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# 1 Diagnosis of bovine dictyocaulosis by 2 bronchoalveolar lavage technique: a 3 comparative study using a Bayesian 4 approach.

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## 5 **Authors**

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7 Thibaut LURIER<sup>a\*</sup>, Marie Laure DELIGNETTE-MULLER<sup>a</sup>, Benoit RANNOU<sup>b</sup>, Christina STRUBE<sup>c</sup>, Marie-  
8 Anne ARCANGIOLI<sup>d</sup>, Gilles BOURGOIN<sup>a</sup>

9 <sup>a</sup> Université de Lyon, Université Lyon 1, CNRS, VetAgro Sup, UMR 5558, Laboratoire de Biométrie et  
10 Biologie Evolutive, F-69622 Villeurbanne, France.

11 <sup>b</sup> Université de Lyon, VetAgro Sup, 1 avenue Bourgelat, BP 83, F-69280 Marcy l'Etoile, France.

12 <sup>c</sup> Institute for Parasitology, Centre for Infection Medicine, University of Veterinary Medicine  
13 Hannover, Buenteweg 17, D-30559 Hannover, Germany

14 <sup>d</sup> Université de Lyon, VetAgro Sup, Anses, UMR Mycoplasmes des Ruminants, F-69280 Marcy l'Etoile,  
15 France.

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17 \* Corresponding author : [thibaut.lurier@vetagro-sup.fr](mailto:thibaut.lurier@vetagro-sup.fr)

18

## 19 **Abstract**

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20 Bovine dictyocaulosis is a pulmonary parasitic disease present in temperate countries, with potential  
21 important clinical and economic impacts. The Baermann technique is routinely used despite its low  
22 sensitivity in adult cows. Recently developed serological tests seem to offer better sensitivity, but  
23 validations of these tests in field conditions are few. We aimed to study two non-previously  
24 evaluated diagnosis methods of dictyocaulosis based on bronchoalveolar lavage sampling (BAL),  
25 which allows finding lungworm stages in the lungs as well as determination of eosinophilia. We  
26 compared them to the Baermann technique and serological tests. As no gold standard was available,  
27 we performed a Bayesian analysis by the simultaneous use of latent class and mixture models. The  
28 study was carried out during the 2015 pasture season on 60 adult cows originating from 11 herds  
29 with clinical signs of dictyocaulosis, and 10 apparently healthy cows originating from the teaching  
30 herd of VetAgro Sup, in France. Prevalence of infection was highly variable among herds with clinical  
31 signs (10-90%). Despite a maximal specificity (100%), the sensitivity of parasitological methods was  
32 low (7.4% for the Baermann sedimentation and 24.7% for the examination of BAL fluids). Better  
33 results were observed with serology (Se = 74.9%, Sp = 85.5%) with an optimal cut-off value estimated  
34 at 0.397 for the optical density ratio. Even better results were obtained with the count of eosinophil  
35 in BAL (Se = 89.4%, Sp = 85.2%) with an optimal cut-off value estimated at 4.77% for the eosinophil  
36 proportion. The BAL is a relevant diagnostic method of dictyocaulosis for practitioners due to the  
37 opportunity to perform two analyses (direct parasitic research and the eosinophil count) and to its  
38 good sensitivity and specificity.

### 39 **Highlights:**

- 40 - Parasitological methods for dictyocaulosis in field lack sensitivity
- 41 - Bronchoalveolar lavage allows diagnosis of dictyocaulosis by direct observation of the parasite and
- 42 indirect through eosinophil proportion in fluids

43 - Bronchoalveolar cytology has a better sensitivity than the serology to diagnose dictyocaulosis at the  
44 individual level and allows the detection of pre-patent infection

45

46 **Keywords:** Baermann, bronchoalveolar cytology, *Dictyocaulus*, ELISA, latent class model, mixture  
47 model

48

49

## 50 **Introduction**

51 Parasitic bronchitis caused by *Dictyocaulus viviparus* is a clinically and economically important  
52 parasitic disease in temperate countries. In Europe, recent studies in dairy cattle revealed the  
53 circulation of this parasite in at least 17.1 to 64.5% herds (Schunn *et al.*, 2013; Bloemhoff *et al.*, 2015)  
54 with an individual prevalence ranging from 6.0 to 13.75% in infected herds (Borgsteede *et al.*, 2000;  
55 Chartier *et al.*, 2013). The disease occurs during the pasture season and main clinical signs are  
56 coughing, nasal discharge and severe dyspnoea resulting in increased mortality in the herd and  
57 important economic losses for the farmer (Holzhauer *et al.*, 2011). *Dictyocaulus viviparus* has a direct  
58 life cycle with cows being infected by swallowing third-stage larvae (L3) present on contaminated  
59 pastures. Historically, first grazing season animals were clinically the most affected by this parasite.  
60 However, since the 1990's, the epidemiology of dictyocaulosis changed in dairy herds with an  
61 increasing occurrence of outbreaks in older animals, mainly explained by weak contact with the  
62 parasite during previous years of life (Ploeger, 2002; van Dijk, 2004).

63 The routine diagnostic method used in veterinary clinics and diagnostic laboratories is the Baermann  
64 method, based on the isolation and morphological identification of larvae in the faeces (Baermann,  
65 1917). This method has a high sensitivity (100%) for primary infection in young animals (Eysker,  
66 1997), but it seems to be lower for older animals (Eysker, 1997; Ploeger *et al.*, 2012). Different  
67 hypotheses have been proposed to explain this change of sensitivity with age, such as the higher  
68 dilution of larvae in the faeces of adults and the previously acquired immune response, limiting the  
69 development of *D. viviparus* to the adult stage and the egg-laying in the lungs (Eysker, 1997; Ploeger  
70 *et al.*, 2012). The low sensitivity of this method to diagnose dictyocaulosis in clinically affected adult  
71 dairy cattle reveals the need to develop more sensitive tools for practitioners.

72 Since 1993, two serological tests have been developed, both based on the detection of specific  
73 antibodies against adult lungworms by ELISA. In a first approach, a low molecular-weight fraction  
74 enriched for a 17 kDa protein, close to the major sperm protein (MSP), was extracted from adult

75 lungworms and used as antigen (de Leeuw and Cornelissen, 1993). Later, an ELISA based on  
76 recombinant expressed MSP was developed (Von Holtum *et al.*, 2008). These tests were promising  
77 due to their high sensitivity and specificity (>99%) during patent infections as determined from  
78 experimental infections. However, recent studies under field conditions, using the Baermann  
79 technique as a gold standard, demonstrated lower sensitivity and specificity of the method  
80 developed by Von Holtum *et al.* (2008) with seasonal variations (Se=58.7% and Sp=94.9% in spring  
81 and Se=80% and Sp=83.7% in autumn; Ploeger *et al.*, 2012, 2014). Considering the observed  
82 differences in the diagnostic abilities of these methods between experimental and field conditions  
83 and the potential biases associated with the use of the weakly sensitive Baermann test as a gold  
84 standard, further evaluations under field conditions are necessary.

