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Piet A. van Rijn

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## Accepted Manuscript

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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**

4  
5  
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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  $\mu$ l of  
80 buffer and 50  $\mu$ l 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  $\mu$ l 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  $\mu$ l TMB substrate. Colour development was  
84 stopped by the addition of 100  $\mu$ l 0.5 M H<sub>2</sub>SO<sub>4</sub>. 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  $\mu$ l skimmed milk and 50  $\mu$ l  
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  $\mu$ l anti-ruminant peroxidase conjugate for 30 min at rT. After  
93 washing, wells were incubated for 15 min at rT with 100  $\mu$ l TMB substrate. Colour  
94 development was stopped by the addition of 100  $\mu$ l 0.5 M H<sub>2</sub>SO<sub>4</sub>. 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  $\kappa$ 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  
295 different and in too many herds. Tijdschr Diergeneeskd. 131, 649 - 654.

296 **Table 1.**  
297

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

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

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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

343

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.**

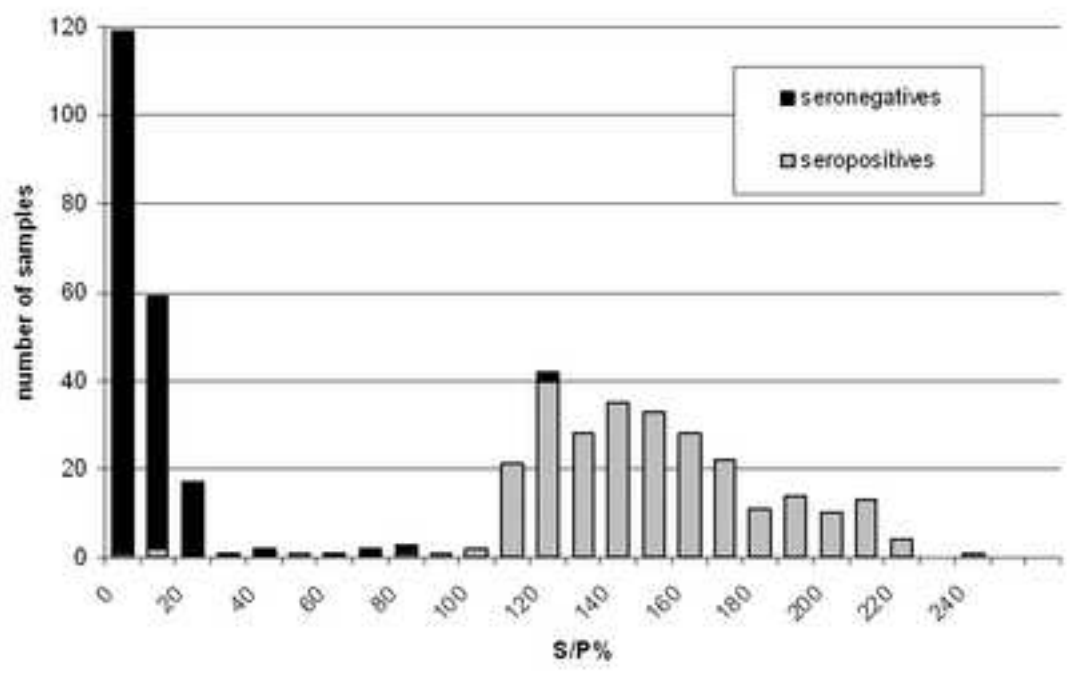
361 Typical dose response curves of serially diluted milk samples (7 positives and 1  
362 negative) tested in the mELISA.

