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UNIVERSITÀ DEGLI STUDI DI TORINO

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Characterization of caprine herpesvirus 1 (CpHV1) glycoprotein E and glycoprotein I ectodomains expressed in mammalian cells

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Abstract

Caprine herpesvirus 1 (CpHV1) is a member of ruminant alphaherpesviruses antigenically related to the prototype bovine herpesvirus 1 (BoHV1). Although cross reactivity between the two viruses involves many structural glycoproteins, the use of two competitive BoHV1 ELISAs detecting anti gB and gE antibodies has been proposed for CpHV1 infection, resulting mainly in a gB+/gE- reactivity and leading to suppose that CpHV1 gE may represent an useful target for the development of specific diagnostic test. Since CpHV1 gE gene has been only partially characterized so far, in this study the genome fragment of the short unique unit (Us) encompassing gI and gE gene was amplified and sequenced. Gene fragments encoding the ectodomain of both glycoproteins were subcloned into pSECTag2/ Hygro and expressed in HEK293T cells as secreted form in serum free medium. Due to the lack of specific monoclonal antibodies (Mabs), the same recombinant glycoproteins were obtained from BoHV1 and used as positive control with a panel of specific gE and gI Mabs as well as in some ELISA assays. Results clearly indicate that the ectodomain of CpHV1 gE, immobilized on solid face in an indirect ELISA format, represents a sensitive and specific marker of infection, when compared with neutralization test, with absence of very low degree of cross-reactivity with BoHV1 gE counterpart, while the use of CpHV1 gI-ELISA or a combination of gE/gI complex did not significantly improve the sensitivity of the assay. In addition, in the rare event in which cross species barrier occurs for both viruses from their natural host to other species, the use of both BoHV1 and CpHV1 gE in a comparative assay may be proposed.

Introduction

Caprine herpesvirus 1 (CpHV-1) causes systemic disease in young kids characterized by high morbidity and mortality rates, while in adult goats the infection leads to vulvovaginitis or balanoposthitis (Engels and Thiry, 2000; Horner et al., 1982; Tempesta et al., 1999, 2000). Less frequently, abortion may occur during the second half of pregnancy (Chenier et al., 2004; Tempesta et al., 2004).

Goat represents the natural host reservoir of CpHV1, with a pathogenesis very similar to BoHV-1 infection in calves (Engels and Ackermann, 1996). However CpHV-1 infection can be experimentally induced in other ruminant species (i.e. cattle or lamb), leading to suppose that, under natural condition, cross-species infection may occur in countries where mixed cattle-goat population are reared in close contact (Engels et al., 1992; Six et al., 2001). CpHV1 belongs to the alphaherpesvirinae subfamily and is genetically and antigenically closely related to other member of ruminant alphaherperviruses, including, among others, Cervid herpesvirus 1 (CvHV1), bubaline herpesvirus 1 (BuHV1) (De Carlo et al., 2004), bovine

herpesvirus 1 and 5 (BoHV1, BoHV5) (Thiry et al., 2006). These close relationships reflect a certain degree of cross-reactivity among the envelope structural glycoproteins, which hampers the use of currently available diagnostic test for specific discrimination of CpHV1 from BoHV1 infection. For instance the percentage of nucleotide sequence identity for gB gene among different herpes-viruses is greater than 78% (Griffin, 1991), while limited information are available for more divergent genes of CpHV1. Serological diagnosis of CpHV1 infection can be accomplished using serum neutralization (SN) test and ELISA assay. The SN test is considered the gold standard and exhibits high sensitivity, while specificity can be ascertained in one way cross seroneutralization test, being homologous titer greater than heterologous by a factor of four, on average (Thiry et al., 2008). On the other hand, the SN test is expensive, time consuming, and require equipped laboratories and trained personnel to be performed. An indirect ELISA has been developed using whole virus antigen and proved to be as sensitive as SN test, although cross reaction with goat experimentally infected with BoHV1 was detected (Marinaro et al., 2010). Competitive gB and gE ELISA have been developed for BoHV1 and are widely used in eradication program in cattle herd (Kramps et al., 1994). The same tests found limited application in other ruminant herpesvirus infections. In fact, due to the conserved immunodominant epitope of gB among different ruminant alphaherpesviruses, the gB blocking ELISA allows the identification of such infections but is not able to make a differentiation. On the other hands, the gE blocking ELISA is specific for BoHV1 and ruminant infected with alphaherpesviruses other than BoHV1 is expected to score gB+/gE-. This has been proven for BoHV5 (Wellenberg et al., 2001) and CpHV1 (Thiry et al., 2008) although, in the latter study, a substantial number of goat serum samples which neutralized CpHV-1 to a much greater extent than BoHV-1 were found gB+/gE+. Hence a specific serological test able to detect CpHV1 with low or limited cross reactivity with other ruminant alphaherpesviridae, is highly desirable. Genetic information on structural proteins of CpHV1 is still limited, for the Us region, upstream to gI gene and downstream to the transmembrane domain of gE, being not enough to carried out a recombinant-based approach to overcome this diagnostic drawback. Since gE is one of the most divergent glycoprotein among ruminant herpesviruses, we focused on the genetic characterization of CpHV1 gE, providing the full length ectodomain sequence and evaluate the diagnostic potential of its recombinant form, expressed as secreted protein in a mammalian expression vector. Preliminary results indicate that the ectodomain of gE is a sensitive and specific marker of infection with very limited or absent cross-reactivity with BoHV1 gE, expressed in a similar manner.

