Surveillance of Infectious Bovine Rhinotracheitis in marker-vaccinated dairy herds: Application of a recombinant gE ELISA on bulk milk samples

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25 April 2024

The publisher's version is available at:
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Link to this full text:
http://hdl.handle.net/2318/1624395

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1 **Title:**

2 Surveillance of Infectious Bovine Rhinotracheitis in marker-vaccinated dairy herds: application of a recombinant

3 gE ELISA on bulk milk samples

4

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38

42   **ABSTRACT**

43   Infectious Bovine Rhinotracheitis (IBR) occurs worldwide, requiring significant resources for eradication programs

44   or surveillance purposes. The status of infection is usually detected by serological methods using the virus

45   neutralization test (VNT) or enzyme-linked immunosorbent assay (ELISA) on individual sera. The gE DIVA

46   (Differentiating Infected from Vaccinated Animals) vaccines approach, adopted in order to reduce the virus

47   circulation and prevent clinical signs, have tightened the range of available methods for the serological diagnosis.

48   Different gE blocking ELISA could be performed to detect specific antibodies in sera of infected or whole virus-

49   vaccinated animals but with less sensitivity if applied to bulk milk samples, especially in marker-vaccinated herds.

50   A new rec-gE ELISA was recently developed in Italy and applied with good performances on blood serum samples.

51   The present paper focuses on the application of a rapid protocol for purification/concentration of

52   immunoglobulin G (IgG) from bulk milk and on the use of the new rec-gE indirect ELISA. The study involved three

53   different partners and 225 herds (12800 lactating cows) with different official IBR diagnostic statuses. The

54   diagnostic specificity of the method was demonstrated closed to 100% while the diagnostic sensitivity was strictly

55   related to the herd-seroprevalence. Considering 2.5% as the limit of detection of within-herd seropositivity

56   prevalence, the diagnostic sensitivity showed by the proposed method was equal to 100%. A single reactivation of

57   a whole strain vaccine in an old cow was detected inside a group of 67 lactating cows, showing the field

58   applicability of the method.

59

INTRODUCTION

Infectious Bovine Rhinotracheitis (IBR) is a widespread animal disease caused by Bovine Herpesvirus (BoHV-1) type 1, with a substantial impact on animal health and livestock productivity (Raaperi et al., 2014). Upon primary infection, BoHV-1 replicates in the mucous membranes of either the respiratory or the genital tract. From there, it will gain access to local sensory neurons for establishment of latency in the corresponding ganglia, reactivating itself in the presence of stress, immunosuppression or treatments with corticosteroids (Ackermann and Engels, 2006; Winkler et al., 2000).

DIVA-vaccines, based on BoHV-1 glycoprotein E (gE) deleted strain, have been reported to be a safe and effective strategy against IBR that can be successfully used in countries with high prevalence of infection (Kaashoek et al., 1995).

Currently, different diagnostic tools are suitable for the surveillance of BoHV-1 infection. Evidences of viral circulations can be detected using the gold standard virus neutralization test (VNT), or indirect ELISAs based on whole-virus antigens. Diagnostically, specific ELISA protocols must be performed in herds where marker vaccines are used, to correctly differentiate between infected and vaccinated animals. Because gE deleted vaccines represent the most commonly used approach (Mars et al., 2001; Rijsewijk et al., 1999), serological investigations are based on the combination of a whole-virus-based indirect ELISA or a glycoprotein B (gB) based competitive ELISA with a gE -based competitive ELISA. An indirect ELISA test was recently developed based on the identification of anti BoHV1-gE antibodies (Bertolotti et al., 2015). In all previously mentioned cases the diagnostic approaches are based on blood serum and on the individual testing of the whole herds.

Bulk milk (BM) can represent a readily available sample matrix for screening tests. Indeed, the BM sampling procedure is non-invasive for the animals, avoiding the negative effects, such as stress, that an invasive blood sampling can cause (Reber et al., 2012); moreover, the lower expenses for farm visits, blood sampling procedures and necessary materials identify it as a valuable and inexpensive sampling method (Reber et al., 2012).

In Countries or regions where IBR eradication has already been achieved, surveillance can be easily performed by indirect ELISA test on BM samples. Unfortunately, if BM samples are tested by competitive gE ELISA when vaccination is still ongoing, IBR diagnosis can feature limited sensitivity (Kramps et al., 2004). Even if individual milk was shown to be a reliable biological matrix for diagnosis of BoHV-1 (Schroeder et al., 2012), blocking ELISAs

87   can prove unable to detect the seroconversion caused by an IBR wild strain infecting a vaccinated herd because of

88   the required dilution of the samples and the lower concentration of antibodies in milk. Some methods for

89   improving the sensitivity of ELISA tests were recently proposed (Schroeder et al., 2012) based on the

90   concentration of immunoglobulins from the milk.

