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1 **Validation of a commercial ELISA for the detection of bluetongue**
2 **virus (BTV) specific antibodies in individual milk samples of Dutch**
3 **dairy cows**

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24 **Abstract**

25

26 A recently developed indirect ELISA for the detection of bluetongue virus (BTV)-
27 specific antibodies in bovine milk samples was compared to that of the routinely used
28 competitive ELISA on serum samples.

29 During the bluetongue outbreak in the Netherlands in 2006, caused by BTV serotype
30 8, coupled serum- and milk samples were obtained from 470 individual cows from 10
31 BTV-infected farms with an average seroprevalence of 57%. In addition, bulk milk
32 samples of the same farms, and historically BT-negative samples were tested.

33 Compared to the ELISA for sera, the relative specificity and sensitivity of the ELISA
34 for milk samples is 96.5% and 98.9%, respectively when using a S/P% cut-off value
35 of 50% as advised by the manufacturer. The optimal cut-off value was found at S/P%
36 of 90% revealing an optimal specificity (99.0%) combined with an optimal sensitivity
37 (98.1%). Titres in positive individual milk samples ranged from 1 to 2048 with a peak
38 titre of 128. Bulk milk samples contained antibodies with titres ranging from 64 to
39 512.

40 The ELISA for milk samples was found to be a reliable and robust test. This
41 diagnostic tool is very useful, and may replace the ELISA for serum samples as first
42 choice in order to get insight into the status of lactating individual animals and
43 therewith of the entire herd with respect to BTV infection.

44

45

46 *Keywords:* Bluetongue virus; Specific antibodies; ELISA; Individual milk; Serum;
47 Sensitivity; Specificity.

48 Introduction

49 Bluetongue is a vector-borne viral disease of ruminants, including sheep,
50 goats and cattle, caused by bluetongue virus (BTV). Clinical signs are seen mainly in
51 sheep whereas the infection in cattle often occurs asymptomatic (Verwoerd and
52 Erasmus, 1994; Gibbs and Greiner, 1994). BTV is a member of the *Orbivirus* genus
53 of the family *Reoviridae*, and consists of 24 serotypes with considerable
54 immunological cross-reaction (Gorman, 1990). Animals can become infected by
55 BTV-infected midges of the genus *Culicoides* that have had a blood meal from
56 viraemic animals (Erasmus, 1990).

57 In 2006, an outbreak of bluetongue occurred in the Netherlands, Belgium,
58 Germany, France and Luxembourg by a BTV variant of serotype 8 (Wuijckhuise van
59 et al., 2006; Mehlhorn et al., 2007). Routine laboratory diagnosis has successfully
60 been performed in the Netherlands by an in-house real-time RT-PCR (reverse
61 transcription and polymerase chain reaction) on EDTA-blood samples (manuscript in
62 preparation) and by serology using a commercial competitive ELISA (ID.VET,
63 Montpellier, France). Positive PCR results can be found in cattle a few days post
64 infection till 200 days post infection or more. Using the ID.VET ELISA,
65 seroconversion has been detected starting 9-14 days post infection.

66 In accordance to EU legislation, control measures must be taken and disease
67 monitoring and surveillance is of fundamental importance to assess the risk posed by
68 animal movements. For dairy cattle, testing for antibodies in milk samples rather than
69 in serum samples would be very cost-effective and acceptable because of the easy
70 availability of the former sample type.

71 In this study, the performance of an indirect ELISA developed by ID.VET for
72 the detection of BTV-specific antibodies in bovine milk samples (mELISA) was

73 compared to that of the routinely used competitive ELISA on serum samples
74 (sELISA).

75

76 **Materials and Methods**

77 *Serum and milk BTV antibody ELISAs*

78 The commercially available competitive ELISA for serum (sELISA; ID.VET,
79 Montpellier, France) was performed as described in the kit manual. Briefly, 50 µl of
80 buffer and 50µl of serum were added to the wells of a BTV-VP7 coated microtitre
81 plate. After incubation for 45 min at room temperature (rT), 100 µl anti-VP7
82 peroxidase conjugate was added and incubated for 30 min at rT. After washing, wells
83 were incubated for 15 min at rT with 100 µl TMB substrate. Colour development was
84 stopped by the addition of 100 µl 0.5 M H₂SO₄. S/N ratios ($OD_{\text{sample}}/OD_{\text{negative control}}$)
85 were calculated using optical density values measured at 450 nm (OD_{450}). S/N ratios
86 <0.4 were considered as positive.

