and negative reactions with NMabs 6 and 8 with aa changes from positions 318 to 324 (hydrophilic peak B). The 91 168 and 94 432 viruses are the first vvIBDVs to present aa changes in peak B.

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Infectious bursal disease virus (IBDV) is a major pathogen of young chickens that replicates in the differentiating lymphocytes of the Bursa of Fabricius. It is responsible for a fatal or immunosuppressive disease causing heavy losses to the poultry industry [12]. IBDV belongs to the genus *Avibirnavirus* in the family *Birnaviridae*, which groups non-enveloped icosahedral viruses with a

*The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ databases under the accession numbers Y 14955 (strain 94432), Y14956 (89163), Y 14957 (91168), Y 14958 (F52/70), Y14959 (variant A), Y14960 (Bursine 2 derivative), Y14961 (CT derivative), Y 14962 (D78 derivative) and Y14963 (strain EM3).

bi-segmented double-stranded (ds) RNA genome [6]. The larger genome segment of the *Birnaviridae* encodes a N-VP2-VP4-VP3-C polyprotein, which is co-translationally processed into the main capsid components, VP2 and VP3 (for a review see [10]).

VP2 is a 454 amino acid (aa)-long polypeptide that builds up the external virus capsid [10]. Expression/deletion studies have shown VP2 aa positions 206 to 350 to represent a major conformational, neutralizing antigenic domain [1]. Most aa changes between IBDV strains are clustered in this region, thus referred to as ≪ VP2 variable domain ≫ [2]. This domain is composed of hydrophobic aa, flanked by two hydrophilic peaks, A and B, which span aa 210 to 225 and aa 312 to 324, respectively [1]. Variations in IBDV antigenicity have been shown to depend on changes in peaks A and B. Serotype 2 strain 23/82 [15], North-American antigenic variants, A, E, GLS-5 and DS326 [9, 11, 16] and neutralization resistant escape mutants [15, 18] all exhibit aa changes in these hydrophilic peaks, whereas only changes in the hydrophobic domain are found in typical serotype 1 strains [16]. Two smaller hydrophilic areas of VP2 variable domain, aa 248 to 252 and 279 to 290, were recently reported to also influence IBDV antigenicity [19].

The term ≪ very virulent ≫ (vv) IBDV has been introduced to refer to IBDV strains which cause mortality rates double those of European reference pathogenic strain Faragher 52/70 (F52/70) [5]. Such viruses have emerged in Europe in 1987 and are currently widespread [3, 5, 17]. Although vvIBDVs can break through high vaccine-induced antibody titers [17], their antigenicity is still considered as typical of serotype 1 [14, 19, 20]. The aa sequence of VP2 variable domain has been determined in several vvIBDVs [4, 13, 19]. Four to seven aa changes occur in vvIBDVs, but only two are located in the hydrophilic peaks. These are the substitution of a Proline for an Alanine at position 222 (P222A), which is common to all vvIBDVs, and the S251R change which is specific to strain 849VB [19]. The antigenic significance of both changes is unclear. Similar changes have been reported in laboratory-selected mutant IBDVs that resist neutralization by different monoclonal antibodies (Mabs), however the reactivity of these Mabs versus vvIBDVs has not been documented on a large scale [15, 18]. Changes at position 222 also occur in North-American antigenic variants, but no Mab-defined antigenic site has been mapped to this position in variant viruses [9, 16].

We recently characterized several anti-VP2 neutralizing Mabs (NMabs) in classical and vvIBDVs. NMab 3 binds to the F52/70 strain, but not to 58 vvIBDV isolates in an antigen-capture ELISA (AC-ELISA). Lack of binding of NMab 3 hence appears associated with typical vvIBDV antigenicity. Two other NMabs, 6 and 8, recognize different VP2-located epitopes and bind to both classical and vvIBDV strains, but not to the 94 432 and 91 168 vvIBDVs, respectively. The latter strains thus appear atypical among recent French vvIBDV isolates [7, 8]. The present study was undertaken to assess whether typical or atypical antigenicity in vvIBDVs were associated to aa changes in VP2 variable domain.