91   In this paper we describe a cost-effective approach for IBR surveillance in vaccinated herds. Immunoglobulins (Ig)

92   in the BM samples are purified, concentrated and subsequently tested with a new indirect ELISA test based on the

93   reactivity against the BoHV-1 gE (Bertolotti et al., 2015). In particular, the recombinant indirect gE ELISA was used

94   in association with a rapid protocol for the concentration and purification of IgG from BM samples. The aim of this

95   study was the evaluation of the performances of this new serological method on samples collected from three

96   Italian Provinces (Turin, Cuneo and Trento).

97

98   **MATERIALS AND METHODS**

99   *Purification and concentration of bulk milk IgG*

100   A protein G (pG) based affinity matrix was prepared following a standard recombinant approach. Briefly, the

101   Streptococcal pG gene subunit, lacking the albumin-binding region, was cloned into pGEX-6His, expressed as

102   GST/pG fusion protein and purified under native condition by immobilized metal ion affinity chromatography

103   (Smith and Johnson, 1988). The purified protein was covalently bound to agarose beads using low density ABT

104   glyoxal resin (Agarose Bead Technologies MADRID, Spain) following manufacturer's recommendation and at 6mg

105   of GST/pG per ml of settled gel ratio. Following bond stabilization, the affinity matrix was washed with PBS and

106   stored as 50% slurry in 25mM phosphate buffer (pH 7.0) 20% ethanol at $4^{o}C$.

107   The purification and concentration protocol was applied to each BM adapting an immunoprecipitation protocol,

108   with minor modification (Harlow and Lane, 1988). Briefly, 10ml of BM were subjected to rennet-based casein

109   precipitation. After curd breaking and 5 minutes of incubation on ice, the sample was centrifuged at 3600g for 10

110   minutes at 4°C. Following the separation from lipids and curd, about 6ml of milk whey were decanted into a new

111   tube and incubated with 100µl of 50% affinity matrix for 10 minutes at room temperature under gentle agitation.

112   The adsorbed matrix was centrifuged and the pellet was loaded into a mini spin column. The matrix was washed

113   twice (Tween 0.05%, EDTA 0.5 mM, NaCl 160 mM) and the IgG were eluted in 200µl of 0.1M glycine buffer pH 2.8

114 onto a collection tube preloaded with 1/10 volume of Tris 1M, pH 8.2. A Bradford quantification assay was

115 performed to confirm presence of at least 1.5 mg/ml of IgG in the purified/concentrated (PC) eluted sample, using

116 Bovine Gamma Globulin (BIORAD Quick Start) standard curve. Only samples with a sufficient IgG concentration

117 have been considered suitable for testing in the indirect ELISA. Moreover, the effectiveness of purification was

118 initially confirmed in a consistent subset of samples by SDS-PAGE.

119

120 *Indirect gE ELISA*

121 In a previous work, we developed and validated an indirect ELISA assay based on the reactivity of bovine sera

122 against BoHV-1 glycoprotein E (gE) expressed in recombinant form. Briefly the ectodomain of gE was cloned into a

123 mammalian expression vector and expressed as secreted protein in protein free medium as described (Bertolotti

124 et al., 2013). The test showed very good performances, especially in terms of specificity. Compared to the original

125 protocol, the ELISA assay was slightly modified and adapted to milk samples. Briefly, each sample, represented by

126 purified/concentrated bulk milk (PC-BM) was diluted 1:2 directly in plate in PBS 1.25% casein and placed into two

127 adjacent wells, the first coated with the recombinant BoHV-1 gE while the second one with a negative antigen.

128 The plates were incubated at room temperature for 60 minutes. After four washing cycles, a peroxidase labeled

129 secondary antibody, diluted at 10 ng/ml in PBS 1.25% casein, was added to each well. After 45 minutes of

130 incubation at room temperature, four washing cycles were performed before the addition of the substrate

131 solution (3,3',5,5'-tetramethylbenzidine, TMB). The reaction was stopped with 0.2M H_2SO_4 after 15 minutes at

132 room temperature, and the absorbance value determined at 450 nm. The net reactivity (net optical density, or OD)

133 was calculated for each sample as the difference between the absorbance of the gE and negative antigen wells.