87

88 A recently developed, indirect ELISA for milk (mELISA) was performed according to
89 instructions of the manufacturer (ID.VET). Briefly, 50 µl skimmed milk and 50 µl
90 'wash solution' (final sample dilution 1:2) were added to the wells of a BTV-VP7
91 coated microtitre plate. After incubation for 45 min at rT, plates were washed and
92 incubated with 100 µl anti-ruminant peroxidase conjugate for 30 min at rT. After
93 washing, wells were incubated for 15 min at rT with 100 µl TMB substrate. Colour
94 development was stopped by the addition of 100 µl 0.5 M H₂SO₄. S/P%
95 ($OD_{\text{sample}}/OD_{\text{positive control}} \times 100\%$) were calculated using optical density values
96 measured at 450 nm (OD_{450}). S/P% $\geq 50\%$ were considered as positive.

97 Milk samples were also tested in the mELISA after a previously prepared dilution of
98 1:4 in the kit wash solution (final sample dilution 1:8).

99 Titres of individual milk samples were determined in the mELISA by testing two-fold
100 serial dilutions prepared in negative bulk milk. The titre of a milk sample was defined
101 as the highest dilution that gave a positive response (S/P% \geq 50%).

102

103 *BTV-PCR*

104 Viral dsRNA was automatically isolated from EDTA-blood (MagNA Pure isolation
105 robot, Roche). First strand cDNA–synthesis (RT), PCR, and real time detection was
106 performed in an one-tube, closed system (Light Cycler 2.0, Roche). The target for
107 amplification is located on genome segment 10 encoding NS3, and real time
108 detection was performed by hydrolysis of a Taqman probe. A weak positive control
109 served as cut-off for positivity for the PCR-test. This test is able to give a positive
110 signal up to 7 days before antibodies can be detected after experimental infection
111 (manuscript in preparation).

112

113 *Test Samples*

114 In this study different types of samples were analysed:

115 (1) Historically negative individual milk samples (n=55) and bulk milk samples
116 (n=88) from Dutch dairy cows.

117 (2) Coupled samples (blood, EDTA blood and a milk sample) were taken
118 simultaneously of each lactating cow from 10 dairy farms selected based on a
119 high BTV seroprevalence (Table 1). In addition, from each of these 10 farms a
120 bulk milk sample was taken.

121 Milk and bulk milk samples, containing Na-azide as preservative, were defatted by
122 centrifugation and stored at -20°C in aliquots to circumvent repeated freezing and
123 thawing. Serum samples were stored at -20°C before testing. EDTA-blood samples
124 were stored at 4°C for up to 2 days before processing and testing by PCR.
125 Bulk milk taken from two BT negative farms, was used to make serial two-fold
126 dilutions of individual milk samples.
127 To test the effect of a preservative on the performance of the mELISA, either Na-
128 azide or BSM (Broad Spectrum Microtabs, D&F Control Systems Inc., San Ramon,
129 CA, USA) was added to this bulk milk.

130

131

132 *Determination of relative sensitivity and specificity, statistical analysis*

133 The relative sensitivity of the mELISA is defined as the proportion of sero-positive
134 animals giving a positive response by testing the corresponding individual milk
135 samples:

$$\frac{\text{Seropositive animals with a positive milk sample}}{\text{All seropositive animals}}$$

139 The relative specificity of the mELISA is defined as the proportion of sero-negative
140 animals giving a negative response by testing the corresponding individual milk
141 samples:

$$\frac{\text{Seronegative animals with a negative milk sample}}{\text{All seronegative animals}}$$

144

145

146 Relative sensitivities and relative specificities of the mELISA were calculated at
147 different S/P% cut-off values. These cut-off values were used to classify milk

148 samples as either positive or negative. Curves were constructed by plotting the
149 relative specificity and the relative sensitivity against the corresponding S/P% cut-off
150 values ranging from 0% to 240%.

151 Receiver operating characteristic (ROC) curves (Kraemer, 1992) of the mELISA were
152 constructed by plotting the sensitivity on the ordinate as a function of the specificity at
153 different cut-off values. The area under the ROC curve is a quantitative measure of
154 the test's performance. These curves were plotted to assess the relative performance
155 of the mELISA when milk samples were tested undiluted or 4 times diluted.

