

16 **Abstract**

17 Diagnostic tests for foot-and-mouth disease (FMD) include the detection of antibodies
18 against either the viral non-structural proteins or the capsid. The detection of antibodies against
19 the structural proteins (SP) of the capsid can be used to monitor seroconversion in both infected
20 and vaccinated animals. However, SP tests need to be tailored to the individual FMD virus
21 serotype and their sensitivity performances may be affected by antigenic variability within each
22 serotype and mismatching between tests reagents. As a consequence, FMD Reference
23 Laboratories need to maintain contingency to employ multiple type-specific assays for large-
24 scale serological surveillance and post-vaccination monitoring in the event of FMD outbreaks.
25 In this study, a highly conserved region in the N terminus of FMDV capsid protein VP2 (VP2N)
26 was characterised using a panel of intertypic-reactive monoclonal antibodies. This revealed a
27 universal epitope in VP2N which could be used as a peptide antigen to detect FMDV-specific
28 antibodies against all types of the virus. A VP2-peptide ELISA (VP2-ELISA) was optimised
29 using experimental and reference antisera from immunized, convalescent and negative animals
30 (n=172). The VP2-ELISA is universal, simple and provided sensitive (98.6 %) and specific
31 (93%) detection of antibodies to all FMDV strains used in this study. We anticipate that this
32 SP test could have utility for sero-surveillance during virus incursions in FMD-free countries
33 and as an additional screening tool to assess FMD virus circulation in endemic countries.

34

35 **Keywords:** FMDV, conserved capsid epitope, ELISA, diagnosis, serology.

36

37 **Introduction**

38 Foot-and-mouth disease (FMD) is an economically devastating viral disease of cloven-
39 hooved animals with a global distribution. It limits access to markets for developing countries
40 and outbreaks in otherwise FMD-free countries are expensive to control (as in the UK in 2001,
41 Japan in 2010 and the Republic of Korea in 2010 and 2011) [1, 2]. FMD virus (FMDV) is a
42 single-stranded, positive-sense, RNA virus belonging to the genus *Aphthovirus* in the family
43 *Picornaviridae*. The virus exists as seven serotypes (O, A, C, Asia 1, South African Territories
44 (SAT)1, SAT2 and SAT3) as well as numerous and constantly evolving strains showing a
45 spectrum of antigenic diversity.

46 The non-enveloped picornavirus capsid has icosahedral symmetry, a diameter of
47 approximately 30 nm and is composed of 60 copies of each of the capsid proteins VP1, VP2,
48 VP3 and VP4. VP1, VP2 and VP3 are the major components of the capsid, while VP4 is a
49 small (approximately (12 kDa) internal protein which lies on the inside surface of the capsid
50 around the five-fold axes of symmetry, where it is thought to stabilise interactions between
51 pentameric capsid subunits [3, 4]. During the replication cycle of FMDV, eight different viral
52 non-structural proteins (NSPs; and additional precursors) are generated which are potential
53 serological targets for diagnostic assays [5]. The presence of antibodies against NSPs can be
54 used to differentiate infected and vaccinated animals (DIVA) because such antibodies are only
55 produced by infection and are not elicited after administration with purified vaccines. In
56 addition, the inter-serotypic conservation of the NSPs means this type of test is compatible with
57 all serotypes of FMDV. Hence, NSP tests can be used as generic screening tools to support
58 national programs to attain the OIE status of FMD-freedom with or without vaccination [6, 7,
59 8]. However, the specificity of these tests is less than 100% [9] and testing algorithms that are
60 designed to confirm absence of FMDV circulation in large populations usually adopt screening
61 and confirmatory serological assays with covariant rates of false positivity [7, 8, 9]. In this

62 context, ELISAs that measure FMDV-specific antibodies directed at capsid structural proteins
63 (SP) are widely used to augment NSP tests for sero-surveillance activities [10, 11, 12, 13]. One
64 of the international standard tests for FMDV antibody detection is the virus neutralisation test
65 (VNT) [14]. However, the VNT is laborious, rendering large scale serological testing difficult.
66 In addition, the procedure requires live virus, thus confining the test to high containment
67 laboratories in non-endemic countries. SP ELISAs with high diagnostic sensitivity are also
68 available for certification of animals as free from FMD prior to import and export, for
69 serological confirmation of FMDV infection, for post vaccination monitoring and for the
70 demonstration of vaccine efficacy [14]. However, SP assays need to be tailored to individual
71 serotypes and as a consequence FMD Reference Laboratories need to maintain parallel assay
72 systems to accommodate the possibility of FMD outbreak due to different virus serotypes.

73 A number of monoclonal antibodies (mAbs) have previously been reported with cross-
74 reactivity against multiple FMDV serotypes [15, 16, 17]. The recognition sites for some of
75 these mAbs have been mapped to a highly conserved region at the N-terminus of VP2 [15, 16,
76 17]. In this study, a highly conserved region in the N terminus of FMDV capsid protein VP2
77 (VP2N) was characterised using a panel of cross-reactive mAbs. This revealed a universal
78 epitope in VP2N which has been investigated as a peptide antigen to detect FMDV-specific
79 antibodies in serum samples from animals infected or vaccinated with any of the FMDV
80 serotypes.

81

82 **Material and Methods**

83 **Cells lines and Viruses**

84 The IBRS-2 (pig kidney) cell line and the BHK-21 (baby hamster kidney 21) cell line, used
85 for FMD viruses propagation and immunoassays, were maintained either in Dulbecco's
86 modified Eagle's medium or in Minimum Essential Medium, (DMEM; Thermo-Fisher
87 Scientific, UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Thermo-
88 Fisher Scientific, UK) and 100 U of penicillin-streptomycin (Sigma) per ml. FMDV strains
89 used are indicated in each relevant paragraph.

90 **Peptides**

91 Peptides representing the N-terminal 15 (VP2N15), 30 (VP2N30) or 45 (VP2N45) amino
92 acids of FMDV VP2 were synthesised (Peptide Protein Research, UK) without modifications
93 except for the addition of 6 lysines at the C-terminus of the peptides to increase the solubility.
94 VP2N45 was used for the development of the peptide ELISA. A control peptide equivalent to
95 a capsid sequence from the related picornavirus human rhinovirus was used [18]. Eight
96 peptides (15mer each) overlapping by ten amino acids , covering the first 45 amino acids
97 from the N-terminus of the FMDV capsid sequence, were used for the fine mapping of the
98 epitope (Fig.1a).