Eleven IBDV strains were studied. These included the F52/70 virus, the 89 163, 91 168 and 94 432 French vv strains, North-American variant strains A, E and GLS-5, and the intermediate vaccine strains D78 (Intervet), Gumboral CT

(Mérial) and Bursine 2 (Solvay). Variant E and GLS-5 were a kind gift from Dr Willem Posthumus (Intervet, Boxmeer, The Netherlands). The origin of the other strains was as previously reported [7, 8]. Prior to study, the vaccine strains were cloned three times by limiting dilution in chicken embryo fibroblast cell cultures (CEF), and were then produced as freeze-thaw supernatants of infected CEF layers. vvIBDV isolates were cloned three times by limiting dilution in specific pathogen free (SPF) embryonated chicken eggs. The embryo-cloned 94 432 and 91 168 viruses were shown to be representative of their uncloned parent isolates, as demonstrated by identical pathogenicity in White Leghorn SPF chickens, and identical antigenicity in AC-ELISA. The cloned viruses were then propagated in 6-week-old SPF White Leghorn chickens, and produced as bursal homogenates as described [7]. IBDV strain EM3, a laboratory-selected mutant virus, was the last strain to be studied. For its production, $10^{6.3}$ mean tissue culture infectious doses ($TCID_{50}$) of the CT vaccine were mixed with 0.5 ml of NMab3-containing ascite [7], neutralized for 2 h at room temperature, and inoculated to CEF ($10^{6.4}$ cells/25 ml). An IBDV-like cytopathogenic effect appeared on day 6 post-inoculation (PI). The virus was passaged once in presence of NMab 3, then was cloned as already described.

The reactivity of NMabs 3, 6 and 8 versus pathogenic viruses was assessed in AC-ELISA, with a dose of captured virus ranging from 30 to 70% of the maximum captured dose [8]. With cell-culture adapted viruses, neither CEF supernatants nor infected bursal homogenates contained enough antigen to allow for AC-ELISA (a lower antigenic content of bursae infected with vaccine IBDVs, compared to pathogenic strains, had already been reported in AC-ELISA in a previous study [20]). Hence, for strains CT and EM3 only, AC-ELISA was applied to 1 200-fold concentrated supernatants which were produced by dialysis against polyethylene glycol 20 000, and subsequent pelleting of viruses by ultracentrifugation. Antigenic characterization of the other CEF-adapted viruses was performed in constant-virus serum-diluting neutralization (VN) assays [7].

The nucleotide (nt) sequences of US variants E and GLS-5 were obtained from the GenBank and EMBL databases (respective accession numbers D10065 and M97346). For other viruses, nucleic acids were extracted from infected 75 cm^2 CEF cultures at 5 days PI, or from 0.700 ml infected bursal homogenates. The samples were chloroform-treated overnight at 4 °C. The resulting aqueous phase was digested for 1h at 37 °C with 1% Sodium Dodecyl Sulfate and 1 mg/ml Proteinase K (Boerhinger), and was then extracted three times v:v with phenol/chloroform (1:1) and once v:v with phenol/chloroform/isoamylic alcohol (25:24:1). For reverse transcription (RT), ethanol precipitated nucleic acids were resuspended in RNase free distilled water, added 20% dimethylsulfoxide (Sigma Chemicals, St. Louis, MO, USA), boiled for 3 min at 92 °C and snap-chilled on ice. RT was performed for 60 min at 42 °C, in 20 µl volumes. Reaction mixes contained 1.25 µl of denatured template, 200 UI of Moloney murine leukemia virus-reverse transcriptase with 4 µl of its 5 × first-strand buffer (Gibco-BRL, Gaithersburg, MD USA), 2 µl of 0.1 M DTT, 0.25 µl of each 10 mM deoxyribonucleotide triphosphate and 320 pmol of the L2 oligonucleotide primer

