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Development of specific diagnostic test for small ruminant lentivirus genotype E

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Abstract

Small ruminant lentivirus (SRLV) belonging to the highly divergent genotype E has recently been identified in the Italian goat breed Roccaverano. In this report we have developed a specific serological test based on recombinant matrix/capsid antigen fusion protein. Performance has been evaluated and compared with a similar test based on genotype B antigen. Herds under study were selected according to the infectious status characterized by blood PCR and sequencing. Results clearly showed that B and E based recombinant ELISA only detected homologous infection and an apparent cross-reactivity was recorded in a herd in which co-infection was present. Three commercially available ELISAs showed different abilities in detecting genotype E infection, being the whole virus-based immunoassay the best choice. Genotype E-recombinant antigen was not detected in ELISA by three commercially available Mabs known to be cross-reactive among CAEV and MVV capsid antigens, further supporting the high divergence of the E genotype from others. Finally, a SRLV-free herd according to commercial ELISA testing, was analysed in the same area where genotype E was identified and few animals belonging to Roccaverano breed were found slightly reactive with the E antigens. Our results suggest that the prevalence of genotype E in other small ruminant populations may be conveniently estimated using a comparative assay based on a combination of genotype specific recombinant antigens and may highlight a wider space in which SRLVs evolve.

Keywords

Small ruminant lentivirus, genotype E, recombinant antigen, diagnosis

Introduction

Small ruminant lentiviruses (SRLVs) are a heterogeneous group of viruses affecting sheep and goat and are responsible for chronic debilitating diseases known as Maedi Visna (MV) and Caprine Arthritis-Encefalitis (CAE) (Pepin et al., 1998). Viral isolates characterized so far, show different genetic, antigenic and biological properties and are no longer considered species-specific (Pisoni et al., 2005; Shah et al., 2004b). From an antigenic point of view, most SRLVs can be classified as MVV-like or CAEV-like, corresponding to genotype A and B respectively (Shah et al., 2004a). Previous studies have suggested that early serological diagnosis can be achieved using homologous antigen (Lacerenza et al., 2006). Nevertheless, most of the currently available diagnostic tests are produced using a single strain-based antigen preparation which is believed to detect cross-reacting antibodies against epitopes located in structural proteins (Gogolewski et al., 1985). Recently, a novel genotype E has been identified in the local breed Roccaverano in north-west Italy. First sequences were obtained by chance in a caprine herd, using a set of degenerated primers designed to amplify a *gag* fragment from the majority of known genotypes, encompassing major linear capsid antigen epitopes. Following a preliminary sequence screening, it seemed quite clear that this viral cluster might have escaped diagnosis in the field using conventional antigen preparations, likely due to the low similarity found in the major immunodominant regions (Grego et al., 2007). A viral strain was subsequently isolated from an apparently healthy goat highly reactive by ELISA against genotype E-derived major capsid antigen epitopes (Reina et al., 2009). Genetic features of this genotype have been described in three epidemiologically unrelated herds. The complete genome (~8,4Kb) presented two major deletions corresponding to the dUTPase subunit of the *pol* gene and to the *vpr* accessory gene. Based on previous studies in which such subunits were independently deleted from a pathogenic infectious clone, these deletions could explain why the viral cluster is not related to any known clinical signs, representing a natural, well host-adapted, low pathogenic lentivirus (Harmache, 1996; Turelli, 1996; Zhang, 2003). Moreover, preliminary

epidemiological data suggest that infection can persist in the population through familiar lineage, with a low tendency to spread horizontally. To date, no information is available on the prevalence of genotype E in larger goat populations due to the lack of a specific antibody detection system. To address this issue, in this report we have developed genotype E specific recombinant antigens which were tested with a panel of monoclonal antibodies (Mabs) known to be reactive against CAEV and MVV capsid antigens. These genotype E derived antigens were also used to develop an indirect ELISA in order to test a panel of goat sera belonging to herds in which the SRLV infectious status was determined by PCR product sequence analysis. Serological and sequence data were in agreement highlighting the importance of using genotype specific tests when determining SRLV seroprevalence, assessing SRLV-free status and searchng for epidemiological information.

Material and Methods

Virus and plasmids

Roccaverano strain was originally isolated using mammary gland explants from an adult goat and a complete proviral sequence was obtained (Genbank accession number EU293537). The *gag* gene was amplified by concatenating overlapping PCR fragments and cloned in pCRTopoXL (Invitrogen). The gene fragment coding for Matrix (P16) and major Capsid Antigen (P25) was subsequently amplified and was subcloned between the *BamH1/EcoR1* sites of pGEX6His following site-directed PCR-mediated mutagenesis suppressing an internal *EcoR1* restriction site. This plasmid, derived from pGEX6P prokaryote expression vector (GE Healthcare), was modified by inserting an in frame 6Xhis-tag between *EcoR1* and *Sal1* restriction sites thus allowing a double step affinity purification.

Expression and purification of P16-25 recombinant antigens and P25-B3 subunit epitopes

Transformed *E.coli* BL21 bacteria were induced at early log phase for 2 h with 0.5mM IPTG under agitation. Bacterial cells were recovered by centrifugation and lysed by physicochemical methods. Recombinant GST/P16-25/6H fusion protein was recovered in the soluble fraction and the first

affinity step was carried out in batch using glutathione Sepharose 4B (GE Healthcare). GST cleavage was achieved in pooled eluted fractions using PreScission Protease (2U/mg) (GE Healthcare). Solution containing GST and P16-25/6H was dialyzed for 24h to remove reducing and chelating agents and loaded into a Hi-Trap chelating HP column (GE Healthcare), positively charged with nickel ions. Following immobilised metal chelate affinity chromatography, purity and yield of recombinant antigen was estimated by SDS-PAGE and DC protein assay (BioRad).