99 **Serum samples**

100 Sera from infected cattle with FMDV O/UKG 34/2001 [19] was used to optimise the ELISA.
101 Reference sera from experimentally vaccinated or infected animals were supplied by FAO
102 World Reference Laboratory for FMD (WRLFMD, The Pirbright Institute). The parameters
103 of selecting serum samples were as follows: Negative (n=100): samples that been collected
104 from negative coherent country (during the UK 2007 outbreak). These samples are from non-
105 vaccinated animals and proved to be negative using NSP-ELISA. Positive (n=72): samples

106 that are known to be infected or vaccinated with FMDV. Selection of the positive samples
107 was based up on more than 7days post vaccination or infection to ensure a positive response.
108 See supplementary table (1) for more details.

109 **Production of mAbs**

110 The following FMD viruses were used as immunogens to produce mAbs in mice and for the
111 following selection of heterotypic cross-reactive mAbs: serotype A Malaysia 16/97, C1
112 Brescia 1964, Asia 1 Nepal 29/97, A24 Cruzeiro and O UK 31/2001.

113 For each immunogen, BALB/c mice were primed subcutaneously with 20µg of purified FMD
114 virus in Freund's complete adjuvant and boosted intraperitoneally with the same antigen in
115 phosphate buffered saline (PBS) once or twice at one-month intervals. Three days after the
116 last boost, mice were humanely sacrificed and hybridomas were generated by fusion of
117 splenocytes with NS0 myeloma cells following standardized procedures [20]. Briefly, at least
118 10^8 spleen cells were recovered from each mouse and fused with NS0 myeloma cells at a
119 10:1 ratio using PEG 4000. Fused cells diluted in Dulbecco's modified Eagle medium,
120 supplemented with hypoxanthine/aminopterin/thymidine and 20% fetal calf serum, were
121 distributed over five microplates (200µl per well). Growing colonies were observed in all
122 wells; in order to select hybridomas secreting monoclonal antibodies specific for the
123 immunogen, the supernatants were screened by trapping ELISAs against the homologous
124 virus strains. Selection of the inter-types cross reactive mAbs was based on results of the
125 trapping ELISA against the homologous and heterologous virus serotypes, as previously
126 described [21]. The selected hybridoma cells were cloned by limiting dilution in order to
127 obtain antibodies from one single cell. The supernatant from exhausted cultures was then
128 used as source of mAb.

129

130 **Immunofluorescence confocal microscopy**

131 IBRS-2 cells on 13-mm glass coverslips (VWR) were infected with FMDV type O1
132 Kaufbeuren (MOI = 2) for 3.75 hours and then washed with PBS and fixed with 4%
133 paraformaldehyde for 40 min at room temperature (RT). The cells were then permeabilized
134 for 20 min with 0.1% Triton X-100 prepared in blocking buffer (Tris-buffered saline
135 supplemented with 1 mM CaCl₂, 0.5 mM MgCl₂, 10% normal goat serum, and 1% fish skin
136 gelatin). The cells were then incubated with primary antibody (mouse mAb 4A3) diluted
137 1/1000 in blocking buffer for 1h at RT. Subsequently, the cells were washed and incubated
138 with Alexa-Fluor-conjugated secondary antibody (goat anti-mouse IgG Alexa-568; Thermo
139 Fisher Scientific, UK) in blocking buffer for 45 min at RT. After washing, the cells were
140 mounted using Vectashield mounting medium with DAPI (4,6-diamidino-2-phenylindole)
141 (Vector Labs) and the coverslips sealed with nail varnish. All data were collected sequentially
142 using a Leica SP8 confocal laser scanning microscope.

143

144 **SDS-PAGE and western blot**

145 Initial tests to verify the reactivity in western blot of each mAb with the homologous partially
146 purified strain were performed as previously described [21]. Later on, the cross-reactivity of
147 one representative mAb (4A3) with all FMDV serotypes was confirmed as follows.

148 Virus lysates from IBRS-2 cells infected cells with different FMDV serotypes were denatured
149 and reduced by heating at 95°C for 5min in Red Loading Buffer and DTT (NEB). The samples
150 were resolved through 12% Tris-glycine gels and transferred to nitrocellulose membrane
151 (0.45µM, GE Healthcare) using a Mini-Protean tetra cell (BioRad). Membranes were placed in
152 blocking buffer (20mM Tris, 150mM NaCl pH7.6 with 0.1% v/v tween-20 (TBS-T) with 1%
153 bovine serum albumin (BSA) w/v (Melford)) for 1h at RT followed by incubation with
154 hybridomas supernatants (mAbs) and anti-mouse HRP-conjugated secondary antibody (Dako)

155 (1/5000 in blocking buffer) in sequence for 1h at RT. Each incubation was separated by cycles
156 of three washings with TBS-T. West Pico chemiluminescent substrate (Thermo Fisher
157 Scientific, UK) was added to the membrane and exposures of the membrane were collected
158 and visualised using a G: Box Chemi XX6 (Syngene).

159 **Serological standard tests: virus neutralisation test (VNT), liquid-phase blocking**
160 **ELISA (LPBE), solid-phase competition ELISA (SPCE) and commercial kits**
161 **(PrioCHECK™ FMDV Type O, Type A and Type Asia 1 Antibody ELISA kits)**

162 VNT was carried out in microplates against 100 TCID₅₀ of the homologous or heterologous
163 viruses and results were reported as the final dilution required to neutralize 50% of the
164 inoculated cultures [14]. The LPBE and the SPCE were carried out as described by Hamblin
165 *et al.*, (1986) [12] and by Paiba *et al.*, (2014) [13] respectively. The cut offs used in the VNT
166 (log titre 1.65), LPBE (log titre 1.95) and SPCE (40% of inhibition) were according to the
167 standard operating procedures for the WRLFMD (The Pirbright Institute, UK). PrioCHECK
168 ELISAs for FMDV type O, A and Asia 1 antibody were carried out according to the kits
169 instructions, with 50% of inhibition as cut-offs.