(5′-GATCCTGTTGCCACTCTTTC-3′) which anneals to positions 1 194 to 1 213 of the positive strand of IBDV segment A (numbering according to Bayliss et al. [2]). For the polymerase chain reaction (PCR), chimeric oligonucleotide primers were synthesized, by coupling the sequences of the ReverseM13 and 21M13 standard primers to the 5′ end of the IBDV-specific L2 and U2 sequences, respectively [21]. The U2 sequence (5′-GGTATGTGAGGCTTGGTGAC-3′) corresponds to nts 658 to 677 of IBDV segment A. The resulting primers amplify a 604 base pair (bp) product which encompasses 516 IBDV-specific bp and spans the *Acc*I-*Spe*I region of the VP2 gene. The *AmpliTaqGold* polymerase was used in standard PCR reactions (Perkin Elmer, Branchburg, NJ, USA), in a Perkin Elmer thermal cycler, with the following programme: initial denaturation 94 °C 10 min; 35 cycles with denaturation: 95 °C 30 sec, annealing: 64 °C 45 sec, elongation: 72 °C 45 sec; and final elongation: 72 °C 7 min. The RT-PCR products were finally purified using the Qiaquick PCR or Qiaquick Gel purification kits (Qiagen, Chatsworth, CA, USA), according to the supplier's instructions. The purified RT-PCR products were sequenced in both directions with an automated 373 ABI DNA sequencer (Applied Biosytems Inc., Perkin Elmer). Sequencing reactions were performed using the *AmpliTaq* DNA polymerase FS (Perkin Elmer), The ABI-Prism Dye Terminator cycle sequencing kit, and the 21M13 and ReverseM13 specific primers. The sequence were checked and analyzed using the SeqEd 1.03 (ABI) and MacDNAsis Pro v3.5 (Hitachi software, San Bruno, CA, USA) softwares, respectively.

In VN or AC-ELISA, NMab 3 reacted significantly with strains D78, CT and F52/70 only (Table 1). In both assays, strain EM3 did not exhibit any reactivity versus NMab 3, hence producing clearly different results than its parent CT strain. North-American variant viruses E and GLS-5 did not react with any of the three NMabs in AC-ELISA. However, a broadly reactive Mab, NMab 1 [7], had been included in the same assays as a control, and positive reactions with this Mab demonstrated the AC-ELISA to be indeed applicable to the variant viruses (data not shown). The E and GLS-5 viruses excepted, the 94 432 and the 91 168 viruses were the only other strains with no reactivity to NMabs 6 and 8, respectively.

Over the 516 bp that were sequenced, all nt sequences shared at least 92.8% identity (Fig. 1). CT, EM3, and D78 were most similar, the only differences being a C in EM3 for a G in CT at position 798 (non-silent), and an A in D78 for a G in CT at position 980 (silent). The F52/70 clone used in the present study had the same nucleotide sequence as previously determined by Bayliss et al. [2], and was very similar to the CT, EM3 and D78 clones (mean homology 95.8%). Variants A, E and GLS-5 composed a distinct group with at least 95.9% identity between virus pairs. The cell-culture adapted variant A used in the present study differed from the previously published sequence of the wild-type variant A at four positions (890, 981, 1 078 and 1 079), all changes corresponding to non-silent mutations [11] the biological significance of which is unknown. As already reported for other vvIBDV sequences [4], the vv viruses were very similar one to the other, and differed by only 2, 8, and 10 nt changes, for the 89 163/91 168, 89 163/ 94 432

Table 1. Reactivity of eleven IBDV strains versus neutralizing monoclonal antibodies 3, 6 and 8, in virus neutralization assays (*VN*) and/or antigen-capture ELISA (*AC*)