Materials and methods

Viruses and cells

BA.1 strain of CpHV1 was isolated and characterized in previous study (Buonavoglia et al., 1996).

BoHV1 field isolate Caselette was obtained from a nasal swab of a seropositive cattle after dexamethasone treatment. The isolate was characterized using a set of specific PCRs targeting gE, gC and TK genes. Vaccine strain of BoHV1, deleted for gE gene, was obtained from commercially available marker vaccine (Risposal IBR marker vivum, Pfizer, United Kingdom). All viruses were propagated in Madin-Darby bovine kidney cell line (MDBK; ATCC CCL-22) were cultured in Dulbecco's modified essential medium (DMEM) (Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 IU/ml of penicillin (Sigma), 100 mg/ml of streptomycin (Sigma-Aldrich), and 2.5 mg/ml of amphotericin B. Cells were incubated at 37 °C with 5% CO₂. Human Embryo Kidney cell line (HEK293T; ATCC, CRL-1573) was cultured as for MDBK cells and used for transfection experiments.

Polymerase chain reaction (PCR), sequencing and cloning

To obtain the full length of the CpHV1 gI and gE genes, primers were designed from the available flanking regions, released from the Genbank database under accession numbers AY437088 (strain E/CH, covering the first 44 residues of gI) and EF624470 (strain BA.1, covering 198 residues of the third medium of gE). DNA was extracted from infected MDBK cells using DNeasy Blood and Tissue kit (Qiagen, Germany) and used as template in a PCR reaction, performed using LongRange PCR Kit (Qiagen) following the standard protocol proposed by the manufacturer. The amplified product of the expected length (inferred from BoHV1 counterpart) was column purified (NucleoSpin1 Extract II kit, Macherey-Nagel, Germany) and subjected to direct sequencing (BMR Genomics, Padua, Italy), using PCR and sequence derived primers. Sequence have been submitted to Genbank data base under accession number JX308226.

To clone and express the ectodomain of both proteins primer set were designed downstream to the signal peptide (predicted with SignalP4.0 software) and 14 residues upstream to the putative transmembrane domain (Expasy TMpred tool). To facilitate directional cloning, each primer contained at 50 terminus appropriate restriction site (see Table 1 for details). According to the same strategy, the ectodomain of BoHV1 gI and gE was amplified using specific primer sets, following reference sequences available on the Genbank database. The complete list of primers used in the study is shown in Table 1. All gene fragments were PCR amplified, digested with appropriate restriction enzymes and ligated with pSecTag2/Hygro plasmid (Invitrogen, USA). This eukaryotic expression vector allows the in-frame cloning of the protein of interest with the Ig kappa chain leader sequence for efficient intracellular sorting and secretion in the medium of transiently transfected mammalian cells. Ligations were used to transform competent Escherichia coli strain JM109 and ampicillin resistant colonies were subjected to PCR for rapid screening and sequencing to confirm the authenticity and in frame insertion of each fragment. Plasmid purification from 25 ml LB culture (about 100 mg) was carried out using Qiagen Plasmid Midi kit.

