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1 **Title** — Genetic and splice variations of *Bos taurus* CD46 shift cell permissivity to BVDV,
2 the bovine pestivirus

3

4 **Running title** — Allelic/splice variations alter CD46 usage by BVDV

5

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ABSTRACT

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The pestivirus bovine viral diarrhea virus (BVDV) is known to bind to the CD46 molecule, which subsequently promotes entry of the virus. Mapping of the BVD-virion-binding site has shown that two peptides, 66EQIV69 and 82GQVLAL87, located on antiparallel beta sheets in the most distal complement control protein module (CCP1), provide the attachment platform. In the present study, we reveal the existence of ten distinct allelic versions of the CCP1 module, varying significantly in frequency among taurine and indicine races. A complex mRNA splicing pattern was also evidenced for bovine CD46, generating three different serine-threonine-proline segments and five different cytoplasmic domains. The four most frequent allelic variants and the six splice variants were then expressed in BVDV-nonpermissive porcine cells and the quantity of progeny virions generated by each cell preparation was measured 48 hours post-infection. As expected, ectopic expression of the 10 bovine CD46 isoforms rendered the PK15 cells permissive to BVDV, as attested by the 100,000-fold greater recovery of virions from these cells than from non-transfected cells. This permissivity increase was significantly lower (-33%, $P < 0.001$) when the canonical CCP1 was replaced with the variant most frequent in zebus, suggesting positive or negative selection of this allele in the latter and in the former, respectively. The predicted secondary structure of this variant suggests that the measured loss of function is due to the disappearance of one of the two beta sheets constituting the BVDV attachment platform. On the other hand we showed that for a given CCP1, the titer recovered at 48 hpi also depended on the nature of the CD46 cytoplasmic domain ($P < 0.001$). This result implies that virus binding generates a cytoplasmic-tail-dependent outside-in signal that determines permissivity to BVDV.

Keywords — CD46, Bos Taurus, BVD/MD, polymorphism

1 INTRODUCTION

2

3 *Bovine viral diarrhea virus* (BVDV) is an enveloped, positive-stranded RNA virus of the
4 genus *Pestivirus* in the family *Flaviviridae*. Other species within this genus are the classical
5 swine fever virus and border disease virus of sheep. Pestiviruses are small (40- to 60-nm)
6 enveloped RNA viruses, which together with members of the genera *Flavivirus* and
7 *Hepacivirus* constitute the family *Flaviviridae* (Lindenbach, 2000). The genome consists of a
8 single open reading frame of about 12,300 nucleotides, which is translated into a polyprotein
9 that is co- and post-translationally cleaved into structural and non-structural proteins (Thiel *et*
10 *al.*, 1993). The structural proteins are the nucleocapsid C protein and three envelope
11 glycoproteins: E^{ms}, E1, and E2. While the first is not required for virus entry, formation of
12 E1-E2 heterodimers is essential (Ronecker *et al.*, 2008), as is virus binding to bovine CD46,
13 identified as a cellular receptor for BVDV (Maurer *et al.*, 2004).

14 CD46, also known as membrane cofactor protein or MCP, is a type 1 transmembrane
15 glycoprotein expressed on all cells. The extracellular portion of CD46 consists of four short
16 consensus repeats (complement control proteins or CCPs 1-4) containing binding sites for
17 C3b and C4b, and a heavily O-glycosylated region (STP) rich in serine, threonine, and proline
18 residues. This fragment is adjacent to a ~10-amino-acid region of unknown relevance, a
19 transmembrane segment, an intracytoplasmic anchor, and a COOH-terminal cytoplasmic tail
20 (Marie *et al.*, 2002). The primary function of CD46 is to regulate complement activation:
21 CD46 binds the C3b and C4b complement products deposited on host cell membranes and
22 serves as a cofactor for their proteolytic inactivation by the plasma serine protease factor I.
23 This process, which irreversibly prevents C3 and C5 convertase formation, protects host cells
24 from lysis by autologous complement (Liszewski *et al.*, 1991). Before its recent recognition
25 as a BVDV receptor, CD46 was identified as a receptor for other viruses and bacteria

1 (Cattaneo, 2004). These pathogens recognize different structures in the CD46 ectodomain:
2 measles virus binds to the two external CCP modules (Buchholz *et al.*, 1997), human
3 herpesvirus 6 binds to modules 2 and 3 (Mori *et al.*, 2002; Santoro *et al.*, 2003), adenovirus
4 strains form contacts with different CCPs (Segerman *et al.*, 2003; Wu *et al.*, 2004),
5 pathogenic *Neisseria* relies on the STP domains (Kallstrom *et al.*, 2001), and BVDV binds to
6 the most external CCP (CCP1) for cell attachment (Krey *et al.*, 2006).

7 In the light of this crucial role of CD46 in BVDV biology, we were prompted to examine the
8 polymorphisms of bovine CD46 and their possible functional consequences. In this paper, we
9 show that CCP1 is highly variable among *Bovidae* and that alternative splicing results in
10 multiple CD46 isoforms, each containing one of five distinct cytoplasmic tails. We also show
11 that CCP1 allelic diversity and splicing-subordinated variation of CD46 cytoplasmic tails
12 control cell permissivity towards BVDV.

13

14

MATERIALS AND METHODS

15

16 **Cells, virus, antibodies, and bioinformatics** —Madin-Darby bovine kidney cells (MDBK,
17 ATCC#CCL-22), and porcine kidney cells (PK-15, ATCC#CCL-33) were cultured in Eagle's
18 Minimum Essential Medium (EMEM) supplemented with 5% (v/v) BVDV-free FCS (heat-
19 inactivated, from Lonza), 1% (v/v) penicillin/streptomycin (120 U/mL), and 0.5% (v/v)
20 fungizone (1.5 U/mL). Cells and FCS were routinely tested for the absence of pestiviruses by
21 reverse transcription PCR (RT-PCR) targeting the 5'UTR (Elvander *et al.*, 1998) and FCS
22 was tested for the absence of functional antibodies to BVDV.

23 The BVDV strain NADL (ATCC#VR-1422) was propagated in MDBK cells to produce de
24 stock solution. An ~80% confluent cell monolayer in a 25-cm² tissue culture flask was
25 inoculated with BVDV. After adsorption for one hour, the cell layer was washed with

1 EMEM, then overlaid with supplemented EMEM and finally incubated at 37°C in 5% CO₂
2 until a cytopathic effect was clearly visible over ~50% of the cell monolayer surface (48-72
3 h). Freeze-thawed cells were centrifuged and supernatants with virus particles were pooled
4 and kept at -80°C. The viral stock was titrated in triplicate in standard median tissue culture
5 infectious dose assays using MDBK cells. The viral titer, calculated according to Reed and
6 Muench (Reed and Muench, 1938) , amounted to 13.4 10⁶ TCID₅₀/mL. The stock solution
7 was aliquoted and stored at -80°C to serve as an inoculum in the functional tests described
8 hereafter.

9 The planned experiments required the use of a primary antiserum appropriate for detecting, by
10 indirect immunofluorescence, expression of the different variants of bovine CD46 at the
11 surfaces of transfected PK15 cells, without simultaneously detecting the constitutive
12 expression of porcine CD46. Because of the close phylogenetic relationship between bovine
13 and porcine CD46, it was necessary to exhaust the available polyclonal rabbit antiserum
14 raised against boCD46 (kindly provided by Pr. Till H. Ruemenapf of Justus Liebig
15 University, Giessen, Germany) to remove the antibodies recognizing epitopes common to
16 both CD46 isoforms. This was done by incubating 1 mL of the crude antiserum diluted
17 1:2000 in PBS on 10⁷ PK15 cells at 37°C for 60 min with gentle shaking. The absence of
18 cross-reactivity between the resulting exhausted antiserum and PK15 cells was then checked
19 by showing that PK15 staining was abolished when the crude antiserum was replaced with the
20 purified antiserum in the staining procedure (Fig. 1).

21 Computer-assisted analyses included primer design with Primer_3 (Rozen and Skaletsky,
22 2000) sequence analyses and comparisons with Chromas v.2.21 and BLAST (Altschul *et al.*,
23 1990) respectively, translation of nucleotide stretches in amino acid sequences with the
24 ExPASy-translate tool, alignments of protein sequences with GeneDoc v.2.6.002, splice site
25 predictions with ASSP accessed at <http://es.embnet.org/~mwang/assp.html> (Wang and Marin,

1 2006), and hydrophobic cluster analysis with the DrawHCA program accessed at [http://www-](http://www-int.impmc.upmc.fr/~callebau/hca_method.html)
2 [int.impmc.upmc.fr/~callebau/hca_method.html](http://www-int.impmc.upmc.fr/~callebau/hca_method.html) (Callebaut *et al.*, 1997).