IBDV strain	Propagation[a]	Assay	Reactivity with		
			Mab 3	Mab 6	Mab 8
D78	CEF	VN	17.0[b]	16.5	16.0
CT	CEF	VN	17.0	17.5	17.0
EM3	CEF	VN	< 3.3	16.5	13.0
Bursine 2	CEF	VN	< 3.3	13.6	8.3
A	CEF	VN	< 3.3	11.0	8.3
CT	CEF	AC	107[c] (101–111)[d]	89 (77–98)	129 (114–140)
EM3	CEF	AC	1 (0–2)	80 (74–87)	125 (122–130)
E	Ch	AC	2 (1–3)	6 (4–11)	2 (1–2)
GLS-5	Ch	AC	0 (0–1)	0 (0–0)	0 (0–0)
91 168	Ch	AC	4 (2–5)	125 (118–131)	3 (2–3)
94 432	Ch	AC	5 (4–6)	10 (8–12)	93 (88–98)
89 163	Ch	AC	1 (0–5)	109 (100–125)	74 (62–84)
F52/70	Ch	AC	91 (86–95)	110 (105–120)	96 (87–112)

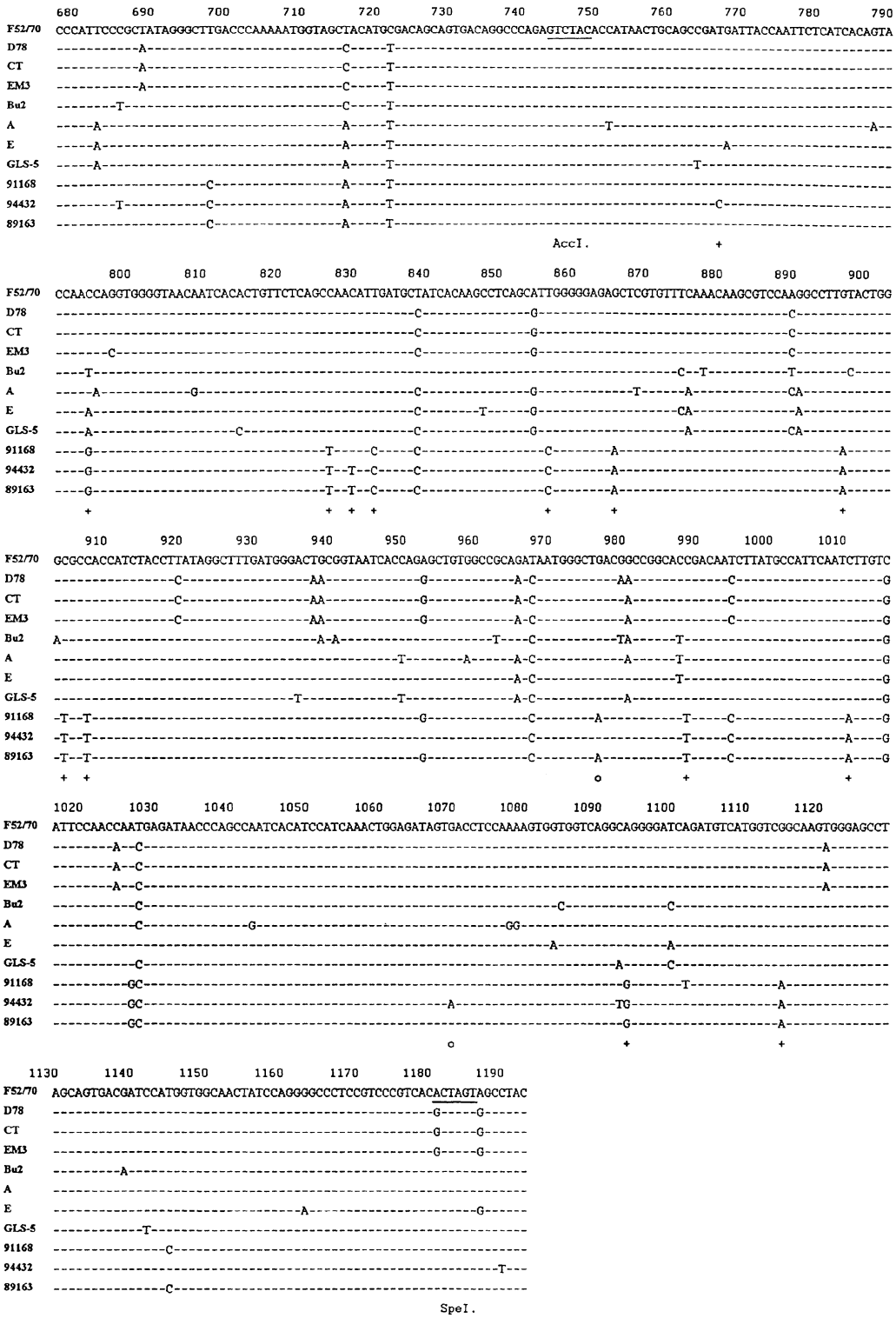
[a] *CEF* Chicken embryo fibroblasts cell cultures, *Ch* 6-week-old SPF chickens

