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Journal of Virological Methods Volume 207, October 2014, Pages 16–21

DOI: 10.1016/j.jviromet.2014.06.023

Expression and antigenic characterization of Bubaline Herpesvirus 1 (BuHV1) glycoprotein E and its potential application in the epidemiology and control of alphaherpesvirus infections in Mediterranean water buffalo

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Keywords: Bubaline Herpesvirus 1, recombinant glycoprotein E, ELISA

Abstract: Bubaline herpesvirus 1 (BuHV1) is a member of ruminant alphaherpesviruses antigenically related to the prototype bovine herpesvirus 1 (BoHV1).The impact of BuHV1 infection in Infectious Bovine Rhinotracheitis control program is difficult to establish to date, due to the lack of specific diagnostic test able to differentiate between the two infections. In this study the ectodomain of glycoprotein E of BuHV1 was amplified, cloned and expressed as secreted protein in eukaryotic system and used in indirect ELISA as well as in a discriminatory test using the BoHV1 counterpart with a panel of well characterized bovine and water buffalo sera. A panel of monoclonal antibodies (Mabs) was also produced against BuHV1 and 6 out of 7 anti-gE Mabs specifically recognize the BuHV1 gE ectodomain. Results indicated that recombinant BuHV1 gE is a sensitive marker of infection compared to SN test or blocking ELISA. When both recombinant gEs (BoHV1 and BuHV1) were immobilized in different wells of the same ELISA microplate, bovine and water buffalo sera were more reactive, by a factor of two, against the respective infecting virus. This was true even in case of experimental cross-infection. In addition we found that about one third of seropositive buffaloes with no history of contact with cattle and having higher SN titers, reacted in BoHV1 gE blocking ELISA, possibly because of steric hindrance. Since in two occasions BuHV1 was also isolated from water buffalo scoring gB+/gE+ BoHV1 blocking ELISA, we conclude that the combination of the two blocking ELISAs, proposed for other alphaherpesvirus infections, are not suitable to differentiate between BoHV1 and BuHV1.

Introduction

Ruminant alphaherpesviruses related to Bovine herpesvirus 1 (BoHV1) includes several host-adapted viruses found in bovine, bubaline, caprine, elk and cervids (Thiry J. et al., 2006). These viruses share common antigenic and serological features with the prototype BoHV1, the etiological agent of Infectious Bovine Rhinotracheitis (IBR), an economical important disease of cattle which has been eradicated or is currently under control in many European Countries. The presence of related alphaherpesviruses in ruminant species reared in the same area where IBR is under control may impose an unjustified restriction in ruminant trade. This is mainly due to the lack of specific diagnostic test able to differentiate between BoHV1 and the other ruminant alphaherpesviruses. Mediterranean water buffalo (*Bubalus bubalis*) is gaining increased interest and importance as farm animal due to the use of water buffalo milk, exclusively used for Mozzarella cheese, which has obtained the European Protected Denomination of Origin (DOP) mark in 1996. To date, more than 400.000 heads are reared in central Italy (National Data Base-IZSAM). Water buffalo is the primary host and reservoir of Bubaline herpesvirus 1 (BuHV1), an alphaherpervirus originally isolated in 1971 in Australia and more recently in Italy (St. George and Philpott, 1972; De Carlo et al., 2004). The partial genome sequence of BuHV1 suggests that this virus is highly related to BoHV5 and to a lesser extent to BoHV1 (De Carlo et al., 2004; Thiry et al., 2006). Limited information are also available on pathogenic potential since it has been associated to subclinical genital infection, although more recently, is has been detected in aborted fetuses and in buffalo calves with respiratory symptoms (St George and Philpott, 1972; Amoroso et al., 2013, Petrini et al., 2012). The impact of BuHV1 infection in IBR control program is also difficult to establish to date. Both cattle and water buffalo are susceptible to heterologous infection. In addition water buffaloes infected with BuHV1 may react at various degree with BoHV1 serological test, including SN test and competitive assays. According to previous studies alphaherpesviruses may be differentiated from the prototype BoHV1 using cross-neutralization test (i.e. Caprine Herpesvirus 1) or a method combining two blocking ELISAs for detection of antibodies directed against glycoproteins B and E of BoHV1. In the latter method most ruminant infected with viruses other than BoHV1 are expected to score gB+/gE- in blocking ELISA, due to the higher degree of conservation of glycoprotein B

immunodominant epitope compared to glycoprotein E (Griffin et al., 1991; Kramps et al., 1994). This method has been proposed for BoHV5 infection, providing that animals were not vaccinated with gE negative vaccine (Wellenberg et al., 2001). The application of the same serological tests (cross neutralization test and gB/gE blocking ELISA) to define the infectious status of water buffalo has been documented in few cases raising conflicting results (Scicluna et al., 2006). To date no serological tests are available for the identification of BuHV1 infection, nor for the differentiation between BoHV1 and BuHV1 infections in bovine and water buffalo species. The aim of the present study was to characterize the glycoprotein E of BuHV1 by genetic and antigenic point of view and to evaluate the diagnostic potential of a recombinant based immunoassay to specifically detect BuHV1 infection.