3 **Identification of BVDV receptor allelic polymorphisms** — Genomic DNA was extracted
4 and purified from semen (n=114), hair follicles (n=24), dried blood spots (n=26), or whole
5 blood (n=2) according to the NucleoSpin® Tissue method (Macherey-Nagel). These samples
6 were collected from the following bovine breeds : Angus, Bazadaise, Belgian-Blue, Blonde
7 d'Aquitaine, Brown Swiss, Charolais, Chianina, Frisian, Gelbvieh, Hereford, Jersey,
8 Limousin, Lulu, Maine-Anjou, Montbeliard, MRY, Normande, Pezzata rossa, Pie rouge de
9 l'est, Piemontese, Friesian-Holstein, Red-Holstein, Rouge de Flandre occidentale, Salers,
10 Shorthorn, Simmental, Tarentaise, Gouransi, and Turkish Gray for *Bos taurus*, and Hariana
11 (India), Tonga (Zambia), Biratnagar (Nepal), Malgache (Madagascar), and Nepalese hill
12 (Nepal) for *Bos indicus*. Taurine DNAs were obtained from Belgian, French, Dutch, English,
13 Hungarian, and German farmers, indicine DNAs from African, Indian, and Nepalese farmers,
14 yack DNA from a Nepalese farmer, bison DNA from a Belgian farm, and the wapiti and
15 wildebeest DNAs from a local animal park (by courtesy of Dr Bertho). Amplification of a
16 189-bp genomic segment containing the entire exon 2 of the *Bos taurus* CD46 gene was done
17 by PCR with the 19-mer forward primer 5'-GCCACAGTCCCTGTCTGGT-3' and the 22-mer
18 reverse primer 5'-CGTTCACATGAGAAAACGATCA-3'. The reaction mixture (20 μ L)
19 consisted of 1 μ L genomic DNA, 0.5 μ L (10 μ M) of each primer, 10 μ L Master Mix
20 (Fermentas), and 8 μ L H₂O. The process was carried out in a Mastercycler EPgradient-S
21 machine (Eppendorf) according to a program consisting of 5 min of initial denaturation at
22 94°C, 35 cycles, each of 30 s denaturation at 94°C, 30 s annealing at 58.4°C, 60 s for
23 polymerization at 72°C, and a final extension at 70°C for 5 min. Each purified PCR product
24 was then sequenced on both strands by the dideoxy chain-termination method with BigDye

1 terminators, starting with the same primers. The termination products were resolved and
2 detected with the ABI Prism 3100 automated DNA sequencer (Perkin-Elmer).

3

4 **Searching for CD46 splice variants** — Attempts to generate full-length *Bos taurus* CD46
5 cDNA from peripheral blood leucocytes revealed a set of splice variants. Total RNA was
6 extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions from
7 peripheral leucocytes from two calves. It was then reverse transcribed with the SuperScript®
8 III First-Strand Synthesis System (Invitrogen) and PCR was performed with either the primers
9 5'-TTGGGTGTCCCGCTGGTCTC-3' (fwd) and 5'-AGCAAAGTCCCCTCAGGTCT-3'
10 (rev) or the primers 5'-GCTGGACTCAGCAAGGTCTC-3' (fwd) and 5'-
11 CATGGTAGCCACTGTGCTGT-3' (rev). The reaction was carried out with the BD
12 Advantage™ PCR kit (BD Bioscience) at 94°C for 2 min, followed by 35 cycles of 94°C for
13 60 sec, 55°C for 60 sec, and 68°C for 60 sec, and finally 68°C for 5 min (PE-9700
14 thermocycler). The PCR products were TA-ligated into the pCRII-TOPO vector (Invitrogen)
15 and the resulting constructs used to transform *Escherichia coli* Top10. Five to six cloned
16 cDNAs were sequenced on both strands by the dideoxy chain-termination method with
17 BigDye terminators, starting with the M13 forward and reverse primers.

18

19 **Construction of expression vectors** — To generate the constructs used in functional studies,
20 the *EcoRI/BamHI* fragments from 9 pCR2.1-TOPO, each containing the total span of a
21 specific allelic or splice variant of CD46, were subcloned directionally into the mammalian
22 expression vector pcDNA4/TO (Invitrogen) at the corresponding MCS *XbaI* and *BamHI*
23 restriction sites. The corresponding recombinant expression plasmids were used to transform
24 *E. coli* Top10. Transformants were selected for ampicillin resistance, identified by restriction

1 mapping, and confirmed by sequence analysis. Construction and validation of pcDNA4/TO,
2 encoding eGFP, have been described previously (Palm *et al.*, 2007).

3

4 **Expression of ectopic boCD46 variants on a BVDV-resistant cell surface** — Semi-
5 confluent PK-15 cells were trypsinized, pelleted, washed 3 times, and nucleofected with 3.5
6 μg plasmid DNA at a cell density of 1×10^6 cells per 100 μl Nucleofector® solution V. For
7 this the Amaxa electroporation device was used with program V-001. After nucleofection, the
8 cells were immediately transferred into prewarmed EMEM culture medium supplemented as
9 mentioned above. Each 100- μL electroporation aliquot was split 1:1 and each of the two
10 resulting cell subpopulations was seeded into one of two wells of a 6-well plate ($\sim 5 \times 10^5$ cells
11 per well) and incubated for 16 hours at 37°C in a humidified 5% CO₂ atmosphere. The first
12 well was used to determine the transfection efficiency typical of each electroporated cell
13 population and the second was enrolled in the functional study (see after). Transfection
14 efficiencies were determined by flow cytometric detection of membrane expression of
15 boCD46 variants. For this, control (MDBK and PK-15) and principal (electroporated PK-15)
16 cells were harvested by trypsinization and pelleted at 300g for 5 min. The cells were then
17 resuspended in ice-cold PBS-1%BSA for 20 min, probed for 30 min with the purified rabbit
18 polyclonal anti-boCD46 primary antiserum diluted 1:2000 and, after 3 thorough washing
19 steps, incubated for a further 45 min with relevant Alexa 488-conjugated secondary
20 antibodies diluted 1:1000 (Molecular Probes). All immunostaining steps were carried out on
21 ice. After 3 additional washing steps, immunolabeled cells were finally resuspended in PBS
22 and analyzed with the BD-Canto flow cytometer, with gating on the forward and side scatter
23 to exclude debris and fluorescence measurement in FL1. A minimum of 10,000 events was
24 acquired and analyzed with the BD-FACSDiva software, v4.1.1.

25

1 **Functional testing** —Cell preparations expressing the various distinct boCD46 isoforms
2 encoded by the allelic and splice variants detected in this study were compared for
3 permissivity to BVDV infection. Test preparations (principals) consisted of a set of
4 transfected PK-15 cell cultures, each characterized by expression of a different boCD46
5 isoform on the cell membrane, and control preparations consisted of MDBK cells (permissive,
6 positive control), PK-15 cells (nonpermissive, negative control-1), and transfected PK-15
7 cells expressing eGFP (negative control-2). The test readout was the BVDV titer measured in
8 control and principal supernatants sampled 48 h after standardized infection of the cell
9 monolayers with BVDV (48 hpi). Sixteen hours after electroporation, the cell monolayers
10 (~500 000 cells per well, 6-well plates) were infected with an aliquot of the viral stock diluted
11 1:30, giving a multiplicity of infection of ~1. In practice the incubation medium was
12 discarded and 1 mL ice-cold inoculum was added to each well. The plates were then kept for
13 60 min on ice and then incubated for 30 min at 37°C. Next, the inoculum was discarded, the
14 cell monolayers were thoroughly washed three times with PBS, 2 mL supplemented EMEM
15 was added to each well, and the cells were incubated for another 48 hours at 37°C. The
16 BVDV-containing cell lysates were then titrated in triplicate by an indirect method using a
17 commercially available BVDV NS3 antigen ELISA (Bio-X BVDV antigen Test Kit). To
18 establish the calibration curve, 5 successive ten-fold serial dilutions of the virus stock were
19 prepared and assayed according to the ELISA conditions described by the manufacturer
20 (<http://www.biox.com/>). The dilutions were chosen so as to cover the entire working range
21 and each dilution was assayed in 3 replicates within the plate.

22

23 **Data analysis** — Three or four independent functional testing campaigns were performed for
24 comparison of allelic or splice variants, respectively. All values (boCD46 expression rates and
25 BVDV titers) are presented as means \pm SD for these 3-4 independent campaigns. Statistical

1 analysis was performed by one-way ANOVA with post-hoc pairwise testing by Fisher's
2 protected least significant difference multiple range test, used to establish (i) whether the
3 transfections resulted in reproducible boCD46 expression rates, (ii) whether there was an
4 effect of the expressed boCD46 variant on BVDV titers 48 hpi, and (iii) which boCD46
5 variants significantly exacerbated/reduced permissivity to BVDV. Comparisons yielding P
6 values below 0.05 were considered statistically significant. All statistical analyses were
7 performed with GraphPad PRISM® software, version 4.0.

8

9

RESULTS

10

11 **Identification and breed origins of the allelic CCP1 variants** — A total of 166 animals
12 were screened for variation in the coding region of the CCP1 in the CD46 gene: 137
13 representatives of taurine breeds and 23 of indicine breeds of *Bos taurus*, 1 yack (*Bos*
14 *grunniens*), 2 bisons (*Bison bison*), 2 wildebeest (*Conochaetes taurinus*), and 1 wapiti
15 (*Cervus elaphus canadensis*). This resulted in the identification of 8 novel point mutations
16 (G84C, T87C, G97A, T100C, G101A, T104G, G114A and G141A, numbered according to
17 NM_183080). Six of the eight point mutations were nonsynonymous changes resulting in
18 amino acid alterations and two were synonymous (G101A and T104G). Sequencing of 39
19 PCR amplicons revealed several homozygous chromatograms yielding 3 haplotypes: Val69-
20 Tyr70-His73-Leu74-Val79-Val88, Val69-Tyr70-Arg73-Leu74-Ile79-Val88, and Val69-
21 Tyr70-Arg73-Leu74-Val79-Val88. Assuming that one of these 3 haplotypes necessarily
22 contributed to one allele in all the remaining heterozygous chromatograms and imposing a
23 minimum number of haplotypes, successive deductions led to presume the existence of three
24 supplementary haplotypes, Val69-Tyr70-Arg73-Leu74-Ile79-Ile88, Leu69-His70-Arg73-
25 Pro74-Ile79-Val88, and Val69-Tyr70-His73-Leu74-Val79-Val88. Sequencing of the yack and

1 gnu CCP1 coding regions repeatedly yielded biallelic chromatograms that were not
2 compatible with any of the 6 above-mentioned haplotypes, thus suggesting 4 additional
3 combinations. PCR amplicons obtained from 3 homozygous bovines, three heterozygous
4 bovines, two gnus, and the yack were then cloned in *E coli*, five clones were resequenced
5 from each animal, and the 7 presumed CCP1 haplotypes were indeed retrieved. They were
6 nicknamed following the Greek alphabet (Fig. 2), assigning α to the first haplotype to have
7 been made publicly available (#Q6VE48). Six of the haplotypes were found in the taurine
8 breeds and only 4 among the indicine breeds. The higher frequency of haplotype α in the
9 genus *Bos* and its detection in both bisons and even the wapiti suggest that it might be
10 ancestral (Table 1). On the other hand, the far higher frequency of haplotype δ among taurine
11 breeds is compatible with an origin in *Bos taurus*, with recent admixture to zebus.
12 Observation of haplotype ϵ in both bisons and cattle suggests an ancient origin too; its far
13 higher frequency among indicine than taurine breeds is appealing, as it suggests positive
14 selection in the former or negative selection in the latter. Sequencing of the PCR amplicon
15 retrieved from MDBK cells revealed a homozygous chromatogram typical of the β haplotype.