156 Statistical analysis (sensitivity, specificity, confidence intervals and the agreement
157 index κ D) was performed using Win Episcopy 2.0
158 (<http://www.clive.ed.ac.uk/winepiscopy/>).

159

160 **Results**

161 Specificity of the mELISA was determined by testing 55 historically negative
162 individual milk samples and 88 historically negative bulk milk samples. All samples
163 tested negative with a mean S/P% \pm SD of 11.0% \pm 2.8% and 10.2% \pm 0.8%,
164 respectively. Based on the mean S/P%+3SD of these negative samples the cut-off of
165 the mELISA should be around an S/P% of 20%.

166 The relative sensitivity and relative specificity of the mELISA was determined
167 using the sELISA as the gold standard test. Coupled serum- and milk samples
168 obtained from individual cows (n=470, see Table 1) from 10 BTV-infected farms were
169 analysed. The overall results are shown in Table 2. The relative specificity of the
170 mELISA is 96.5% (195/202 x 100%) with a 95% confidence interval (CI) between
171 94.0% and 99.1%. The 7 animals with discordant results were negative in the BTV-
172 PCR test.

173 The relative sensitivity amounts to 98.9% ($265/268 \times 100\%$; 95% CI: 97.6% - 100%).

174 Two of the three animals with a discordant result showed a positive BTV-PCR

175 response. The overall accuracy is 97.9% ($460/470 \times 100\%$; $\kappa_D=0.96$).

176 A previous 4x dilution of milk samples in wash solution revealed a somewhat higher

177 specificity (97.5%; 95% CI: 95.4% - 99.7%) and a somewhat lower sensitivity (98.1%;

178 95% CI: 96.5% - 99.8%) at the prescribed cut-off SP% value of the mELISA of 50%.

179 To verify the positive/negative cut-off value of the mELISA when milk was

180 tested undiluted, a frequency distribution diagram was constructed based on the

181 results of the 470 milk samples from the 202 BT-antibody seronegative and 268 BT-

182 antibody seropositive animals (see Figure 1). In addition, relative sensitivity and

183 relative specificity at different SP% cut-off values were calculated and plotted (see

184 Figure 2). Highest relative sensitivity (98.1%; 95% CI: 96.5% – 99.8%), combined

185 with highest relative specificity (99.0%; 95% CI: 97.5% - 100%) was observed at a

186 cut-off SP% value of 90% (see also figure 3).

187 ROC curves were constructed to compare the performance of the mELISA

188 when milk samples were tested either undiluted or 4x diluted in wash solution (Figure

189 3). The test with undiluted milk samples shows a slightly greater area under the ROC

190 curve compared with the test performed with 4x diluted samples indicating a

191 somewhat better performance with undiluted milk samples.

192 To determine the BTV-specific antibody titres in the individual milk samples,

193 two-fold serial dilutions in negative bulk milk were tested in the mELISA. Typical dose

194 response curves of 8 serially diluted milk samples (1 mELISA negative and 7

195 mELISA positive samples) are depicted in Figure 4. Similar curves were found when

196 dilutions were made in bulk milk containing either azide or BSM as preservative.

197 Figure 5 depicts the frequency distribution diagram of the antibody titres of the 470

198 individual milk samples. Milk samples from seropositive animals showed a mean 2log
199 titre of 7. The 7 positive milk samples from seronegative animals had 2log titres of 0
200 (n=5) and of 1 (n=2).

201 The bulk milk samples collected from the 10 BT infected farms showed 2log
202 titres of 6 (n=5), 7 (n=4) and 9 (n=1) in the mELISA. The bulk milk sample with the
203 highest titre (2^9) originated from the farm with the highest seroprevalence (95%).

204

205 **Discussion**

206 The commercial ID.VET mELISA is a recently developed indirect ELISA able
207 to detect BTV-VP7 specific antibodies in milk samples.

208 The performance of the mELISA was investigated using the ID.VET sELISA as the
209 reference test. In a previously performed study, the ID.VET sELISA showed both a
210 high specificity (100%) and a high sensitivity (98%) relative to BTV-PCR results on
211 field samples obtained at the early stage (Sept. and Nov. 2006) of the BT outbreak in
212 the Netherlands (manuscript in preparation). Moreover, the ID.VET sELISA showed a
213 good performance in proficiency testing (Workshop on bluetongue diagnostics and
214 epidemiology, Brussels, Belgium, November 28-29, 2006). In our laboratory (CIDC-
215 Lelystad), the sELISA is an ISO17025 accredited test and used for routine serum
216 testing of both cattle and sheep.