Using the same protocol, the same gag antigens derived from strain It-Pi1 (genotype B) were employed to generate the antigenic CAEV-like counterpart.

Subunit immunodominant capsid antigen epitopes of MVV and CAEV had previously been characterised. In this study a third version using genotype E (P25-B3) derived sequence was produced, generating a GST fusion protein as previously described (Grego et al., 2002; Rosati et al., 1999).

Blood samples, polymerase chain reaction and sequencing

Twelve caprine herds were selected in this and in a previous study (Grego et al., 2007). Heparinized blood samples were obtained from a number of adult animals, representative of each herd: and DNA was extracted from white blood cells using DNA blood kit (Qiagen). A *gag* nested PCR previously developed (Grego et al., 2007) and known to detect the highest number of SRLV genotypes/subtypes was applied to each sample and all positive results with suitable bands were sequenced. After, this preliminary screening, five herds of Roccaverano breed were selected: a first herd, BL (n=52) in which only genotype E was detected; a second herd, NG (n=40) in which genotype B and E were detected; and a third group of goats, TM (n=20), BM (n=18) and CF (n=6) in which only genotype B was present. In this study, all caprine herds were retested when possible at completion and additional sequences were obtained.

To date, the presence of genotype E had been limited to few herds of the Roccaverano breed. Therefore, an additional three-breed long term SRLV negative herd (n=400) was also included for

123 serological testing. The herd consisted of 109 Roccaverano, 107 Saanen and 184 French Alpine
124 goats.
125
126 *Serum samples and ELISAs*
127 For P16-25 recombinant ELISA, microplates (Immunomaxi TPP) were coated with 100 ng of P16-
128 25 derived from B and E genotypes or water as negative antigen (Fig. 1). Plates were allowed to dry
129 overnight at 37°C and then blocked with 2.5% bovine casein for 1h at 37°C. After four washes,
130 serum samples were diluted 1/20 in phosphate-buffered saline containing 1.25% casein and
131 incubated for 1h at 37°C. Subsequently to the washing step, anti-sheep/goat IgG peroxidase labelled
132 Mab diluted in the same buffer was added and the plates incubated as above. After a final washing
133 step, the reaction was developed with ABTS and plates were read at 405 nm. Net absorbances were
134 obtained by subtracting the absorbance of negative antigen from the absorbance of each
135 recombinant antigen and visualized by box plots. Cut off was previously defined for genotype B as
136 having a reactivity of >40% relative to the positive control serum reactivity included in each plate
137 (Lacerenza et al., 2006). The same absorbance value was applied for genotype E, due to the lack of
138 a truly negative flock regarding this genotype.
139 For subunit ELISA (P25-B3 epitope), microplates were coated with 220 ng of GST-B3 derived
140 from genotype B (sequence KLNEEAERWRRNNPPPP), genotype E (sequence
141 KLNKEAETWMRQNPQPP) and an equimolar amount of GST as negative control. Net
142 absorbances were obtained by subtracting the GST antigen absorbance from that of each
143 recombinant subunit.
144 Three commercially available ELISAs, based on whole virus (brand A), double recombinants
145 (brand B) or recombinant and synthetic (brand C) antigens, were used to detect and quantify the
146 infection status in BL herd. Assays were carried out according to the manufacturer's protocols.
147 Three commercially available monoclonal antibodies (Mabs), namely 5A1, 10A1 and 8B1, known
148 to be reactive against CAEV and MVV capsid antigen (McGuire et al., 1987) were obtained from

149 VMRD, Inc. (Pullman WA, USA) and tested in P16-25 recombinant ELISA. Mabs were used at
150 dilution of 1µg/well, using mouse-specific secondary antibody. In the latter experiment, a
151 previously produced recombinant capsid antigen from the MVV strain K1514 (Rosati et al., 1999),
152 was used to confirm Mabs cross-reactivity between genotype A and B.
153
154 *Western blot*
155 The genotype B and E recombinant P16-25 proteins were also tested by Western blot using the
156 same three Mabs (5A1, 10A1 and 8B1), as well as a polyclonal serum from mice immunized with
157 recombinant P16-25 of genotype E (positive control) and serum from the same goat from which the
158 Roccaverano strain (genotype E prototype) had originally been isolated.
159 *Sequence analysis and phylogenetic trees*
160 In order to create phylogenetic trees, a model of molecular evolution was estimated using a
161 hierarchical likelihood ratio test approach and the Akaike information criterion (Akaike, 1973)
162 implemented in the software ModelTest ver. 3.7 (Posada and Crandall, 1998, , 2001). Bayesian
163 methods implemented in the computer program MrBayes ver. 3.1.1 (Huelsenbeck and Ronquist,
164 2001. Ronquist and Huelsenbeck, 2003) were used to create phylogenetic trees and to assess
165 statistical support for clades. A Markov chain Monte Carlo search for 1,000,000 generations using
166 two runs with four chains (temperature = 0.05) was performed and results were represented as a
167 50% majority rule consensus tree. Tree statistics and phylogenetic manipulations were calculated
168 from the computer program PAUP* ver. 4.0b10 (Swofford, 2000). Genetic diversity was expressed
169 as nucleotide diversity (Nei, 1987), or the mean proportion of nucleotide differences among
170 sequences.
171 *Statistical analysis*
172 Maximum expected prevalence of both genotypes in the herds was estimated using WinEpiscope
173 2.0 (http://www.clive.ed.ac.uk/winepiscope/).Agreement beyond chance between genotype E

specific ELISA and commercial assays was analysed using Fleiss'κ coefficient (Fleiss, 1981). Differences in O.D. values between tests were evaluated using Wilcoxon test.