363

364 **Figure 5.**

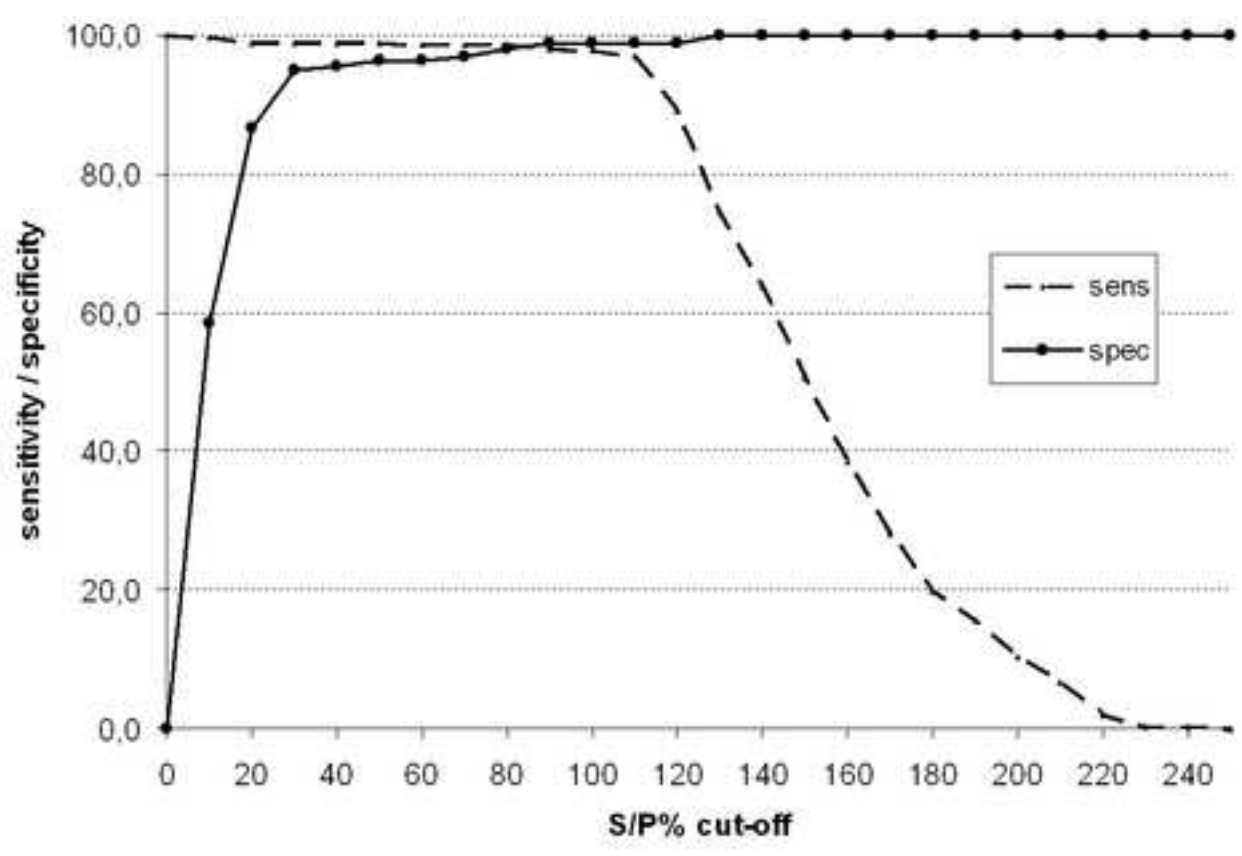
365 Frequency distribution of  $2\log(\text{titres})$  of 470 individual milk samples collected from  
366 seropositive and seronegative animals of 10 BTV-infected farms.