16

17 **Identification of *Bos taurus* CD46 splice variants** — The alternative splicing pattern of the
18 bovine CD46 gene was analyzed by bioinformatics first, then by cloning/sequencing cDNAs
19 from peripheral blood mononuclear cells (PBMCs) purified from 2 Belgian-Blue calves.
20 Confirmation was finally obtained by RT-PCR and sequencing of PCR products. The splicing
21 pattern appears rather complex, arising from exon skipping (exons 6, 7, 8, 9, and/or 13) and
22 from the usage of alternative 3' exon splice sites for exons 11 and 12 (Fig. 3 and Table 2),
23 yielding six alternative transcripts, of which 3 new ones. Skipping of exon 6, 7, 8, 9, or 13
24 does not lead to a frame-shift in the encoded protein, but usage of an alternative donor splice
25 site for exon 11 does. Diversity among the predicted protein isoforms was observed for the

1 STP segment, with three different lengths, and for the cytoplasmic tail, with five different
2 versions. The CCP1, CCP2, CCP3, CCP4, and transmembrane modules were always present
3 and organized in the same way (Fig. 3).

4

5 **Effect of CCP1 allelic variation on cell permissivity towards BVDV** — To determine
6 whether the allelic variation affecting the CCP1 module within the CD46 surface glycoprotein
7 might alter the biological cycle of BVDV, full-length cDNAs encoding CD46 splice isoform
8 2 (sv2) with included CCP1- α , - β , - δ , or - ϵ were transiently expressed in the porcine PK-15
9 cell line, which is 500 times less susceptible to BVDV virus infection than MDBK cells
10 (Maurer et al., 2004). Only cell preparations with roughly similar membrane expression rates
11 of boCD46 variants were kept for functional testing. The boCD46 membrane expression rates
12 were not statistically different ($P>0.05$) among cell populations selected for the functional
13 studies and, within the expression window selected, correlation between expression rate and
14 retrieved virus titers was excluded (Spearman's correlation, $P>0.20$). Typical flow cytometric
15 expression patterns are shown (Fig. 4). The titer of BVDV measured 48 hpi in culture
16 supernatants was $>10^5$ -fold higher for boCD46-expressing than for eGFP-expressing PK15
17 cells ($p<0.001$). The permissivities of cell populations expressing the boCD46 CCP1- β , - δ , or
18 - ϵ allelic variant were compared, on the one hand, with that of PK15 control cells expressing
19 eGFP and, on the other hand, with that of PK15 cells expressing boCD46 with inserted CCP1-
20 α (the permissivity of this last population was set at 100%). In this experiment the
21 permissivity of the eGFP-expressing PK15 cells appeared similar to that of the parental PK15
22 cells (\sim zero), but the permissivity of cells expressing boCD46-[CCP1- α] reached about half
23 the level displayed by MDBK cells. Replacement of CCP1- α with CCP1- β or CCP1- δ did not
24 alter the permissivity significantly ($P>0.05$), but insertion of CCP1- ϵ consistently led to a
25 lower ($P<0.05$) permissivity towards the virus (Fig. 5).

1
2 **Effect of alternative CD46 splicing on cell permissivity towards BVDV** — To determine
3 whether alternative splicing of *Bos taurus* CD46 transcripts affects the BVDV receptor
4 function of the resulting surface glycoproteins, full-length cDNAs encoding CD46 CCP1- α
5 splice isoforms 1-6 (sv1→sv6) were transiently expressed in the BVDV-resistant porcine PK-
6 15 cell line. Again, only cell preparations with similar membrane expression rates of boCD46
7 splice variants were kept for functional testing (see typical flow cytometric expression
8 patterns in Fig. 6). The BVDV titer measured 48h pi in the culture supernatants of cells
9 expressing any of the boCD46 splice variants was more than 10^5 -fold higher than the titer
10 measured in culture supernatants of eGFP-expressing PK15 cells ($p < 0.001$). The
11 permissivities of cell populations expressing the boCD46 sv1, sv3, sv4, sv5, or sv6 splice
12 variant were compared with those of PK15 eGFP-expressing control cells and PK15 cells
13 expressing boCD46 sv2, whose permissivity was set at 100%. Membrane expression of sv3
14 and sv4 conferred a permissivity similar to that afforded by sv2, while cells expressing sv1
15 ($p < 0.05$) or especially sv5 or sv6 ($p < 0.01$) showed significantly lower permissivity (Fig. 7).

16

17 DISCUSSION

18

19 BVDV uses the membrane glycoprotein CD46 as a platform for attachment to bovine cells
20 (Maurer *et al.*, 2004). This is in keeping with its status as a « microbe magnet » established on
21 the basis of its use by the measles virus, human herpesvirus 6, adenoviruses of different
22 serotypes, *Streptococcus pyogenes*, *Neisseria meningitidis*, and *N. gonorrhoeae* (Cattaneo,
23 2004). The distal end of CD46 consists of several successive CCP modules that each of the
24 microbes just mentioned uses in its own way. In the case of the ligand-receptor interaction

1 between BVDV and CD46, the attachment platform is provided by CCP1 alone, and
2 specifically by its 66-EQIV-69 and 82-GQVLAL-87 peptides (Krey *et al.*, 2006).

3

4 **CCP1 allelic diversity** — As the porcine version of CD46 is far from providing an optimal
5 attachment platform for BVDV (Maurer *et al.*, 2004), it is tempting, although one should
6 remain cautious, to propose a causal link between infinitesimal CD46-dependent virus
7 attachment and the fact that BVDV is asymptomatic in swine. Should this hypothesis be at
8 least partially verified and should a nonfunctional variant of the CCP1 module of bovine
9 CD46 be found, then the severity of the disease caused by BVDV in bovines might depend, to
10 some extent at least, on the genotype of the infected animal. This prospect led us to inventory
11 the allelic variations affecting exon 2 of the bovine CD46 gene, which encodes the whole
12 CCP1 module. This exon 2 was systematically sequenced in our single cervine and 165
13 bovine samples. Among the animals of the genus *Bos*, many breeds were represented in the
14 DNA library so as to maximise its representativity (see list in the methods section). Eight
15 nucleotide substitutions were detected, of which six cause an amino acid substitution. The
16 amino acid positions concerned within CCP1 are 69, 70, 73, 74, 79, and 88. The ten
17 haplotypic variants observed result from various combinations of these substitutions (Fig. 2).
18 Four of these variants are frequent (α , β , δ , ϵ), two of them (α , β) even being present in the
19 phylogenetically most distant animal (wapiti). Two variants seem rather rare (γ and θ), and
20 the last four are probably species-specific (2 in yack and 2 in wildebeest). By comparison
21 with other similar studies, it seems somewhat exceptional to have detected ten different
22 versions at protein level in a module containing only 60 amino acids. The observation that
23 75% of the mutations cause an amino acid substitution is likewise unusual. The reason for this
24 variability is unknown, but the fact that CD46 is used as a receptor by a multitude of different
25 pathogens suggests that several, possibly contradictory microbial pressures may have been

1 exerted, simultaneously or successively, on this locus. Furthermore, the haplotype frequencies
2 listed in Table 1 reveal a disparity between taurine and indicine breeds: the majority
3 haplotype is the same (α), but zebus are distinguished by an undeniable concentration of
4 haplotype ε , differing from haplotypes α , β , γ , and δ by the Tyr70His substitution, which is
5 exceptional since it is absent even from *Sus scrofa*, *Mus musculus*, and *Homo sapiens*. Again
6 it seems plausible that zebus and taurine breeds have been exposed to different microbial
7 pressures, which might explain positive selection of CCP1- ε in the former. This observation
8 makes it all the more interesting to study the BVDV receptor function of different CCP1s.
9 The most frequent version of the CCP1 module is the variant present in the CD46 initially
10 sequenced and cloned from Stratagene bovine ganglion cDNA (Maurer *et al.*, 2004). This is
11 why we named it « alpha ». This is also the variant used in experiments showing that the
12 receptor BVDV function relies on the peptides 66-EQIV-69 and 82-GQVLAL-87 (Krey *et al.*,
13 2006). At first glance, one would not expect the receptor function to be very different for the
14 different identified allelic variants of CCP1, as 5 of the 6 highlighted positions do not lie in
15 these “strategic” peptides. As for the sixth position, it is occupied by a glutamate in the
16 resistant porcine CCP1, a valine in the reputedly sensitive bovine CCP1- α (Krey *et al.*, 2006),
17 and a leucine in five of the new CCP1s detected here. A priori, as leucine and valine belong to
18 the same group of amino acids, one would expect the replacement of one by the other to have
19 little functional impact. Yet in several CCP1s whose function is unknown, there is an
20 additional Tyr70His substitution at position 70, directly adjacent to the strategic peptide 66-
21 EQIV-69. We thus observe two doubly substituted pentapeptides, 66-EQIVY-70 (CCP1
22 variants α , β , γ , δ) and 66-EQILH-70 (ε , ζ , η), in a context where 66-EQIV-69 is known to
23 support, and its porcine equivalent 66-DRVE-70 is known not to support, the receptor
24 function (Krey *et al.*, 2006). As residue Tyr70 is widely conserved in bovines (#NP_898903
25 and CCP1s α , β , γ , δ , θ , ι , κ), swine (#NP_999053), mouse (deduced from the GI31981632