Nucleotide sequence accession numbers

Nucleotide sequences reported in this paper were deposited in the GenBank database with accession numbers FJ547242-46. A subset of additional sequences (EF675997-6026; EU726488-525) had been generated in previous studies (Grego et al., 2007; Reina et al., 2009).

Results

Recombinant antigen derived from genotype E was successfully expressed and purified. SDS-PAGE of the purified product revealed a single protein band of a molecular weight corresponding to the expected size of matrix and capsid antigen fusion protein (41kDa) (Fig 1).

Out of 124 blood DNA samples, 56 were PCR positive and the 39 suitable for sequence analysis, were used to construct a phylogenetic tree using CAEV-CO (M33677), Roccaverano (EU293537) and K1514 (M10608) as reference strains. As shown in Fig. 2, all sequences, representative of herd BL belonged to genotype E (8 sequences out of 12 positive PCR reactions). This sample size allowed us to estimate a maximum expected prevalence of B genotype lower than 25% (CI 95%) within this herd.

Conversely, sequences obtained from herds TM, BM and CF were grouped into the B1 subtype (15 sequences out of 22 PCR reactions), allowing an estimation of a maximum expected prevalence of E genotype lower than 13.5% (CI 95%). Finally, both infections were detected in herd NG with a high degree of co-infections (B and E genotypes) within the same animal, as a previously described genotype specific PCR had revealed (Grego et al., 2007).

Box plots of serum reactivity against B and E recombinant antigens are shown in Fig 3. In herd BL and the TM-BM-CF herd group, only one homologous antigen was clearly detected among infected animals based on infectious status determined by phylogenetic analysis, while an apparent cross-reactivity between the two antigens was present only in herd NG. To further study the serological reactivity against genotype specific gag antigens, sera were also tested by subunit ELISA, using

P25-B3 epitope from genotypes B and E. Indeed, several samples reacted in a type specific manner (Fig. 4). Serum samples belonging to herd BL showed a distribution mainly oriented towards the E derived epitope and on the contrary, B1 infected herds (TM-BM-CF) were clearly reactive only with the B derived epitope (Fig 4 a and 4b). Herd NG showed a wider distribution of absorbances reaching high values against both peptides (Fig 4 c).

Genotype E derived p16-25 antigen reacted also with few sera from the long term negative herd tested and these sera were from Roccaverano goats, while no reactivity was recorded in Saanen and French Alpine goats cohabiting in the same herd (not shown).

Commercial ELISAs showed a different capacity in detecting infection in herd BL, being the whole virus ELISA in perfect agreement with genotype E derived ELISA (κ= 1), followed by the double recombinant ELISA (κ= 0.83) and the recombinant/synthetic ELISA (κ= 0.47).

Distribution of absorbance values in infected animals of the same herd revealed that the best signal - to - noise ratio belongs to genotype E antigen and is significantly higher than those obtained from the three commercial ELISAs ($p<0.05$) (Fig.5).

None of the three Mabs showed reactivity against recombinant antigen derived from genotype E in ELISA while antibody cross-reaction was confirmed when using CAEV and MVV capsid antigens (Fig. 6a). Western blot analysis (Fig. 6b) showed a strong reaction when using p16-25 from genotype B and a weak signal against p16-25 of genotype E, likely due to intrinsic higher sensitivity of WB compared to ELISA under the test conditions applied.

Discussion

In this study we demonstrate that the highly divergent SRLV genotype E can be detected in a type specific manner by a comparative assay using *gag* derived antigens from different genotypes. The true prevalence of viral E genotype might have been underestimated so far for two reasons: i) commercial ELISAs may detect infection without yielding specific serotype information; and ii) some animals may escape diagnosis based on immunodomiant epitopes highly divergent from E

226 genotype antigens. The development of a first serological tool specific for genotype E has become
227 therefore essential to monitor natural infection by this genotype in the field. We chose *gag* derived
228 structural proteins since it is well documented that anti-gag derived antibody response remains
229 detectable for a long time, if not life long (de Andres et al., 2005), and cross-reactivity between
230 MVV and CAEV P16-25 is acceptable except in the early stage of infection when homologous
231 antigen is more quickly recognised (Lacerenza et al., 2006). Lack of sensitivity of CAEV P16-25 in
232 detecting genotype E infection and vice versa was not surprising. All the diagnostically relevant
233 epitopes characterized so far in the *gag* encoded proteins (summarized in Table 1), show
234 divergences between genotype E and CAEV or MVV. Commercial ELISA based on whole virus
235 preparations demonstrate that genotype E can be detected, likely due to the presence of a larger
236 panel of cross reacting epitopes which may compensate the high variability between genotype E and
237 ELISA antigen. The other commercial ELISAs used are based on a more restricted antigen display
238 and we cannot exclude that, for example, the TM epitope of genotype E might elicit cross-reacting
239 antibodies recognised by heterologous antigen preparations.
240 We previously described P25 immunodominant epitope and matrix protein as specifically detecting
241 A or B genotype infections (Rosati et al., 1999; Grego et al., 2005). Here, we show that genotype E
242 derived P25 immnodominant epitope also recognises only homologous infection. Lack of reactivity
243 of three Mabs, known to be reactive against CAEV and MVV, with genotype E P25 protein was
244 surprising and demonstrates that there are at least three different type specific epitopes in P25. This
245 is relevant when aiming for antigenic detection of a wide spectrum of SRLVs. The hypothesis that
246 genotype E infection could have escaped through traditional diagnostic pressure (test and slaughter
247 policy), was evaluated in a SRLV free herd in which few old aged animals of the Roccaverano
248 breed were slightly reactive against genotype E recombinant antigen. We cannot rule out a false
249 positive reaction since an attempt to amplify viral sequences from the same animals gave
250 inconclusive results. It should be taken into account that no positive reaction was recorded in any of
251 the 292 animals belonging to other breeds of the same herd. On the other hand, animals with a low