Protein expression and quantitation

Sub-confluent HEK293T cells (70–80%) cultured in 75 cm² flasks were transfected with 6 ml of DMEM containing 9 mg of plasmid and 21 ml of LTX transfection reagent (Invitrogen) according to standard protocol (Donofrio et al., 2006). After 6 h at 37°C, 5%CO₂, the transfection medium was replaced with 9 ml of protein free medium (50% DMEM–50% F12) and flasks incubated as above for additional 48h. Medium was then collected, centrifuged at 3000 \times g for 100 to remove cell debris and stored at -80 8C until used.

In order to promote the gI/gE complex, known to form an immunodominant conformational epitope in BoHV1 infection (Tyborowska et al., 2000), co-transfection with both plasmids was also carried out. As negative antigen, used in all ELISA assays, supernatant from cell transfected with empty plasmid was also obtained with the above protocol.

Since all proteins were expressed in fusion with a 6xHis tail, protein concentration was roughly estimated using serial twofold dilution of each supernatant coated on solid face and probed with anti-6xHis Mab in indirect ELISA. A known amount of serially diluted recombinant 6xHis tail fusion protein was used as positive control, obtaining a standard curve. A dilution between 1/5 and 1/20 was found optimal for all proteins.

BoHV1 monoclonal antibody panel

A panel of monoclonal antibodies (Mabs), known to be directed against BoHV1 gE or gI has been previously characterized (Egyed et al., 1992). They were used to evaluate (if any) cross-

reactivity with the CpHV1 protein counterpart in immunocytochemistry assay. Briefly, MDBK cells, cultured in 24 wells microplates, were infected with BoHV1 field strain, BoHV1 gE-deleted vaccine strain, CpHV1 or mock infected. After 48 h cells were fixed in acetone/methanol and probed with each Mab. After washing, peroxidase labeled secondary antibody was added and, after final washing step, reaction was developed using fast orange DAB (KPL, MD, USA). The same panel was used to characterize the recombinant gI and gE in indirect ELISA assay. Since no reactivity was observed against CpHV1 recombinant proteins (see below), a western blot was carried out using an anti-6xHis monoclonal antibody, in order to assess the presence and the size of each expressed protein.

Serum samples and ELISA procedure

A panel of 53 bovine and 169 caprine sera was used. In more details, the first group included 19 sera from BoHV1 infected cattle, as detected by virus isolation, and/or a major seroconversion episode during periodical serosurvey. Fifteen sera were randomly collected from long term vaccinated herds using inactivated marker vaccine strain, continuously tested for gB and gE blocking ELISA, according to IBR Regional Control Plan. Finally 19 serum samples were collected from officially BoHV1-free farms, as detected by gE and gB blocking ELISA and SN test. Among goat sera, 145 were from CpHV1 infected herds (47 with negative SN titer and 98 with an homologous neutralization titer between 1/2 and 1/128) and 24 were from negative flocks as detected by standard SN test (Deregt et al., 2005; Tempesta et al., 2000). According to animal species, a cross-SN test was performed using homologous and heterologous virus.

ELISA microplates (Nunc Maxisorp) were coated overnight at 4 °C with 50 ng of each recombinant protein (even wells) or negative antigen (odd wells) diluted in 0.1 M carbonate/bicarbonate buffer pH 9.6. After blocking with 2.5% bovine casein, primary antibody, diluted 1/20 in PBS 1.25% casein, was added and plate incubated for 1 h at 37 °C. After washing step, peroxidase labeled protein G, diluted at 10ng/ml in the same buffer was added and plates incubated as above. After final wash, reaction was developed with TMB and stopped with 0.2 M H₂SO₄. Cut off was determined for each ELISA as the mean + 3 standard deviations of negative samples' reactivity enclosed in each plate.

Statistical analysis

ELISA test performances in terms of sensitivity and specificity were calculated by using contingency tables and confidence intervals of each parameter were calculated by using Exact Binomial test. Levels of agreement between SN and ELISA tests were expressed by Cohen's Kappa. Correlation between SN titer and ELISA reactivity was evaluated using Spearman's Rho Correlation test. All statistical analyses were performed using R statistical software (R Core Team, 2012).