170 The frequency distribution of values generated by various serological assays for the negative
171 and the positive (vaccinated and infected animals) serum samples were plotted using
172 GraphPad Prism (V7). Statistical analysis was performed using GraphPad Prism V7 for
173 Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

174 **Indirect ELISAs and the development of the VP2 ELISA**

175 Plastic 96-well plates (Maxisorp –Nunc) were coated with 100µl per well of the peptides in
176 0.05M standard carbonate/bicarbonate coating buffer (pH 9.6) at 4°C overnight. Different
177 peptides concentrations, ranging from 125ng/ml up to 4µg/ml, were initially evaluated for
178 test optimization. Wells were washed three times with phosphate buffered saline (PBS)

179 containing 0.1% Tween 20 (PBS-T) between all incubations. Wells were blocked with 200µl
180 blocking buffer (1% w/v BSA in PBS-T) at 37°C for 1h, and incubated either with 100µl of
181 mAb (hybridoma supernatants, 1/5) or bovine sera (diluted 1:50 to 1 in 400 in blocking
182 buffer) at 37°C for 1h. Antibody binding was detected by incubation at 37°C for 1h with
183 100µl of species specific HRP conjugated secondary antibodies (Dako), diluted in blocking
184 buffer 1:1,000 in case of anti-mouse Ig conjugate or 1:15,000 for the anti-bovine-Ig conjugate
185 . The chromogen development was mediated by the addition of 50µl of HRP substrate (OPD:
186 Sigma FAST, Sigma, UK). The reaction was stopped after 20min by addition of 50µl of
187 1.25M sulphuric acid and the optical density (OD) was measured at 490nm.

188 **Results**

189 **Characterisation of an FMDV-VP2 conserved epitope by cross reactive mAbs**

190 Among the multiplicity of mAbs generated from mice independently immunized with four
191 different FMDV serotypes (A Malaysia 16/97, C1 Brescia 1964, Asia 1 Nepal 29/97, A24
192 Cruzeiro, or O UK 31/2001), seven mAbs were selected because of their cross-reactivity with
193 the seven FMDV serotypes. All mAbs were characterised as non-neutralising. Five of these
194 mAbs strongly recognised the capsid protein VP2 by western blot and showed a weaker
195 reaction with VP0, while two mAbs reacted with P1 (Table 1).

196 Previous studies have identified the conserved N-terminus of VP2 as a site for recognition by
197 cross-reactive mAbs [15, 16, 17]. We therefore tested the reactivity of the seven mAbs
198 against peptides equivalent to the first 15 (VP2N15), 30 (VP2N30) or 45 (VP2N45) amino
199 acids of the N-terminus of VP2 from FMDV O1K (Fig.1a). The N-terminus of VP2 is known
200 to be most highly conserved within the first 15 amino acids. The five mAbs (4D1, 1D6, 4A3,
201 5B2 and 5F10) identified as VP2-specific by Western blots also reacted strongly with the
202 VP2 peptides in ELISA (Fig.1b). Among them, two mAbs (4A3 and 5B2) showed an

203 equivalent reactivity with the three peptides, while the three remaining mAbs recognized the
204 VP2N15 peptide with lower intensity (Fig.1b). The mAb 4A3 was taken forward for further
205 characterisation. In particular, fine mapping using 15mer peptides with 10 amino acids
206 overlaps (Fig.1a) showed that mAb 4A3 reacted with the 15mer peptide that corresponded to
207 the N-terminus of VP2 and not with a 15mer starting at amino acid 6, confirming the
208 presence of an epitope at the N-terminus of VP2 (Fig.1c). The mAb 4A3 specifically detected
209 a protein band in western blot of the expected size for VP2 in cell lysates from infections
210 with all 7 serotypes (Fig.1d) confirming that the epitope is linear, conserved and specific for
211 VP2. MAb 4A3 also recognised virus infected cells when used as the primary antibody in
212 indirect immunofluorescence microscopy of IBRS-2 cell cultures infected with type O
213 FMDV (Fig.1e).

214 **VP2N peptides detect antibodies in sera from animals infected with all serotypes of** 215 **FMDV**

216 An indirect ELISA using peptides VP2N15, VP2N30 or VP2N45 was used to assess the
217 presence of antibodies against the N-terminus of VP2 in a representative serum from an animal
218 infected with type O FMDV. All three peptides captured antibodies, with the longer peptides
219 producing a slightly higher signal (Fig.2a). A control peptide equivalent to a capsid sequence
220 from the related picornavirus human rhinovirus gave a low signal consistent with background.

221 The longer peptide VP2N45 was then used to test monovalent sera from different animals
222 vaccinated against the seven serotypes of FMDV; this showed that the same peptide was able
223 to detect antibodies against all the serotypes (Fig.2b).

224 **Development of a VP2 ELISA for universal detection of FMDV antibodies**

225 A VP2 ELISA using peptide VP2N45 was developed using reference sera. The optimal
226 concentration of peptide and dilution of sera to be used in the test was first evaluated by

227 checkerboard titrations using bovine sera known to be negative or strongly positive or weakly
228 positive for antibody by existing tests. The best signal to noise ratio (positive: negative) was
229 obtained using a serum dilution of 1 in 100 and peptide concentration of 2 μ g/ml (Fig S.1). At
230 these optimised conditions, the cut off for distinguishing between positive and negative
231 signals was set as 0.4 OD units, calculated using the average value of three independent tests
232 using the standard negative reference serum sample used by WRLFMD for routine FMDV
233 diagnostics.

234 Using the optimized assay conditions, a collection of previously characterized serum samples
235 was tested in triplicate and repeated twice independently, representing naïve cattle (n=100)
236 and cattle vaccinated (n=38) or infected (n=34) with all seven serotypes of FMDV. The
237 majority of vaccinated and infected (positive) samples gave a relatively strong signal
238 (average absorbance value of 1.4) and the majority of naïve (negative) samples gave a
239 relatively low signal below 0.4 (Fig.3a).

240 Seven negative sample exceeded the cut off of 0.4 OD units (ranging between 0.4 and 1.0
241 OD) and would be considered false positive, therefore producing a diagnostic specificity for
242 the test of 93%. The signal for one positive sample (type A vaccinated) was below this cut off
243 and would be considered a false negative in this test giving a sensitivity of 98.6%.

244 **Comparison of the VP2 ELISA with existing tests (VNT, LPBE, SPCE and** 245 **PrioCHECK)**

246 For the positive serum samples analyzed by VP2 ELISA in Fig.3a, pre-existing WRLFMD
247 data generated using established diagnostic tests was accessed retrospectively and used to
248 compare the performance of the VP2 ELISA. The pre-existing data was generated with four
249 tests: VNT to quantitate neutralising antibodies, LPBE, SPCE and PrioCHECK to quantitate

250 anti-capsid antibodies. The sensitivity of the VNT, LPBE and SPCE are dependent on close
251 antigenic match between reagents used (virus/antigen and antibodies) and the serum sample
252 being tested. Therefore, the data from VNT and LPBE were subdivided into groups carried
253 out with homologous (same virus used to vaccinate or infect the animal) or heterologous
254 (same serotype but strain different than those used to vaccinate or infect the animal) reagents.
255 The data obtained with PrioCHECK kits was only available for samples from infections with
256 serotypes O, A and Asia 1.