252 proviral load could be misdiagnosed using PCR. Interestingly, young animals of the three breeds
253 (Roccaverano, Saanen and French Alpine) were negative, confirming that horizontal transmission is
254 probably hampered due to genetic deletions present in this low pathogenic virus. Thus, artificial
255 feeding of kids, a prevention strategy for SRLV control still used in the herd, is an efficient way to
256 co-eradicate both CAEV and genotype E infections.
257 To date we could identify only three herds infected with genotype E and no more than about a
258 hundred E-infected animals, all belonging to the Roccaverano goat breed (total heads 1500) which
259 was at risk of extinction in the seventies. We are therefore aware that the geographical distribution
260 of genotype E may have a very limited impact in other countries. However, this study shows that
261 the lack of E specific serological tests does not allow the determination of the true prevalence of
262 genotype E infection. Molecular tools such as PCR are now available for genotype E as well as a
263 congruous number of GenBank deposited sequences. The low agreement between PCR and ELISA
264 in detecting genotype E infected animals was not surprising, since this has been widely
265 demonstrated by other authors regarding A or B genotypes. This is likely due to low viral load or to
266 high heterogeneity at the primer binding site (de Andres et al., 2005). It should also be noted that
267 not all the recently described PCRs developed for generic detection of SRLVs may be used for
268 genotype E identification. Primers described by Shah (Shah et al., 2004a) designed to amplify 1.2kb
269 of *pol* gene, will result in a product of 0.8kb, resembling unspecific, due to internal dUTPase
270 deletion. In addition, due to the same deletion, PCR primers described by Eltahir (Eltahir et al.,
271 2006) will fail to amplify as a result of the lack of forward primer binding site.
272 In conclusion, our results demonstrate that, despite the limited information on the distribution of
273 genotype E in small ruminant population, specific antigen design is required for accurate diagnosis.
274 Subunit ELISA using a panel of A, B and E immunodominant region of capsid antigens can be used
275 as a rapid preliminary screening test that would allow serotype determination. On the other hand,
276 evidence regarding this highly divergent genotype has widened the space in which SRLVs have
277 evolved with potential implications for control strategies.

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285 Fig legends

286 Fig 1

287 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing expression and purification of
288 recombinant antigens in *E.coli.* MW, molecular-weight standard; lane 1 *E. coli* lysate; lanes 2 and 4
289 bacterial lysate expressing GST/P16-25 of It-Pi1 (genotype B) and Roccaverano (genotype E)
290 respectively; lanes 3 and 5, affinity purified recombinant P16-25 of the same strains after GST
291 cleavage and IMAC.

292 Fig 2

293 Phylogenetic tree of amplified *gag* region from different herds. The reference strains of genotype A
294 (M10608), B (M33677) and E (EU293537) are shown.

295 Fig 3

296 Box plots of net absorbances in ELISA testing of sera from BL herd (panel A), NG (panel B) and
297 TM-BM-CF group (panel C) against recombinant P16-25 of genotypes B (left box) and E (right
298 box). Bottom and top of each box represent the first and the third quartiles of the distributions. The
299 median is represented by the line inside the box.

300 Fig4

301 Net absorbance against P25-B3 subunit ELISA: each serum sample was tested against the B derived
302 epitope (X axis) and E derived epitope (Y axis). Samples are from BL herd (panel A), NG (panel
303 B) and TM-BM-CF group (panel C).

304 Fig 5

305 Distribution of O.D. values in BL herd using different serological tests. Sera which were positive to
306 at least two ELISA tests were considered as true positive. Brand A: whole virus based ELISA (O.D.
307 450nm); brand B: double recombinant based ELISA (O.D. 450nm-595nm); brand C recombinant
308 and synthetic based ELISA (O.D. 450nm-595nm); genotype E specific recombinant P16-P25
309 ELISA (O.D. 405nm). Bottom and top of each box represent the first and the third quartiles of the
310 distributions repectively. The median is represented by the line inside the box.

Fig.6

a) Monoclonal antibody reactivity in ELISA (O.D. 405 nm) against capsid antigen of CAEV isolate (McGuire et al. 1987). Net absorbances against recombinant P16-25 of genotypes E and B and recombinant P25 of genotype A are shown.

b) Western blot analysis of the same Mabs (5A1, 10A1 and 8B1) against P16-25 of genotypes B (lanes 1, 2 and 3) and E (lanes 4, 5 and 6). Lanes 7 and 8 were developed using a polyclonal mouse antiserum raised against E derived fusion protein, and a serum from an E infected goat respectively. MW, molecular weight marker.

Table: Immunodominant linear epitopes of SRLV identified so far along the structural proteins in genotypes A, B and E. Genbank accession numbers, source references and amino acid sequences from each genotype are indicated. Dashes represent the same amino acid as that of genotype B.