134 The results were expressed as the percentage of reactivity against the net OD of the positive control included in

135 each plate.

136 Considering that samples represent a pool of individual milks, we modified the cut-off of the test compared to the

137 previous work as suggested (Böttcher et al., 2012). The samples with a reactivity percentage greater than 40%

138 were classified as positive, those with reactivity percentage between 30% and 40% as doubtful, and those with a

139 reactivity percentage lower than 30% were considered negative.

140

141 *Evaluation of analytical and diagnostic performances*

142 A selection of 7 individual milk samples belonging to IBR-positive farms was used to evaluate the efficacy of IgG

143 purification/concentration method in rec-gE ELISA protocol. The purified/concentrated milk samples were

144 obtained as described above. Twofold dilution of each sample was tested by rec-gE ELISA and compared to the

145 reactivity of the corresponding untreated milk. The yield was defined as the dilution showing the same reactivity

146 of the untreated sample.

147 The limit of detection of the proposed method was estimated using spiked samples obtained diluting an IBR

148 positive individual milk in increasing number of negative individual milks. Each test was performed in triplicate.

149 Sensitivity and specificity values were calculated considering the official IBR status as the gold standard and the

150 95% confidence intervals of these parameters were calculated by using Exact Binomial test with R statistical

151 software (R Core Team, 2015).

152

153 *Milk samples*

154 Bulk milks were collected from dairy herds of three Italian Provinces (Turin, Cuneo and Trento), where IBR

155 surveillance and eradication programs have been in effect since 2004 (Turin and Cuneo) and 2002 (Trento). Milk

156 samples included a total of 225 dairy farms covering all different official IBR statuses.

157 Three different sets of BM were used in order to evaluate the performances of the proposed diagnostic protocol.

158 Details about farm numbers and IBR statuses are reported in Table 1.

159 The Department of Veterinary Science of the University of Turin (DVS) conducted a preliminary validation based

160 on laboratory tests and field data. The field sample set included BM collected from 76 herds belonging to the

161 Turin province corresponding to 4784 lactating cows. In case of unexpected results (i.e disagreement with official

162 IBR status) further analyses were conducted at individual level, including milk and blood sampling.

163 A larger field application was conducted in the second part of the study, involving three different partners: the

164 Department of Veterinary Sciences of the University of Turin (DVS), the Official Diagnostic Laboratory *Istituto*

165 *Zooprofilattico Sperimentale di Piemonte Liguria e Valle d'Aosta* (IZSPLV) in Turin, and the *Istituto Zooprofilattico*

166 *Sperimentale delle Venezie* (IZSVe) in Legnaro. A total of 149 farms were included in this phase, for a total of 8016

167 lactating cows.

168 In all cases data about the farm's official IBR status (evaluated at the same time of milk collection or based on the

169 most recent official serological investigation) and the number of lactating cows were collected. Unfortunately, the

170 ear tag identification numbers, useful for the estimation of seroprevalence, were available only during the

171 preliminary validation.

172

173 **RESULTS**

174 *Evaluation of analytical sensitivity*

175 The reactivity of the PC milks was compared to the reactivity of the corresponding untreated sample. In all seven

176 cases the PC milks diluted 1:16 showed higher reactivity than the untreated one (fig. 1). The average yield of the

177 PC protocol was identified as 27.3x. On the other hand, spiked samples are classified as IBR positive if positive

178 individual milk is diluted in 39 negative samples. This result suggests the theoretical prevalence of 2.5% in

179 lactating cows as detection limit (i.e. diagnostic sensitivity) of the method (fig. 2).

180

181 *Field application*

182 The first set of PC-BM were collected from 76 herds from the Turin province (30 IBR-positive herds, 29 IBR-

183 marker-vaccinated farms, and 17 to IBR-free farms).

184 The results for the positive herds were strictly dependent on the intra-herd prevalence (Fig. 3).

185 Among the 30 IBR-positive farms, 14 have an estimated internal prevalence lower than 2,5%: thirteen of them

186 resulted negative while a single herd, whose data on the intra-herd prevalence had not been updated, showed

187 full-scale reactivity due to a recent BoHV-1 viral circulation. Of the remaining 16 positive herds, 15 PC-BM showed

188 a positive outcome in the rec-gE ELISA test, reflecting the true infectious status; just one farm, where a few

189 animals had been vaccinated many years before using a whole-virus strains, resulted negative.