257 As mentioned above, the VP2 ELISA data (Fig.3a) contained a single false negative
258 equivalent to a sensitivity of 98.6%. In comparison, the homologous VNT data (n=37) had no
259 false negatives (sensitivity of 100%) while the heterologous VNT data (n=72) had a
260 sensitivity of 73.2% (Fig.3b and Table 2). Similarly, the homologous LPBE data (n=30) had
261 no false negatives (sensitivity of 100%) and the heterologous LPBE data (n=72) had several
262 false negatives (sensitivity of 93.0%) (Fig.3c and Table 2). The SPCE data (n=72) had a
263 single false negative (sensitivity of 98.6%) (Fig. 3d and Table 2) and the PrioCHECK data
264 (n=29) had two false negatives (sensitivity of 93.1%) (Fig. 3d and Table 2).

265 The single false negative sample (A Eritrea 3/98- 41dpv) in the VP2 ELISA was also a false
266 negative in both the heterologous VNT (log titer =1.04) and heterologous LPBE (log
267 titre=1.6), but was positive in homologous VNT (log titer of 2.06) and weakly positive in the
268 SPCE (52 % inhibition) and PrioCHECK (65 % inhibition).

269 Overall these results show that the VP2 ELISA detected antibody to all serotypes and the OD
270 values may provide an estimate of the level of antibodies. The sensitivity of the new test
271 resulted equivalent to or better than PrioCHECK kits and SPCE; sensitivity was significantly
272 higher than LPBE and VNT when such assays are carried out with heterologous reagents.

273 **Discussion**

274 This study describes the development of a novel assay for the detection of antibodies against
275 the FMDV capsid that can be used to test for seroconversion in infected or vaccinated animals.
276 The benefits of this assay are that FMDV-specific SP antibodies from all seven serotypes can
277 be detected without the requirement for individual specific antigen or antibody reagents that
278 are required for existing tests such as VNT, LPBE, SPCE.

279

280 This assay targets a capsid epitope at the N-terminus of VP2 that exhibits high sequence
281 conservation among all seven serotypes of FMDV. Cross-reactive mAbs and overlapping
282 peptides were used to show that the minimum sequence required for this linear epitope was
283 VP2-N 1-DKKTE-5. This is consistent with previous studies, where structures of the FMDV
284 capsid suggested that the N-terminus of VP2 is an internal component but may be flexible
285 allowing it to be present at the surface to contribute to antigenicity [22, 23, 24]. In addition, the
286 production of monoclonal antibodies to VP2 N-terminus in response to immunisation with
287 FMDV, suggested that capsid flexibility may expose some of the internal domains of the capsid
288 proteins to the surface enabling them to become antigenic sites [15,16, 17]. It has also been
289 reported that a purified recombinant 1AB (VP4/VP2) capsid protein was detected by antisera
290 against all seven FMDV serotypes, indicating that the VP4/VP2 protein contained a highly
291 conserved epitope. Peptides containing the VP2 N-terminal epitope were reactive with
292 antibodies against all seven FMDV serotypes and one (VP2N45) was selected as the basis of a
293 novel VP2 ELISA that was evaluated with a panel of reference sera from naïve (n=100),
294 vaccinated (n=38) and infected (n=34) cattle, representative of all the seven FMDV serotypes.
295 Results demonstrated that the VP2 ELISA detected antibody to all serotypes with a diagnostic
296 specificity of 93% and sensitivity of 98.6%. The sensitivity of the new ELISA was equivalent

297 to or better than existing tests, such as PrioCHECK kits and SPCE; sensitivity was significantly
298 higher than LPBE and VNT carried out with heterologous reagents.

299 The VP2 ELISA is suitable for detection of antibodies against the capsid of FMDV either post
300 vaccination or post infection. The capture antigen contains a universally conserved viral
301 epitope that is expected to be present on any isolate of FMDV, this ensures that the VP2-ELISA
302 is able to detect FMDV antibodies regardless of the viral strain. In contrast to the biological
303 reagents necessary in many other ELISA, the VP2 capture antigen is a synthetic peptide, greatly
304 facilitating standardisation, continuity of supply and reproducibility. More importantly, it does
305 not require the optimisation and re-validation when serum from antigenic distant strains needs
306 to be tested.

307 Serological testing is a suitable tool for FMD surveillance. Detection of NSP antibodies
308 currently offers the advantages of a DIVA and cross-serotype test. However, the VP2 ELISA
309 can be used as a complementary or confirmatory test to the NSP ELISA, which is especially
310 useful in obtaining FMDV free status after an outbreak. As for the NSP ELISA, the VP2 ELISA
311 can also be used as (1) a front-line sero-surveillance assay in areas which are normally free
312 from FMD without vaccination, (2) for areas conducting surveillance to achieve free from
313 vaccination status, and (3) at the point of import and export to confirm the freedom of animals
314 from FMDV antibodies. The test may also provide a simple approach for evaluating vaccine
315 efficacy in experimental and field trials, although additional studies would need to be carried
316 out to determine the cut-off that correlates to protection.

317 In conclusion, the results suggest that the VP2 ELISA developed for the detection of antibodies
318 to FMDV has potential applications as a rapid, simple and inexpensive test in the sero-diagnosis
319 of FMDV and in sero-surveillance programmes. Further validation and standardisation will be
320 required to confirm the potential benefits of the VP2 ELISA.

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325 BB/E/I/00007036)

326 **Conflict of interest**

327 The authors declare that there are no conflicts of interest.

328

329

330 **List of tables and figures**

331 **Table 1.** FMDV mAbs showing cross-serotype reactivity and viral protein (VP) specificity

332 **Table 2.** Comparative sensitivities of VP2 ELISA and other existing serological tests

333 **Fig 1. FMDV heterotypic-reactive mAbs recognise the N terminus of VP2. (a)**

334 Overlapping peptides representing the VP2 N-terminal 45 amino acids. The (K)₆ denotes to
335 addition of 6 lysine residues at the C-terminus of the peptide to increase peptide solubility.