ript



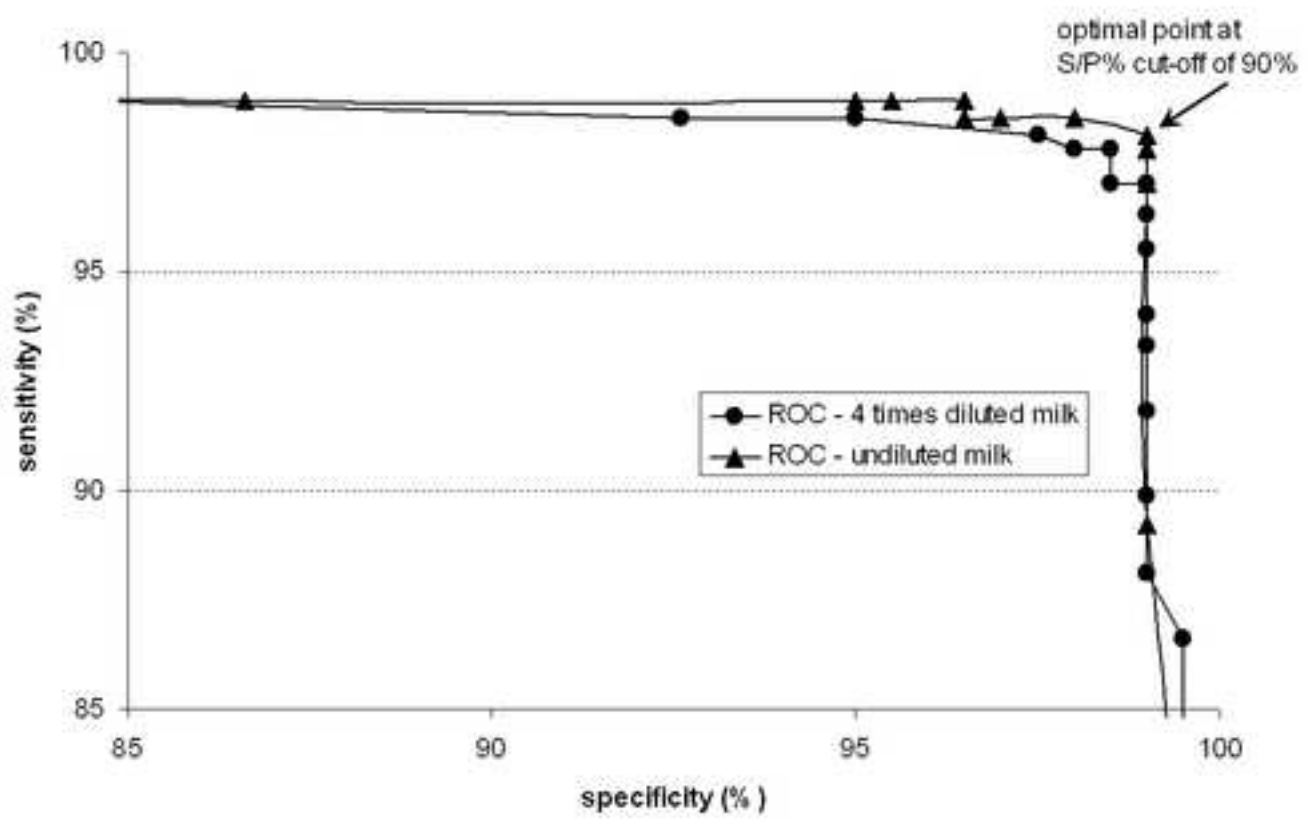
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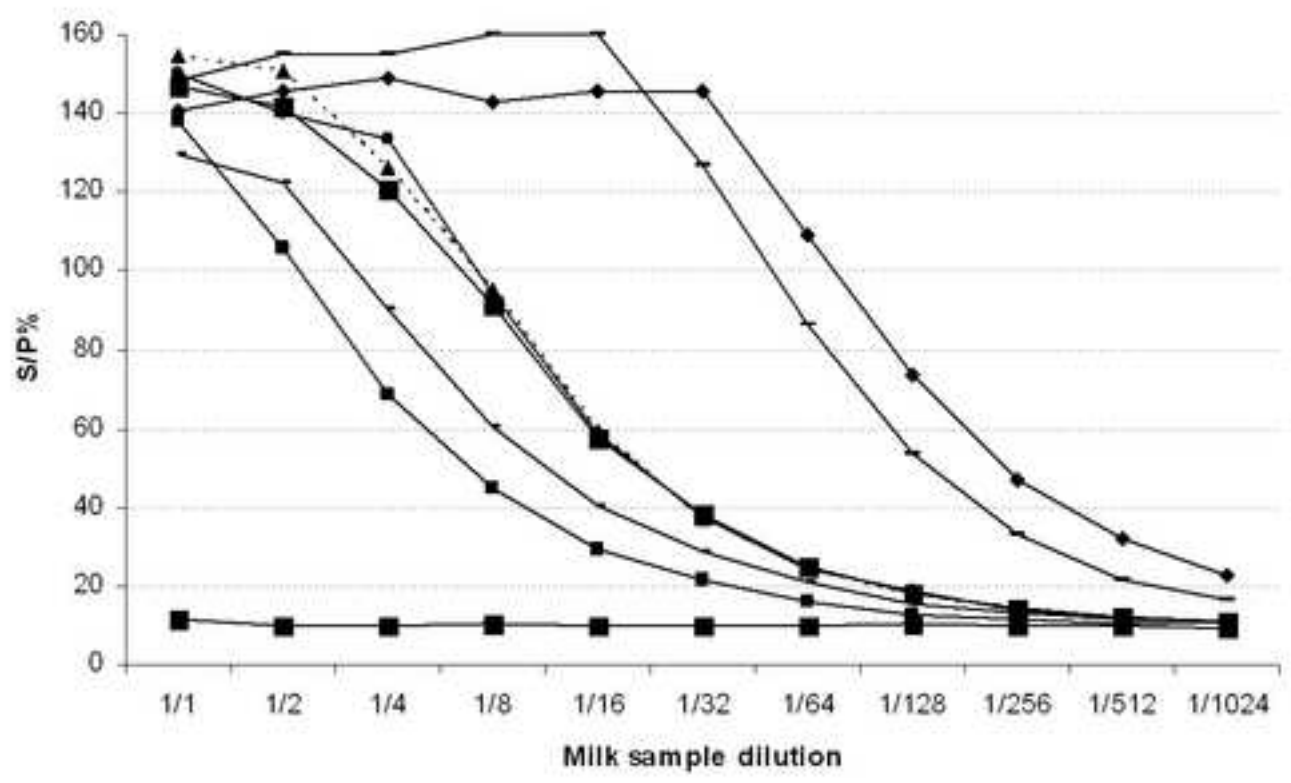


