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Title: Immunoblotting for the serodiagnosis of alveolar echinococcosis in alive and dead Eurasian beavers (*Castor fiber*)

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1 Immunoblotting for the serodiagnosis of alveolar echinococcosis in alive and dead Eurasian beavers  
2 (Castor fiber)

3

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12

13 Abstract

14 A novel species-specific anti-beaver-IgG-alkaline-phosphatase conjugate was synthesized for the  
15 development a of a new serological test for echinococcosis in beavers. Two different ELISAs  
16 conventionally used for human *Echinococcus multilocularis* serology (Em18-ELISA and Em2-ELISA)  
17 yielded diagnostic sensitivities of 0% and 46%, respectively. In contrast, the subsequently developed  
18 immunoblotting assay gave an 85% diagnostic sensitivity (11 out of 13 beavers with alveolar  
19 echinococcosis were immunoblotting-positive, i.e. showed reactivity with a specific 21 Mr band), and  
20 maximal specificity. In conclusion, this immunoblotting assay should be the method of choice for use in  
21 serological studies on *E. multilocularis* in Eurasian beavers, and the test proved suitable to investigate  
22 both animals alive and post-mortem.

23

24 Keywords

25 *Echinococcus multilocularis*; beaver; Em2-ELISA; Em18-ELISA; immunoblotting; EmVF-antigen

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30

## 30 Introduction

31 *Echinococcus multilocularis* is one of the most pathogenic parasitic zoonoses prevalent in central  
32 Europe. The definitive (final) wildlife hosts in Europe are canids, including predominantly the red fox  
33 (*Vulpes vulpes*), but the non-native raccoon dog (*Nyctereutes procyonoides*) and the domestic dog are  
34 affected as well (Carmena and Cardona, 2013; Carmena and Cardona, 2014). Adult tapeworms live in  
35 the small intestine of the definitive hosts, from which gravid parasite proglottids and eggs are shed with  
36 the faeces into the environment. Intermediate hosts are infected when they ingest parasite eggs, which  
37 upon release of an oncosphere, develop into the larval stage called metacestode. Metacestodes show a  
38 distinct predilection for the liver. At a later stage of infection, metastases develop predominantly in the  
39 lungs and brain, though other organs can be affected. The metacestode proliferates continuously and  
40 leads to a cancer-like disease in affected intermediate hosts. The sylvatic cycle is completed by  
41 carnivore predation of such infected intermediate hosts. In Europe several species of small microtine  
42 and arvicolid rodents are the principle intermediate hosts, as well as two larger introduced species, the  
43 coypu (*Myocastor coypus*) and the muskrat (*Ondatra zibethicus*) (Mathy et al., 2009). The Eurasian  
44 beaver (*Castor fiber*) is another intermediate host. The first cases were reported from Switzerland  
45 (Janovsky et al., 2001; Wimmershoff et al., 2012) and Austria (Cronstedt-Fell et al., 2010), and more  
46 recent cases were described in the United Kingdom (Barlow et al., 2011) and in Serbia (Cirovic et al.,  
47 2012). Humans are paratenic (“accidental”) intermediate hosts.

48 A beaver reintroduction to Britain is currently underway as a scientific trial in Scotland, with a large  
49 population of free-living beavers now established on the east coast. Further releases have been  
50 proposed in Wales, and feasibility investigations are undertaken in England. However, there is now  
51 another growing population of free-living beavers, which have either escaped from captivity or been  
52 purposely, released in Scotland and England but are not part of government-sanctioned trials. The origin  
53 of beavers for importation has been subject to academic debate (Halley 2011, Rosell et al. 2012), the  
54 health status of imported animals being a key parameter. Current prevention measures include a six  
55 months quarantine period, which is deemed sufficient to prevent the associated entry of rabies.  
56 Screening for other infectious organisms is not required during this period, but additional health  
57 screening recommendations have been made (Goodman et al. 2012). Among others, the risk of  
58 introduction of *E. multilocularis* to non-endemic regions via importation of beavers originating from  
59 endemic areas has been assessed following the Office International des Epizooties (OIE) risk assessment  
60 framework (Kosmider et al., 2013; Defra, 2012). Subsequently, cases have been detected among beavers  
61 meant for reintroductions (Cirovic et al., 2012; Barlow et al., 2011), and it is now recognized that both  
62 captive and wild-caught beavers from central Europe represent a risk to import *E. multilocularis* to  
63 presently *E. multilocularis*-free areas (Barlow et al. 2011; Campbell-Palmer et al., 2012; Pizzi et al., 2012).