Genotype	GenBank	Matrix (Grego et al., 2005)	Capsid antigen B (Grego et al., 2002)	Capsid antigen D (Rosati et al., 1999)	Nucleoprotein (Lacerenza et al., 2008)	Transmembrane (Bertoni et al., 1994)
B	M33677	KLLTPEESNKKDFMSL	LNEEAERWRRNNPPPPA	VQQASVEEKMQACRDVGSE	GNGRRGIRVVPSAPPME	ELDCWHYHQYCITS
A	M10608	-N-----TS-RE-A--	--D-----V-Q---G-N	----T----------	--N---P---------L	-------QH--V--
E	EU293537	RSM----ESR---V--	--K---T-M-Q--Q--GP	A-TST----L----E-E-S	--SQ--P---------Q	-I------G--V--

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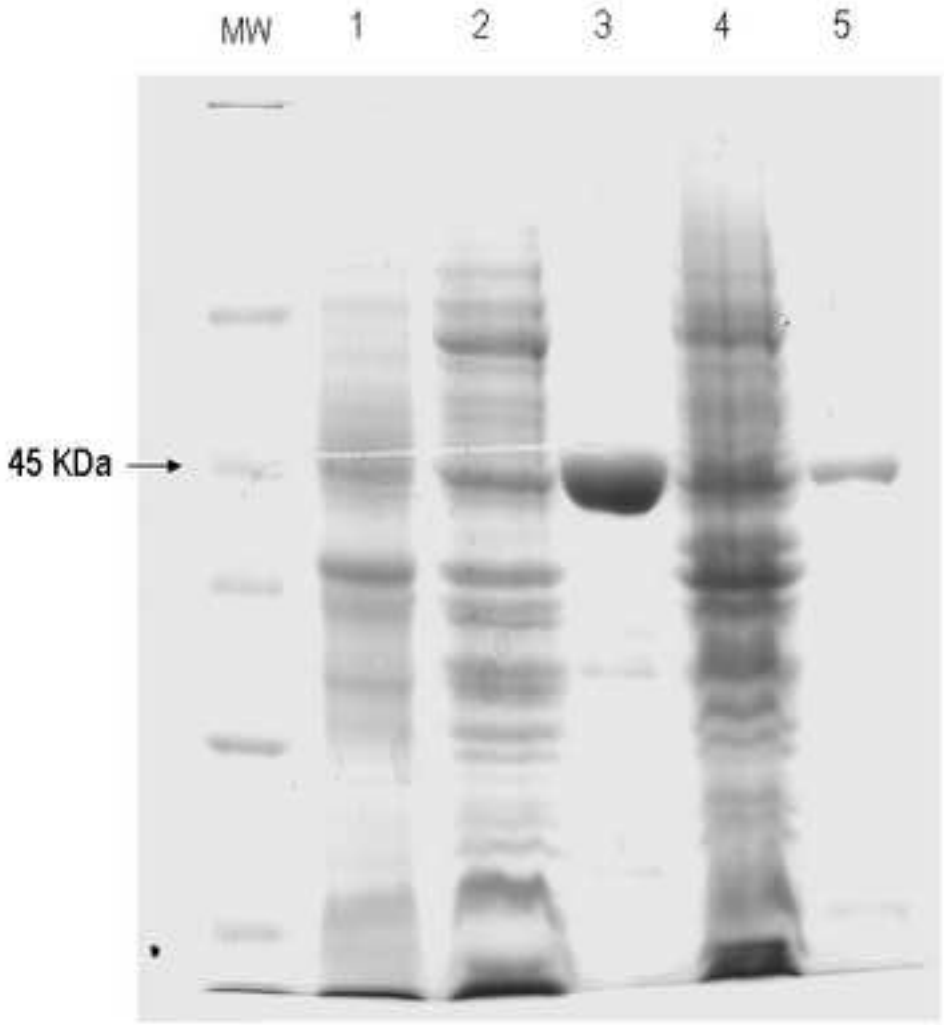
Figure 1