Materials and methods

Virus and cells

The Australian strain B6 of BuHV1, kindly provided by Sandro Cavirani, Veterinary University of Parma, was isolated and characterized in previous study (St George et al., 1971). The Italian isolate of BuHV1 strain MR077 was isolated from water buffalo vaginal swab after dexamethasone treatment and characterized by PCR and sequencing (Nardelli S., personal communication). Viruses were propagated in Madin-Darby bovine kidney cell line(MDBK; ATCC CCL-22), cultured in Dulbecco's modified essential medium (DMEM) (Sigma–Aldrich, Germany) containing 10% fetal bovine serum (FBS),2mM of L-glutamine, 100 IU/ml of penicillin (Sigma–Aldrich),100 mg/ml of streptomycin (Sigma–Aldrich), and 2.5 mg/ml of amphotericin B. Cells were incubated at 37°C with 5%CO_2. 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 BuHV1 gE gene, primers were designed from the available flanking regions: a first forward primer was designed on the consensus sequences obtained aligning BoHV1 and BoHV5 gI genes. Reverse primer was designed referring to C' terminal part of BuHV1 gE ectodomain (accession number EF624469 strain B6)14residues upstream to the putative transmembrane domain (Expasy

97 TMpredtool).Following forward primers were designed basing on sequence data; forward primer used to
98 amplify gE ectodomain was designed downstream to the signal peptide (predicted with SignalP4.0
99 software). To facilitate directional cloning, each primer contained at 5' terminus appropriate restriction
100 site. Primer sequences are reported in table 1. DNA was extracted from infected MDBK cells using DNeasy
101 Blood and Tissuekit (Qiagen, Germany) and used as template in a PCR reaction, performed using LongRange
102 PCR Kit (Qiagen) following the standard protocol proposed by the manufacturer. The amplified product of
103 the expected length(inferred from BoHV5 counterpart) was column purified(NucleoSpin1 Extract II kit,
104 Macherey-Nagel, Germany)and subjected to direct sequencing (BMR Genomics, Padua, Italy), using PCR
105 and sequence derived primers. Sequence has been submitted to Genbank database under accession
106 number KF495996. The ectodomain ofBoHV1 gE was amplified and expressed in a previous study and used
107 in some experiments (Bertolotti et al., 2013).
108 All gene fragments were PCR amplified, digested with appropriate restriction enzymes and ligated with
109 pSecTag2/Hygro plasmid (Invitrogen, USA) as described. This eukaryotic expression vector allows the in-
110 frame cloning of the protein of interest with the Ig kappa chain leader sequence for efficient intracellular
111 sorting and secretion in the medium of transiently transfected mammalian cells. Ligations were used to
112 transform competent *Escherichia coli* strain JM109 and ampicillin resistant colonies were subjected to PCR
113 for rapid screening and sequencing to confirm the authenticity and in frame insertion of each fragment.
114 Plasmid purification from 300 ml LB culture (about 800 mg)was carried out using Macherey-Nagel Plasmid
115 endotoxin free kit.
116 *Protein expression and quantitation*
117 Sub-confluent HEK293T cells (70–80%) cultured in 55 cm^2dishes were transfected with 6 ml of DMEM
118 containing 9 mg of plasmid and 21 µl of LTX transfection reagent (Invitrogen) according to standard
119 protocol (Donofrio et al., 2006). After 6 h at 37°C, 5% CO_2, the transfection medium was replaced with 6 ml
120 of Ex-cell293serumfree medium (Sigma) and dishes incubated as above for additional 42 h. Medium was
121 then collected, centrifuged at 3000*g* for 10min to remove cell debris and stored at -80°C until used. As
122 negative antigen, used in some ELISA experiments, supernatant from cell transfected with empty plasmid

was also obtained with the above protocol. Since both BuHV1 and BoHV1 gEs were expressed in fusion with a 6xHistail, protein concentration was estimated as previously described, 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 1/10 and 1/60 was found optimal for BoHV1 and BuHV1gEproteins respectively.

Monoclonal antibodies

A panel of 60 monoclonal antibodies (MAbs) against BuHV-1 were produced in Balb/c mice according to an internally standardized method using as immunogen the viral strains MR077. Screening and characterization of the hybridomas were carried out by indirect immunofluorescence (IFAT) performed in parallel using MDBK cells infected with the homologous BuHV-1 and BoHV-1. IFAT positive MAbs were further tested in recombinant gEELISA and positive hybridomas were selected and cloned by limiting dilution to ensure monoclonality and stability. Two monoclonal antibodies reactive against BoHV1 glycoprotein E, obtained using reference strain BoHV1.1, were available from previous studies and were used in some experiments (Egyed et al., 1992).

Serum samples and ELISA procedure

A panel of 91 bovine and 84 water buffalo sera was used. In more details, the first group included 30 sera from BoHV1infected cattle, as detected by virus isolation, and/or a major seroconversion episode during periodical serosurvey. Thirty 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 30 serum samples were collected from officially BoHV1-free farms, as detected by gE and gB blocking ELISA and SN test. A serum sample from cattle experimentally infected with BuHV1 and collected at day 50p.i. was available from previous study (De Carlo, unpublished).

Among water buffalo sera, 82were from different herds with no history of contact with cattle and never subjected to BoHV1 vaccination (neither whole nor gE negative vaccines). Buffalo were divided into three main groups according to reactivity in SN test against both BoHV1 and BuHV1 strains and gB/gE blocking ELISA. First group (n=26) was defined as true negative resulting negative to both SN tests and gB/gE