190 Regarding the 29 IBR-marker-vaccinated farms, all their PC-BM showed negative outcomes in the rec-gE ELISA.

191 Among the 17 farms classified as IBR-free, 16 PC-BM were tested as negative, confirming their negative

192 serological status for BoHV-1 gE. The case of the remaining herd that recently acquired "IBR-free" status was

193 more interesting and further investigated, because its PC-BM showed full-scale reactivity in the rec-gE ELISA test.

194 A new BM and 67 individual milk samples were collected a month later and tested by rec-gE ELISA. The new PC-

195   BM confirmed the first positive outcome. Only a single individual milk sample showed a high reactivity, while the

196   remaining milks were negative. Epidemiological investigation, as well as individual blood serum tests (IDEXX IBR

197   gE ab ELISA), confirmed a seroconversion in this animal due to reactivation of whole-virus vaccine administered

198   more than 10 years before (fig. 4).

199   A second sample set included BM from 86 farms belonging to the provinces of Turin and Cuneo and 63 from the

200   province of Trento.

201   Among the positive farms (n=21), 3 samples resulted negative. Further investigations confirmed a seroprevalence

202   lower that 2.5% among lactating cows in two farms, and the presence of a single whole-virus vaccinated animal in

203   the last farm.

204   Only 2 out the 128 expected negative farms showed a positive results in rec-gE ELISA. In both cases, the BM

205   included a very small number of lactating cows (5 and 9 animals respectively).

206

207   *Evaluation of diagnostic specificity and sensitivity*

208   Performances of the proposed method were evaluated. Diagnostic specificity of the test was high (dSp = 98.85%

209   95CI: 95.90% - 99.86%) whereas the diagnostic sensitivity is strictly related to the seroprevalence in lactating cows.

210   As expected, the antibodies of few positive animals diluted in a large number of negative milks are not always

211   detectable by the proposed test. However, when antibody titer increases because of a viral circulation as well as a

212   whole virus vaccine reactivation, BM can be a very good sample for IBR surveillance. Indeed, the 2.5% as limit of

213   prevalence was previously suggested as possible limit of detection in IBR positive farm (Casarin et al., 2016), field

214   data suggested good performances of the test if used for surveillance purposes in gE negative herds. However,

215   considering 2.5% as the limit of detection of within seropositivity prevalence (Casarin et al., 2016), the diagnostic

216   sensitivity showed by the test is equal to 100% (95%CI: 89.4% - 100%).

217

218   **DISCUSSION**

219   Blocking gE ELISAs were demonstrated to be less sensitive if milk is used as diagnostic matrix due to the lower

220   concentration of antibodies (0.6 mg/ml) compared to blood (10 mg/ml) (Król et al., 2010; Marnila and Korhonen,

221   2002). Indeed, Wellenberg and colleagues demonstrated that a commercial blocking gE ELISA is able to detect a

222 minimum prevalence of 10-15% in a bulk milk sample (Wellenberg et al., 1998). Obviously, the purification and

223 concentration of IgG from bulk milk can overcome this limitation. Moreover, the use of an indirect ELISA could

224 represent a possible diagnostic solution.

225 The aim of this study was the evaluation of the performances of a new diagnostic method in the detection of

226 antibodies against BoHV-1 gE in bulk milk samples.

227 Currently, the directive no. 1964/432/EEC of the European Union allows the use of commercial ELISA for Brucella

228 and Enzootic Bovine Leukosis antibodies research in bulk milk of 100–200 cows. In the case of IBR, according to

229 decision n. 2004/558/EC, to date, the largest bulk pool size of the licensed kits corresponds to 50 cows. This

230 approach can be easily applied to IBR-free farms using BHV-1 ab indirect ELISA (as IDEXX Trachitest Serum

231 Screening Ab Test). In fact, antibody response against several structural proteins may achieve the expected

232 sensitivity and may lead to the detection of a few positive animals. In contrast, the IBR surveillance in marker-

233 vaccinated animals is more complex. The anti-gE response investigated using the blocking ELISA approach can

234 suffer in sensitivity (Kramps et al., 2004) and it may not fit with EU regulations. Moreover we cannot exclude that

235 IgG concentration may negatively affect the blocking ELISA specificity due to the steric hindrance caused by the

236 high antibodies concentration: indeed the role of antibodies hindrance was highlighted in hyper-vaccinated

237 animals tested by blocking ELISA, showing false positive results (Kramps et al., 2004). In those farms IBR

238 surveillance is possible only by individual blood serum testing. In this framework, the proposed approach

239 including the IgG concentration protocol and the rec-gE ELISA can solve these drawbacks and can help in IBR

240 surveillance.