85 Due to the pulmonary location of most developmental stages of *D. viviparus*, bronchoalveolar lavage  
86 (BAL) could be a promising method for the diagnosis of dictyocaulosis. The analysis of collected BAL  
87 fluids (BALF) may allow both direct and indirect diagnosis of dictyocaulosis: the observation of  
88 macroscopical lungworm stages (Hagberg *et al.*, 2005; Holmgren *et al.*, 2014) and the measurement  
89 of BAL's eosinophil proportion (EP). The latter measure points out the local immune response  
90 developed by the host when the parasite arrives into its lung (Schnieder and Dauschies, 1993;  
91 Hagberg *et al.*, 2005; Holmgren *et al.*, 2014). To our knowledge, BAL has only been used to  
92 characterize the immune pulmonary response of cattle to *D. viviparus* (Schnieder and Dauschies,  
93 1993; Hagberg *et al.*, 2005; Holmgren *et al.*, 2014), but tests on its suitability to diagnose parasitic  
94 bronchitis are lacking yet. On healthy calves, eosinophil proportion represents less than 1% of the  
95 total pulmonary white cells (Trigo *et al.*, 1984; Taylor *et al.*, 1989; Hagberg *et al.*, 2005), while it  
96 increases to 20-50% during primary infection and up to 70-80% during secondary infection  
97 (Schnieder and Dauschies, 1993; Hagberg *et al.*, 2005; Holmgren *et al.*, 2014). This eosinophilia  
98 appears as of 2 weeks after infection and remains for at least 12 weeks without re-infection  
99 (Schnieder and Dauschies, 1993; Hagberg *et al.*, 2005; Holmgren *et al.*, 2014). To our knowledge,  
100 there is no other disease which leads to pulmonary eosinophilia in cattle except when an acute

101 hypersensitivity reaction occurs, e.g. after fungal infections with *Micropolyspora faeni* (Wilkie, 1978;  
102 Gershwin *et al.*, 1990). Nevertheless, the epidemiology of these infections differs from dictyocaulosis  
103 as they are observed mostly in animals housed during wintertime.

104 None of the potential diagnostic methods could be considered as a gold standard as the true status  
105 of each animal remains uncertain, except when the parasite is detected by the Baermann method  
106 and/or in the BALF. In this context, we could use a latent class model to estimate the sensitivity and  
107 the specificity of each test by maximum likelihood (MLE) (Hui and Walter, 1980) or within a Bayesian  
108 framework (Enøe *et al.*, 2000; Chaka *et al.*, 2015; White *et al.*, 2016). Serology and pulmonary  
109 eosinophilia supply quantitative results for which cut-off values need to be estimated during natural  
110 infection (previously estimated only for serology after experimental infections; Von Holtum *et al.*,  
111 2008). Estimating such cut-off values could be done using mixture models, as it has been widely  
112 suggested for serological diagnosis either by MLE (Greiner *et al.*, 1995; Gay, 1996) or within a  
113 Bayesian framework (Nielsen *et al.*, 2007; Opsteegh *et al.*, 2010; Peel *et al.*, 2013).

114 The present study aims to compare the four diagnostic methods (Baermann technique, serology,  
115 detection of the parasite and eosinophil proportion in the BALF) for detecting infected adult cows  
116 (clinical and subclinical dictyocaulosis) in herds with clinical signs of parasitic bronchitis. Bayesian  
117 approach is proposed to estimate the prevalence of infection in each herd, the cut-off values for the  
118 two quantitative methods (serology and pulmonary eosinophilia) and the characteristics of the four  
119 methods (sensitivity and specificity). This approach is based on a model simultaneously describing  
120 the results obtained with the four diagnostic methods using both latent class and mixture model  
121 principles.

## 122 **Materials and Methods**

### 123 Study design and animals

124 The study is a prospective field study performed in France during the 2015 pasture season. We  
125 collaborated with **treating veterinarians from five veterinary clinics near Lyon (France) to report**  
126 **pasturing dairy herds with an enzootic coughing (at least 30% of coughing cows) and supporting**  
127 **presumption of dictyocaulosis**. Eleven herds were reported and identified by a number from 1 to 11.  
128 Once we trapped all the cows from the herd at the feeding fence, five or six individuals were  
129 randomly sampled in each herd to get a representative sample of the herd (including clinically  
130 healthy and primiparous cows). We sampled a maximum of six **individuals per herd to ensure of the**  
131 **farmer approval**. Ten additional cows from the teaching herd of VetAgro Sup, without clinical sign,  
132 were included in the study (the 12<sup>th</sup> herd). This last herd was included to have a sufficient diversity of  
133 herd prevalences in order to insure the model convergence. **Even if this herd does not fit the**  
134 **inclusion criteria (herds with enzootic coughing), we assume that individual characteristics of the**  
135 **diagnostic methods for cows originated from this herd are similar to those of non-clinical and non-**  
136 **infected cows originated from the other herds**. We collected blood samples, faeces and  
137 bronchoalveolar lavage fluids (BALF) on each sampled cow. The experimental protocol was approved  
138 by the ethical comity of VetAgro Sup (n° 1539-2015).

### 139 Bronchoalveolar lavage technique

140 BAL was performed on non-sedated animals. After cleaning the nostril (chlorhexidine solution diluted  
141 at 2%), a naso-tracheal tube (Cook®) was passed to the lungs. Two 60 ml aliquots of 0.9% sterile NaCl  
142 solution were introduced by the catheter and 30 to 40 mL of fluid was re-aspirated immediately. The  
143 collected fluid was then split into two 4 ml EDTA tubes for cytological analysis and the remaining fluid  
144 was placed in a sterile container for parasitological examination. Samples were stored 24-48 h at 4°C  
145 before laboratory analyses.



146 Cytology

147 We pipetted 200 µl of homogenized BALF from one of the EDTA tubes to prepare one  
148 cytocentrifugated smear (10 min, 106g) which was automatically stained (Aerospray 7150, Wescor®).  
149 We then assessed the eosinophil proportion (EP) by performing a differential count on at least 400  
150 leucocytes on each cytospin.