149 blocking ELISA; second group included 38 animals positive to both SN tests and scoring gB+/gE- or doubt;
150 the last group comprised18animals positive to both SN tests and scoring gB+/gE+. Two sera from water
151 buffaloes experimentally infected with BoHV1 and BuHV1 and collected at day 48 p.i. were available from
152 previous study (De Carlo, unpublished).
153 According to animal species, a cross-SN test was performed using homologous and heterologous virus.
154 Two ELISA procedures were employed. In the first assay (BuHV1 gE indirect ELISA), microplates
155 (NuncMaxisorp) were coated overnight at 4°C with 50 ng of BuHV1 gE recombinant protein(even wells) or
156 negative antigen (odd wells). In the second test (BoHV1-BuHV1 gE indirect ELISA) microplates were coated
157 overnight at 4°C with 50 ng of BoHV1 gE recombinant protein(even wells) and an equal amount of BuHV1
158 gE recombinant protein (odd wells). Antigens were diluted in0.1 M carbonate/bicarbonate buffer pH 9.6.
159 After blocking with 2.5% bovine casein, primary antibody, diluted 1/20 (1/10 for hybridoma culture
160 supernatants) in PBS 1.25% casein, was added and plate incubated for 1 h at RT. After washing step,
161 peroxidase labeled protein G (or anti-mouse IgG for hybridoma culture supernatants),diluted at 10 ng/ml in
162 the same buffer was added and plates incubated as above. After final wash, reaction was developed with
163 TMB and stopped with 0.2 M H_2SO_4. Cut off was determined for BuHV1 gE indirect ELISA as the mean + 3
164 standard deviations of negative samples' reactivity enclosed in each plate. For BoHV1-BuHV1 gE indirect
165 ELISA, reactivity of each serum against both antigens was displayed in a dispersion plot and classified
166 according to the infectious status.
167 *Statistical analysis*
168 Levels of agreement between BuHV1 gE indirect ELISA and SN or gB blocking ELISA were expressed by
169 weighted Cohen's Kappa (Cohen, 1968). Association between SN and gE blocking ELISA results was
170 evaluated comparing SN titers showed by negative and positive sera, by using Wilcoxon Rank Sum test. All
171 statistical analyses were performed using R statistical software (R Core Team, 2012).
172
173 **Results**

The amplified product of the expected length was successfully amplified and subjected to direct sequencing using the PCR primers and the sequence-derived primers. The entire ectodomain sequence was generated but a small stretch of 5 residues within a proline rich region, which generate a secondary structure hampering the primer extension step during cycle sequencing, remains unresolved. Comparing BuHV1 gE ectodomain sequence with BoHV1 and BoHV5 homologues regions, nucleotide similarity was 85.65% and 97.01% respectively whereas amino acid sequences were 77.20% and 97.01% similar. All cysteine residues were highly conserved while the most divergent amino acid sequence was located between the two cysteine rich regions (Fig. 1). Despite the non resolved residues, the gE gene fragment lacking the leader peptide and TM region was amplified and cloned into eukaryotic expression vector and successfully expressed in HEK-293T. A protein of >60kDa was immunostained by anti-6xHis as well as Mab 1G4 (see below) and found compatible with a 43kDa unglycosylated protein plus 8 putative O-glycosylated sites (Fig. 2).

Among the IFAT positive hybridoma culture supernatants, seven were found reactive against BuHV1 gE indirect ELISA. In detail Mab 5E4 was cross reactive between BoHV1 gE and BuHV1 gE, Mab 1G4recognized a linear epitope of BuHV1 gEas detected by western blot and Mabs 3F7, 3C4, 4D2, 5D4 and 1C9 were only reactive against BuHV1 gE under non denaturing condition (Fig. 3).

When water buffalo sera were tested in BuHV1 gE indirect ELISA, perfect agreement was obtained with SN test (K = 1.00, 95%CI 0.65-1.00) and nearly perfect agreement with gB ELISA (K = 0.944, 95%CI 0.70-1.00). Association between blocking gE ELISA and SN titers was statistically significant: ELISA positive sera showed SN titers higher than negative ones, both considering BoHV1 (Wilcoxon Rank Sum test $p<0.001$) and BuHV1 SN results ($p<0.005$). A single sample was positive in gB ELISA and negative to both SN assays or indirect ELISA and another sample was doubtful in gB ELISA and positive to all other assays. Most of bovine samples which were positive to homologous gE ELISA were found reactive against BuHV1 indirect ELISA. However when bovine and buffalo sera were tested in a combined assay (BoHV1-BuHV1 gE indirect ELISA), a clear discrimination was possible. All bovine sera displayed a reactivity against homologous antigen at least double compared to BuHV1 gE antigen, while all buffalo sera were more reactive with similar strength ratio

against BuHV1 gE antigen and independently from the negative or positive status versus gE ELISA (Fig 4). On the other hands, when sera from bovine and water buffalo experimentally infected with BuHV1 and BoHV1 were tested, reactivity was again more evident against homologous infecting virus. Finally cross neutralization test was not able to differentiate the two infections being the SN titers very similar and somewhat conflicting with the true infectious status(Fig 5).

Discussion

In this study the ectodomain of BuHV1 glycoprotein E was amplified and expressed as secreted protein in a protein free medium and directly used for the development of an indirect assay. Results clearly suggest that this recombinant antigen is as sensitive as SN test for detection of wild BuHV1 infection in the field. In addition we propose a method to specifically differentiate BoHV1 from BuHV1 infection based on double antigen ELISA format. A similar strategy was used in a previous study where CpHV1 infection could be easily differentiated from BoHV1, based on reactivity against each recombinant gE (Bertolotti et al., 2013). However in the cited study, the true infectious status of bovine and caprine tested samples was clear, based on cross SN, being homologous titer greater than heterologous by a factor of four (Thiry et al., 2008).Moreover the natural occurrence of CpHV1 infection in cattle has never been proved. By contrast water buffalo can be susceptible to both BuHV1 and BoHV1 infections. The latter virus has been recently isolated and characterized from an aborted fetus (Fusco et al., 2012), rising concern on the role of water buffaloes in the epidemiology of BoHV1 infection (Scicluna et al., 2010). For this reason, the need of reliable diagnostic tools is highly desirable especially in countries where IBR is under control program and water buffalo farms has gained increasing interest. Both bovine and water buffalo-adapted alphaherpesviruses share common antigenic properties as revealed by cross SN test which was unable to discriminate homologous infection (Scicluna et al, 2006). This may reflect the high degree of cross reactivity among viral protein that elicit neutralization antibodies, such as gB, gD or gC. To further support this finding, the gB blocking ELISA is generally considered a marker of all ruminant alphaherpesvirus infections being based on highly conserved immunodominant epitope on this glycoprotein (Ros et al, 2002). On the other hand,