241 Despite the fact that different Italian regions have undertaken voluntary or compulsory programs to eradicate IBR,

242 the Province of Bolzano is the only one that has achieved this goal. The milk sampling procedures are easier and

243 cheaper than the collection of blood serum. For this reason the method described here could be employed in the

244 programs for the control of vaccinated and IBR-free herds.

245 In the first validation, we evaluated the field applicability of the method. The ELISA cut-off was slightly modified

246 (40% instead of 80% of the reactivity of positive control) and the method's limit of detection in terms of

247 seroprevalence among the lactating cows was identified as 2.5 % (equivalent to a single infected cow in a pool of

248 40 animals). Indeed, the sensitivity of the newly proposed method seems to be mainly affected by the within-herd

249   prevalence. Details on the prevalence of IBR-positive animals within each farm revealed the presence of few

250   positive individuals among the lactating cows and suggested the 2.5% as the seroprevalence limit detected by this

251   method. The same limit in detecting positive animals in milk pool was recently identified (Casarin et al., 2016). No

252   false positive results were obtained in this phase; in contrast, the method was actually able to identify a single

253   vaccine reactivation within a farm officially classified as IBR-free, supporting its helpfulness during IBR surveillance.

254   We further investigated the epidemiological situation of that farm, collecting individual milk samples from

255   lactating cows, detecting a single positive animal in a group of 67. This animal was vaccinated with a live whole-

256   virus in 2003, with a consequent lifelong latency of the vaccine strain in the spinal ganglia. To confirm the

257   seroconversion, a commercial gE blocking ELISA test was performed on blood serum from this animal. The

258   positive outcome was confirmed, as well as the absence of successful virus excretion in the herd, considering that

259   all the remaining 66 animals were still negative to the same test one month after the first positive outcome.

260   In the second part of the study, out of the 21 IBR-positive farms only three resulted as negative but the lack of

261   detailed information about the IBR status of lactating cows that were part of those pools made it impossible to

262   evaluate the real seroprevalence of those samples. All the vaccinated herds (n=106) were correctly classified.

263   Among the 22 IBR-free herds, only two resulted positive. The two cases were similar both for the number of

264   lactating cows (9 and 5 respectively) and for the presence of a single doubtful milk sample diluted in a limited

265   number of negative samples. We speculated that this combination could lead to false positive results.

266

267   **Conclusions**

268   Bulk milk sample testing is a non-invasive method that is not stressful for the animals. Moreover, milk samples are

269   taken routinely during milk quality assurance programs, so the use of the same sampling infrastructure would be

270   extremely helpful in providing a cost-effective way of monitoring herd status (Reber et al., 2012). Financial

271   comparison between the two methodologies was possible in Switzerland, where the costs were reduced by 50%

272   without any major impact on the quality of the surveillance program (Reber et al., 2012). One limitation of bulk

273   milk as diagnostic matrix is that it only represents the cattle delivering milk to the bulk tank on the day of

274   sampling, therefore excluding any non-milking heads such as dry cows, diseased cows and cows in the colostral

275   period. The use of milk samples can however increase the flexibility of the surveillance programs, for example by

276   increasing the intensity of surveillance in case of disease events or increase in the early detection capabilities of

277   the survey design. The main limitation experienced in this work was the impossibility to calculate beforehand the

278   prevalence within each bulk milk sample. To overcome this limit, a further investigation is taking place, through

279   an important collaboration with the sanitary authorities and the Regional farmers association. The evaluation of

280   the ideal pool sample size should be conducted as previously suggested (Schroeder et al., 2012). As demonstrated

281   in this work, pooled milk from few samples is more susceptible to a false positive result in rec-gE ELISA test;

282   conversely milk pools comprising around 40 animals represent the best target sample for this test. A more robust

283   evaluation of the field applicability of this method could be performed, supported by the suggestions described

284   above, to confirm the preliminary limit of detection of 2,5%. Nevertheless, this work represents an important first

285   step towards a validation pathway of an innovative serological assay to detect antibodies against BoHV-1 gE.