217 In the study described it was investigated whether the mELISA can be used as
218 a tool to identify BT-seropositive dairy cows. Analysis of historical negative individual
219 (n=55) and bulk milk samples (n=88) indicated a specificity of 100% in this small set
220 of data.

221 Testing individual milk samples obtained from seropositive (n=268) and seronegative
222 cattle (n=202) revealed a high sensitivity and a high specificity relative to serology

223 testing. Of the 470 milk samples tested in the mELISA, only 10 discordant results
224 (=2.1%) with the sELISA were found at the prescribed positive/negative cut-off S/P%
225 value of 50%. From the results as shown in Figure 2 it can be concluded that the test
226 shows a high sensitivity and a high specificity that change minimally between cut-off
227 values of 30% and 110%. Highest sensitivity combined with highest specificity
228 revealed 7 discordant results (5 false negatives and 2 false positives) and was found
229 at a cut-off S/P% value of 90%. A 4x dilution of milk samples before testing did not
230 result in a better performance (Figure 3): highest accuracy revealed 9 discordant
231 results (6 false negatives and 3 false positives) at a cut-off value of 80%.

232 Samples investigated were from farms with a high seroprevalence ranging
233 from 34% to 95%. These farms were not very recently infected (samples were taken
234 probably more than 4 months after primary infection), and most seropositive animals
235 have reached their maximum antibody levels resulting in a minimum number of false
236 negative test results. In case of a recently infected farm, at least part of the sera
237 taken from infected animals may contain relatively low antibody titres giving weaker
238 responses in the ELISA. For this reason a positive/negative cut-off value should be
239 chosen resulting in a test with an optimal sensitivity combined with a specificity
240 producing an acceptable low number of false positives (high sensitivity combined
241 with an acceptable good specificity). As can be deduced from the results of this study
242 and presented in Figures 1 and 2 using a S/P% cut-off value of 50% resulted in 3
243 false negatives (sensitivity = 98.9%), two of which were confirmed by a positive BTV-
244 PCR and 7 false positives (specificity = 96.5%), all of which were confirmed by a
245 negative BTV-PCR. In view of the impact of these false positives, one may decide to
246 confirm (false) positive milk test results by serum antibody testing and/or by BTV-

247 PCR. When a higher specificity is required, the cut-off value can be changed to a
248 S/P% of 90% with minimal effects on sensitivity.

249 Antibody levels in the individual milk samples were determined by testing two-
250 fold serial dilutions, prepared in negative milk, in the mELISA. Figure 5 shows a titre
251 distribution of positive milk samples between 2^0 and 2^{11} with a peak at 2^7 . Of the 8
252 milk samples with the lowest titre (2^0 and 2^1), 7 showed discordant results with the
253 serum test (positive milk samples from seronegative animals). This suggests that
254 animals develop BTV-specific antibody mELISA titres of 2^2 or higher and that by bulk
255 milk detection of a herd prevalence of 25% or lower seems to be feasible. BTV-
256 specific antibody titres of the bulk milk samples of the 10 farms ranged from 2^6 to 2^9 .
257 A weak positive correlation was observed between the seroprevalence and the bulk
258 milk titre showing the highest titre in the bulk milk sample obtained from the farm with
259 the highest seroprevalence. The application of the mELISA for bulk milk testing is
260 now being validated by testing bulk milk samples from BTV infected farms with
261 different percentages of seropositive lactating animals.

262 Due to immunological cross reaction with BTV-VP7 (Gorman, 1990) it can not
263 be excluded that the presence of antibodies directed against BTV-related Orbiviruses
264 like epizootic haemorrhagic disease virus (EHDV) may result in positive responses in
265 the mELISA and/or sELISA. In case animals are tested positive in the mELISA one
266 should consider to further investigate these animals by more specific serum tests
267 and/or PCR. However, up to this moment EHDV never has been detected in this part
268 of Europe.