Results

To cover the full length gI and gE genes of CpHV1, primers were designed to anneal flanking regions, giving rise to a single band of 2.1 kb, slight lower than expected based on BoHV1 consensus sequence. The complete sequence of PCR fragment covered from 50 to 30,1094 bp of gI, an intergenic region of 160 bp and the first 834 bp of gE. Considering gI and gE ectodomain sequences, nucleotide and aminoacidic identity (similarity) between BoHV-1 and CpHV-1 were 66.4% and 53.6% (gI), 70.1% and 60.7% (gE) respectively. Similarity along gI and gE ectodomains between BoHV-1 and CpHV-1 is reported as similarity plot, provided as supplementary Figs. 1 and 2, showing a higher heterogeneity in the most N0-terminal region.

The ectodomain of CpHV1 gI and gE and the BoHV1 counterpart was then cloned into mammalian expression vector, in frame with the Ig kappa chain leader sequence for efficient secretion of recombinant protein into protein free medium. Sequence analysis reveal the correct in frame orientation of each construct. Despite the low degree of similarity between BoHV1 and CpHV1, all Cys residues were conserved suggesting a similar conformation of each glycoprotein. We then evaluated the usefulness of a panel of Mabs, elicited against BoHV1, for potential cross reactivity with CpHV1 gI and gE. Neither native antigens, evaluated in immunocytochemistry (Fig. S3), nor recombinant antigens, evaluated in ELISA (Fig. S4), were recognized by any of the Mab panel, while BoHV1 native antigens were reactive according to each Mab specificity (see Table 2 for details). Recombinant BoHV1 gE and gI were reactive against a subset of Mabs, while Mabs 1H10 and 1D11, known to react against the gE/gI complex, were only slightly reactive against supernatant obtained from cells co-transfected with pSEC/Bo-gE and pSEC/Bo-gI plasmids (Fig. S4).

To evaluate expression and size of CpHV-1 gI and gE ectodomains, western blot was carried out using an anti-6xHis Mab. Both proteins showed a similar yield and size, corresponding to ~50 kDa, displaying the gI ectodomain a 13 putative O-glycosylation sites spanning the serine/ threonine-rich region (Fig. 1).

Recombinant protein concentration was roughly estimated in indirect ELISA using serial dilution of each supernatant and anti-6xHis tail Mab. Standard curve created using serial dilution of a known positive control showed a good linearity ($R^2 = 0.975$, $p < 0.0001$) and allowed to quantify each expressed protein (Fig. S5).

The reactivity of the serum panel used in the study is summarized in Fig. 2. Recombinant gE ELISA showed a greater reactivity versus homologous infection in goat sera and the best signal to noise ratio. Although the reactivity of bovine sera against the homologous antigen seems to be suboptimal, plotting the reactivity of positive samples versus BoHV1 gE and CpHV1 gE, a clear recognition of homologous infection is evident in both animal species, according to the corresponding cross-SN assay (Fig. 3). The use of CpHV1 gI-ELISA or a combination of gE/gI complex did not significantly improve the sensitivity of the assay. When SN test was used as gold standard, sensitivity and specificity of CpHV1 gE ELISA was 97.11% (CI95% 91.80– 99.40%) and 98.57% (CI95% 92.30–99.96%) respectively and a Cohen's Kappa value (chance-corrected concordance) of 0.952 (CI95% 0.906–0.999). By using gB/gE blocking ELISA as proposed by Thiry et al. (2008), goat sera were classified as gB- (negative samples) and gB+/gE- (animals infected by CpHV-1). One goat resulting gB+/gE+ showed a higher SN titer against CpHV-1 than against BoHv1. According to the latter classification, sensitivity and specificity of CpHV1 gE ELISA was 97.89% (CI95% 92.60– 99.74%) and 98.53% (CI95% 92.08–99.96%) respectively and a Cohen's Kappa value (chance-corrected concordance) of 0.962 (CI95% 0.920–1.00). Finally, correlation between ELISA CpHV1 gE absorbance results and SN titer was evaluated considering goat sera (Fig. 4), resulting in a Spearman's Rho equal to 0.88 ($p < 0.001$).