1 nucleotide sequence), and man (deduced from the GI27502404 nucleotide sequence), it is
2 reasonable to suggest that the Tyr70His substitution typical of CCP1s ϵ , ζ , and η may affect
3 the BVDV receptor function. It might influence, for example, the conformation of the
4 adjacent strategic peptide. CCP1s β , γ , δ , ϵ , and η also contain the His73Arg substitution and
5 CCP1- ϵ additionally contains the Leu74Pro substitution. These substitutions bring these five
6 bovine CCP1s focally closer to the porcine version. Yet replacing the bovine His-Leu pair
7 with the porcine Arg-Pro pair within a bovine CD46 does not lead to loss of function (Krey *et*
8 *al.*, 2006), suggesting that these two substitutions do not play an important role. CCP1s
9 β , γ , ϵ , ζ , and η contain the Val37Ile substitution, and CCP1- γ contains also the Val88Ile
10 substitution. Again, in addition to the fact that these positions do not lie within either strategic
11 peptide, the similarity of leucine and valine residues suggests that replacing one with the other
12 will probably have no functional impact. To confirm these speculations, the BVDV receptor
13 function of the four most frequent CCP1s was measured *in vitro*. Firstly, it appeared that
14 expression of a bovine CD46, whether it contains CCP1- α , - β , - δ , or - ϵ , renders PK15 cells
15 very permissive towards BVDV, confirming previous results (Krey *et al.*, 2006; Maurer *et al.*,
16 2004). Secondly, we found that the permissivity conferred by CD46/CCP1- ϵ never reached
17 the level conferred by CD46/CCP1- α , - β , or - δ , the particle production deficit being about
18 33% (Fig. 5). These functional results confirm that the two substitutions characterizing CCP1s
19 β and δ , His73Arg and Val79Ile, do not affect virus attachment as compared to the reference
20 CCP1, α . As for CCP1- ϵ , it carries a combination of 5 substitutions. Assuming that the
21 His73Arg et Val79Ile substitutions do not affect the receptor function and as the substitution
22 at position 69 is from a valine to a closely related leucine, it would seem that the functional
23 deficit associated with CCP1- ϵ is probably caused by either the Tyr70His substitution, the
24 Leu74Pro substitution, or their combination. On the basis that VILFMWY are hydrophobic
25 residues (CCP1- ϵ has thus lost two) and taking local proximities into account, a hydrophobic

1 cluster analysis of CD46 primary sequences ([http://mobyale.rpbs.univ-paris-diderot.fr/cgi-](http://mobyale.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA)
2 [bin/portal.py?form=HCA](http://mobyale.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA)) reveals that the combination of these two substitutions breaks a β -
3 strand shared by CCP1- α , - β , and - δ (Fig. 8) and forming the attachment platform for BVDV
4 (Krey *et al.*, 2006). The general architecture of CCP1- ε is thus different from that of the other
5 CCP1s, and its BVDV attachment platform may be sufficiently modified to explain decreased
6 cell permissivity to BVDV. The NADL strain is a very old BVDV-1a strain whose passage
7 history is mostly not known in detail. For this study, the strain was propagated in MDBK
8 cells. As these cells express the β haplotype, it could be that the virus used here shows a
9 preferential binding to CCP1- β . Duplicating the present study with a strain isolated from a
10 bovine population where the ε haplotype is highly prevalent would be helpful to determine
11 whether a selective pressure is exercised by the CCP1 module on the virus.

12

13 **CD46 splicing-associated diversity** — Six different transcripts resulting from alternative
14 splicing were detected, of which 3 are new. These can be expected to encode six different
15 proteins. It is likely that this inventory of the splicing-dependent diversity of bovine CD46 is
16 not exhaustive, notably because it is based solely on PBMCs from only two animals. This
17 complex splicing pattern comes as no surprise, given the splicing-dependent diversity
18 detected in man (18 variants [(Karosi *et al.*, 2008; Russell *et al.*, 1992)]), mouse (4 variants
19 [(Nomura *et al.*, 1999; Tsujimura *et al.*, 2001)]), and Guinea pig (5 variants [(Hosokawa *et*
20 *al.*, 1996)]).

21 When the use of these splice variants by BVDV was examined (in the context of a constant
22 CCP1, α), significant differences were detected, suggesting that the splicing pattern of bovine
23 CD46 controls cell permissivity towards BVDV. Alternative splicing generates three
24 extracellular versions according to whether exon 9 (sv4), exon 8 (sv5), and exons 6-9 (sv1,
25 sv2, sv3 et sv6) are skipped or not. This differential usage of exons 6 to 9 generates three

1 CD46 proteins equipped with an STP of variable length. As PK15 cells expressing either sv3
2 or sv4 show the same permissivity towards BVDV, despite one having a long STP (sv4) and
3 the other a short one (sv3), it would seem that variations in STP segment length do not affect
4 virus binding. This conclusion is consistent with that of Krey et al. (2006), who observed little
5 effect on BVDV binding and internalization when the segment between the membrane and
6 the four successive CCPs was artificially lengthened by 18 nm. Alternative splicing also
7 generates five different cytoplasmic tails (CT1→CT5) according to the usage of the
8 alternative 3' exon splice sites of exons 11 and 12. These tails are:
9 CCFCRQRKKGIYVTGESHRQDILFLSEKR (CT1, of sv1), VFADRGRKGGKQNALRTP
10 LIRIKQPLQQR (CT2, of sv2), CCFCRQRKKGKAECSATYTTYQDKATTATEQMN
11 (CT3, of sv3 and sv4), VFADRGRKAYT (CT4, of sv5), and CCFCRQRKKGLK (CT5, of
12 sv6). The functional data show that the variants least favorable to the virus are those with a
13 truncated cytoplasmic tail (CT4 and CT5), suggesting that the cytoplasmic tail conditions cell
14 permissivity towards BVDV. Whereas until now CD46 was assumed to intervene only at the
15 level of BVDV binding, our results imply the existence of an outside-in signal adjusting cell
16 permissivity towards BVDV and triggered by virus binding to CCP1. On the basis of the
17 literature, it seems likely that interaction between BVDV and CD46 might indeed trigger
18 outside-in signaling. For example, binding of the measles virus or of a specific antibody
19 triggers IL-6 production in astrocytoma cells (Ghali and Schneider-Schaulies, 1998),
20 enhances immunoglobulin E class switching in the human Ramos B cell line (Imani *et al.*,
21 1999), and enhances both nitric oxide synthesis and the production of type I interferons in
22 macrophage cell lines (Hirano *et al.*, 1999; Katayama *et al.*, 2000) . Furthermore, IL-12
23 production is inhibited in activated human monocytes or macrophages after exposure to the
24 measles virus, CD46 cross-linking, or C3b binding (Karp *et al.*, 1996). Studies on CD46-
25 transgenic mice have shown that measles virus-CD46 interaction inhibits both IL-12

1 production by DCs and the inflammatory response (Marie *et al.*, 2001). Also, upon exposure
2 of epithelial cells to the purified pili of *Neisseria gonorrhoeae* or *N. meningitidis*, a Ca^{++} flux is
3 induced, again suggesting a CD46-dependent cell-signaling event (Kallstrom *et al.*, 1998).
4 Lastly, control of outside-in signaling by the nature of the cytoplasmic domain of CD46 has
5 been demonstrated several times in the human species. Among the 18 splice variants
6 described, two distinct cytoplasmic domains have been detected (Russell *et al.*, 1992): CT1 is
7 16 residues long (TYLTDETHREVKFTSL) and CT2 is 23 residues long
8 (KADGGAEYATYQTKSTTPAEQRG). These two cytoplasmic tails contain different
9 consensus sites for phosphorylation, suggesting that they are responsible for triggering
10 different outside-in signaling pathways and hence different biological responses (Liszewski *et*
11 *al.*, 1994). As expected, CT-dependent biological responses have indeed been evidenced. In
12 the context of contact hypersensitivity, a T-cell-dependent inflammatory response, it was
13 shown that CD46 controls the immune response and that the final outcome depends on CT1
14 and CT2, which exert opposite effects. More specifically, according to whether CD46/CT1 or
15 CD46/CT2 is engaged, CD8-dependent cytotoxicity, CD4 T-cell proliferation, IL-2
16 production, and IL-10 production are differently affected. If CD46/CT1 comes into play, the
17 contact hypersensitivity reaction is diminished, but when CD46/CT2 is engaged, the reaction
18 is exacerbated. Hence, the splicing pattern does indeed control inflammation (Marie *et al.*,
19 2002). There is also a known example of permissivity towards a virus being controlled by the
20 cytoplasmic tail of the CD46 receptor: mouse macrophages expressing a tailless human CD46
21 mutant produce 2 to 3 orders of magnitude more infectious measles virions than mouse
22 macrophages expressing a human CD46 with intact cytoplasmic domains (Hirano *et al.*,
23 1999). The observation that CD46 cytoplasmic domains differentially control cell
24 permissivity towards BVDV raises many questions concerning their mechanisms of action
25 and the role they play in BVDV biology. Although the expression patterns of the different

1 boCD46 splice variants are not known, data on humans suggest that certain tissues or cell
2 lines express more of one cytoplasmic isoform than of another. Should this be the case in the
3 bovine species, predominant expression of one isoform in some animals or in particular cell
4 populations may lead to an altered BVDV replication rate or may control BVDV tropism.
5 Moreover, as BVDV is a serious health problem in cattle, largely because of the immune
6 response suppression associated with this infection, the results gathered here also suggest that
7 BVDV might exploit the CD46-CT-dependent immunoregulatory role to manipulate host
8 adaptive immune responses for its own biological success.