336 **(b)** Peptide ELISA showing cross reactive mAbs recognise peptides equivalent to the N-
337 terminal 15 (N15), 30 (N30) and 45 (N45) amino acids of FMDV VP2. The N-terminal 45
338 amino acids of human rhinovirus VP4 (HRV-VP4) was used as negative control; peptides
339 concentration was 2 µg/ml. **(c)** mAb 4A3 epitope mapping (using peptides shown in panel a)
340 identifies the cross-reactive epitope at the N-terminus of VP2. **(d)** Reactivity of mAb 4A3
341 with capsid protein VP2 of all 7 serotypes in western blot. 4A3 mAb produced a clear intense
342 band for VP2 and a weaker reaction for VP0 **(e)** Immunofluorescence microscopy using mAb
343 4A3 to detect FMDV serotype O infected IBRS-2 cells.

344 **Fig 2. Sera from animals infected with any serotype of FMDV react with VP2 peptides**

345 **(a)** Reactivity of serum from an animal experimentally infected with FMDV serotype O with
346 peptides equivalent to the N-terminal 15 (N15), 30 (N30) or 45 (N45) amino acids of FMDV
347 VP2, or the N-terminal 45 amino acids of human rhinovirus VP4 (HRV-VP4, negative
348 control). **(b)** Reactivity of sera from animals vaccinated with vaccine strains of the seven
349 serotypes with the FMDV VP2N-45 peptide.

350 **Fig. 3. Testing reference negative and positive serum samples to detect the specificity**

351 **and sensitivity of the assay. (a)** Reactivity in VP2 ELISA (OD 490nm) of negative (black
352 squares, n=100) and positive (circles are infected, triangles are vaccinated, serotypes
353 represented by colours as indicated; n=72) reference sera. Peptide was at 2µg /ml and sera

354 diluted 1 in 100. **(b)** Distribution plots comparing results of positive sera tested with VP2
355 ELISA (same as data in a; cut-off of 0.4 OD) and with homologous (n=37) and heterologous
356 (n=72) VNT (cut-off = log titre 1.65).

357 **(c)** Distribution plots comparing results of positive sera tested with VP2 ELISA (same as
358 data in a; cut-off of 0.4 OD) with homologous (n=30) and heterologous (n=72) LPBE (cut-off
359 =log titre 1.95). **(d)** Distribution plots comparing results of positive sera tested with VP2
360 ELISA (same as data in a; cut-off of 0.4 OD), with SPCE (n=30, cut-off =40% of inhibition)
361 and PrioCHECK kits ELISA (n=29, cut-off =50% of inhibition).

362 **Supplementary Figures and tables**

363 **Fig S1. Optimisation of the peptide ELISA using different concentrations of peptide and**
364 **dilution of the serum of serotype O from infected animal. (a)** Checkerboard ELISA with
365 negative (0 d) and positive (42 d) sera diluted from 1:10 to 1:80 (as shown in key) and with
366 peptide concentration in the range 0.125-2µg/ml. The optimal conditions for signal to
367 background are highlighted with a box (2µg/ml of peptide and 1 in 100 serum dilution). **(b)**
368 Reactivity with VP2N45 peptide at 2µg/ml of different dilutions of a strong responder serum
369 sample (type C) and a weak responder serum sample (type SAT3). The asterisk denotes the
370 best conditions of peptide at 2µg/ml and sera diluted 1:100.

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Table 1. FMDV-specific mAbs showing cross-serotype reactivity, viral protein (VP) specificity.

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mAb ID	Parent virus	Trapping ELISA							Western blot
		O	A	C	ASIA1	SAT1	SAT2	SAT3	VP TARGET
5B2	A Malaysia 16/97	+	+	+	+	+	+	+	VP2 (+VP0)
4A3	C1	+	+	+	+	+	+	+	VP2 (±VP0)
5F10	Asia 1 Nepal 29/97	+	+	+	+	+/-	+	+	VP2 (+VP0)
4A10	A24 Cruz	+	+	+	+	+	+	+	P1
5A4	A24 Cruz	+	+	+	+	+	+	+	P1
4D1	O UK 31/01	+	+	+	+	+	+	+	VP2 (+VP0)
1D6	O UK 31/01	+	+	+	+	+	+	+	VP2 (+VP0)

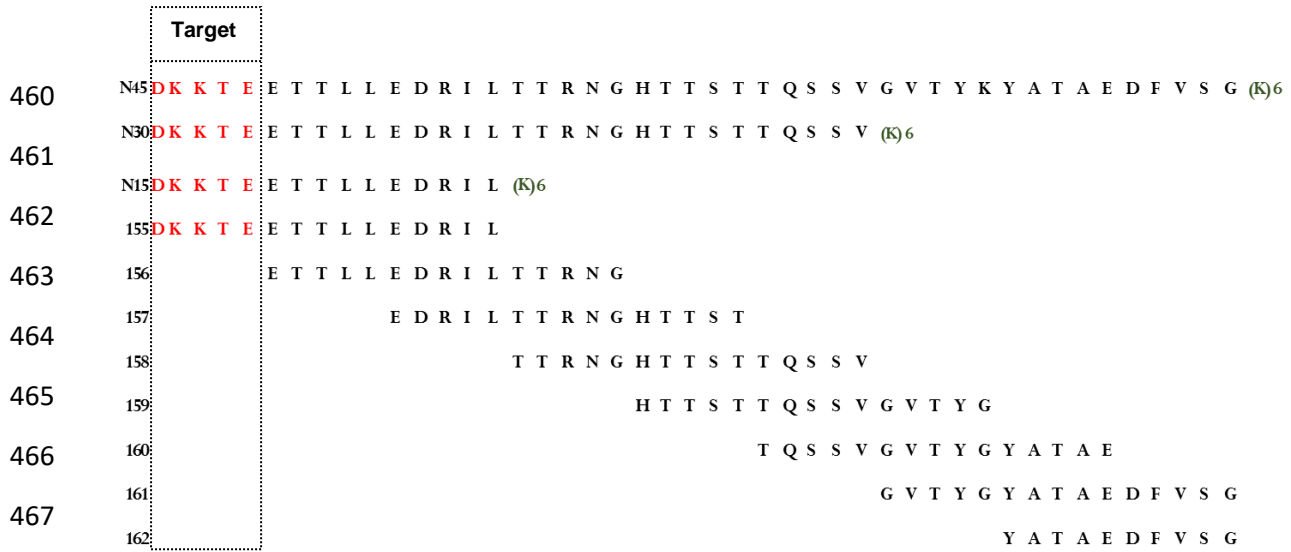
Table 2. Diagnostic sensitivity of the VP2 ELISA compared to other serological tests