151 Parasitological tests

152 Two parasitological tests were performed, (1) the Baermann sedimentation technique on faecal  
153 samples (BS test) and (2) the visual examination of the BALF for last stages of *D. viviparus* (BAL test).  
154 Faeces were sampled directly from the rectum of each selected cow and stored at 4°C. Within 12h  
155 after collection, we placed 50g of faeces in a sieve (1 mm mesh size) with a standard gauze compress  
156 at the surface of the water on a funnel. After 24 h, we collected and centrifuged (5 min, 700g) the  
157 first 12 ml of water at the bottom of the funnel, and performed microscopic examination (x25-40  
158 magnification) of the sediments. As it allows the isolation of *D. viviparus* larvae, the BS test could only  
159 diagnose patent infection with adult breeding parasites in the lungs. The two aliquots of BALF were  
160 directly examined macroscopically for the presence of lungworm stages. As this method could  
161 potentially detect any pulmonary stages of the parasite, we assume that it could diagnose both  
162 patent and pre-patent infection. Given that the identification of the larvae or adults of *Dictyocaulus*  
163 *viviparus* is reliably feasible (Eysker, 1997), and as regard to the definition of the infection status (i.e.  
164 non infected, infected by *D.viviparus* and infected by breeding *D.viviparus*), we assumed the  
165 specificity of the two parasitological tests to be equal to one.

166 MSP Elisa

167 The ELISA analysis was performed at the Institute for Parasitology of the University of Veterinary  
168 Medicine Hannover (Germany) according to the protocol previously described by von Holtum et al.  
169 (2008). All sera were kept at -80°C until analysis. This ELISA developed by von Holtum et al. (2008)

170 uses the major sperm protein from adult male lungworms as recombinantly expressed antigen. Thus,  
171 it could only diagnose patent infection. Results were expressed as optical density ratios (ODRs).

## 172 Statistical analysis

173 We built a global model to describe all the available data for each animal of the twelve herds. The  
174 unknown infection status of the  $j^{\text{th}}$  animal sampled in the  $i^{\text{th}}$  herd, denoted  $Sinf_{ij}$  is equal to 1 for  
175 infected and 0 for non-infected cows. It is supposed to have a Bernoulli distribution, with a Bernoulli  
176 parameter  $Pinf_i$  being the prevalence in the herd. This prevalence is unknown but supposed to be  
177 dependent of the herd.

$$178 \quad Sinf_{ij} : Bern(Pinf_i)$$

179 Given that the BS and the serology only diagnose patent infection, we also defined the unknown  
180 patent status of the  $j^{\text{th}}$  animal sampled in the  $i^{\text{th}}$  herd, denoted  $Spatent_{ij}$ . It is equal to 1 for patent  
181 cows and 0 for pre-patent and non-infected cows. It is supposed to have a Bernoulli distribution  
182 among infected animals, with a Bernoulli parameter  $Ppatent$  being the overall proportion of patent  
183 animals among infected animals that can be defined as a function of the infection status:

$$184 \quad \begin{aligned} &\text{If } Sinf = 0, \quad Spatent_{ij} = 0 \\ &\text{If } Sinf = 1, \quad Spatent_{ij} : Bern(Ppatent) \end{aligned}$$

185 Given that the identification of the larvae or adult of *Dictyocaulus viviparus* is reliably feasible, and  
186 as regard to the definition of this infection status (*i.e.* presence or absence of infection), the  
187 specificity of the two parasitological tests is assumed to be equal to one, and their respective  
188 sensitivities are denoted  $Se^{BS}$  and  $Se^{BAL}$ . Note that the model also includes  $Seth^{BS}$  the theoretical  
189 sensitivity based on the diagnosis of patent infected animals and not all infected animals simply  
190 linked to the true sensitivity ( $Se^{BS}$ ) by  $Se^{BS} = Seth^{BS} \times Ppatent$ . The results of those tests on each

191 animal, denoted  $BS_{ij}$  and  $BAL_{ij}$  (1 if the test is positive, 0 in the other case), can simply be  
 192 expressed as a stochastic function of sensitivities of the test and of the patent or infection status:

$$193 \quad BS_{ij} : \text{Bern}\left(Se^{BS} \times Spatent_{ij}\right) \text{ and } BAL_{ij} : \text{Bern}\left(Se^{BAL} \times Sinf_{ij}\right)$$

194 Concerning the result of the MSP-ELISA, expressed as ODR and denoted  $ODR_{ij}$ , conditioned on the  
 195 latent patent status  $Spatent_{ij}$ , the model assumes that the decimal logarithm of the ODR follows a  
 196 normal distribution, where the mean  $\mu_{ij}$  and standard deviation  $\sigma_{ij}$  are controlled only by the  
 197 patent status:

$$198 \quad \log_{10}(ODR_{ij}) : N(\mu_{ij}, \sigma_{ij})$$

199 The mean of the normal distribution for patent animals was supposed to be greater than that of non-  
 200 patent animals ( $\mu_{non-patent}$ ), and parameterized by  $\theta$ , the positive difference between these two  
 201 means ( $\mu_{patent} = \mu_{non-patent} + \theta$ ). This parameterization was chosen to prevent the classical un-  
 202 identifiability of any mixture model (is group 1 the patent or the non-patent group?). Standard  
 203 deviations for patent ( $\sigma_{patent}$ ) and non-patent ( $\sigma_{non-patent}$ ) groups were not supposed equal.

204 The results of the eosinophil count were also modelled in close way by a Gaussian mixture with two  
 205 components representing infected and non-infected animals, characterized as for the previous  
 206 mixture by parameters  $\mu'_{non-inf}$ ,  $\theta'$ ,  $\sigma'_{inf}$  and  $\sigma'_{non-inf}$ . This mixture Gaussian distribution was used  
 207 to describe the logit distribution of the eosinophil proportion ( $EP_{ij}$ ) in the pulmonary leucocytes.