9

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Table 1 — Frequency distribution of CCP1 allelic variants

Table 2 — CD46 gene splicing sites usage by bovine PBMCs

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FIGURE LEGENDS

1

2

3 **Figure 1** — Upon absorption onto PK15 cells, anti-boCD46 antiserum loses its cross-
4 reactivity towards porcine CD46. Alexa 488 (CD46 staining) fluorescence intensity frequency
5 distribution plot from the flow cytometric analysis of MDBK (bovine) and PK15 (porcine)
6 cells. Cells were probed first with nonpertinent (top 2 plots) or anti-boCD46 (lower 4 plots)
7 crude (top 4 plots) or exhausted (bottom 2 plots) rabbit polyclonal antiserum diluted 1:2000
8 (kindly provided by Pr. Till H. Ruemenapf from Justus Liebig University, Giessen,
9 Germany), then with relevant Alexa 488-conjugated secondary antibodies diluted 1:1000
10 (from Molecular Probes). Fluorescences were collected in FL1, a minimum of 10,000 events
11 were acquired and analyzed with the BD-FACSDiva software, v4.1.1. RAS, rabbit antiserum.

12

13 **Figure 2** — CCP1 allelic variants, as predicted from cloned CD46 cDNAs. Nonsynonymous
14 nucleotide substitutions were found to change amino acids at the six indicated positions
15 (numbering according to GenBank #NP_898903). Ten distinct combinations of amino acid
16 substitutions were detected, yielding ten distinct CCP1 modules. These were nicknamed
17 following the Greek alphabet, assigning α to the first publicly available module (#Q6VE48).
18 Green lozenges refer to residues identical to the corresponding residues in the reference and α
19 CCP1 versions, blue lozenges refer to substituted residues.

20

21 **Figure 3** — The mRNA of *Bos taurus* CD46 has 14 exons (boxes). Alternative splicing
22 results in 6 CD46 splice variants (SV) derived from a single gene. Exons 1 to 5 code for the
23 Complement Control Protein repeats (CCPs). The CCPs are followed by the O-glycosylated
24 Serine-Threonine-Proline-rich region (STP) encoded by exons 6-10. STP is followed by the
25 transmembrane domain (TM, exons 11-12) and the cytoplasmic tail (CYT, exons 12-14). The

1 splicing machinery generates three types of STP depending on the pattern of exon 6-10
2 skipping: STP1 (encoded by exons 6-8 and 10), STP2 (exons 6-7 and 9-10), and STP3 (exon
3 10). There are five types of cytoplasmic tails, depending on the usage of canonic or alternative
4 3' exon splice sites in exons 11-12 and on the skipping of exon 13. The amino acid sequence
5 of each cytoplasmic tail is given in the text. See Table 2 for details at the nucleotide level.

6

7 **Figure 4** — Ectopic membrane expression profiles of four boCD46 allelic isoforms in PK15
8 cells. Typical Alexa 488 fluorescence intensity (bovine CD46 staining) frequency distribution
9 plots from the flow cytometric analysis of MDBK cells (100% boCD46 expression expected)
10 and PK15 cells. PK15 cells were transfected with a pcDNA4 plasmid coding for either eGFP
11 (0% boCD46 expression expected) or the indicated boCD46 allelic variant with its specific
12 CCP1 insert. Cells were probed with either nonpertinent or exhausted anti-boCD46 rabbit
13 polyclonal antiserum diluted 1:2000, followed by relevant Alexa 488-conjugated secondary
14 antibodies diluted 1:1000 (from Molecular Probes). Fluorescence intensities were collected in
15 FL1 and a minimum of 10,000 events was acquired and analyzed with the BD-FACSDiva
16 software, v4.1.1. Membrane expression of ectopic boCD46 variants was always detected in
17 60-85% of the transformed cells. Only cell preparations with 76-82% expression were kept
18 for functional studies. RAS and ARAS refer to primary antiserum, with rabbit antiserum and
19 exhausted rabbit antiserum, respectively. Numerals in each panel refer to the proportion of
20 cells in the P3 area.

21

22 **Figure 5** — CCP1 allelic variants of bovine CD46 differentially control cell permissivity
23 towards BVDV in a nonpermissive background. PK15 cells were transformed to express
24 either eGFP or the indicated bovine CD46 isoform (four tested SV2 isoforms with different
25 CCP1 inserts). Sixteen hours after electroporation, the cells were infected with BVDV

1 (multiplicity of infection: ~ 1). Progeny-virion-containing supernatants sampled 48 hpi were
2 titrated in triplicate. Boxes represent mean values of three independent experiments (two for
3 CCP1- δ) and error bars indicate standard deviations. Only boxes with different letters are
4 significantly different (post-hoc testing, $P < 0.05$). Between-isoform reproducibility of CD46
5 membrane expression was ensured by discarding experiments with irregular membrane
6 expression as judged by the proportion of bovine-CD46-positive cells and the mean Alexa
7 488 fluorescence intensity (MFI) of the CD46-positive cell populations. Among the reported
8 experiments, the percentages of CD46-expressing cells and the MFIs were not statistically
9 different (one-way ANOVA, $P > 0.05$).

10

11 **Figure 6** — Ectopic membrane expression profiles of six boCD46 splice isoforms in PK15
12 cells. Typical Alexa 488 fluorescence intensity frequency distribution plots (bovine CD46
13 staining) from flow cytometric analyses of MDBK cells (100% boCD46 expression expected)
14 and PK15 cells. PK15 cells were transformed with a pcDNA4 plasmid coding for either eGFP
15 (0% boCD46 expression expected) or the indicated boCD46 splice variant. Cells were probed
16 with either nonpertinent or exhausted rabbit polyclonal anti-boCD46 antiserum diluted
17 1:2000, followed by relevant Alexa 488-conjugated secondary antibodies diluted 1:1000
18 (from Molecular Probes). Fluorescences were collected in FL1 and a minimum of 10,000
19 events was acquired and analyzed with the BD-FACSDiva software, v4.1.1. Membrane
20 expression of ectopic boCD46 variants was always detected in 60-85% of the transformed
21 cells. Only cell preparations with 75-82% expression were kept for functional studies. Within
22 this expression window, correlation between expression rate and retrieved virus titers was
23 excluded (Spearman's correlation, $P > 0.20$). RAS and ARAS refer to primary antiserum
24 (rabbit antiserum or exhausted rabbit antiserum, respectively). Numerals in each panel refer to
25 the proportion of cells in the P3 area.

1
2 **Figure 7** — Splice variants of bovine CD46 differentially control cell permissivity towards
3 BVDV in a non-permissive cell background. PK15 cells were transfected so as to express
4 either eGFP or the indicated bovine CD46 splicing isoform (six isoforms tested, all with the α
5 form of CCP1). Sixteen hours after electroporation, cells were infected with BVDV
6 (multiplicity of infection: ~ 1). Progeny-virion-containing supernatants sampled 48 hpi were
7 titrated in triplicate. Boxes represent mean values of three independent experiments and error
8 bars indicate standard deviations. Only boxes with different letters are significantly different
9 (post-hoc testing, $P < 0.05$). Between-isoform reproducibility of CD46 membrane expression
10 was ensured by discarding experiments with irregular membrane expression, as judged by the
11 proportion of bovine-CD46-positive cells and the mean Alexa 488 fluorescence intensity
12 (MFI) of the CD46-positive cell populations. Among the reported experiments, the
13 percentages of CD46-expressing cells and MFIs were not statistically different (one-way
14 ANOVA, $P > 0.05$).

15
16 **Figure 8** — A two-dimensional helical representation of CCP1/CCP2 sequences reveals the
17 disappearance of a β -strand in CCP1- ϵ due to the Tyr70His and Leu74Pro substitutions. The
18 representation was obtained by a hydrophobic cluster analysis carried out with the DrawHCA
19 program accessed at http://www-int.impmc.upmc.fr/~callebau/hca_method.html. A star stands
20 for proline, a diamond for glycine, and a square with or without a point for serine or
21 threonine, respectively.

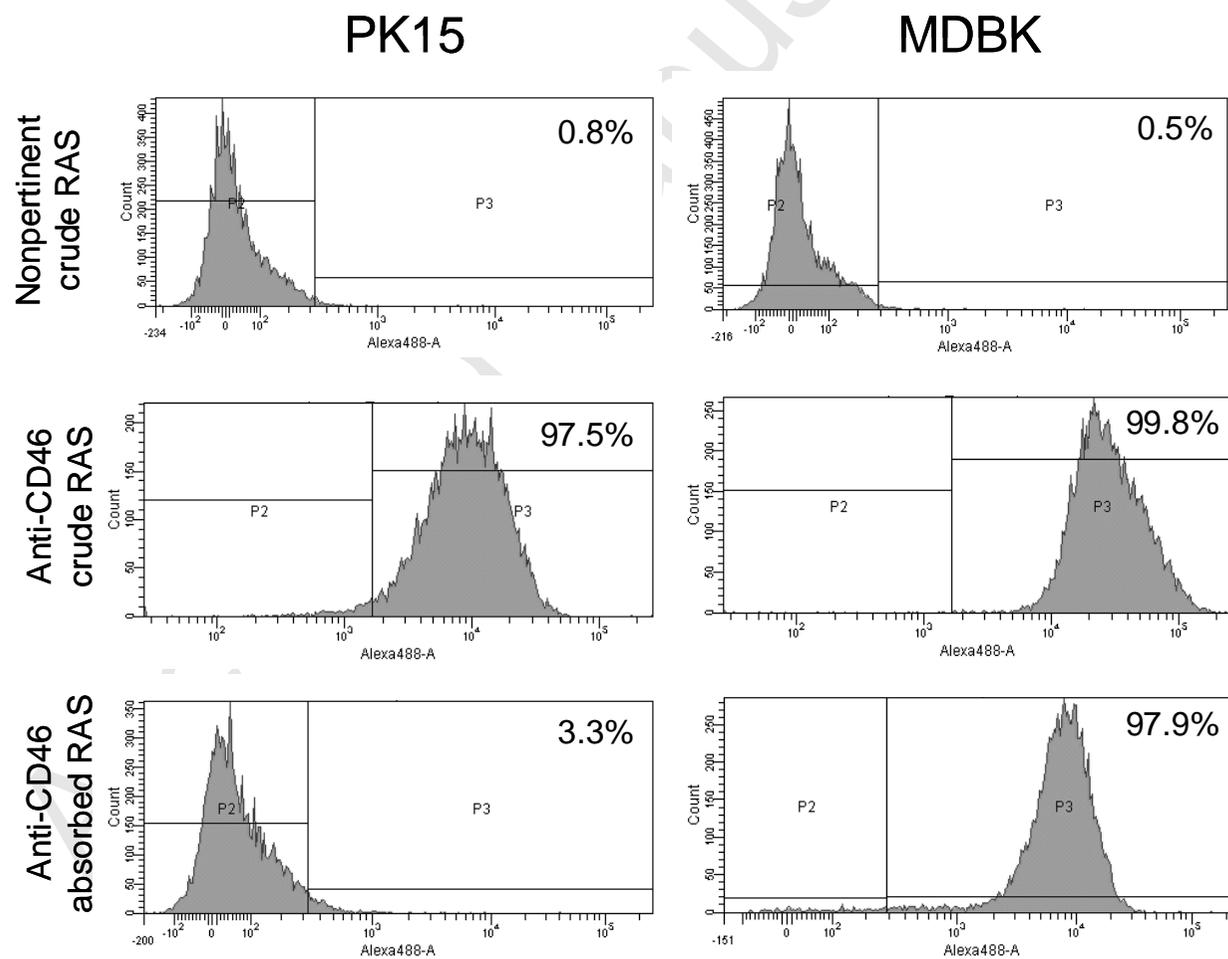
Table 1 — Frequency distribution of CCP1 allelic variants