	VP2-ELISA	VNT hetro	VNT homo	LPBE-hetro	LPBE-homo	SPCE-homo	PrioCHECK	Samples status
Total number of samples	72	72	37	72	30	72	29	Positive
% Sensitivity	98.59	73.23	100	92.95	100	98.59	93.1	
% Specificity	93	ND	ND	ND	ND	ND	ND	Negative

Fig.1 FMDV cross-reactive mAbs recognise the N terminus of VP2

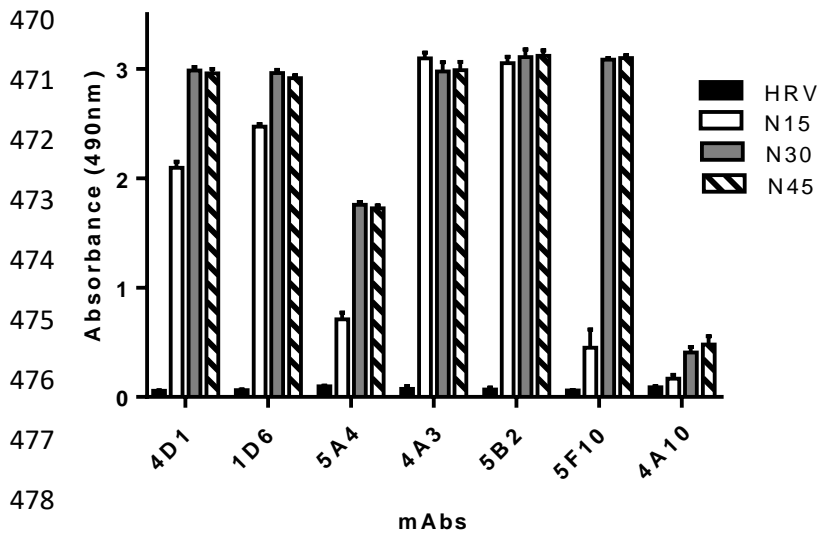
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(a)

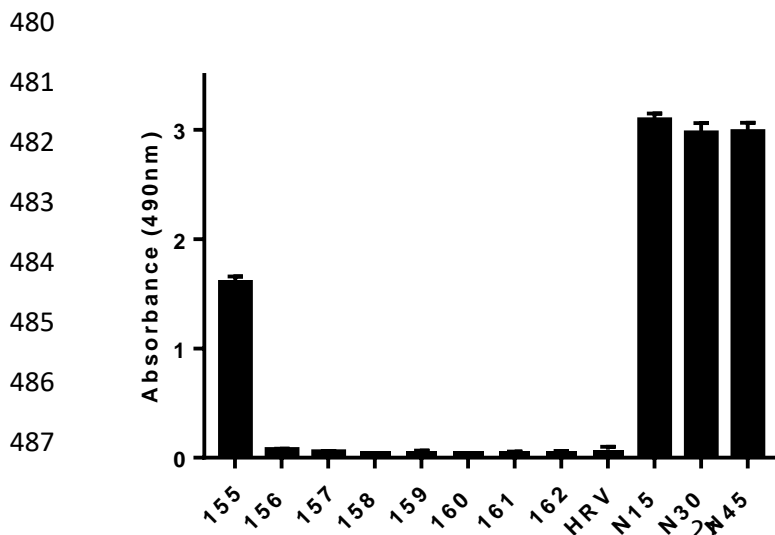


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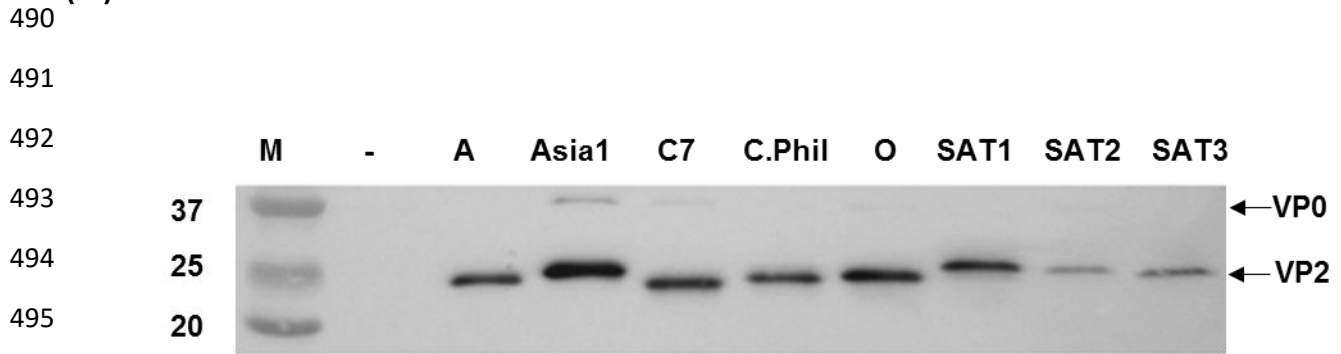
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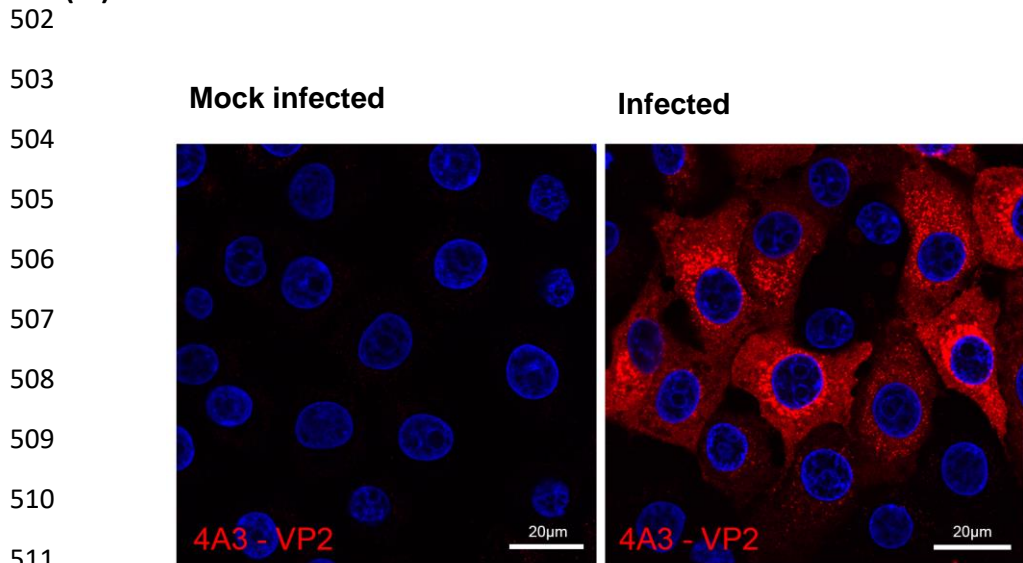
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488 **Fig.1 (continue)**