208 This former variable is not directly measured but estimated on each animal from the count of  $EC_{ij}$   
 209 eosinophils among a total count of  $TC_{ij}$  leucocytes (the total count is indexed by  $i$  and  $j$  and  
 210 considered as a covariate in the model as it was not exactly equal to 400 for each animal). The count  
 211 of eosinophils for each animal  $EC_{ij}$  is thus modelled by a binomial distribution of size parameter

212  $TC_{ij}$  and probability parameter the latent variable  $EP_{ij}$ , the logit of which is defined by the  
213 Gaussian mixture:

$$214 \quad EC_{ij} : Binom(TC_{ij}, EP_{ij}) \text{ with } \text{logit}(EP_{ij}) : N(\mu'_{ij}, \sigma'_{ij})$$

215 Vaguely informative uniform prior were assigned to each parameter allowing variation within a  
216 realistic range and forcing probability parameters ( $Seth^{BS}$ ,  $Se^{BAL}$ ,  $Pinfi$  and  $Ppatent$ ) to be  
217 between 0 and 1 and  $\theta$  and  $\theta'$  to be positive (Table 1). Monte Carlo Markov-Chain (MCMC)  
218 techniques were used to estimate the full joint posterior distribution of parameters from prior  
219 distributions and data. Computations were performed using the JAGS software via the R package  
220 rjags (Plummer et al., 2016) (a corresponding R script is provided in Supplementary material both in  
221 Appendix A and in a runnable script with corresponding data). Three independent MCMC chains  
222 were run in parallel. For each chain 110,000 samples were produced. We discarded the first 10,000  
223 as burn-in. To avoid autocorrelation, we thinned the remaining 100,000 samples by selecting one out  
224 of 20 samples, thus keeping 5000 samples per chain. We checked the convergence again by  
225 displaying MCMC chain traces and autocorrelation plots and by computing the Gelman and Rubin's  
226 statistics as modified by Brooks and Gelman (Brooks and Gelman, 1998). For each parameter, its  
227 point estimate was defined as the median of its marginal posterior distribution, and the 95% credible  
228 interval was defined from the 2.5 and 97.5 percentiles of this distribution.

229 For both Gaussian mixtures, ROC curves accompanied with their 95% credibility bands were built  
230 from the joint posterior distribution using the 15,000 MCMC samples obtained by pooling the 5000  
231 samples of each chain (Details of the computation are given in Appendix B. Supplementary material).  
232 For each of the 15,000 sampled ROC curves, an optimal cut-off was estimated by minimizing the  
233 distance to the upper left corner corresponding to sensitivity and specificity equal to one (Perkins  
234 and Schisterman, 2006). For each test the final optimal cut-off was estimated using the median of the  
235 15,000 sampled optimal cut-off values. We then estimated the sensibility and specificity of each test

236 at this optimal cut-off value using once again the joint posterior distribution. We also computed the  
237 posterior probabilities of each animal to be infected and to be patent using the 15,000 MCMC  
238 samples of the latent variables  $Sinf_{ij}$  and  $Spatent_{ij}$  in the model.

239 For representation of continuous observed data from serology and cytology, we used transformed  
240 data on which we assumed a mixture Gaussian distribution: we represented the optical density ratios  
241 ( $ODR_{ij}$ ) in decimal logarithm and the eosinophil proportions ( $EP_{ij}$ ) in logit. As some eosinophil  
242 proportions were estimated to 0, for representing data we fixed the corresponding values to an  
243 arbitrary value of -7. In fact, these values are censored and only estimated to be below the detection  
244 limit equal to 1/400 (roughly -6 in logit). This censoring was not a problem for modelling as the model  
245 directly describes the raw data (eosinophil count  $EC_{ij}$ ).

246 We performed a posterior predictive check with the fitted model by comparing predicted and  
247 observed values of the numbers of animals in each combination of the four tests, predicted and  
248 observed cumulative distribution functions of both quantitative measures (ODR and EP values), and  
249 predicted and observed numbers of individuals above and below the chosen cut-off values. In order  
250 to check the constant accuracy of tests across population, we also repeat the analysis with the  
251 exclusion of each herd of the study one a time (Kostoulas et al., 2017; Mahmmod et al., 2013).

## 252 **Results**

253 Prior distributions and descriptive statistics of posterior distributions (medians and 95% credible  
254 intervals) of all model parameters are summarized in Table 1 and representation of both Gaussian  
255 mixtures are shown in Figure 3. Posterior distributions of parameters characterizing the two mixture  
256 models and the test sensitivities were highly shrunk using data in comparison to prior distribution, as  
257 it is generally expected in such an inference. The fitted model seems to present a high overall  
258 goodness of fit regarding the results of the posterior predictive check (Table C1 and Figures C1, C2,  
259 and C3 in Appendix C, Supplementary material). Table C1 shows that 12 out of the 16 observed

260 numbers of animals in each test pattern are equal to their posterior median, and none are outside of  
261 the 95% credibility interval. Similarly, the observed numbers of animals above and below the chosen  
262 cut-off values for both continuous tests match their predicted posterior modes (Figure C1).  
263 Furthermore, Figure C2 and C3 also point out that the two modelled Gaussian mixtures well describe  
264 the observed cumulative distribution of ODR and EP values.

265 Infection prevalences of herds were sometimes difficult to estimate, with 95% credible intervals (95%  
266 CI) not far from the maximal interval [0, 1] (herds 1, 2, 4, 9), while the others were more precisely  
267 estimated. The overall proportion of patent animals among infected ones was estimated at 0.75 (95%  
268 CI [0.48; 0.96]). These patent animals are those with relative high EP and ODR values (red circle  
269 points in Figure 3). We estimated the sensitivities of parasitological tests to relatively low values:  
270 7.4% (95% CI [1.7; 19.4]) for the BS test and 24.7% (95% CI [11.9; 42.9]) for the BAL test. Note that we  
271 detected only one or two immature lungworms (less than 2 cm) in each bronchoalveolar fluid of the  
272 eight BAL positive cows. For the serology and cytology tests, the sensitivity (Se) and specificity (Sp)  
273 depend on the chosen cut-off value. For each of these two continuous variables (log(ODR) for the  
274 serology and logit(EP) for the cytology), Se and Sp were plotted as a function of the cut-off within a  
275 classical ROC curve analysis (Figures 1 and 2). The optimal cut-off value for serology was estimated at  
276 0.397 ODR, corresponding to a sensitivity of 74.9% (95% CI [62.7; 79.7]) and a specificity of 85.5%  
277 (95% CI [72.1; 93.8]). We also estimated the theoretical sensitivity of the serology at the optimal cut-  
278 off, if used to diagnose only patent animals and not all the infected animals. This theoretical  
279 sensitivity was estimated at 96.9% (95% CI [80.3; 99.8]).