64 So far, cases of echinococcosis in beavers have mainly been diagnosed by post-mortem investigation,  
65 principally based on methods that have been widely used to identify the larval stage of *E. multilocularis*  
66 in other rodents and in human patients. Beside conventional histopathology, a molecular analysis by  
67 PCR and/or direct immunofluorescence yields a reliable species-specific identification of the parasite.  
68 This is particularly helpful in the rare cases where histology findings are inconclusive (Diebold-Berger et  
69 al., 1997).

70 In contrast, diagnosing alveolar echinococcosis in live beavers is challenging. Imaging procedures may  
71 provide a certain degree of information, as it has been demonstrated in other rarely infected  
72 intermediate hosts, e.g. dogs (Scharf et al., 2004), rats (Asanuma et al., 2005) and non-human primates  
73 (Kishimoto et al., 2009) but it is not reliable enough to rule out an infection. Currently, investigation of  
74 live beavers prior translocation includes a time-consuming combination of clinical examination and  
75 diagnostic imaging, such as detailed abdominal ultrasonography combined with endoscopic surgical  
76 visual examination of the liver and other abdominal organs in anaesthetized animals (Pizzi et al., 2012).  
77 A serological test could be used as a rapid diagnostic tool that could considerably reduce such  
78 investigations in beavers. Besides its application to prevent the entry of the parasite via imported  
79 individuals, serology could also be useful to assess exposure in captive or free-living populations  
80 considered potential sources for translocation projects as well as to estimate prevalence in infected  
81 populations. To our knowledge, serological diagnosis of parasitic infections in beavers has not yet been  
82 reported. The goal of this study was to elaborate and evaluate serological tests regarding their suitability  
83 to diagnose an *E. multilocularis* infection in the Eurasian beaver.

84

## 84 Materials and Methods

85 Study design, animals and samples

86 We comparatively evaluated several conventional antigens presently used to detect anti-E.  
87 multilocularis antibodies in intermediate hosts such as humans and small rodents (crude vesicle fluid,  
88 EmVF-antigen [Müller et al., 2007], Em2-antigen [Gottstein et al., 1991], Em18-antigen [Sako et al.,  
89 2002]).

90 In a first step we developed a new anti-beaver-IgG-specific secondary antibody, because we had found  
91 out in preliminary experiments that heterologous conjugates (anti-mouse IgG; protein A, protein G)  
92 yielded unsatisfactory results with beaver samples. This host-specific antibody, at an affinity-purified  
93 status, was coupled to alkaline phosphatase by using a conventional procedure provided by the  
94 manufacturer (Sigma-Aldrich, [http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-  
95 explorer/analytical-enzymes/alkaline-phosphatase/conjugation.html](http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase/conjugation.html)).

96 In a second step, we compared two ELISAs and immunoblotting as previously evaluated for human  
97 alveolar echinococcosis serology (Müller et al., 2007). Test evaluation was done using the samples from  
98 three different groups of beavers: (1) a “positive” group of 13 beavers confirmed to be infected with E.  
99 multilocularis by post-mortem investigations including histopathology and PCR (nine animals of Swiss  
100 origin and four animals of Austrian origin); (2) a “negative” group consisting of 27 beavers originating  
101 from a region known to be non-endemic for E. multilocularis (Scotland), i.e., animals expected to have  
102 not been exposed to the parasite; (3) another “negative” group including 29 beavers from areas  
103 endemic for E. multilocularis (25 dead animals from Switzerland and four from Austria), i.e., with  
104 possible previous exposure. All negative beavers (groups 2 and 3) were necropsied and did not present  
105 lesions consistent with the presence E. multilocularis infection. For statistical determination of a  
106 negative-positive-threshold value in ELISA, only negative animals from group (2) were used.