286

287   **Abbreviations**

288   IBR: Infectious Bovine Rhinotracheitis

289   DIVA: Differentiating infected from vaccinated animals

290   Ig: immunoglobulins

291   BM: bulk milk

292   PC: purified/concentrated

293   PC-BM: purified/concentrated bulk milk

294

295   **Acknowledgements**

297

298   Table 1. Description of BM collected during the study.

|	Laboratory	Positive farms	IBR-free farms	IBR marker vaccinated farms
Preliminary validation	DSV	30	17	29
Second validation	DSV IZSPLV	5	22	59
	DSV IZSVe	16	4	43
		51	**43**	**131**

299

300

301   Figure 1. Estimation of analytical sensitivity. Mean reactivity of 7 untreated individual milks (nt, white circle)

302   compared to corresponding PC samples (black circles) at different dilutions. Vertical bars: standard deviation.
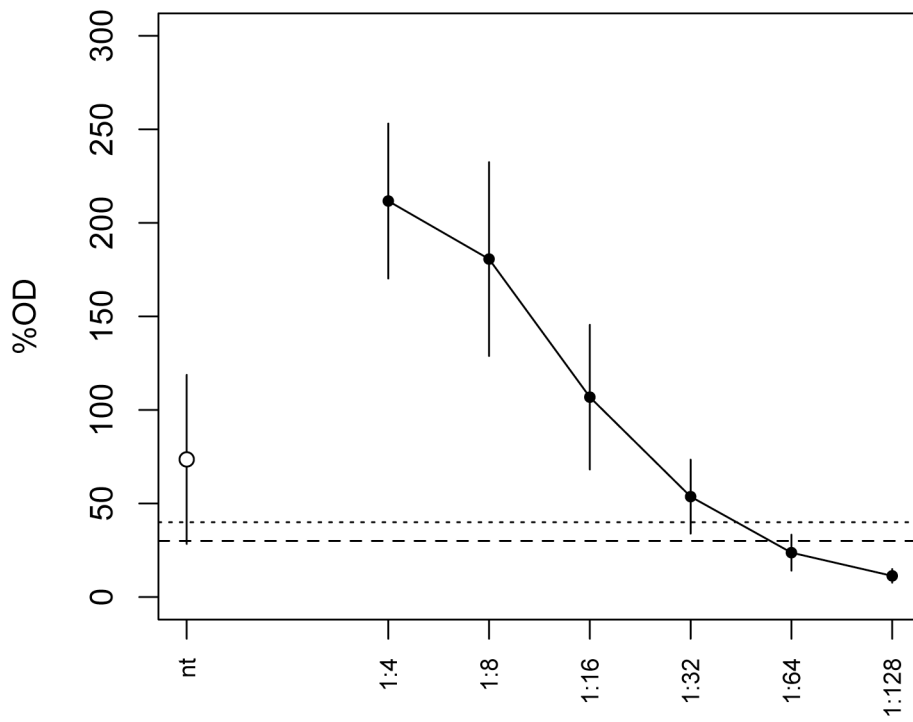

303

304

305 Figure2. Estimation of the analytical sensitivity by testing the reactivity of spiked milks. Individual milk from a IBR

306 positive animal was used to spike different volumes of negative milks (4, 9,19, 29, and 39 volumes). Each sample

307 was tested both untreated and after PC protocol. Gray bars: Untreated spiked milk samples; Black bars: PC spiked