269 We conclude that the mELISA using undiluted milk at cut-off S/P percentages
270 between 30% and 110% is a very reliable diagnostic tool. Because milk samples are
271 easy and cost-effective to obtain this could be the first choice of testing in order to get

272 insight to the status of animals and therewith of the herd concerning bluetongue virus
273 infection.

274

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294 clinical cases and differential diagnosis. Common symptoms just a little
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296 **Table 1.**
297

298 Numbers of samples and anti-bluetongue seroprevalence of farms analysed in this
299 study.

Farm nr.	Number of lactating cows	Seroprevalence
1	50	94% (47/50) ⁰²
2	25	56% (14/25) ⁰³
3	29	41% (12/29) ⁰⁴
4	27	67% (18/27) ⁰⁵
5	55	49% (27/55) ⁰⁶
6	42	50% (21/42) ⁰⁷
7	22	95% (21/22) ⁰⁸
8	76	67% (51/76) ⁰⁹
9	97	42% (41/97) ¹⁰
10	47	34% (16/47) ¹¹
All	470	57% (268/470)¹²

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316 **Table 2.**

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318 Comparison of results obtained with serum ELISA and milk ELISA of 470 coupled

319 serum-milk samples.

320

		Sera tested in sELISA		Total
		Positive (S/N<0.4)	Negative (S/N≥0.4)	
Milk samples tested undiluted in mELISA	Positive (S/P%≥50%)	265	7	272
	Negative (S/P%<50%)	3	195	198
Total		268	202	470

342

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344

345 **Figure captions**

346

347 **Figure 1.**

348 Frequency distribution diagram of 470 milk samples obtained from seropositive and
349 seronegative animals originating from 10 Dutch BTV infected herds. S/P% represent
350 test results obtained with the ID.VET mELISA.

351

352 **Figure 2.**

353 Relative sensitivity and relative specificity of the mELISA at different S/P% cut-off
354 values.

355

356 **Figure 3.**

357 ROC curves based on mELISA test results obtained with undiluted milk and milk 4x
358 diluted in buffer.

359

360 **Figure 4.**

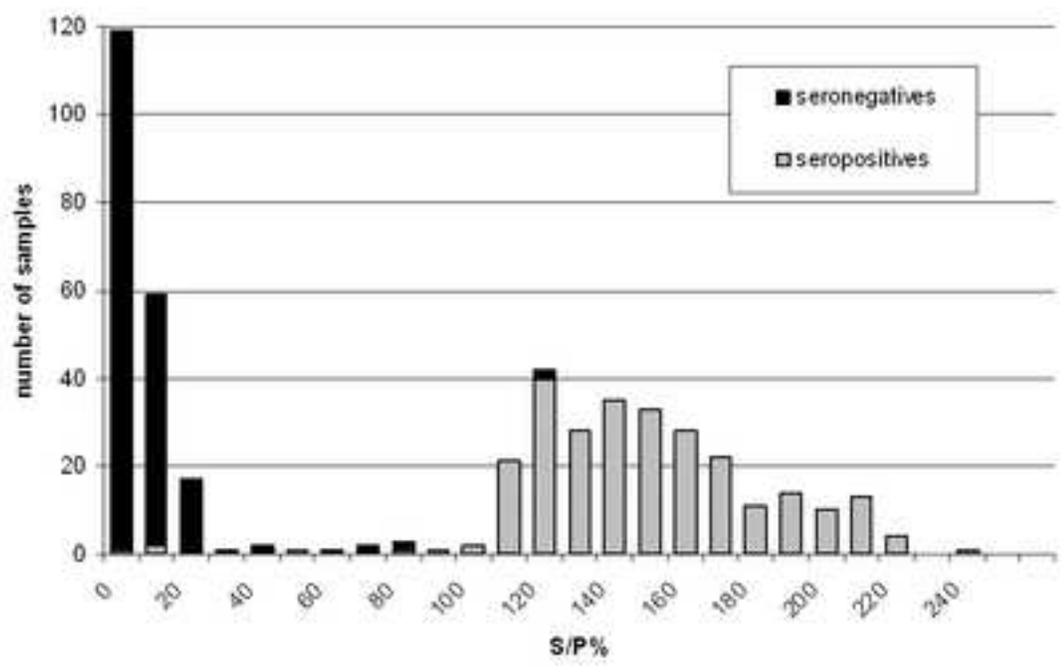
361 Typical dose response curves of serially diluted milk samples (7 positives and 1
362 negative) tested in the mELISA.