280 The optimal cut-off value for the cytology was estimated at 4.77% corresponding to a sensitivity of  
281 89.4% (95% CI [76.9; 96.7]) and a specificity of 85.2% (95% CI [68.8; 99.4]). As shown in Figure 3, the  
282 detection limit of the cytology (-5.99 in logit) was far lower than the optimal cut-off value (-2.85 in  
283 logit). Thus, counting 400 cells during the cytology test seemed sufficient to prevent an impact of  
284 censored values on the diagnosis. As the bronchoalveolar lavage combines two diagnostic methods,

285 the parasite identification in the BALF and the pulmonary eosinophilia, we estimated the sensitivity  
286 and specificity of a global BAL test combining these two methods. We considered an animal positive  
287 if at least one of the two tests was positive, and negative if both tests were negative. Thus, the  
288 sensitivity of the global BAL test was estimated at 91.4% (95% CI [80.7; 97.4]) and the specificity at  
289 85.2% (95% CI [68.8; 99.5]).

290 At the herd level, for the case where these methods would be applied to confirm a dictyocaulosis  
291 presumption (*i.e.* to find at least one positive animals in the herd), we calculated the number of  
292 samples required to reach a herd sensitivity of 95%. Veterinarians would have to perform a minimum  
293 of either 40 Baermann sedimentation tests (95% CI [14; 170]), 11 BAL parasitological tests (95% CI [6;  
294 24]), 3 serology tests (95% CI [2; 4]) or 2 eosinophil proportion counts (95% CI [1; 3]). For the two last  
295 methods, the herd specificity corresponding to the probability to find at least one positive animal in  
296 an uninfected herd would drop to 62.2% (95% CI [37.3; 83.0]) and 72.7% (95% CI [47.7; 98.9])  
297 respectively.

## 298 **Discussion**

299 This study evaluated the performance of four diagnostic methods for dictyocaulosis in adult cattle  
300 under field conditions and in the absence of a known gold standard test. The sensitivity and  
301 specificity of each test, as well as cut-off values for the two quantitative methods, were estimated by  
302 Bayesian inference, fitting a global model describing all the raw data collected in the experiment.  
303 One of the main benefits of such an approach is the global consideration of the four test results to  
304 estimate the individual status of each cow. Likewise, the two mixture models are fitted  
305 simultaneously but not independently, allowing a better consideration of discordant results and  
306 limiting the animal misclassification for both tests. Furthermore, the censoring of low values of  
307 eosinophil proportions is naturally included in the Bayesian approach by directly modelling the count  
308 of eosinophils, without any need of specific methods dedicated to censored data.

309 In a Bayesian framework, the posterior estimation depends upon both prior information and  
310 observed data. In our study, prior information was particularly poor because none of the four  
311 diagnostic methods has been characterized in the field with adult cows. Ploeger *et al.* (2014)  
312 determined in a field study the sensitivity and the specificity of the serology with the faecal larvae  
313 excretion (i.e. the Baermann technique) as reference test, while it has a poor sensitivity in adults.  
314 Similarly, there are few data about the BALF cytology in cows. Even if none of the previous  
315 publications we have had access to mentions healthy cows with an eosinophil rate at over one  
316 percent, we cannot be sure that every healthy cow has an eosinophil rate less than one percent.  
317 Therefore, all prior information was chosen only vaguely informative.

318 One assumption of the latent class analysis is that all diagnostic methods measure the same infection  
319 status. This assumption is however not respected in our study as the Baermann sedimentation and  
320 the serology require mature worms (patent infection) to be positive whereas pulmonary eosinophilia  
321 and BAL test only require the presence of immature or adult parasites (i.e., pre-patent and patent  
322 infection) in the lung. By modelling the three class latent status (non-infected, pre-patent and patent)  
323 the latent class model remains valid. The use of multiple latent disease status is also a method  
324 allowing for the consideration of conditional dependence between tests (Dendukuri *et al.*, 2009).  
325 Indeed, in our case, BS and serology could not have been considered as independent conditionally to  
326 the infection status because they both detect only patent infection. However, conditionally to the  
327 three class latent status, the four diagnostic methods could be considered as independent because  
328 they are based on different biological principles.

329 The model assumes that the overall proportion of patent animals among infected animals is constant  
330 in all herds. It is an important constraint that allows an easy consideration of pre-patent and patent  
331 infections. However, this proportion might vary from one herd to another especially if the outbreak  
332 occurs at different times before the sampling or if cows are well immune against *Dictyocaulus*  
333 *viviparus*. The model could be improved by taking into account this proportion variability for instance



334 using a hierarchical model but the simple proposed model already provides a good fit to the data as  
335 shown by the results of the posterior predictive check (see Table C1 and Figure C1, C2 and C3 in the  
336 supplementary material).

337 The Bayesian latent class model requires herds with different prevalences to ensure a proper  
338 convergence of the algorithm. It led us to include a clinically healthy herd with no history of  
339 dictyocaulosis (the 12<sup>th</sup> herd). Then, the assumption of constant accuracy of tests across populations  
340 might not have been appropriate. We checked this assumption by repeating the analysis with the  
341 exclusion of each herd of the study one at a time and results are presented in the table D in the  
342 supplementary material. The exclusion of each herd led to slightly different point estimate of cutoff,  
343 sensitivity and specificity of each test but every credibility interval stay very close to the original  
344 estimations. In particular, the exclusion of the 12<sup>th</sup> herd led to quite similar point estimate but wider  
345 credibility intervals. Then the assumption of constant accuracy of test across population could be  
346 considered as a reasonable assumption.

347 Parasitological results are quite different compared to previous studies (Ploeger et al., 2012). Only  
348 3.3% of cows originating from clinically positive herds were positive with the BS test, with a low level  
349 of larval excretion (less than 1 larvae per 10g of faeces; data not shown), whereas it was 20.1% in the  
350 quoted study. The climatic conditions could partly explain these differences, as spring and summer  
351 2015 were very dry. These particular climatic conditions may have lowered larvae survival in the  
352 environment and consequently reduced the number of larvae ingested by the cows, resulting in  
353 rather low infection intensities. Furthermore, acquired immunity during the present or previous  
354 pasture season may have prevented invasion and maturation of a large number of ingested parasites,  
355 again resulting in reduced larval excretion by infected cows. We might have found perhaps more  
356 larvae and positive cows by using the McKenna modified method of sedimentation instead of the  
357 Baermann method (McKenna, 1999), even if it could have been compensated by the use of a higher  
358 amount of faeces (50g vs 30g). As the sensitivity of faecal examination may vary according to the