Discussion

Serological diagnosis of CpHV1 infection is still based to date on a first generation of native antigen-based immunoassay, requiring time consuming and expensive procedures for viral growth and viral particles purification. Moreover, despite the high sensitivity of whole virus preparation, cross-reactivity versus other ruminant alpha- herpesvirus glycoproteins may produce doubtful results in relation to which virus is circulating in the population. Indeed, although ruminant herpesviruses have mainly co- evolved with their natural hosts, cross-species transmission may occur under natural condition and spread in the new host at least during the primary infection (Thiry et al., 2006). An alternative approach is based on the use

of two blocking ELISAs based on detection of antibody binding epitopes of gB and gE, the former being cross-reactive among ruminant alphaherpesvirinae and the latter being specific for BoHV1 infection. However the ultimate interpretation of the infectious status, often requires to use of cross seroneutralization test (Thiry et al., 2008). Moreover the extensive use of marker vaccines against BoHV1 infection in cattle population, further complicate the use of these tests. To overcome the problem, a second generation of diagnostic tests is desirable, providing that more genetic information become available. In this context, the present study completes the sequence data of CpHV1 gE and gI genes allowing the development of a recombinant based second generation of diagnostic assays. We chose gE because of its strong immunogenicity and the highest variability among ruminant herpesviruses. In addition, the use of gI in combination of gE was expected to generate a complex harboring an immunodominant epitope well characterized in BoHV1 infection (Tyborowska et al., 2000). Sequence similarity with BoHV1 counterpart, as well as the lack of reactivity of any of the BoHV1 Mabs strongly support that CpHV1 gE is indeed quite different from BoHV1 counterpart. The lack of specific probe, except for an anti-6xHis tail Mab, limited us to precisely evaluate the yield of both CpHV1 recombinant glycoproteins. For this reason we assumed that BoHV1 gI and gE ectodomains would have expressed in a similar way, and were used as control of the expression system. Three mabs strongly bound BoHV1 gE and one the gI ectodomains, confirming that the recombinant antigens were correctly secreted in medium. Although a negative antigen, consisting in culture medium from HEK-293T cell transfected with empty vector, was initially used in each assay, the background level was low, with an average O.D. value of 0.113 (SD 0.01). We therefore assumed that all proteins, expressed in protein free medium, could be used for direct coating without further affinity tag purification. We failed to demonstrate a strong reactivity of Mab 1H10 and 1D11 which specifically bind the gE/gI complex of BoHV1. This was clearly unexpected since previous work suggested that baculovirus expressed secreted ectodomains were able to form complex and the smallest part of gE that still formed a complex was encoded by the first 246 residues of gE, compare to 391 residues of the present study (Tyborowska et al., 2000). A possible explanation could be the composition of the protein free medium used in this study which may hamper for unknown reason the complex formation. Despite this unexpected result, which affects the sensitivity of BoHV1 gE ELISA (7 out of 23 false negatives), the ectodomain of CpHV1 gE was much greater reactive, even as a single antigen, suggesting that, differently from BoHV1 infection, diagnostically relevant epitopes are located on the surface of gE glycoprotein. Indeed 80 out of 82 SN positive sera were clearly above the cutoff level. On the other hand, one sample obtained from SN negative goats showed positive reactivity against CpHV1 gE antigen, slight above the cut off leading to a not absolute specificity of the test; this result could be explained as different kinetics in antibody response toward different structural proteins occurring in different stages of the infection (Marinaro et al., 2010). By contrast gI ELISA was less antigenic in both assays and its use in addition to gE ELISA seems to add doubtful advantages in terms of overall sensitivity. We then evaluated the potential cross-reactivity of gE ELISAs between bovine and goat sera. The use of Protein G as common secondary reagent, which binds with equal strength both bovine and goat IgG molecules, allowed a direct comparison of signal versus homologous and heterologous antigen, being the latter quite lower than that of the homologous antigen in both ways. Data presented suggest that a multi-antigen ELISA, based on recombinant gE ectodomains derived from different ruminant alphaherpesviruses may be proposed, at least at the herd level, to specifically identify the circulating virus. In this context a future characterization of BuHV1 gE would further complete the set of viruses which should be enclosed in the assay. This diagnostic tool seems particularly important in Countries in which

BoHV1 is under eradication program in presence of vaccination. It is not rare the occurrence of cattle herds that, from a free status, turn to marker vaccinated status (gB+/ gE-) without apparent vaccination (Mars et al., 2000). In this situation a seroconversion against attenuated marker vaccine or BoHV1 strain naturally mutated at the immunodominant epitope level has never been demonstrated. The multi-gE ELISA may serve in this situation facilitating a rapid identification of heterologous (if any) herpesvirus infection.