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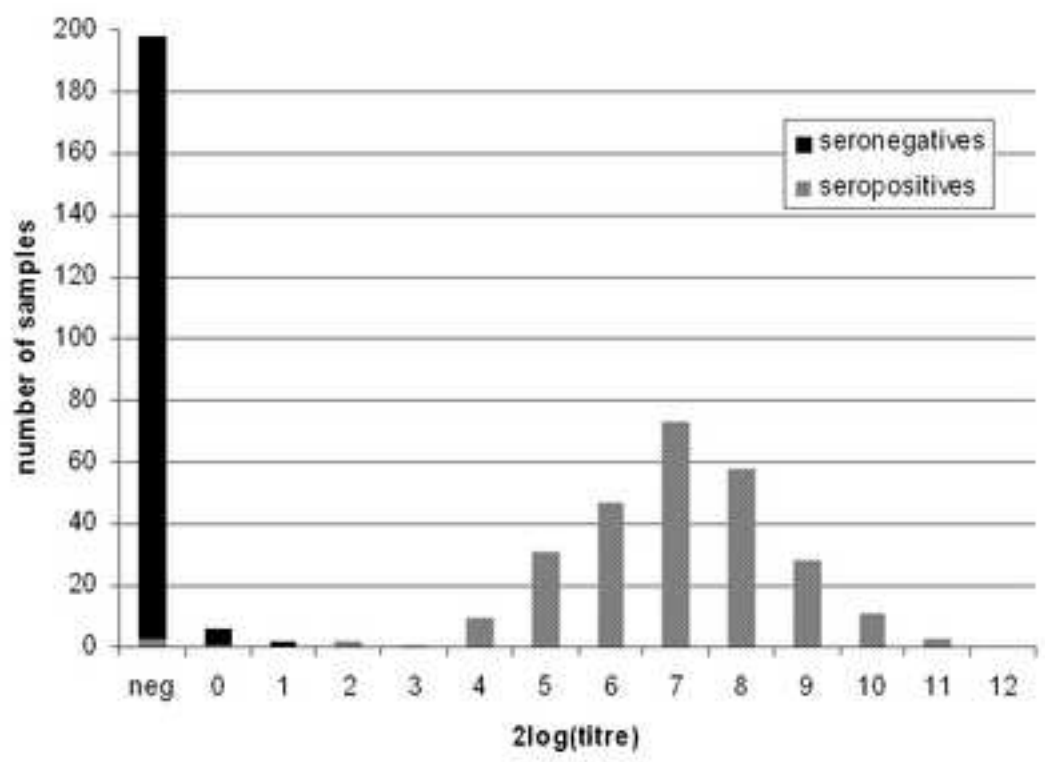
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