107 For all beavers, blood samples consisted in sero-sanguinous fluid collected post-mortem, either  
108 obtained from clotted heart-blood, or (if not available) muscle juice obtained as described elsewhere  
109 (Berger-Schoch et al., 2011). Samples were kept frozen at -20°C until further analysis.

110 Anti-beaver IgG-conjugate

111 One ml of frozen beaver blood was used to purify IgG with the ammonium sulfate precipitation  
112 technique as described by Page and Thorpe (2002), by applying a three-cycle-precipitation at 40%, 40%  
113 and 50% saturation conditions. Purified beaver IgG was sent to Gallus Immunotech Inc. (Ontario,  
114 Canada) for the production of affinity-purified chicken anti-beaver-IgG, based on IgY isolation from eggs  
115 derived from hens immunized with 100µg beaver-IgG emulsified in Freund’s adjuvants (indications refer

116 to one immunization shot; hens received 1 primary immunization and 3 boosters every 10 days). Egg  
117 yolk IgY was subsequently immuno-affinity purified on a solid-phase beaver-IgG column as previously  
118 described (Felleisen and Gottstein, 1993). All subsequent steps to prepare the final anti-beaver-IgG-  
119 alkaline phosphatase-conjugate were carried out as described elsewhere (Baumann and Gottstein,  
120 1987).

121 For a primary validation of the newly synthesized anti-beaver-IgG-conjugate, we electrophoretically  
122 separated the purified beaver-IgG by SDS-PAGE and applied a Coomassie-blue staining, followed by  
123 immunoblotting.

#### 124 ELISA

125 All blood samples were examined for antibodies directed against the Em2- and recEm18-antigen from *E.*  
126 *multilocularis* by ELISA as previously published for foxes (Gottstein et al., 1991) but using the beaver-  
127 IgG-specific alkaline phosphatase conjugate described above. Beaver samples were considered sero-  
128 positive when the ELISA A405nm-values exceeded the average negative control value plus 4 standard  
129 errors (S.E.). The actual threshold value discrimination between negative and positive reactions was  
130 based on a 99.9% range exhibited by the 26 “negative” beaver samples from non-endemic areas, this  
131 was performed for each antigen separately. We used the mean plus 4 S.E. to calculate the threshold  
132 values for the Em2- and the recEm18-antigen, respectively. All values above these cut-offs were  
133 regarded as positive, all others below as negative. A ROC based approach was not feasible due to the  
134 low number of “positive” cases.

#### 135 Immunoblotting

136 Immunoblotting was performed as previously described for *E. granulosus* hydatid fluid (EgHF) antigen  
137 (Poretti et al. 1999), with the exception that EmVF (7 µg per cm slot) was used instead of EgHF (Müller  
138 et al., 2007). The conjugate was identical to that used for the ELISAs described above.

#### 139 Statistical analyses

140 Data were analyzed using the computer program SPSS 17.0. One-way ANOVA and Student’s t-test were  
141 used to determine threshold values and to compare differences between groups.  $P < 0.05$  was considered  
142 as indicating statistical significance.

143

143 Results

144 Anti-beaver IgG-conjugate

145 Electrophoresis (Figure 1A) and Coomassie blue staining revealed the presence of two predominating  
146 bands, the upper one corresponding by relative molecular mass ( $M_r$ ) to the heavy chain of the  
147 antibody, and the lower one to the light chain. Immunoblotting analysis of these two bands upon use of  
148 the anti-beaver-IgG-alkaline-phosphatase conjugate revealed the binding capacity of the conjugate to  
149 both antibody chains, with a stronger activity to the heavy chain (Figure 1B).

150 ELISA

151 Table 1 shows the results obtained with the two ELISAs for the beaver samples from the “positive”  
152 group. With the Em18-ELISA, all samples from this group yielded negative findings. With the Em2-ELISA,  
153 six out of 13 “AE-positive” beavers showed a serological reaction. Thus, the diagnostic sensitivities of  
154 both tests were very low (0% and 46%, respectively).