489 (d)



501 (e)



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Fig. 2. Sera from animals infected with any serotype of FMDV react with VP2 peptides

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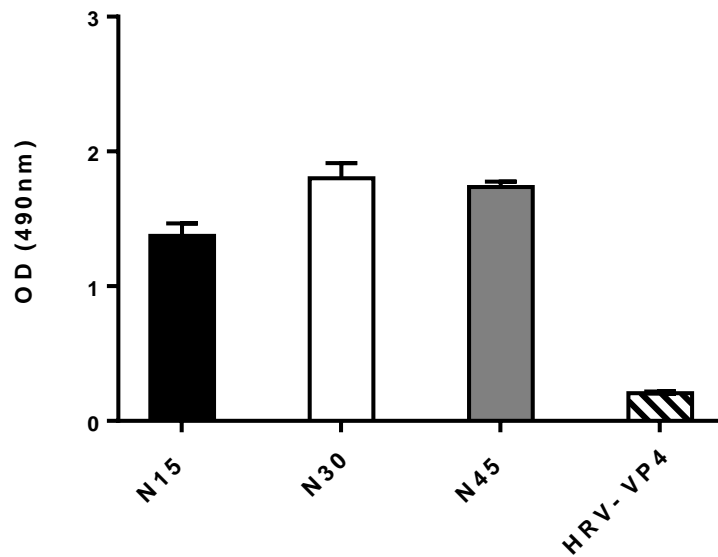
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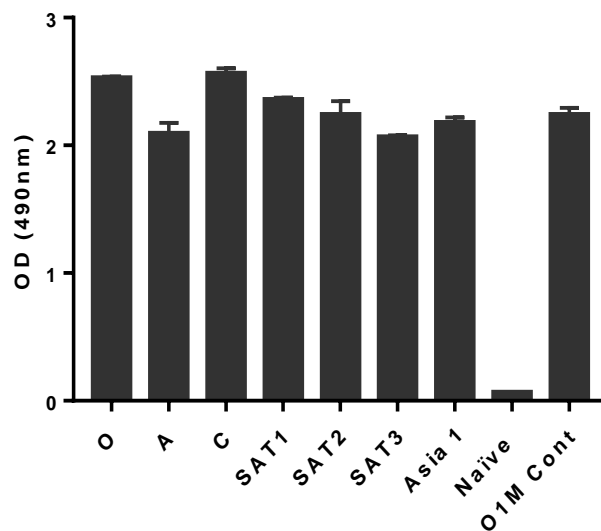
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Fig.3. Testing reference negative and positive serum samples to define the diagnostic specificity and sensitivity of the VP2 ELISA

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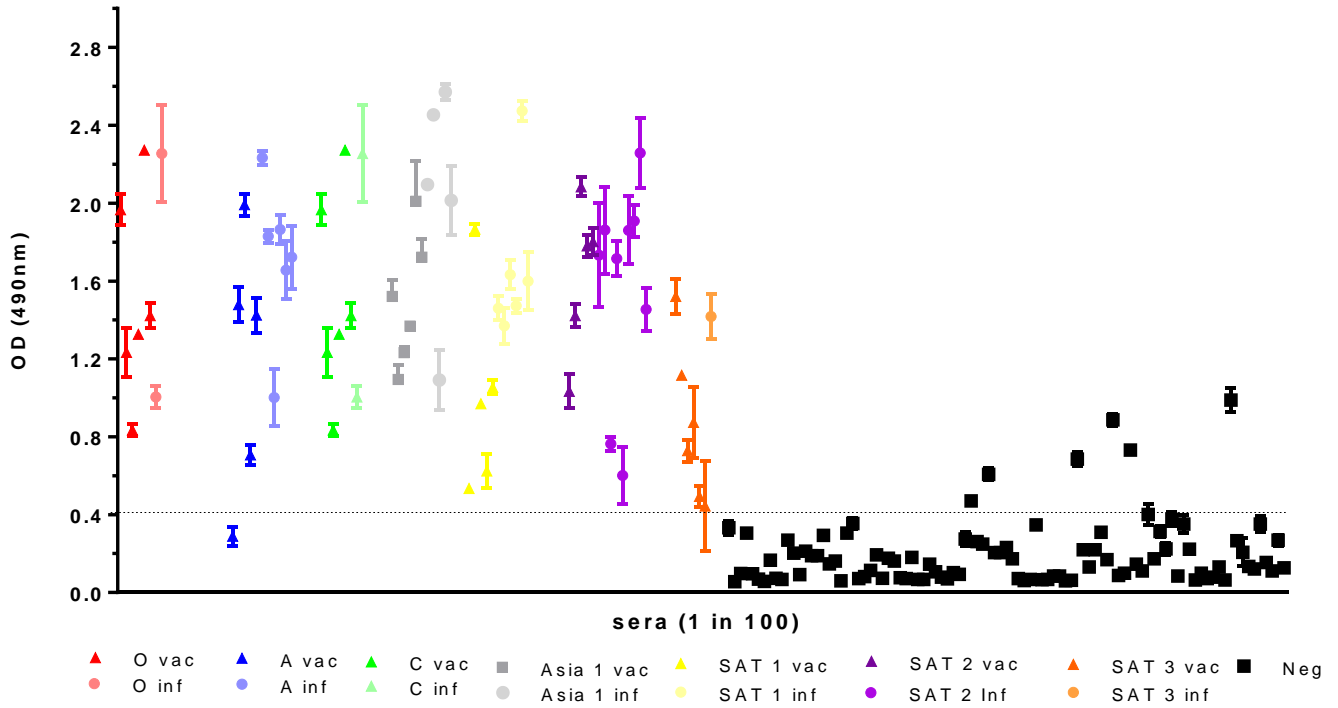
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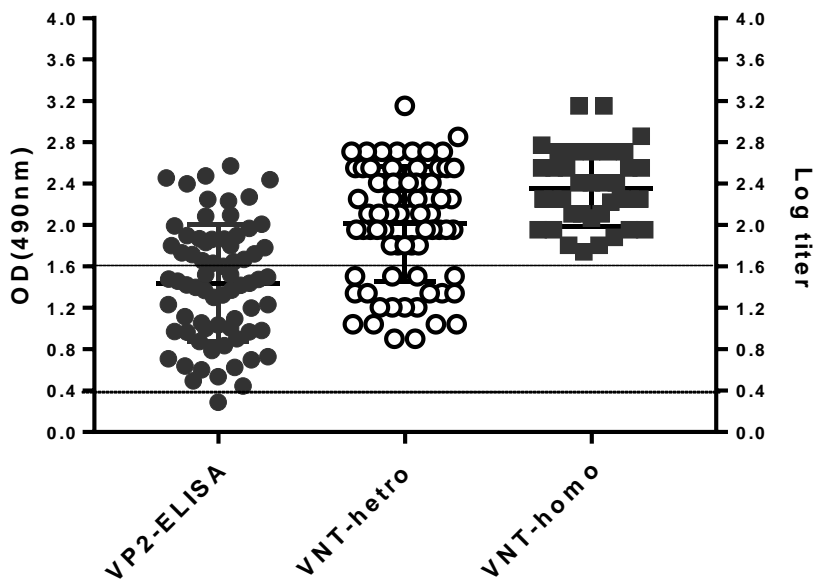
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(a)