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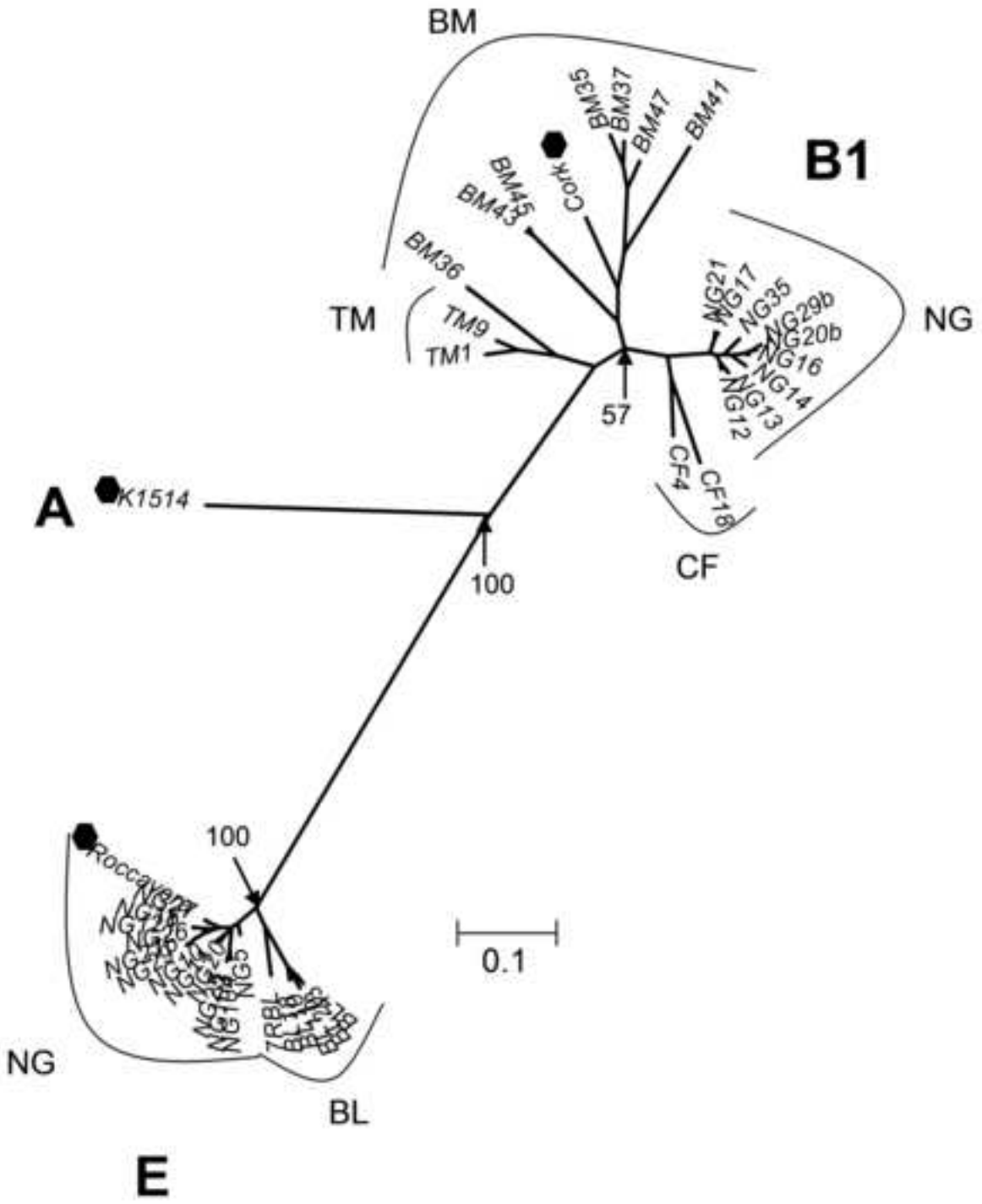
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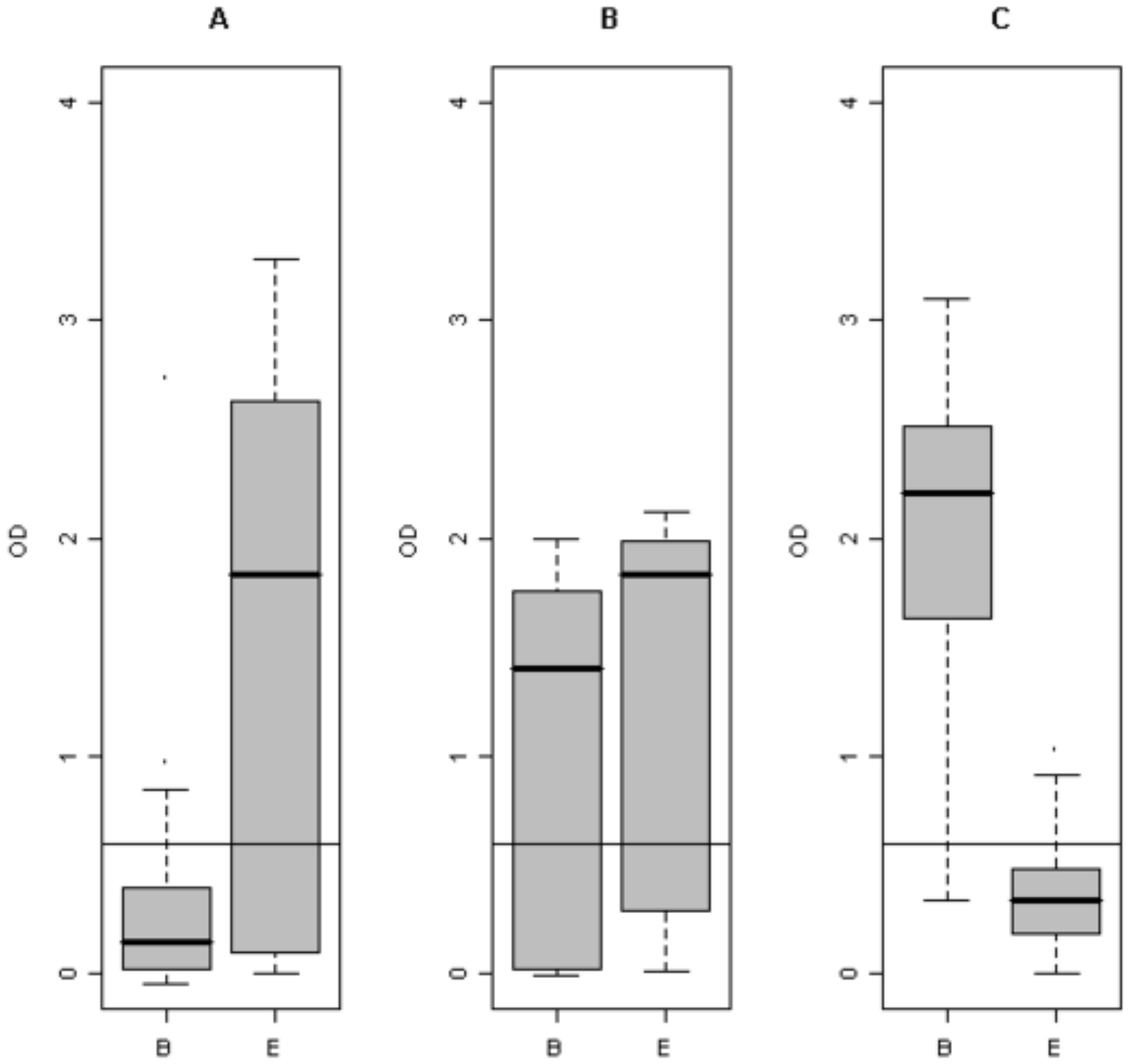
Figure 3