similarity of the gE ectodomain between BoHV1 and BuHV1 was about 77% rising the hypothesis that specie-specific epitopes may be useful for serological discrimination. For this reason, we firstly evaluate a panel of monoclonal antibodies raised against the BuHV1 whole virus as immunogen and found that 6 out of 7 Mabs were reactive against BuHV1-specific gE. Although we cannot exclude at this stage that some of them could recognize the same epitope, at least three different antigenic determinants were detected: a cross reacting epitope between BuHV1 and BoHV1, and two epitopes (one linear and one conformational) specific for BuHV1 gE. Two additional Mabs raised against BoHV1 and characterized in previous study were found reactive against BoHV1 gE but not against BuHV1 gE. Based on these results we conclude that gE specific epitopes are present in the ectodomain of gE and can be used to detect, if any, a differential reactivity based on the specific infectious status. We then examined a panel of bovine sera with known BoHV1 positive infectious status, based on virus isolation and major seroconversion episodes. All samples scored gB+/gE+ in blocking ELISA. According to BoHV5 experience (Wellenberg et al., 2001), a first set of water buffalo sera were selected in a flock with no history of vaccination and based on the gB+/gE- reactivity in blocking ELISA, revealing a true BuHV1 infectious status. In this two groups of sera the reactivity against the homologous antigen was always higher (by a factor of two) than heterologous one. In the same flock, nearly one third of gB+ buffalos were also gE+ in blocking ELISA and, once again, moved towards BuHV1 gE antigen with higher strength. In this second group of animals the true infectious status is more difficult to establish. However we found several arguments to support the hypothesis that they were indeed infected with BuHV1. Bubaline Herpesvirus 1 was isolated in Mediterranean water buffalo in four events: after pharmacological reactivation (De Carlo et al., 2004, De Carlo et al., 2010),during an episode of abortion (Amoroso et al., 2013)and in buffalo calves with respiratory signs (Petrini et al., 2012). In all these cases, animals were found gE+ in blocking ELISA. In our study we used sera from three animals experimentally infected with BuHV1 (one cattle and one water buffalo) and BoHV1 (one water buffalo). The formers were found doubt at gE blocking ELISA at 48 d.p.i. (De Carlo E., personal observations). All experimentally infected animals showed a clear reactivity against homologous antigen with absorbance values two to three times higher compared to heterologous antigen (see fig 4). We then conclude that a

252 proportion of water buffaloes infected with BuHV1 may react in gE blocking ELISA. Since this test is based
253 on a single epitope located in the gE/gI complex of BoHV1 (Tyborowska et al., 2000), it should be noted that
254 monoclonal antibodies specific for this epitope do not recognize similar epitope in BuHV1 infected cells (not
255 shown). However high antibody level directed towards other glycoproteins located close to gE/gI complex
256 may inhibit the binding of labeled Mab, possibly due to steric hindrance. We found a statistically supported
257 evidence that the set of gB+/gE+ water buffalo sera from the selected flock were associated to higher SN
258 titer compared with gB+/gE- panel. Obviously when water buffalo have been experimentally infected with
259 BoHV1 all animals were reactive against gE blocking ELISA two to three weeks after infection (Sciclunaet al.,
260 2010). Thus a positive result in gE blocking ELISA in water buffalo, differently from BoHV5 infection, can be
261 the result of both BoHV1 and BuHV1 infection.
262 Our study revealed the high level of similarity at amino acid level with BoHV5, and a similar method to
263 discriminate between BuHV1 and BoHV5 infections seems not possible at this stage. However, at least in
264 Europe and in particular in Countries with high density of water buffalo farms, this diagnostic drawback
265 seems of limited impact since the occurrence of BoHV5 infection had been rarely or never
266 documented(Thiry J. et al., 2006).
267 In conclusion the antigenic characterization of the ectodomain of glycoprotein E of BuHV1 was carried out
268 and its recombinant form has been used for the development of a sensitive marker of infection with
269 excellent agreement with SN test. This indirect ELISA test can be potentially applied in water buffalo in
270 association with IBR marker vaccine for control of herpesvirus infection (both BoHV1 and BuHV1).In fact,
271 the use of marker vaccine in buffaloes has been hampered for decades due to the lack of diagnostic tools
272 able to differentiate vaccinated from BuHV1 infected animals. Using such an indirect ELISA, vaccinated
273 animal will be expected to score negative. Moreover the possibility to discriminate between BoHV1 and
274 BuHV1 by double antigen indirect ELISA is to our knowledge the sole serological method available to date.
275 According to 64/432/CEE, bovine animal definition includes Bison bison and Bubalus bubalus species: this
276 aspect is particularly important considering movement restriction on gB+/gE+ animals in IBR control plans.
277 Consequently doubts on serological diagnosis are hampering water buffalo movements, especially for

reproduction aims, and leading to economic losses. The application of tests for the identification of BuHV1 infection could improve water buffalo trade, allowing the correct identification of the infectious status. Further validation, especially on sera from water buffalo experimentally co-infected with BoHV1 and BuHV1,may be helpful to clarify the role of water buffalo in the epidemiology of IBR under natural conditions. Future work will be addressed to evaluate the potential application of these assays for both control and epidemiology of BuHV1 infection in the field.