363

364 **Figure 5.**

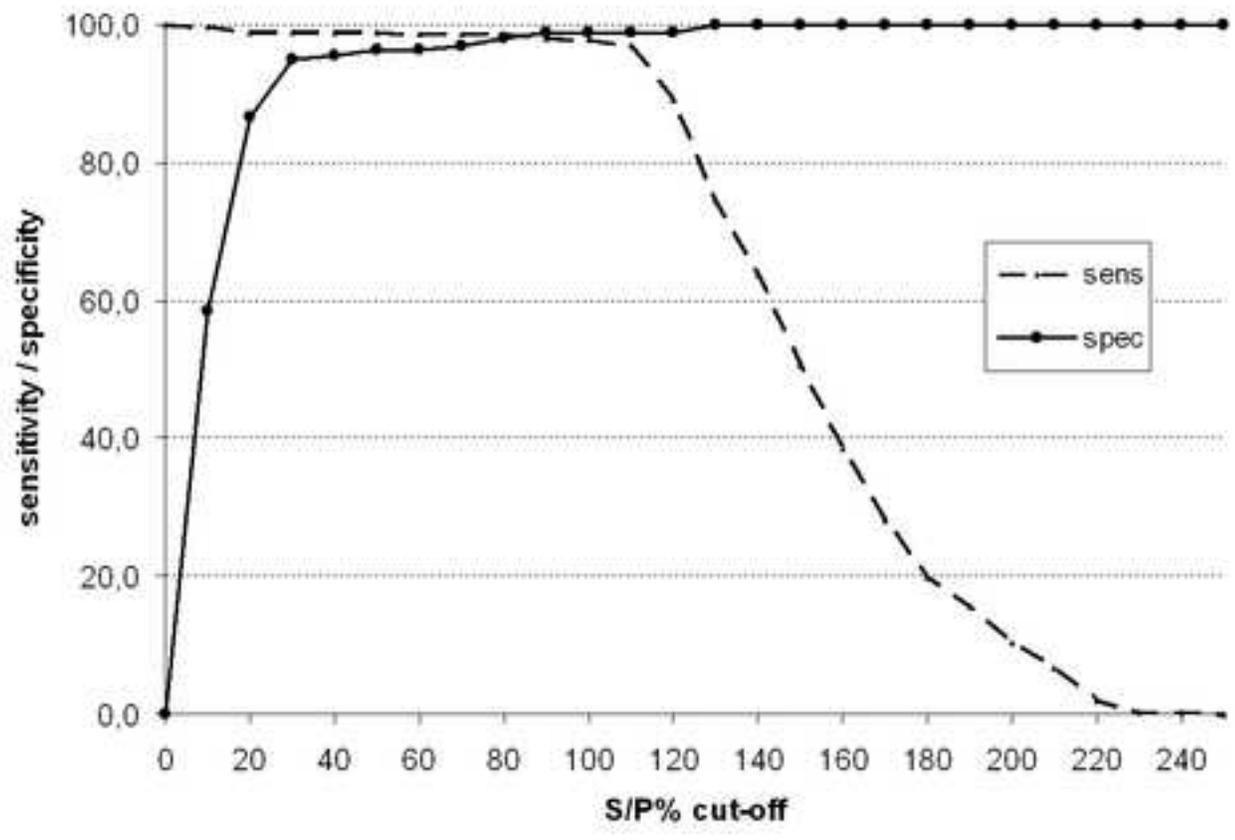
365 Frequency distribution of $2\log(\text{titres})$ of 470 individual milk samples collected from
366 seropositive and seronegative animals of 10 BTV-infected farms.