In conclusion the CpHV1 gE was characterized at genetic and antigenic level and prove to be an useful marker of infection in its recombinant form, expressed as secreted ectodomain in mammalian cells. Data presented open new approach for the development of a new generation of diagnostic tests among ruminant alphaherpesvirinae infections.

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Table 2

Monoclonal antibody reactivity against native and recombinant (rec-) antigen evaluated by immunocytochemistry (IC) and ELISA respectively. (+) Strong reactivity; (±) weak reactivity; (-) no reactivity.

	Test	2A5	5H5	1H10	1D11	5F5	4D7
BoHV1 field strain	IC	+	+	+	+	+	+
BoHV1 ΔgE strain	IC	-	+	-	-	-	-
CpHV1 BA.1	IC	-	-	-	-	-	-
Mock MDBK	IC	-	-	-	-	-	-
rec-BoHV1 gI	ELISA	-	+	-	-	-	-
rec-BoHV1 gE	ELISA	+	-	-	-	+	+
rec-BoHV1 gE+gI	ELISA	+	+	±	±	+	+
rec-CpHV1 gI	ELISA	-	-	-	-	-	-
rec-CpHV1 gE	ELISA	-	-	-	-	-	-
rec-CpHV1 gE+gI	ELISA	-	-	-	-	-	-
pSEC empty vector	ELISA	-	-	-	-	-	-

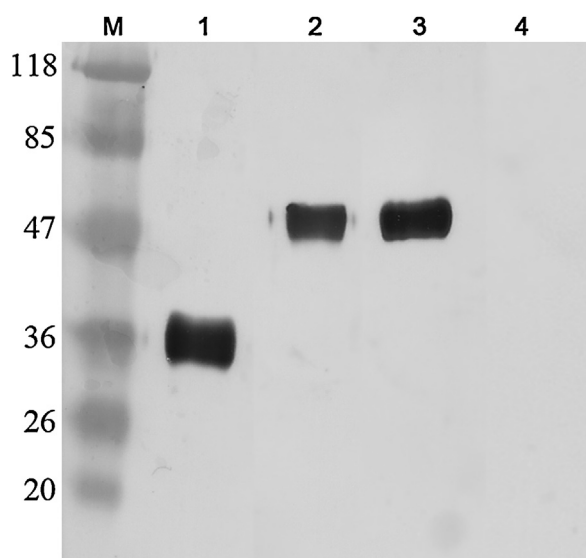


Fig. 1. Western blot of recombinant CpHV1 gE and CpHV1 gI ectodomains secreted in mammalian cells and probed with anti 6xHis mab. Lane M, prestained molecular weight (sizes are expressed in kDa); lane 1, 6xHis tailed positive control; lane 2, recombinant CpHV1 gE; lane 3, recombinant CpHV1 gI; lane 4, supernatant from cells transfected with the empty plasmid.