308 milk samples; Horizontal lines represent the cutoff limits.
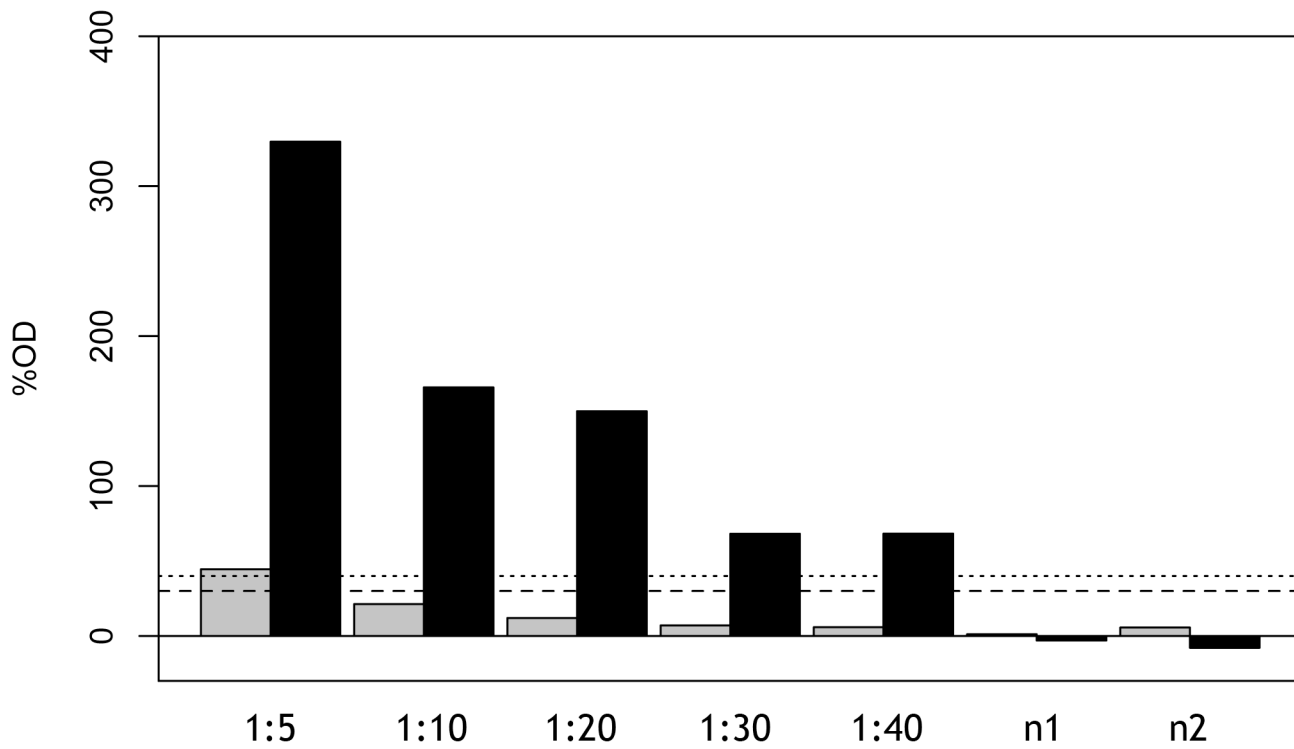

309

310

311   Figure 3. Distribution of rec-gE ELISA results during the preliminary validation. Circles: PC-BM from gE negative

312   farms. Triangles: PC-BM from gE positive farms. Solid-black points represent the three farms that showed

313   unexpected results (see the text for details). Horizontal dashed line: ELISA positivity cutoff. Vertical dotted line:

314   within-herd prevalence of 2.5%.
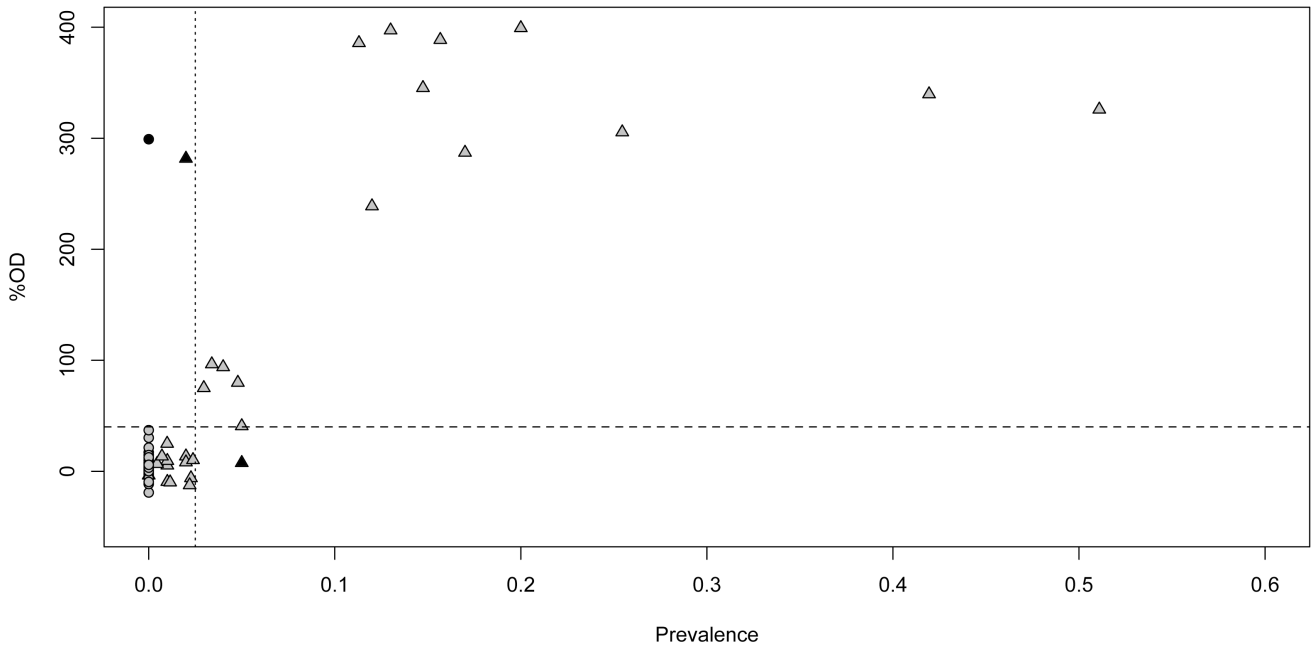
315