Acknowledgments

This work was partly supported by the Italian Ministry of Health – Progetto di Ricerca Corrente 2012 IZS PLV 11/12 12C11.

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343 be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA. Vet.
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345

346 Table1. Primers sequences. Restriction enzyme sites are underlined.

347

348 Figure 1. Amino acid alignment of bovine and bubaline Herpesvirus glycoprotein E ectodomain. Conserved
349 Cys residues are reported in bold. Stars indicate identical amino acids and double dots (:) indicate conserved
350 substitutions and dots (.) indicate semi-conserved substitutions.Sequence unresolved is boxed.

351

352 Figure 2. Western blot of recombinant BuHV1 gEectodomains secreted in mammalian cells and
353 probed with Mab Lane M: prestained molecular weight (sizes are expressed in kDa); Lane 1:
354 recombinant BuHV1 gE probed with Mab 4D2.

355

356 Figure 3. Mab panel reactivity evaluated by indirect ELISA against recombinant antigens. For each Mab,
357 absorbance against BuHV1 gE antigen (black bar) and BoHV1 gEantigen (gray bar) is shown. Mabs 2A5
358 and 5F5 were raised against BoHV1 as immunogen while all others were raised against BuHV1 as
359 immunogen

360

361 Figure 4. Dispersion plot of the ELISA reactivity versus BoHV1 recombinant gE (Y axes) and BuHV1
362 recombinant gE (X axes).Bovine Sera (circles) and water buffalo sera (squares and triangles) were classified
363 according to SN titer, gB/gE blocking ELISA and infecting virus (only experimentally infected animals):
364 bovine seragB+/gE+ with known history of IBR isolation and major seroconversion episode (white circles);
365 water buffalo seragB+/gE- with positive SN titers (black squares); water buffalo sera gB+/gE+ with high SN
366 titers (black triangles); bovine experimentally infected with BuHV1 (black circle and arrow); water buffalo
367 experimentally infected with BoHV1 (white square and arrow); water buffalo experimentally infected with
368 BuHV1 (black square and arrow). Diagonal and envelope lines represent the prefect cross reactivity and the
369 arbitrarydiscriminatory uncertainty respectively.

370

371 Figure 5. Dispersion plot of a subset of water buffalo sera according to SN titer. Cross-SN titer against
372 BuHV1 and BoHV1 are reported on the X and Y axes respectively in log scale. Diagonal represents the
373 perfect cross reactivity.

Figure1

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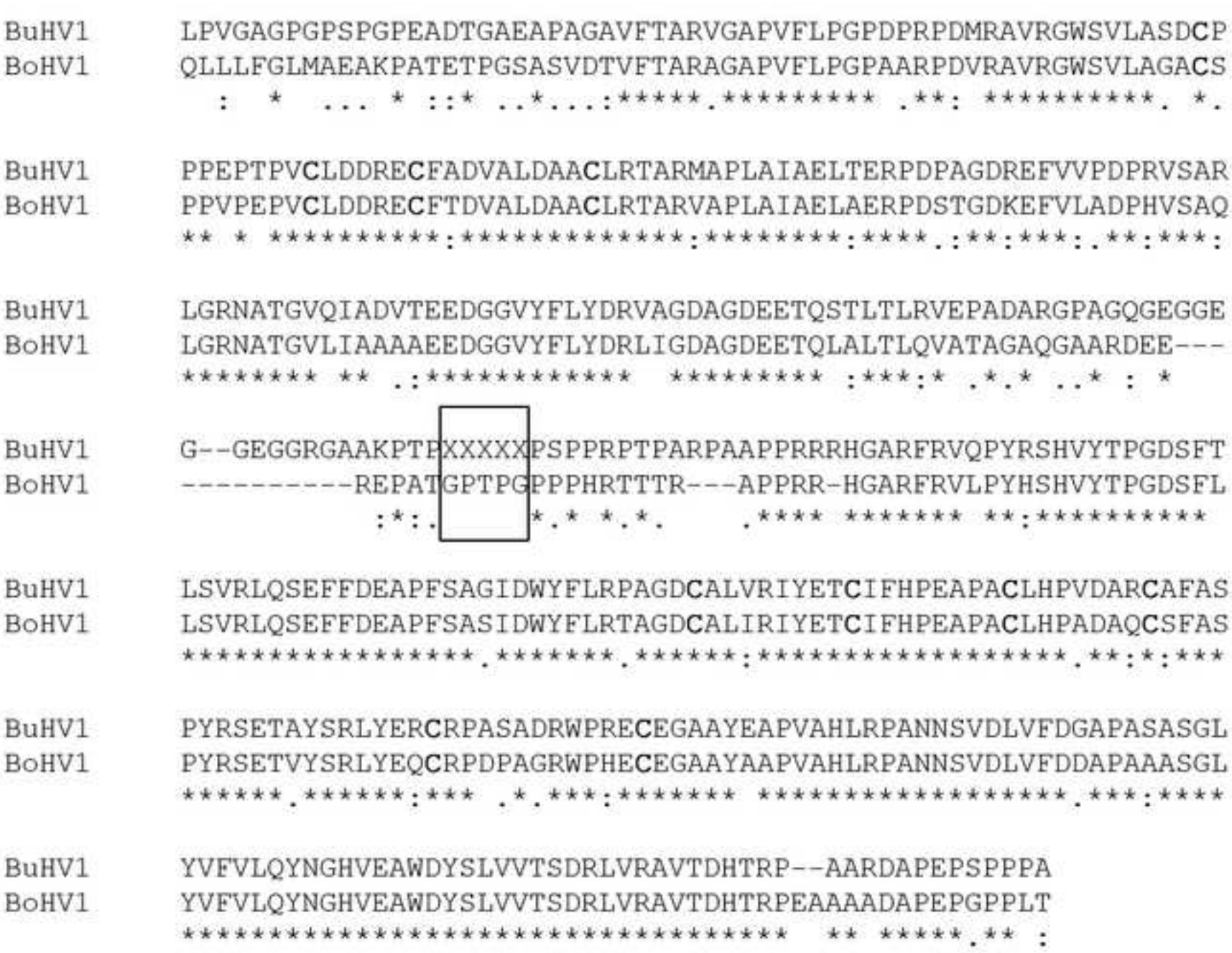