ript



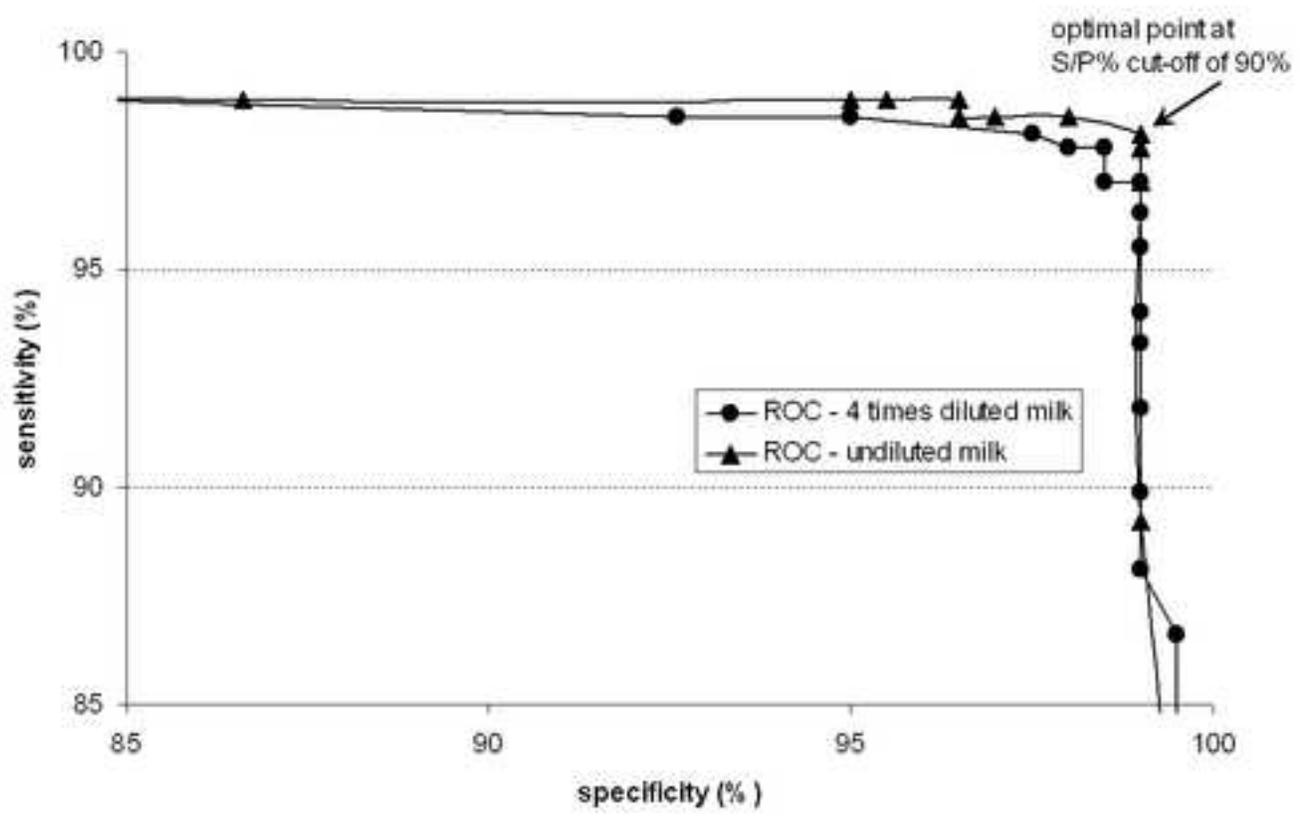
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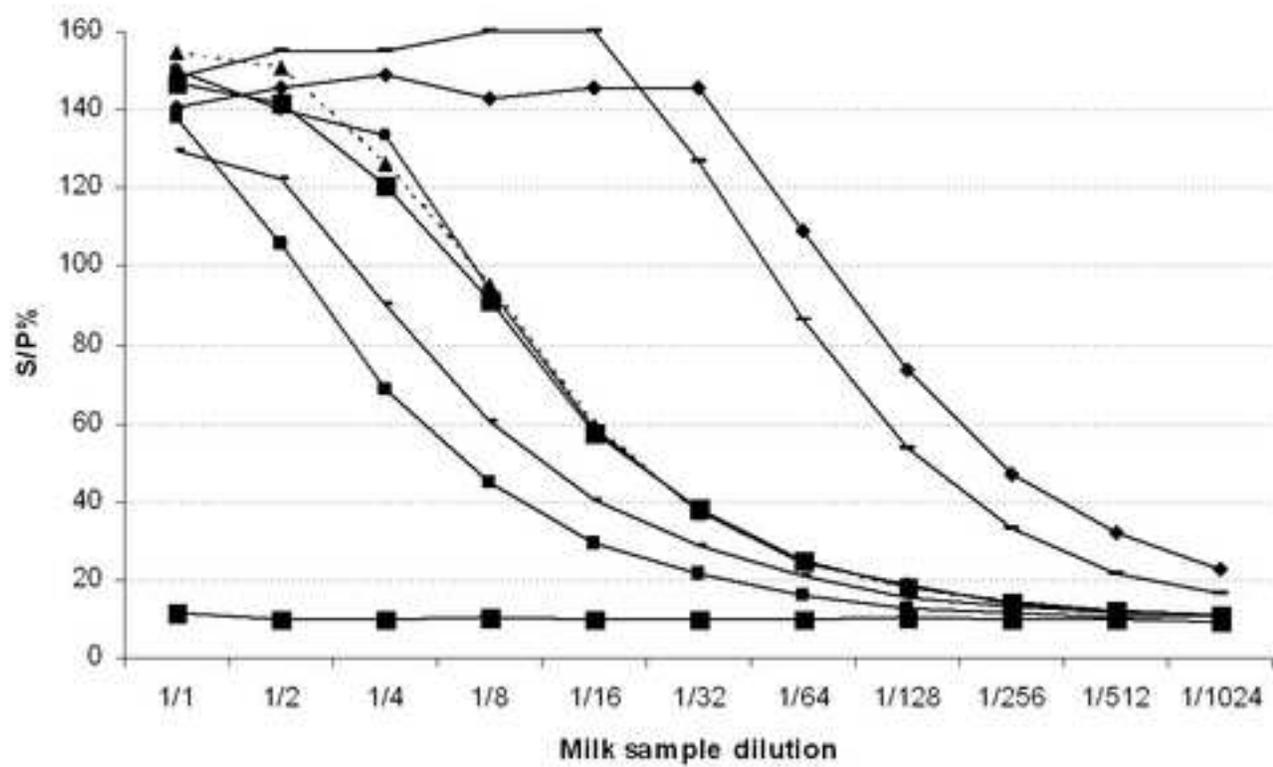