359 technique and the amount of faeces used, the estimated sensitivity of the Baermann sedimentation  
360 in this study may perhaps not be generalised to other faecal examination techniques. The sensitivity  
361 of the BS test was evaluated at 7.4% (95% IC [1.7; 19.4]) in adult cows. Identification of *D. viviparus* in  
362 the BALF (BAL test) seems to be more sensitive (Se = 24.7% (95% IC [11.9; 42.9]) than the BS test.  
363 Despite a relatively high proportion of patent animals (0.75; 95% CI [0.48; 0.96]) which should host  
364 fully developed worms, we only recovered immature *D.viviparus* in the BALF of BAL positives cows. It  
365 seems not to be due to the diameter of the lung catheter as we already recovered adult parasites (5  
366 to 6 cm) with the same device during preliminary tests (*unpublished data*), but rather to the  
367 immunity of adult cows, which may slow down the *D.viviparus* development in the lungs. Then, they  
368 may not be able to reach the mature stage, which could explain the low recovery of adult worms in  
369 the BALF and also the low sensitivity of the Baermann sedimentation.

370 The lung cytology and serology seem to be better-suited diagnostic tools for dictyocaulosis than  
371 parasitological tests. The optimal cut-off value of the serology proposed by the model (*i.e.* 0.397  
372 ODR) is lower than the recommended cut-off (*i.e.* 0.500 ODR) by Von Holtum *et al.* (2008). This  
373 model-proposed cut-off value of 0.397 ODR is probably adapted to adult cows but it seems too low  
374 for reliable differentiation between infected and non-infected calves, as in the study by Von Holtum  
375 *et al.* (2008) the sera from helminth-negative calves reached values up to 0.490 ODR. Otherwise,  
376 according to our model, at the cut-off indicated by Von Holtum *et al.* (2008), the sensitivity would be  
377 61.2% (95% CI [40.8; 82.6]) and the specificity 96.1% (95% CI [87.8; 99.2]). As expected, these values  
378 were lower than those estimated from controlled experimental infections (Se and Sp > 99%; (Von  
379 Holtum *et al.*, 2008)), especially for the sensitivity. Most of the differences observed on the serology  
380 characteristics between field and experimental studies must be due to the presence of pre-patent  
381 infections which reduce the observed sensitivity of the serology. A seasonal variation of the  
382 proportion of pre-patent infected animals could also explain the observed seasonal variation of the  
383 sensitivity and specificity of the serology (Ploeger *et al.*, 2012, 2014).

384 To our knowledge, this study is the first to provide a cut-off (-2.99 in logit or 4.77% eosinophils in  
385 cellular proportion of BAL, designed by the model) as well as sensitivity and specificity values (89.4%  
386 with 95% CI [76.9; 96.7] and 85.2% with 95% CI [68.8; 99.4], respectively) for bronchoalveolar  
387 cytological measurement for the diagnosis of dictyocaulosis in adult cows. The proposed cut-off  
388 (4.77%) is relatively high compared to previously reported eosinophil proportion values in healthy  
389 calves (all <1%, n=15; Hagberg *et al.*, 2005). The combined bronchoalveolar lavage diagnostic method  
390 (the parasite identification in the BALF and the pulmonary eosinophilia) is as specific as the  
391 pulmonary eosinophilia alone but slightly more sensitive (91.4% with 95% CI [80.7; 97.4]). This  
392 difference comes from few cows with EP values under the cut-off value despite we found parasites in  
393 the BALF.

394 At the herd level, we calculated the number of infected animals to sample to reach a herd sensitivity  
395 of 95%. Unfortunately, as we did not monitor the clinical status of cows, we could not predict if  
396 clinical cows were more likely to be positive than sub-clinical ones. It could be interesting to refine  
397 our analysis based on cows with known clinical status. The two parasitological methods seem to be  
398 limited, because they had to be performed on a large number of infected animals to reach 95%  
399 sensitivity. The individual serology seems to be more suitable than the parasitological methods to  
400 diagnose dictyocaulosis infection at the herd level because it only requires 3 samples to reach a herd  
401 sensitivity of 95% even if it has a low herd specificity. Collecting BAL fluid is more complicated than  
402 collecting blood or faeces, but it can be a relevant technique as it may allow diagnosing  
403 dictyocaulosis in a herd with only two samples. Moreover, the bronchoalveolar lavage combines two  
404 diagnostic methods, increasing the opportunities to diagnose dictyocaulosis. If BAL is performed on  
405 two animals suffering from dictyocaulosis in a herd, the probability of isolating *D. viviparus* in the  
406 BALF is 43% (95% CI [22; 68]) and if negative, cytological examination could be performed with a 95%  
407 probability to diagnose dictyocaulosis with an eosinophil proportion superior to 4.77%.

408 Performing analyses on pooled samples could be an interesting alternative to screen more animals,  
409 with limited time and financial costs, but this might be at the expense of the sensitivity. Bulk tank  
410 milk ELISA (Fiedor et al., 2009; Schunn et al., 2012) or ELISA on pooled serums (Chartier *et al.*, 2015)  
411 could also be interesting methods of herd diagnosis. Serology is also easier than the BAL to carry out  
412 on the farm and is more suitable for large epidemiological studies. Furthermore, the serology only  
413 detects patent infections whereas BAL detects both patent and pre-patent infections. Thus, at the  
414 individual level, BAL should be preferred to confirm a dictyocaulosis presumption.

## 415 **Conclusion**

416 This study provides for the first time a combined estimation of the characteristics of different  
417 commonly used methods to diagnose dictyocaulosis, including the first evaluation of the BAL  
418 technique. Overall, we confirmed the rather low sensitivity of the Baermann technique in adult cows  
419 (7.4%). Furthermore, bronchoalveolar lavage proved to be a suitable technique to confirm a  
420 dictyocaulosis suspicion at the individual level in dairy cows. This technique is interesting as it  
421 combines two complementary and relevant diagnostic methods: first, the BAL parasitic test which  
422 can potentially provide a quick result on the farm, and second, the eosinophil proportion, a highly  
423 sensitive diagnostic measure of dictyocaulosis at the veterinary office. Despite a lower sensitivity due  
424 to pre-patent infections, the serology remains a suitable method to confirm a hypothesis of  
425 dictyocaulosis in adult cow herds and it is more practicable for large epidemiologic studies.

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## 431 **Conflict of interest**

432 No conflict of interest to be declared.

433

434 **Supplementary material**

435 Appendices A, B, C and D and a runnable R script with corresponding data can be found in the online

436 version of this article.