Figure2
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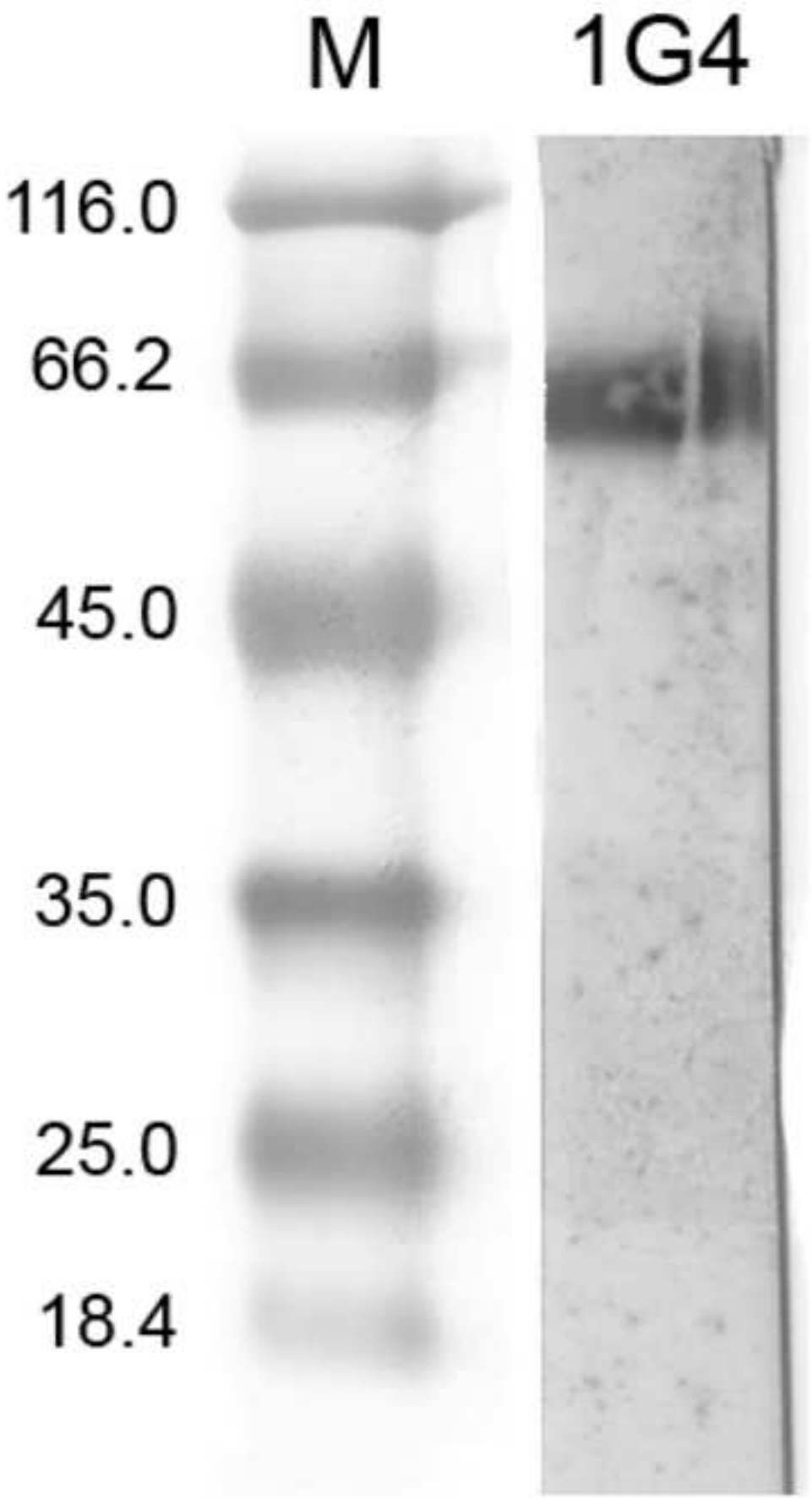