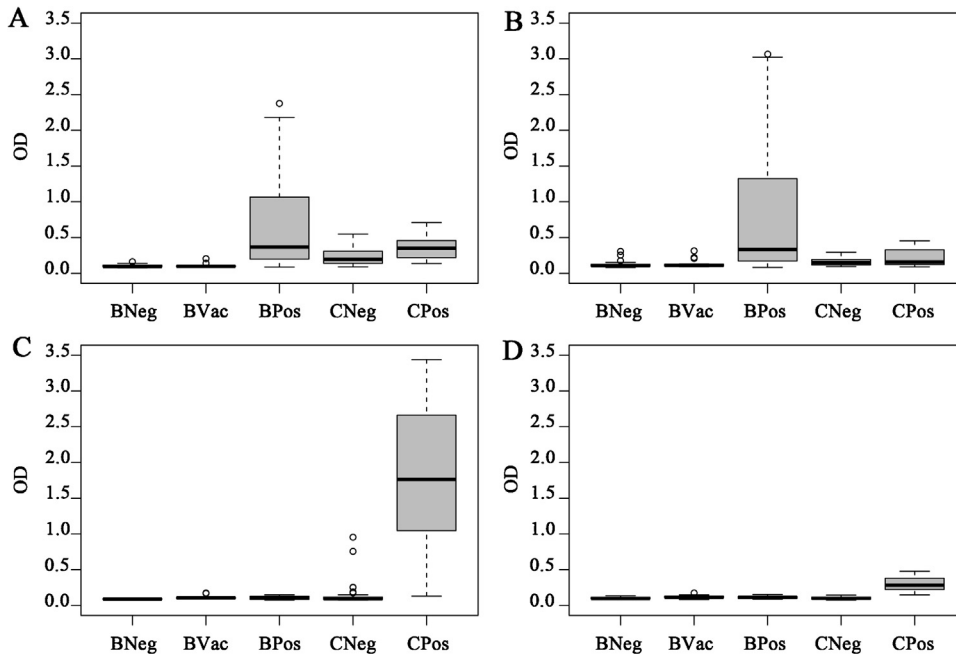


Fig. 2. Recombinant BoHV1 and CpHV1 gE and gI ELISA. Net absorbance boxplots of bovine and caprine sera with known serological status (BNeg, bovine BoHV1 negative sera; BVac, sera from vaccinated bovines; BPos, bovine BoHV1 positive sera; CNeg, caprine CpHV1 negative sera; CPos, caprine CpHV1 positive sera). Sera were tested against the following recombinant antigens: (A) BoHV1 gE; (B) BoHV1 gI; (C) CpHV1 gE; (D) CpHV1 gI.

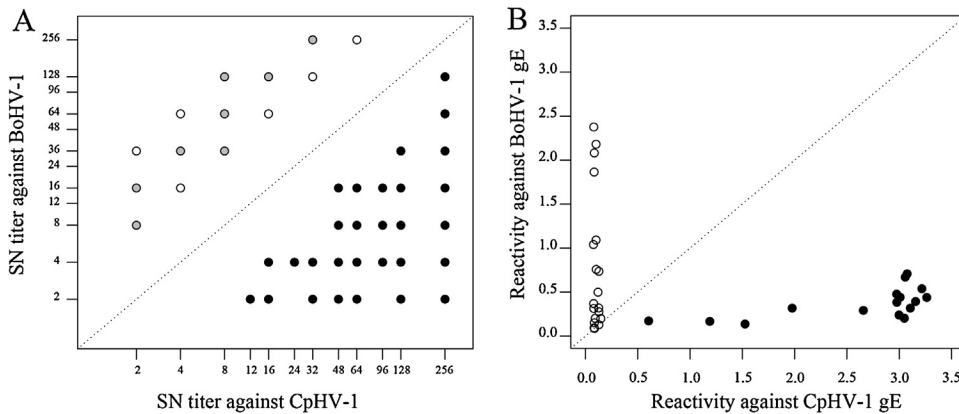


Fig. 3. Dispersion plot of a subset of positive bovine and caprine sera according to SN titer and ELISA reactivity. (A) Cross-SN titer of sera, represented in log scale, from bovines infected by BoHV1 (white circles), vaccinated (gray circles) and goats infected by CpHV1 (black circles); SN titers against CpHV1 and BoHV1 are reported on the X and Y axes respectively. (B) ELISA reactivity of sera from bovines infected by BoHV1 (white circles) and goats infected by CpHV1 (black circles); absorbance versus CpHV1 recombinant gE and BoHV1 recombinant gE are reported on the X and Y axes respectively.

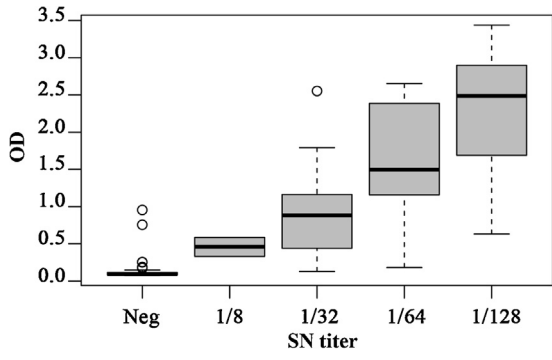


Fig. 4. Correlation between recombinant CpHV1 gE ELISA absorbance and SN titer of caprine sera.