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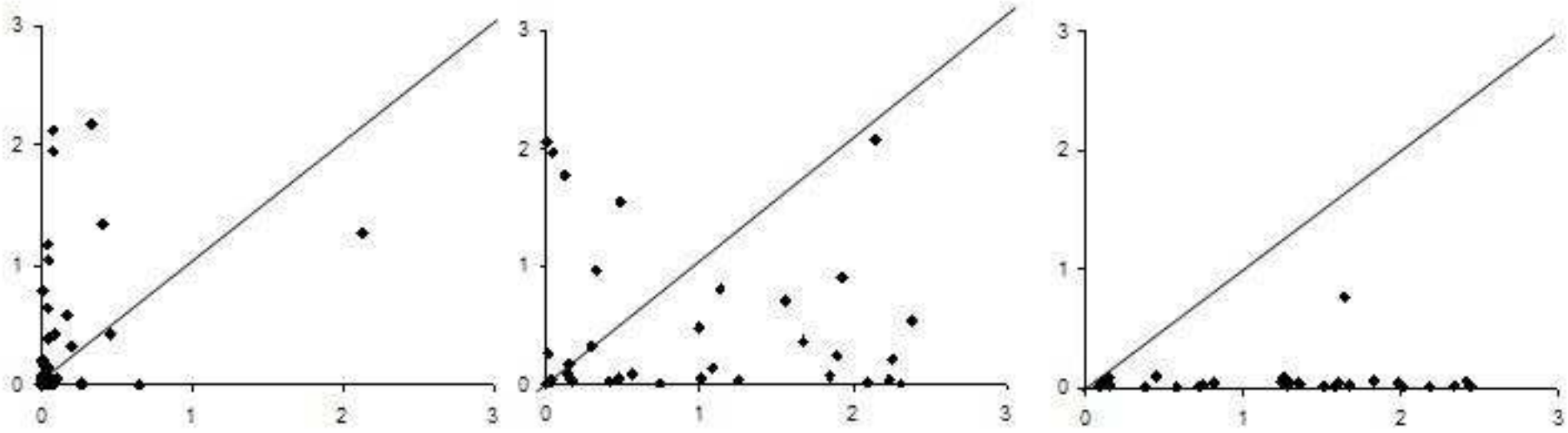

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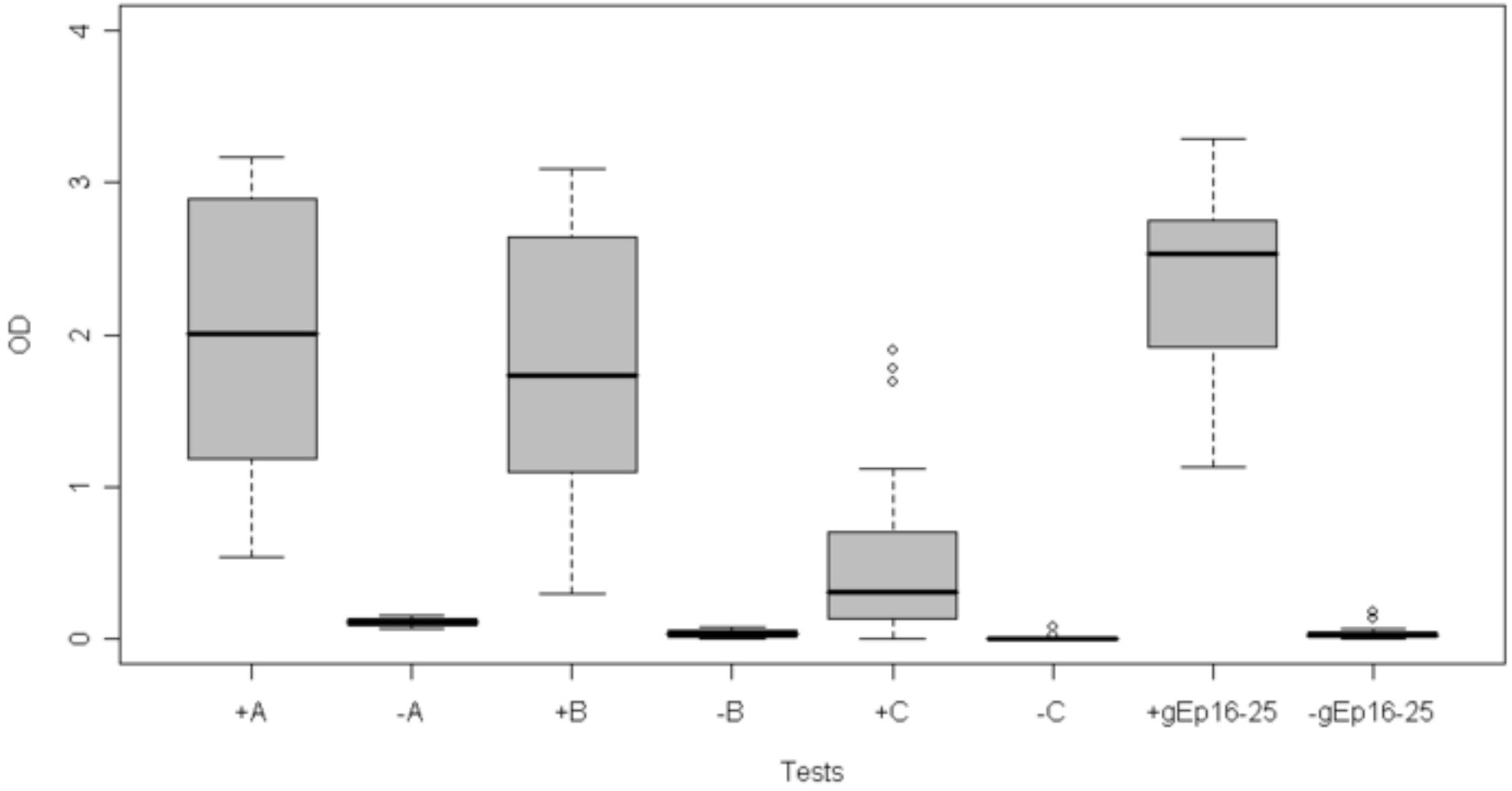