Figure3
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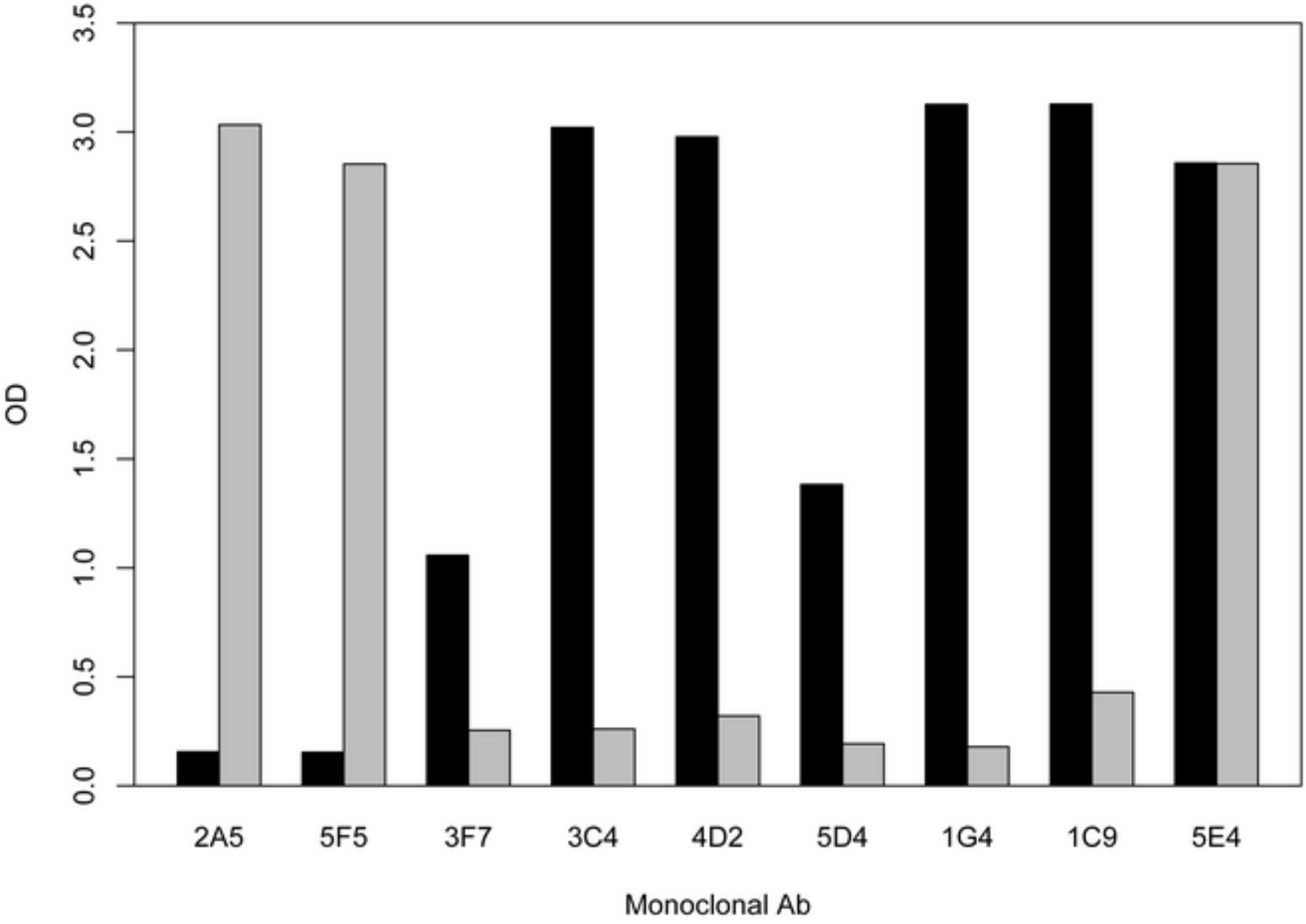

Figure4

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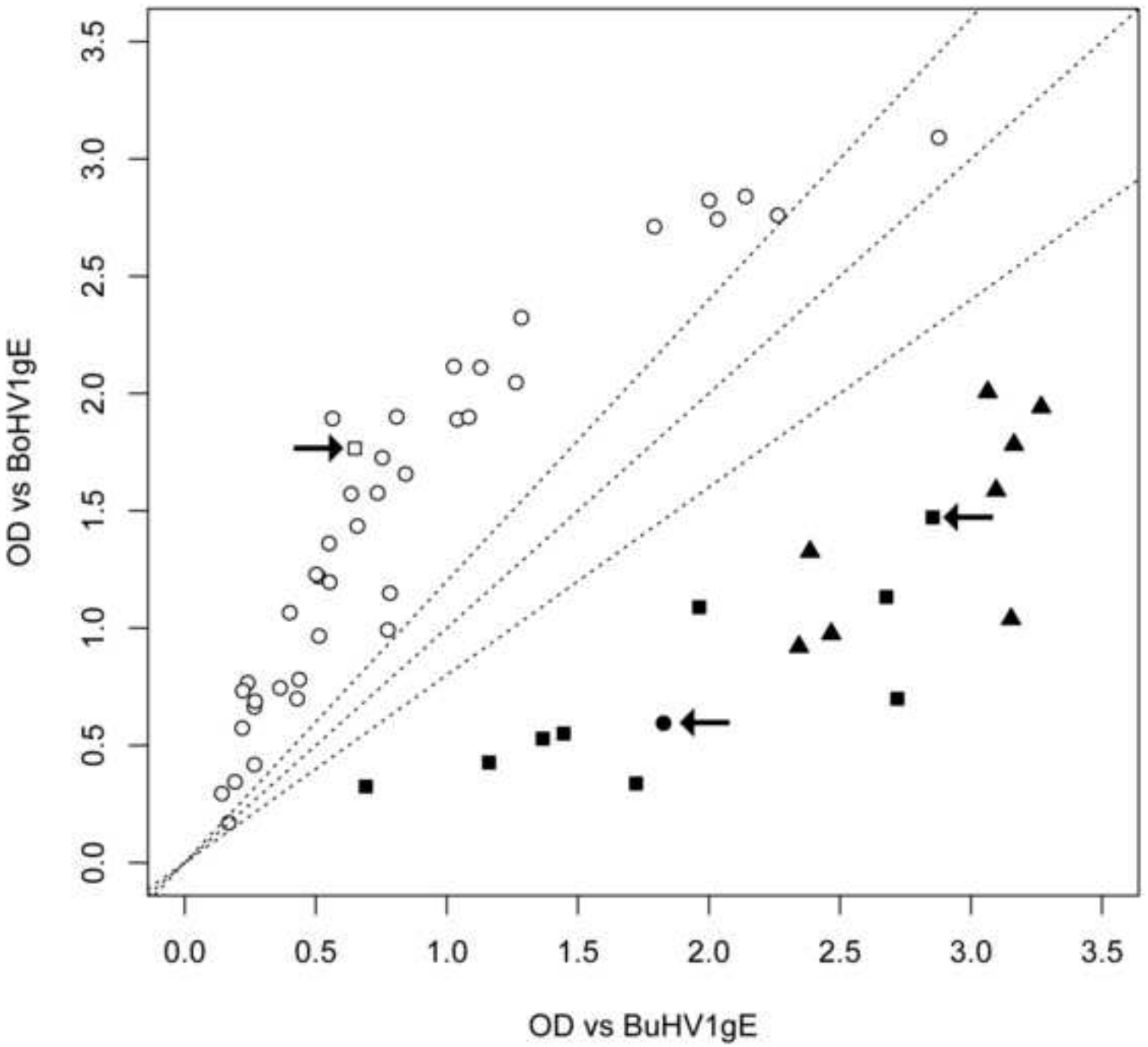