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553



554 Table 1 : Model parameters with corresponding prior distributions and posterior estimates.

|  | Parameter       | Prior distribution | Posterior estimate<br>Median [95% credible interval] |
|--|-----------------|--------------------|--|
| Test sensitivities                               | $Se^{BS}$       | Unif(0, 1)         | 0.074 [0.017; 0.194]                                 |
|  | $Se^{BAL}$      | Unif(0, 1)         | 0.247 [0.119; 0.429]                                 |
| Herd prevalences                                 | $P_1$           | Unif(0, 1)         | 0.395 [0.035; 0.843]                                 |
|  | $P_2$           | Unif(0, 1)         | 0.331 [0.0147; 0.856]                                |
|  | $P_3$           | Unif(0, 1)         | 0.797 [0.28; 0.991]                                  |
|  | $P_4$           | Unif(0, 1)         | 0.665 [0.153; 0.982]                                 |
|  | $P_5$           | Unif(0, 1)         | 0.888 [0.536; 0.996]                                 |
|  | $P_6$           | Unif(0, 1)         | 0.727 [0.337; 0.957]                                 |
|  | $P_7$           | Unif(0, 1)         | 0.102 [0.004; 0.439]                                 |
|  | $P_8$           | Unif(0, 1)         | 0.903 [0.589; 0.996]                                 |
|  | $P_9$           | Unif(0, 1)         | 0.596 [0.172; 0.928]                                 |
|  | $P_{10}$        | Unif(0, 1)         | 0.392 [0.081; 0.77]                                  |
|  | $P_{11}$        | Unif(0, 1)         | 0.311 [0.048; 0.7981]                                |
|  | $P_{12}$        | Unif(0, 1)         | 0.06 [0.002; 0.29]                                   |
| Proportion of patent animals among infected ones | $P_{patent}$    | Unif(0, 1)         | 0.749 [0.479; 0.958]                                 |
| ODR mixture parameters                           | $\mu_{non-inf}$ | Unif(-10, 10)      | -0.587 [-0.640; -0.526]                              |

|                       |                            |               |                      |
|-----------------------|----------------------------|---------------|----------------------|
|                       | $\theta$                   | Unif(0, 10)   | 0.400 [0.316; 0.471] |
|                       | $\sigma_{\text{non-inf}}$  | Unif(0, 5)    | 0.162 [0.128; 0.213] |
|                       | $\sigma_{\text{inf}}$      | Unif(0, 5)    | 0.123 [0.087; 0.195] |
| <hr/>                 |                            |               |                      |
| EP mixture parameters | $\mu'_{\text{non-inf}}$    | Unif(-10, 10) | -4.87 [-5.81; -4.01] |
|                       | $\theta'$                  | Unif(0, 10)   | 3.23 [2.33; 4.12]    |
|                       | $\sigma'_{\text{non-inf}}$ | Unif(0, 5)    | 1.76 [0.94; 2.54]    |
|                       | $\sigma'_{\text{inf}}$     | Unif(0, 5)    | 1.07 [0.83; 1.45]    |
| <hr/>                 |                            |               |                      |