[b] Neutralizing titre, i.e. −log2 of the highest dilution of ascite neutralizing 100 $TCID_{50}$ of virus (mean of two repeated tests)

[c] Mean AC-ELISA reactivity, expressed as previously described [18]

[d] Range in three to five repeated tests

and 91 168/ 94 432 pairs, respectively. The *Acc*I-*Spe*I area was previously reported to encompass 14 nt positions conserved in most vv viruses, differentiating them from all other IBDVs, and two variable positions allowing for the distinction of two vv groups [4]. The present study confirmed variable nts at positions 977 and 1 070 (A or G in both cases), as in both positions 89 163 and 91 168 differed from 94 432. However, two more positions, previously reported as vvIBDV-specific, also proved variable in the vv strains: at position 767, both 89 163 and 91 168 had a T and not a C like most vvIBDVs (they thus resemble the DV86 Dutch vv strain), and at position 830, 91 168 had a C and not a T as found in all other vvIBDVs (Fig. 1, [4]).


680 690 700 710 720 730 740 750 760 770 780 790
F52/70 CCCATTCCCGCTATAGGGCTTGACCCAAAAATGGTAGCTACATGCGACAGCAGTGACAGGCCCAGAGTCTACACCATAACTGCAGCCGATGATTACCAATTCTCATCACAGTA
D78
CT
EM3
Bu2
A
E
GLS-5
91168
94432
89163
AccI.
800 810 820 830 840 850 860 870 880 890 900
F52/70 CCAACCAGGTGGGGTAACAATCACACTGTTCTCAGCCAACATTGATGCTATCACAAGCCTCAGCATTGGGGGAGAGCTCGTGTTTCAAACAAGCGTCCAAGGCCTTGTACTGG
D78
CT
EM3
Bu2
A
E
GLS-5
91168
94432
89163
910 920 930 940 950 960 970 980 990 1000 1010
F52/70 GCGCCACCATCTACCTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAGATAATGGGCTGACGGCCGGCACCGACAATCTTATGCCATTCAATCTTGTC
D78
CT
EM3
Bu2
A
E
GLS-5
91168
94432
89163
1020 1030 1040 1050 1060 1070 1080 1090 1100 1110 1120
F52/70 ATTCCAACCAATGAGATAACCCAGCCAATCACATCCATCAAACTGGAGATAGTGACCTCCAAAAGTGGTGGTCAGGCAGGGGATCAGATGTCATGGTCGGCAAGTGGGAGCCT
D78
CT
EM3
Bu2
A
E
GLS-5
91168
94432
89163
1130 1140 1150 1160 1170 1180 1190
F52/70 AGCAGTGACGATCCATGGTGGCAACTATCCAGGGGCCCTCCGTCCCGTCACACTAGTAGCCTAC
D78
CT
EM3
Bu2
A
E
GLS-5
91168
94432
89163
SpeI.