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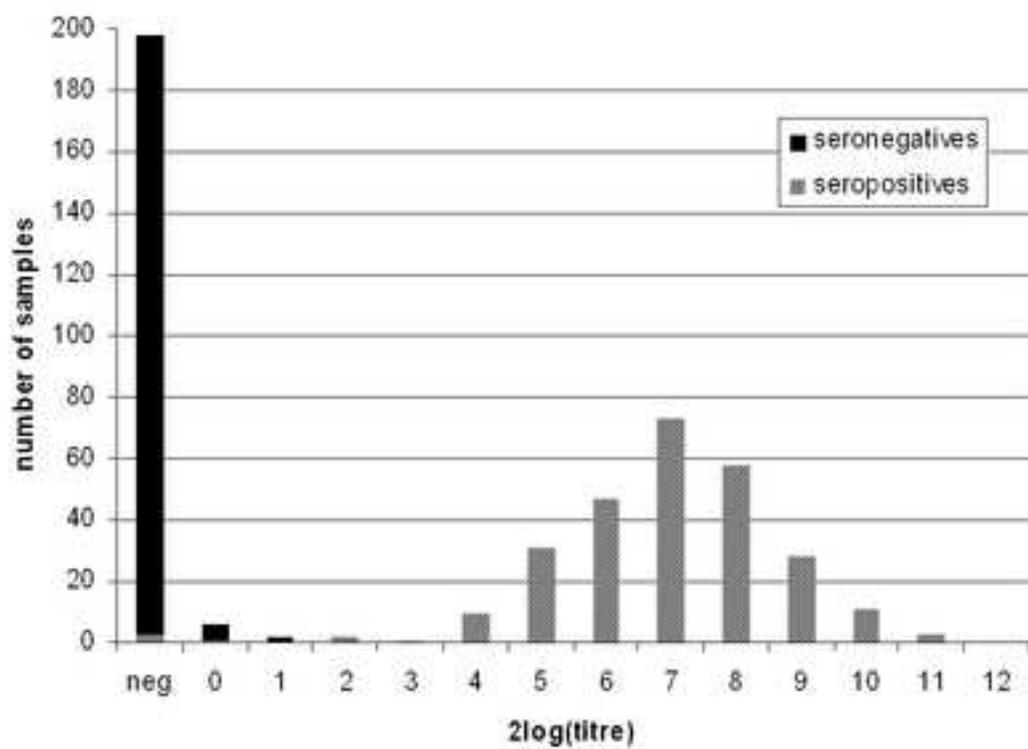


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