316

317   Figure 4. Reactivity of samples from the farm showing vaccine reactivation. White bars: reactivities of the 67

318   individual untreated milk samples; gray bar: reactivity of the untreated BM; black bar: reactivity of the PC-BM

319   sample. Horizontal lines represent the cutoff limits.

320
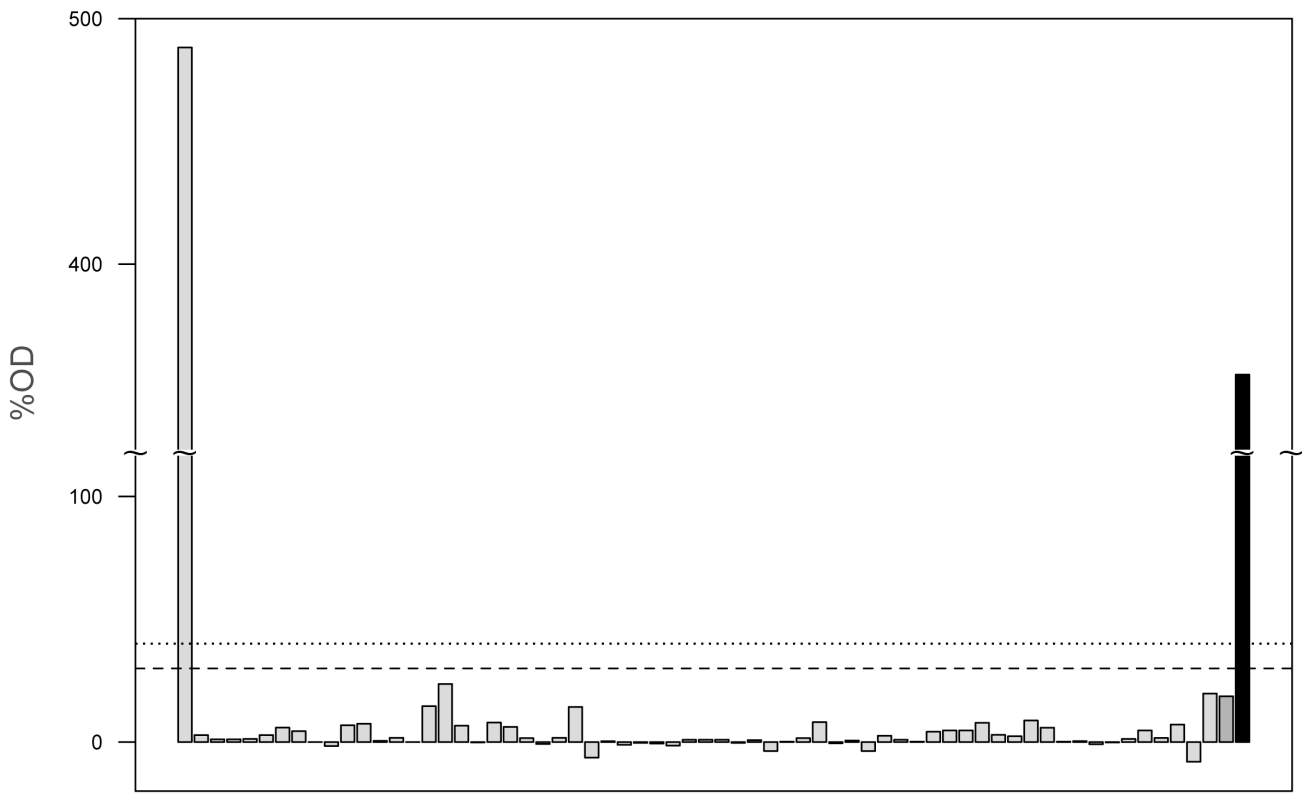
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