Deduced aa sequences are shown in Fig. 2. The F52/70 and D78 clones proved identical to those previously sequenced [2, 16], the former virus differing from the others by only 4 to 9 aa changes (2.3 to 5.2%). The variant A clone used in the present study differed from a previously establised sequence at 3 positions, namely Q253H, A284T and K316R [11]. The two first changes also exist in variant GLS-5, and the exchange of basic hydrophilic aa at position 316 does not change the hydrophilicity of peak B. As already reported, vvIBDVs differed from F52/70 by only 4 vvIBDV-conserved residues: A222, I 256, I 294 and S299 [4]. The SWSASGS heptapeptide was found in both cloned pathogenic viruses and in a cloned derivative of an intermediate vaccine. This confirms that the heptapeptide, although tentatively proposed as a marker for virulence [9], may also be found in egg or cell-propagated attenuated IBDVs [19, 22].

With respect to the reactivity of NMab 3, the only aa changes that were common to all non-reactive vaccine and field IBDVs were those affecting position 222 (P222S/Q/T/A). Consistently, the only change observed in the laboratory-selected EM3 mutant was G223R, in an adjacent position. This last change excepted, modifications at position 222 did not introduce major hydrophilic alterations in peak A. They might however affect VP2 secondary structure, as the Chou, Fassman & Rose algorithm predicts a probable coiled structure at positions 222 to 224 in F52/70, D78 and CT, versus a probable β sheet structure in other strains. With respect to the reactivity of NMabs 6 and 8, the only aa differences between typical and atypical vvIBDVs were located at the C-terminal end of peak B. These were Q324L and A321V in 91 168 and 94 432, respectively. To the authors' knowledge, no vvIBDV sequence with changes in VP2 second hydrophilic peak had been reported so far, and only variant E and GLS-5 exhibit aa changes in a roughly similar location (Fig. 2). Interestingly, these variant strains do not react with NMabs 6 and 8, either. The changes in variant viruses result in a more hydrophobic N-teminal half in peak B, whereas those found in atypical vvIBDVs rather result in a more hydrophilic C-terminal half. Because the present sequence study was restricted to the *Acc*I-*Spe*I region, it cannot be ruled out that other aa changes in segment A may also contribute to vvIBDV typical and atypical antigenicity. The finding that VP2 positions 222–223 and 318–324 are critical for

Fig. 1. Nucleotide sequence of VP2, from positions 678 to 1 193 (VP2 variable domain, numbering according to Bayliss et al. [2]). Presented IBDV strains are the Faragher 52/70 serotype 1 reference strain (F52/70); cloned derivatives of intermediate vaccines D78, CT and Bursine 2 (Bu2); a laboratory-selected mutant virus that escapes neutralization by Mab 3 (EM3); North-American antigenic variants (A, E, and GLS-5), antigenically typical vv strain 89 163, and antigenically atypical vv strains 91 168 and 94 432. Sequence data of the E and GLS-5 strains are from the Genbank and EMBL database accession numbers D10065 and M97346, respectively. A dash indicates positions where the sequence is identical to that of the F52/70 virus. + indicates positions where vvIBDVs were previously reported to differ from all other strains, O positions previously described as allowing for the distinction between two vv groups [4]. The *Acc*I and *Spe*I restriction sites flanking the variable domain are underlined