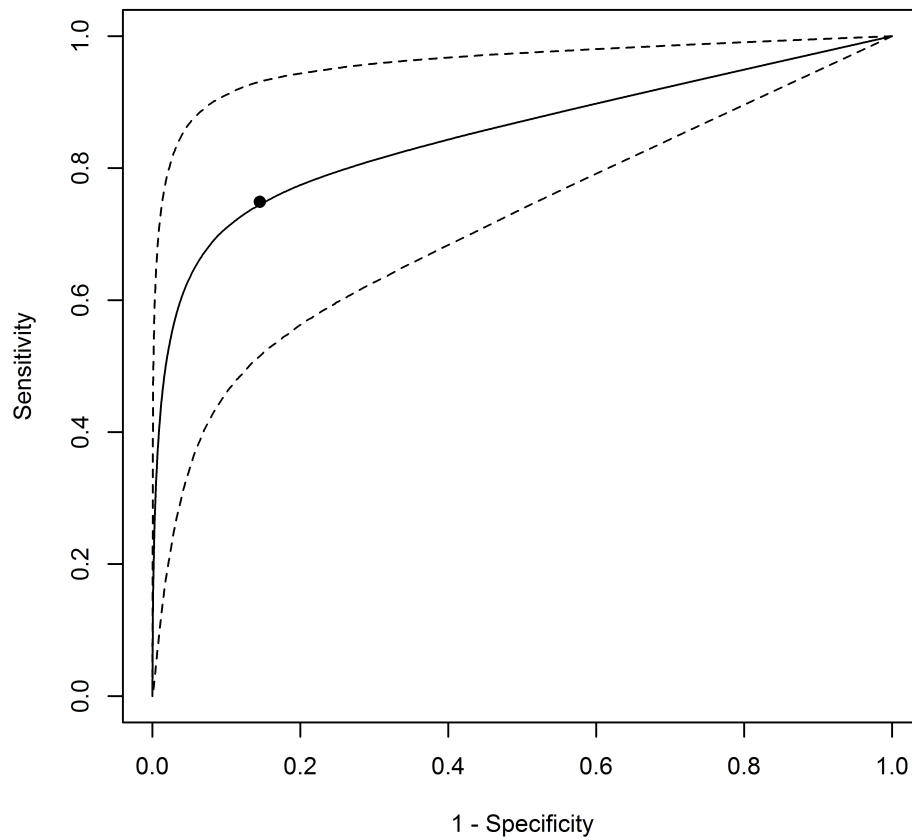
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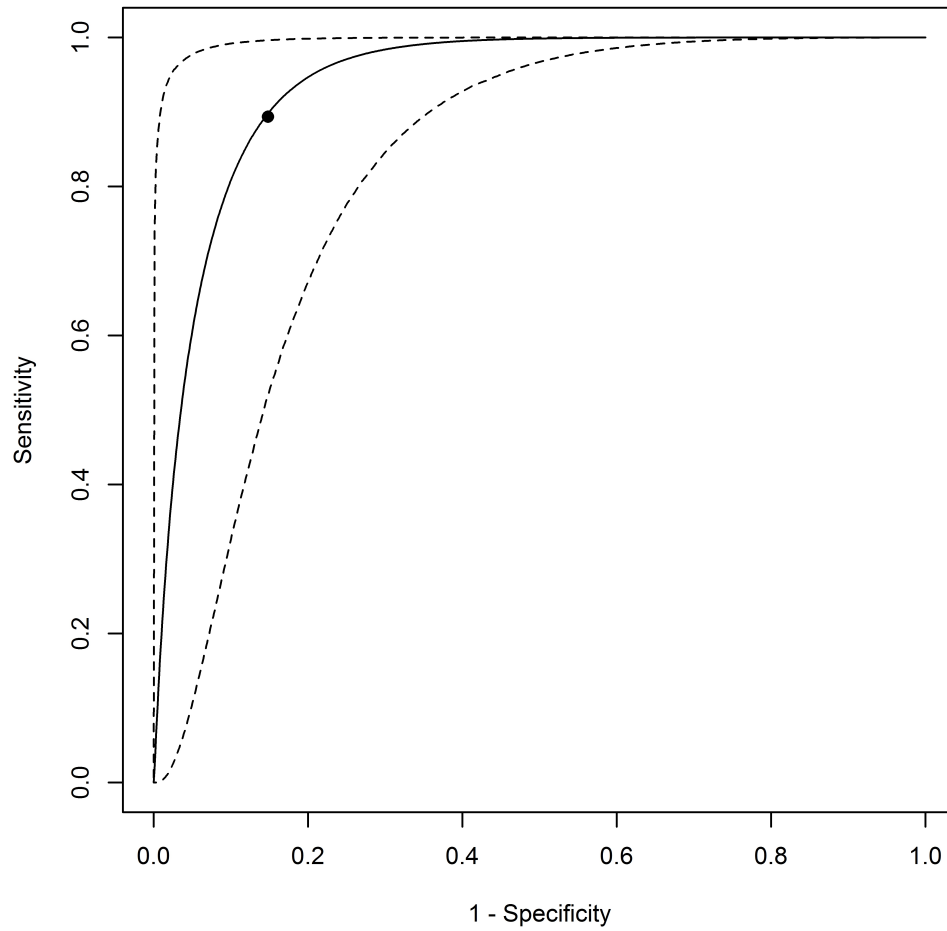
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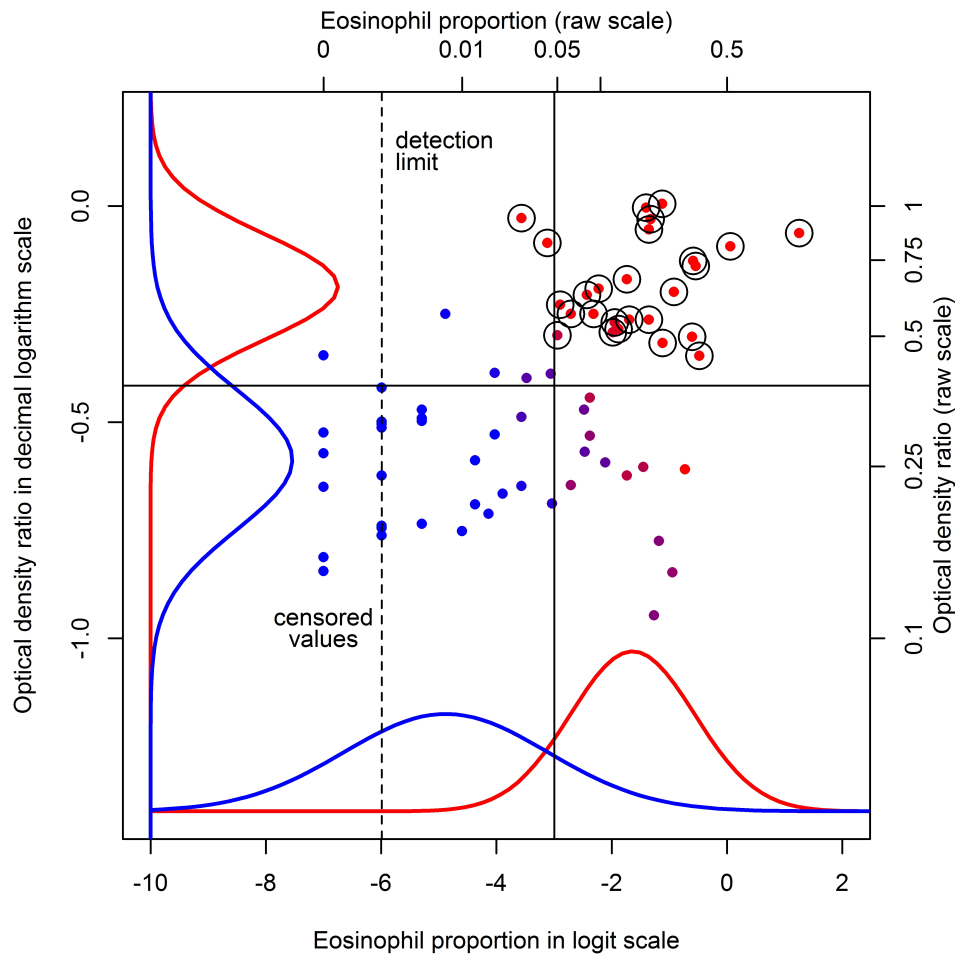
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561 Figure 1: ROC curve for the serology. Dotted lines represent a 95% credible interval around the ROC  
 562 curve and the point on the ROC curve corresponds to the estimated cut-off of 0.397 for the optical  
 563 density ratio (ODR; -0.415 in decimal logarithm).



564

565 Figure 2: ROC curve for the cytology. Dotted lines represent a 95% credible interval around the ROC  
566 curve and the point on the ROC curve corresponds to the estimated cut-off of 4.77% for the  
567 eosinophil proportion (-2.99 in logit scale).



568

569 Figure 3: Scatter plot of observed data from the serology and the cytology tests, with points coloured  
 570 according to the posterior probability of infection of each animal calculated from the model (colour  
 571 gradient from blue for 0 to red for 1). Circular points represent animals with a posterior probability of  
 572 being patent superior to 0.5, triangular points the ones with a probability of infection inferior to 0.5  
 573 and square points the ones with a probability of infection superior to 0.5 but a probability of being  
 574 patent inferior 0.5. These three clusters are surrounded by ellipses respectively coloured in red, blue  
 575 and purple. Red and blue curves represent the estimated density of the two components of the  
 576 Gaussian mixture for each test (the red ones for positive animals and the blue ones for negative  
 577 animals). Plain vertical and horizontal lines indicate optimal cut-off estimates respectively for the  
 578 eosinophil proportion and the optimal density ratio. The vertical dotted line represents the detection  
 579 limit for the eosinophil proportion (one eosinophil out of 400 total cells counted).