(b)



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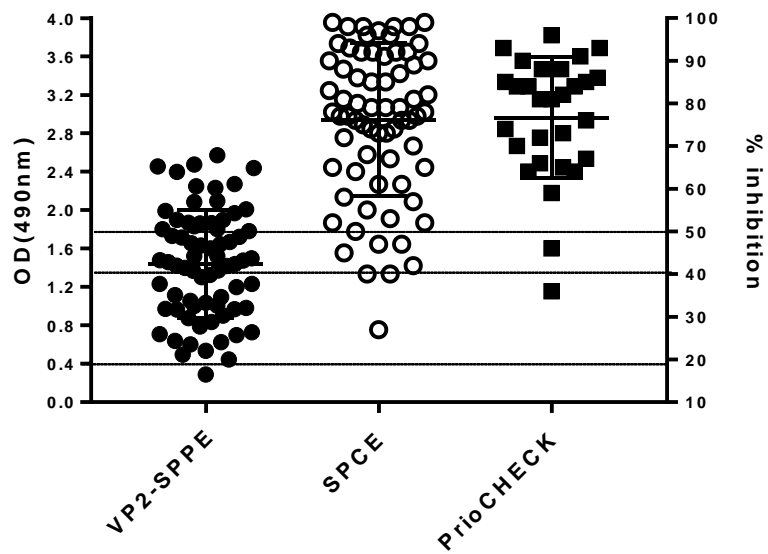
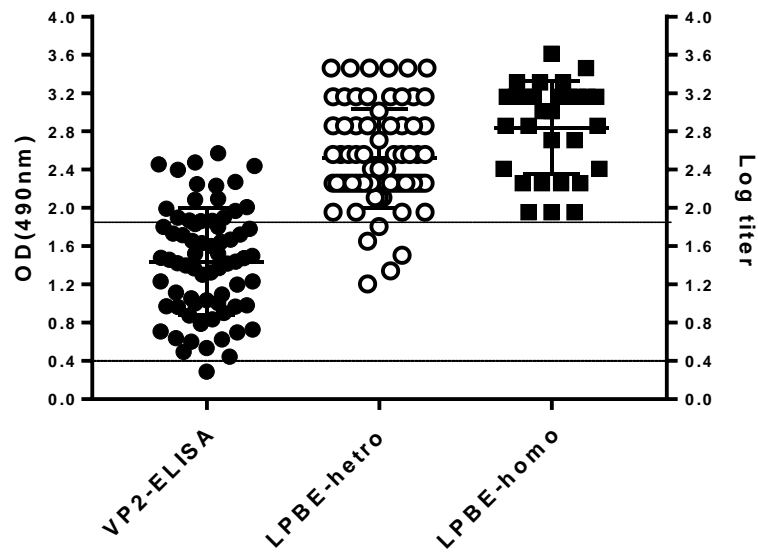
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588 **Supplementary figures and tables**

589 **Fig.S1. Optimisation of the peptide ELISA using different concentrations of**
 590 **peptides and dilutions of the serum**

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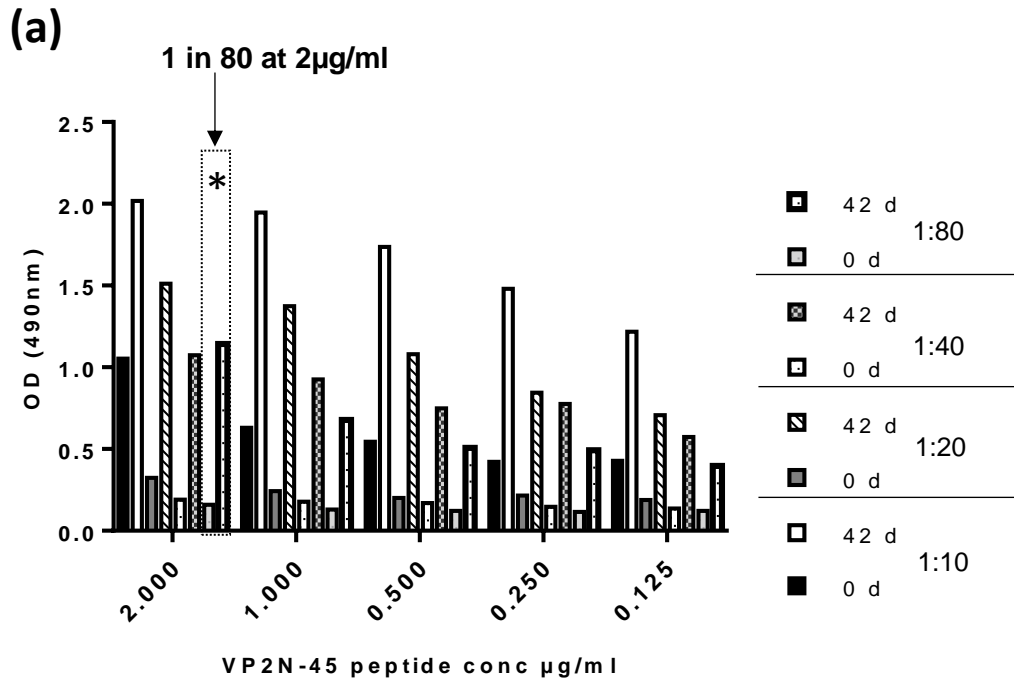
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(b)