| <i>Species</i> | <i>n</i> | CCP1 haplotypes | | | | | | | | | |
|-----------------------------|----------|-----------------|---------|----------|----------|------------|---------|--------|----------|---------|----------|
| | | α | β | γ | δ | ϵ | ζ | η | θ | ι | κ |
| <i>Bos taurus</i> | 137 | 41.6 | 23.7 | 5.1 | 23.0 | 5.8 | 0.0 | 0.0 | 0.8 | 0.0 | 0.0 |
| <i>Bos indicus</i> | 23 | 41.5 | 15.2 | 0.0 | 2.2 | 39.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| <i>Bos grunniens</i> | 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 50.0 | 50.0 | 0.0 | 0.0 | 0.0 |
| <i>Bison bison</i> | 2 | 25.0 | 0.0 | 25.0 | 0.0 | 50.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| <i>Conochaetes taurinus</i> | 2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 50.0 | 50.0 |
| <i>Cervus elaphus</i> | 1 | 50.0 | 50.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

Allelic frequencies are given as percent value

| Exon | Acceptor site | Donor site | Exon length (nt) | Relevant polypeptidic segment | Contribute to isoform(s) ... | CD46 domain | Comment |
|--------------|---|---|------------------|---|------------------------------|-------------|---|
| Not numbered | Not determined | Not determined | Not determined | MRAS (...) PTSS | 123456 | N-term | |
| 1 | ctattcctag ATGCCTGTGA (Constitutive acceptor) | GGCTGTAAAAgtaagtaaaa (Constitutive donor) | 189 | <u>DAC</u> -(... 62aa ...)- <u>GCK</u> | 123456 | CCP1 | |
| 2 | ttgcttttag AAAGACGGTG (Cryptic acceptor) | GTAATAAT GG gtaagtaggc (Unclassified donor) | 103 | <u>KRR</u> -(...33aa...)- <u>NNG</u> | 123456 | CCP2-N | |
| 3 | tctcatttag TTATTACTTA (Constitutive acceptor) | ACATGTGAAAgtaagtagt (Constitutive donor) | 89 | <u>YY</u> -(...29aa...)- <u>TCE</u> | 123456 | CCP2-C | |
| 4 | tcttttaag AGATTTTGTG (Cryptic acceptor) | CAGTGTAAGgtaagtagat (Unclassified donor) | 198 | <u>KIL</u> -(...65aa...)- <u>CK</u> | 123456 | CCP3 | |
| 5 | aattttcag TGGTCAAATG (Constitutive acceptor) | TGTATTAAAGgtaaaagtac (Cryptic donor) | 183 | <u>VVKC</u> -(...61aa...)- <u>CIK</u> | 123456 | CCP4 | |
| 6 | tgttttcag TGTCGATTCC (Cryptic acceptor) | AGTGTCTCAGgttagtaac (Cryptic donor) | 66 | <u>VS</u> IPSTQSPISTQPPVPS <u>VS</u> | 4&5 | STP | always skipped, excepted by SV4 and SV5 |
| 7 | ttccccag TGTCGACTCG (Constitutive acceptor) | AATGTCTCAGgttagtaac (cryptic donor) | 42 | <u>VSTR</u> STQHPVP <u>NVS</u> | 4&5 | STP | always skipped, excepted by SV4 and SV5 |
| 8 | ttccccag TGTCGACTCG (Constitutive acceptor) | AATGTCTCAGgttagtaac (Cryptic donor) | 42 | <u>VSTR</u> STQHPI <u>NVS</u> | 4 | STP | always skipped, excepted by SV4 |
| 9 | tccattccag ATTCCAAGCC (Cryptic acceptor) | AGTCATCCAGgttcagggc (Cryptic donor) | 48 | <u>DSKPT</u> SPTMTSGL <u>SHP</u> | 5 | STP | always skipped, excepted by SV5 |
| 10 | tttttctag GACATCCCC (Constitutive acceptor) | GAGGGTTTAGgtattat (Cryptic donor) | 54 | <u>GH</u> PPRPDASPPNGA <u>EGL</u> | 123456 | STP | shared by all SVs |
| 11 [long] | ttttcacag GTGCAGGATA (Constitutive acceptor) | GTTGCTGTACgtaagtgca (Constitutive donor) | 39 | <u>GAGYIVLVIVAV</u> | 1346 | TMB | Thus two versions of exon 11, a short and a long |
| 11 [short] | | CATTGTTGCTgtacgtaagt (Cryptic donor) | 35 | <u>GAGYIVLVIVA</u> | 2&5 | TMB | |
| 12 [short] | tttctcagtag TTATTGGCGT (Cryptic acceptor) | GGAAGAAA GG gtaaaagtaca (Cryptic donor) | 61 | <u>LIGV</u> LLLLCLYCCFCRQR <u>KKG</u> | 1346 | TMB/CT | Thus two versions of exon 12, a short and a long. The short version yields 2 different amino acid stretches because frameshift. |
| 12 [long] | | AAAGGGTAAAgtagcagcatg | 66 | <u>LIGV</u> LLLLCLYCCFCRQR <u>KKG-Stop</u> -(...) | 6 (end) | TMB/CT | |
| 12 [short] | tttctcagtag TTATTGGCGT (Cryptic acceptor) | GGAAGAAA AG gtaaaagtaca (Cryptic donor) | 61 | <u>LLALDYCS</u> ACTAVFADR <u>GRK</u> | 2&5 | TMB/CT | |
| 13 | ctctgttcag CATATACGTA (Cryptic acceptor) | TTGACCTTTGgtcagactgt (Cryptic donor) | 348 | <u>IYVTG</u> ESHRQDILFLSEKR- <u>Stop</u> -(...) | 1 (end) | CT | Exon gives two different amino acid stretches due to frameshift (SV1 vs. SV5). Skipped for giving SVs 2, 3 & 4. |
| | ctctgttcag CATATACGTA (Cryptic acceptor) | | 348 | <u>AYT</u> - <u>stop</u> -(...) | 5 (end) | CT | |
| 14 | tcccttag GAAAGCAGAA (Constitutive acceptor) | TCAGGACAAAgtgaatgctg (Cryptic donor) | 1170 | <u>KA</u> ECSATYTTYQDKATTATEQMN- <u>Stop</u> -(...) | 3&4 (ends) | CT | Exon gives two different amino acid stretches due to frameshift (SV3/SV4 vs. SV2). |
| | tcccttag GAAAGCAGAA (Constitutive acceptor) | | 1170 | <u>GKQ</u> VALRTPILIRIKQLQQR- <u>Stop</u> -(...) | 2 (end) | CT | |