Figure 6a

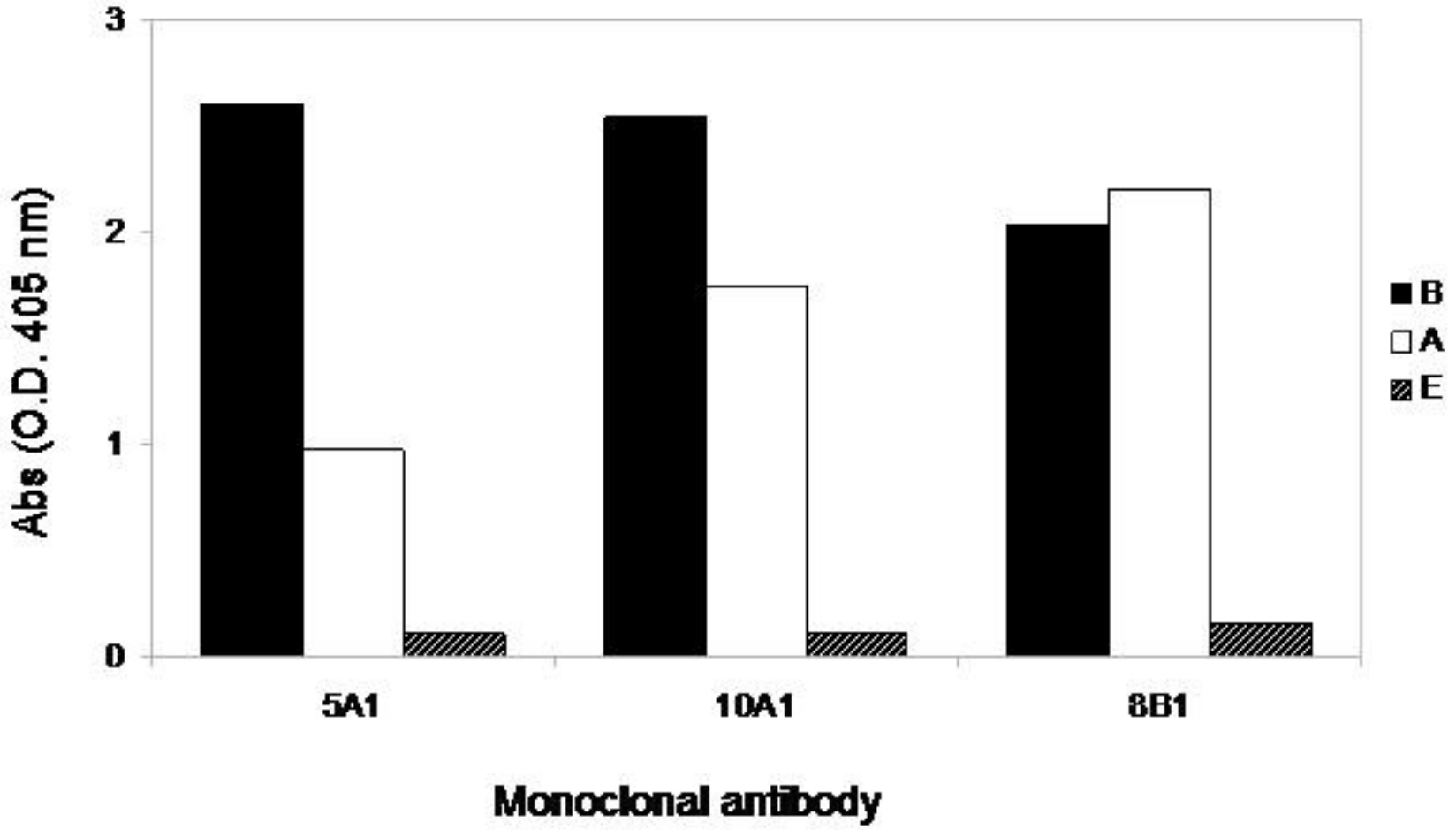

Figure 6b