Figure5

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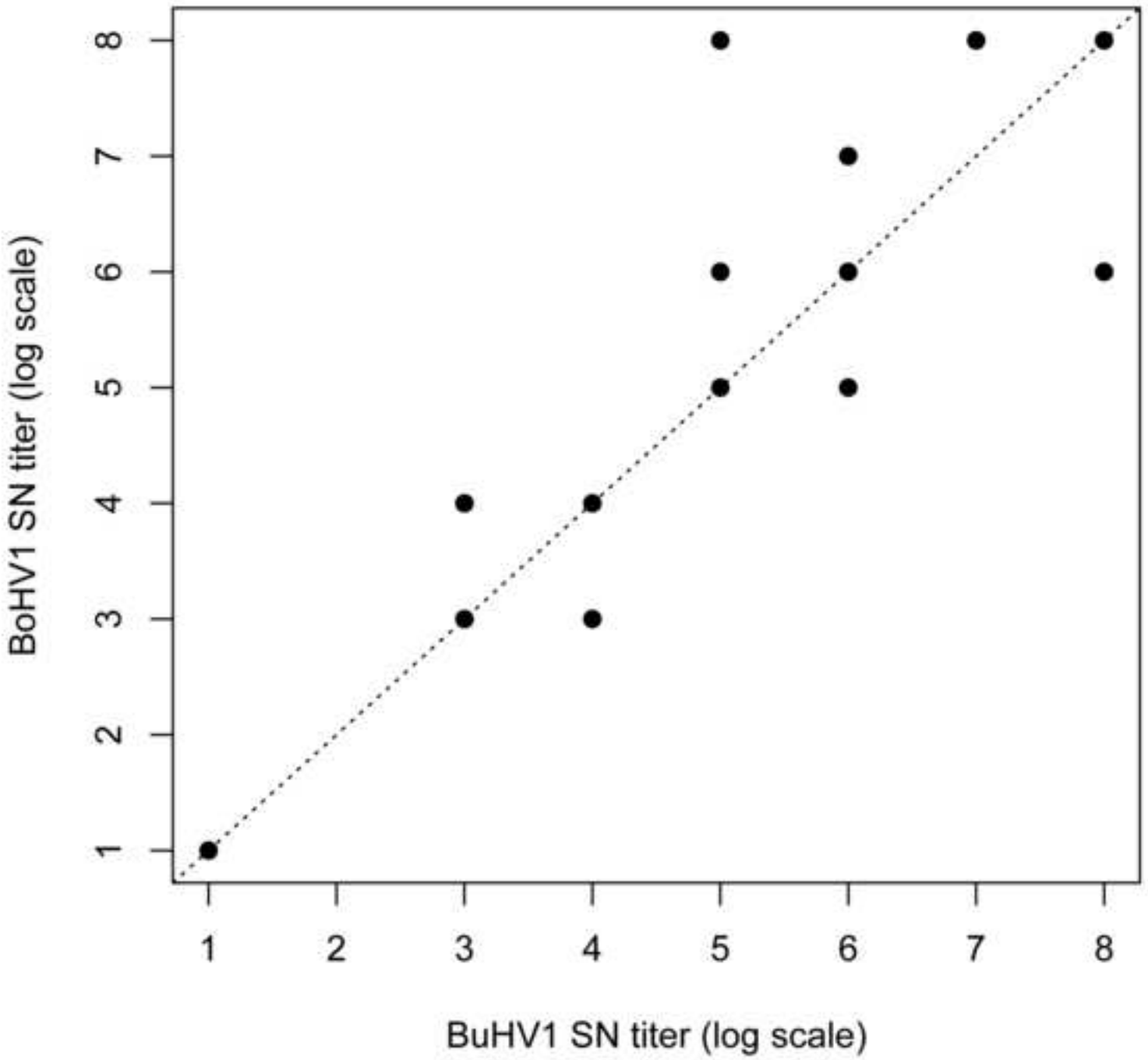

Table1. Primers sequences. Restriction enzyme sites are underlined.

Primer name	Sequence
BuHV1gIc2	5'-ATCAGCGAAGAATAAASGCCGC
BuHV1gIf	5'-GAGAGGCATGGGCTGTGCGAAAGG
BuHV1HgEf	5'-TTAAGCTTTCTACCGGTCGGGGCCGGCCCC
BuHV1XgEr	5'-TTCTCGAGTGGCGGGCGGCGGGCTCGGCTC