Figure 1



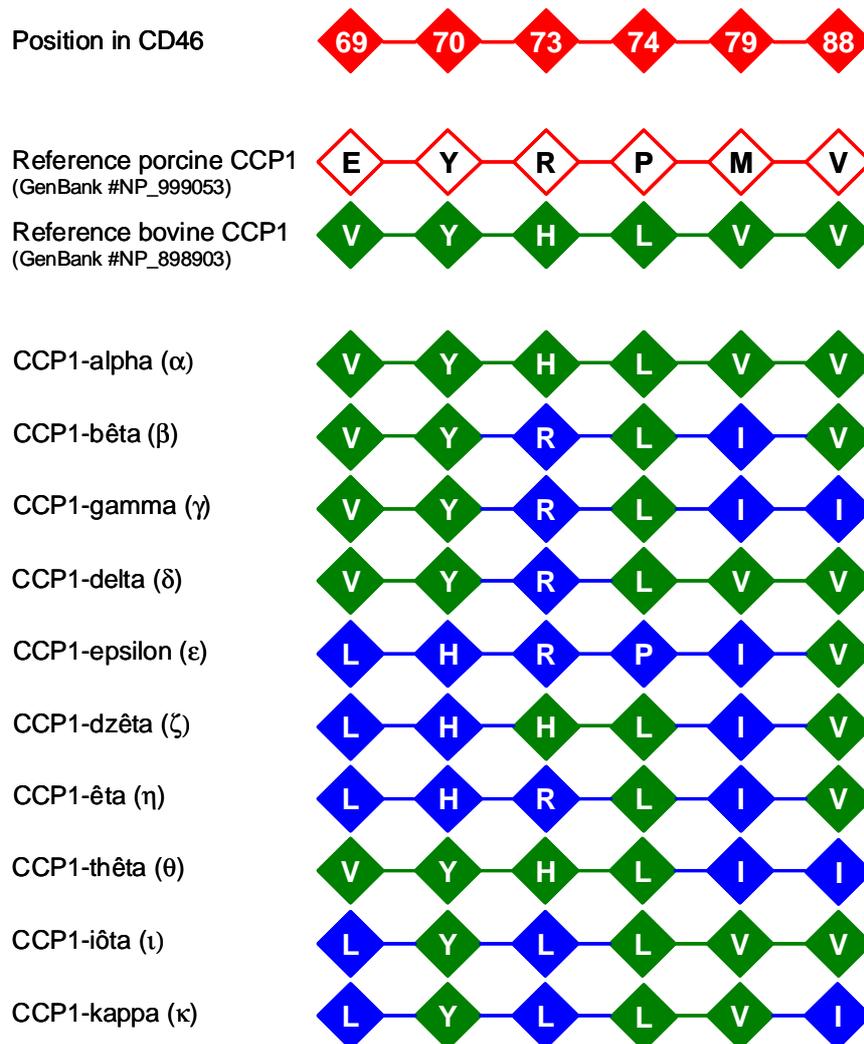


Figure 3

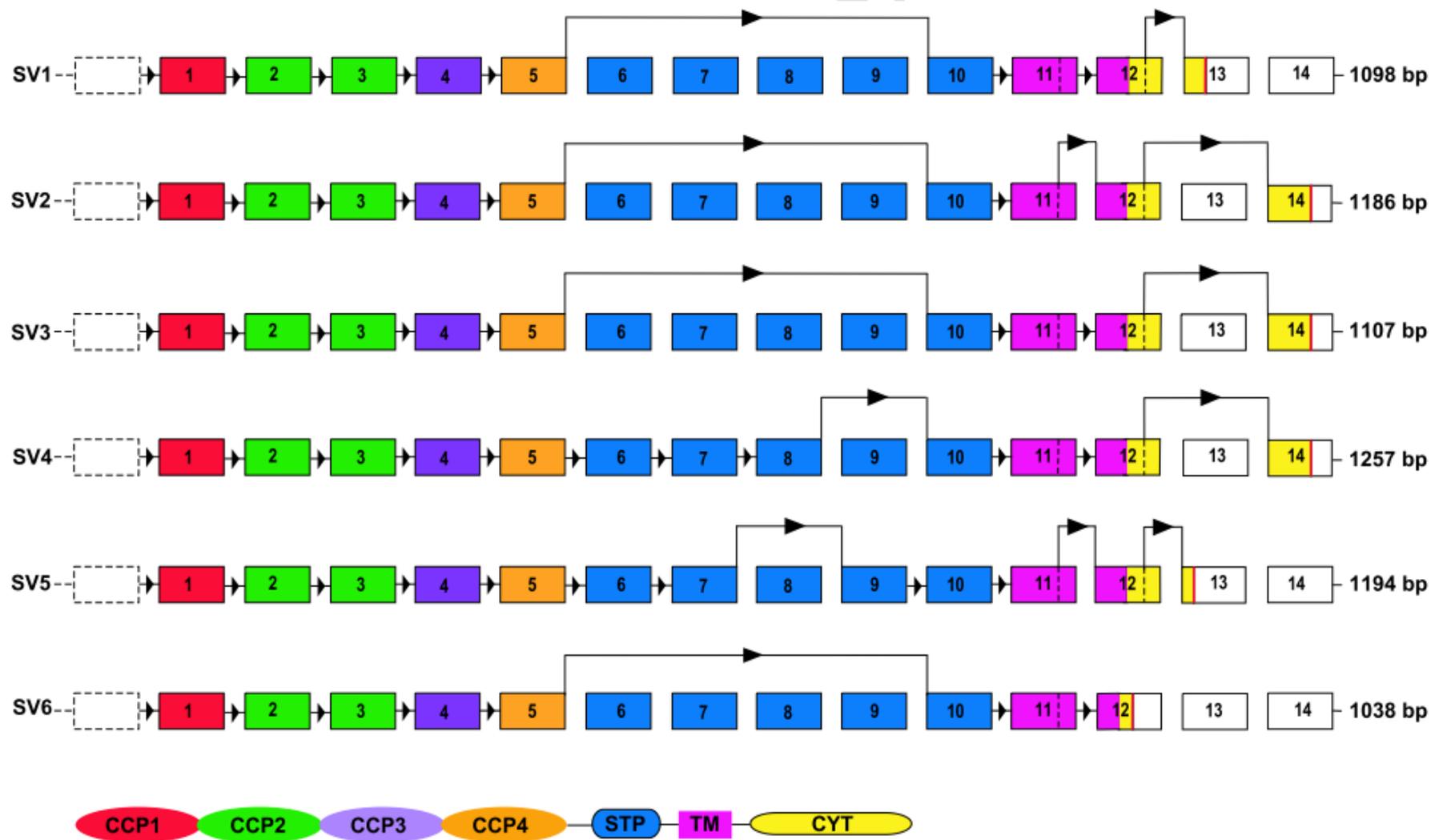


Figure 4

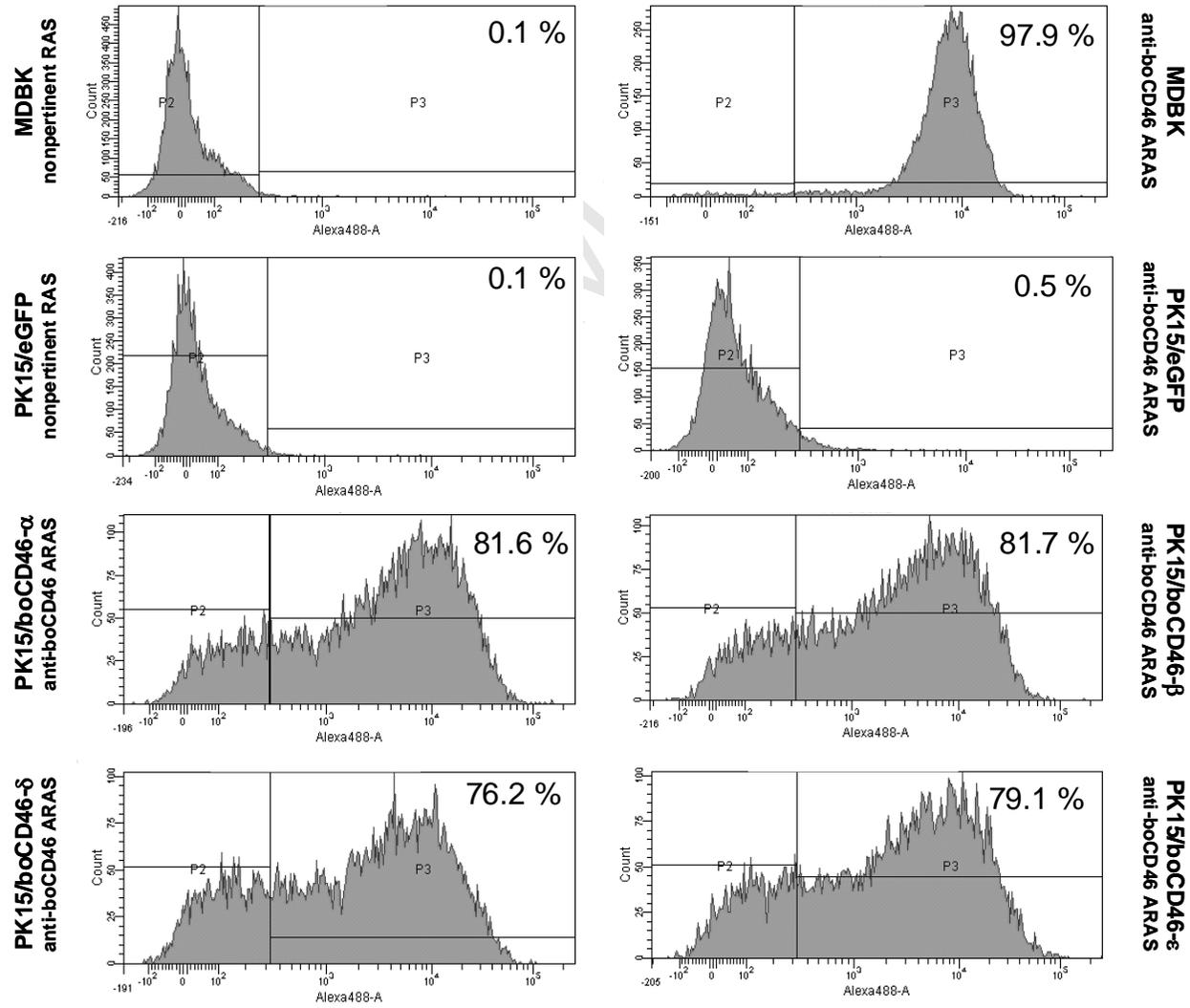