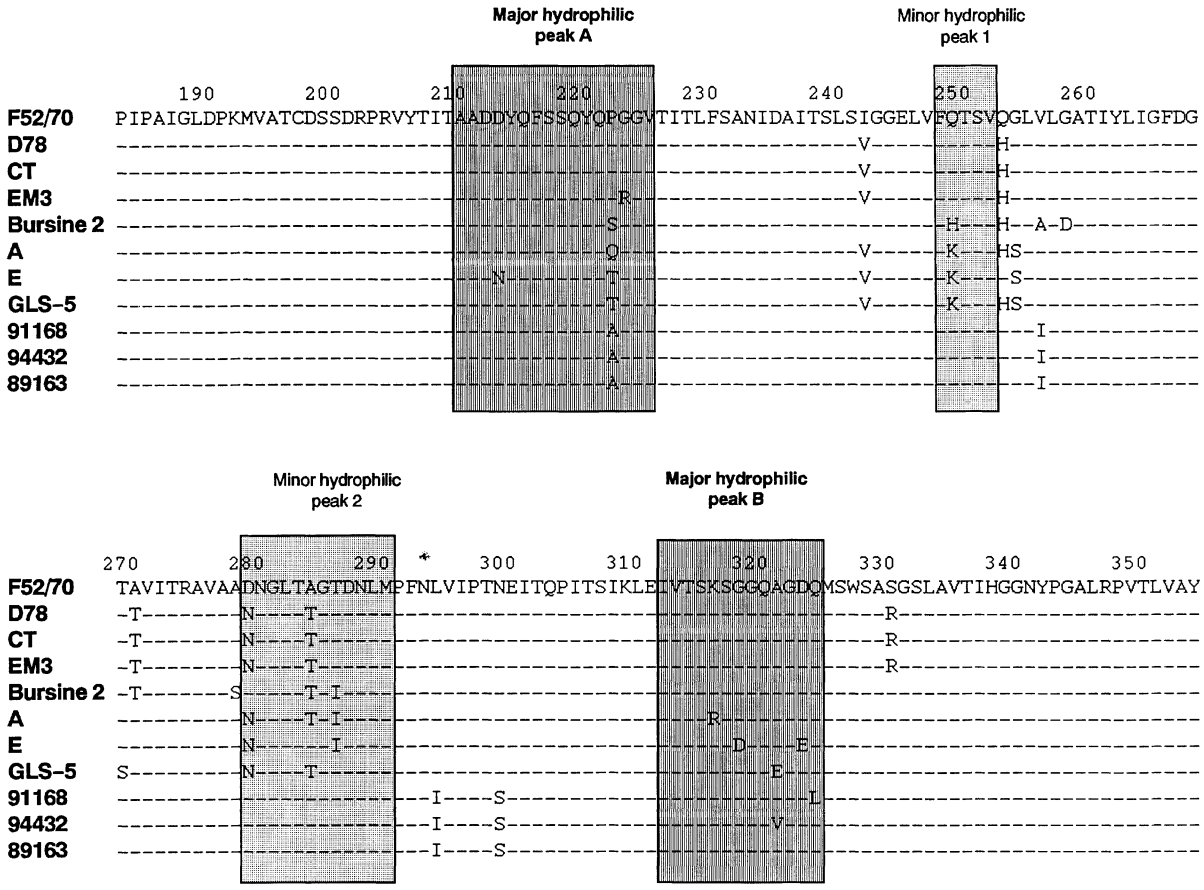


Fig. 2. Deduced amino acid sequence of VP2, from positions 183 to 354 which correspond to VP2 major immunogenic domain (numbering according to Bayliss et al. [2]). Presented IBDV strains are as shown at Fig. 1. A dash indicates positions where the sequence is identical to that of the F52/70 virus. Dark shading indicates VP2 central major hydrophilic peaks A and B [1], pale shading indicates minor hydrophilic peaks 1 and 2 also reported to influence IBDV antigenicity [19]

vvIBDV typical and atypical antigenicity, respectively, is however corroborated by the fact that these positions had already been identified as ≪hot-spots≫ for mutations in several laboratory-selected NMab-resistant escape mutants [15, 18]. Besides, NMab3 proves to have for target an epitope involving hydrophilic peak A, whereas NMabs 6 and 8 recognize different peak B-dependent epitopes that involve tightly clustered aa (positions 321 and 324, respectively). Such a model is indeed consistent with ELISA additivity results which showed that NMabs 3, 6 and 8 probe different antigenic domains [7]. Overall, these results strongly suggest that critical aa positions in VP2 central hydrophilic peaks A and B are of paramount importance for vvIBDV antigenicity, as previously demonstrated in less pathogenic virus strains.

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Authors' address: Dr. N. Eterradossi, Unité de Virologie Immunologie et Parasitologie Aviaires et Cunicoles, CNEVA, B. P. 53, F. 22 440 Ploufragan, France.

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