Figure 5

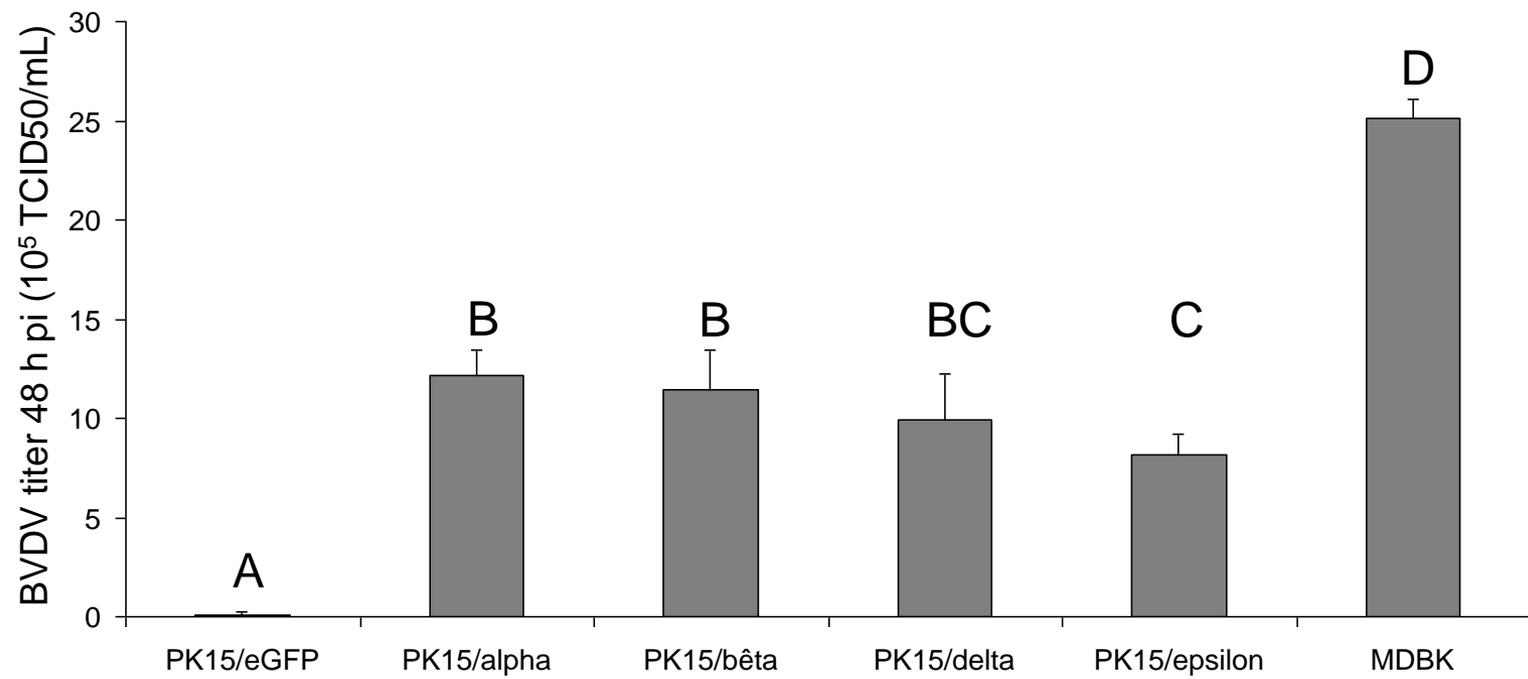


Figure 6

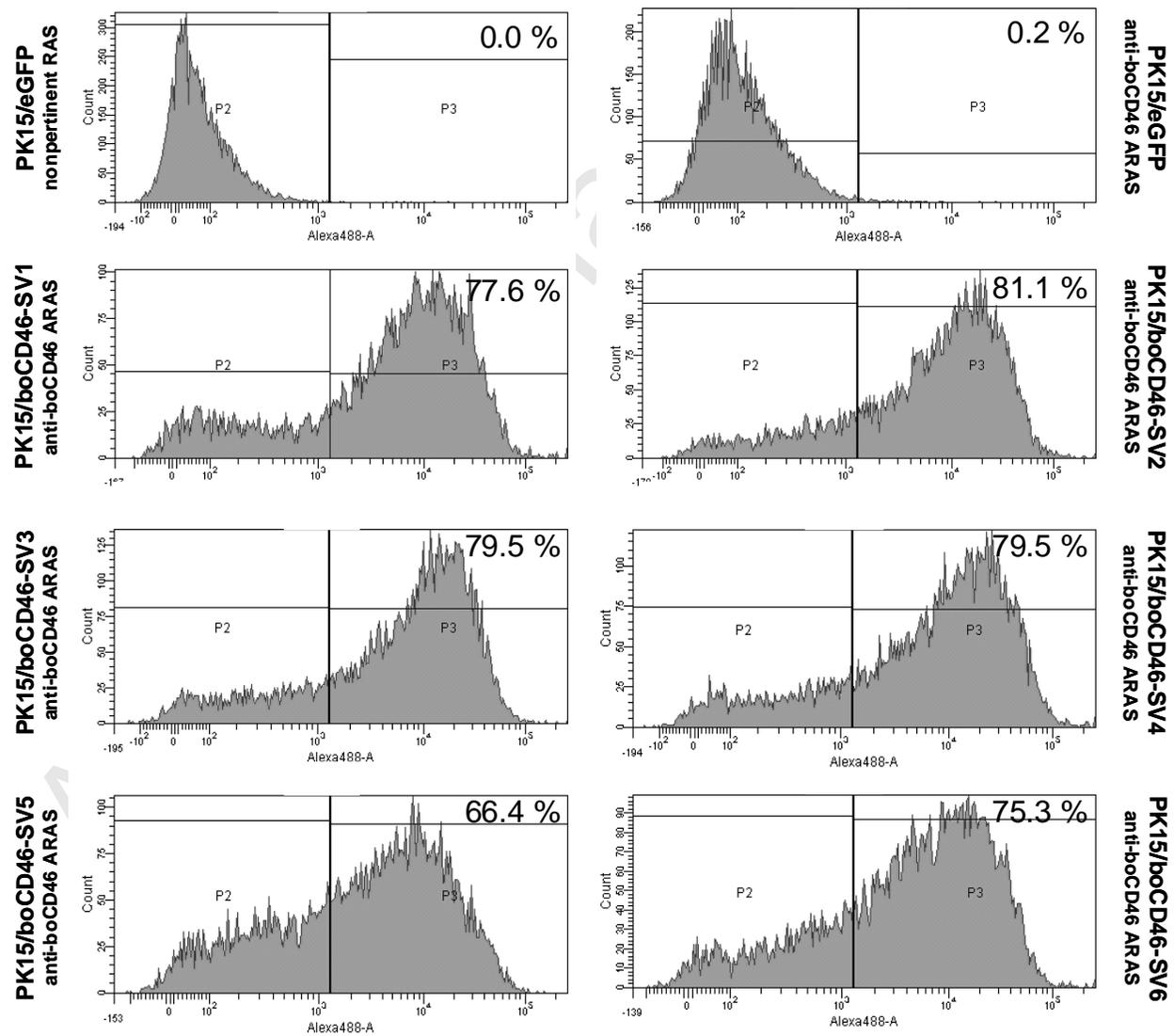


Figure 7

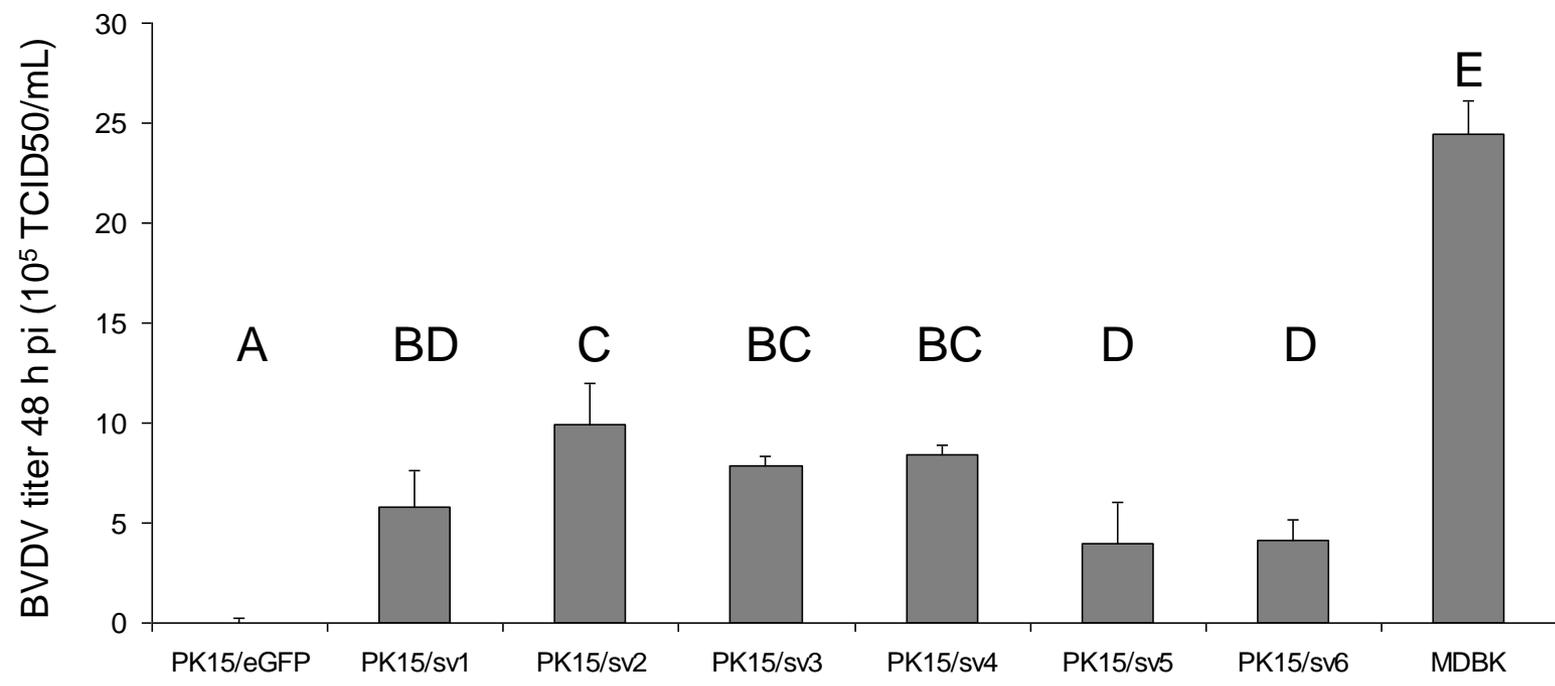


Figure 8