155 All 29 beaver samples of the “negative” group from endemic areas were serologically negative, i.e., the  
156 obtained values were all in the same range as the 27 “negative” samples from non-endemic areas used  
157 to determine the cut-off point. Statistically, there was no difference between the median value of the  
158 negative sera from non-endemic areas and those from endemic areas.

159 Immunoblotting

160 Immunoblot profiles, as shown for two samples from the “positive” group (beavers B1 and B2, Figure 2),  
161 demonstrated antibody reactivity with one major immunoreactive band and two minor side bands of  
162 approximately  $M_r$  21 (major band), and  $M_r$  19 and  $M_r$  40 (minor bands). The localization of these three  
163 bands corresponded to the localization of bands obtained with a positive control of human origin (H1).  
164 This human serum banding pattern also matched the one described earlier in a large human serological  
165 study (Müller et al. 2007). Based on the detection of an anti-21 $M_r$ -banding activity, the immunoblotting  
166 approach yielded an 85% diagnostic sensitivity, as 11 out of 13 beavers from the “positive” group were  
167 seropositive, (Table 1). All “negative” and “true negative” beaver samples were clearly seronegative,  
168 considering the absence of any band (exemplified by samples B3 – B5 in Figure 2).

169

169 Discussion

170 The purpose of this study was to develop and evaluate the suitability of serological tests for the  
171 detection of *E. multilocularis* infection in the Eurasian beaver. Such a rapid diagnostic tool is urgently  
172 needed to facilitate the procedures aiming at minimizing the risk of introducing the parasite via  
173 translocated animals, which requires both the testing of translocated individuals in vivo prior to release,  
174 and screenings of potential source populations.

175 A serological test would be applicable on blood samples from both live and dead beavers, making it  
176 suitable for in-vivo testing of animals prior translocation and for serological surveys using samples  
177 collected post-mortem. The beaver-specific conjugate developed in this study operates methodically  
178 very well in ELISAs and also in immunoblotting assays, but the diagnostic performances turned out very  
179 different between the two test systems.

180 None of the beavers from the “positive” group showed a seropositive reaction in the Em18-ELISA,  
181 indicating that beavers do not develop a humoral immunity against this antigen. Results obtained with  
182 the Em2-ELISA were very unsatisfying a well. Thus, the evaluation of these two tests revealed that they  
183 are not suitable for diagnosis of *E. multilocularis* infections in beavers. One possible explanation for this  
184 phenomenon may not be related to the antigen itself, but may be due to the quality of the beaver  
185 “blood” used for serology. Post-mortem decay and degradation may decrease the serological quality of  
186 the fluids recovered from the dead animals with regard to application in ELISAs that use highly purified  
187 antigens, but may be not with regards to immunoblotting that uses the complex mixture of a crude  
188 metabolic antigen. We plan to investigate this aspect upon direct comparison of the Em2-ELISA and  
189 EmVF-IB with sera obtained from beavers captivated for translocation. However, such a study may be  
190 very lengthy in time, as we would need to investigate their livers post-mortem to get a conclusive  
191 diagnosis regarding the presence or absence of AE-lesions and *E. multilocularis* infection, respectively.

192 In contrast, a diagnostic sensitivity of 85% was observed with immunoblotting, which, in terms of  
193 serodiagnosis, reaches an acceptable level, especially as specificity reached 100% in our study. Overall,  
194 while a seropositive result in immunoblotting unambiguously indicates an infection (high positive  
195 predictive value), a negative serological result has to be considered with caution.

196 Interestingly, the tested seropositive beavers showed a very weak banding pattern in immunoblots.  
197 While a distinct and rather complex pattern of antigen bands is identified in samples of most human  
198 patients with alveolar echinococcosis, beavers exhibit a binding activity with a maximum of three  
199 different antigens. This suggests that the *E. multilocularis* metacestode antigens are of very weak  
200 antigenicity, which may partially explain why all beavers demonstrated negative results with the  
201 conventional Em18-antigen, and why only a very weak diagnostic sensitivity was obtained with the

202 conventional Em2-antigen. As discussed for the ELISAs above, one of the reasons why a few samples  
203 from infected beavers were negative in immunoblotting may have been a decreased quality of the  
204 beaver blood due to post-mortem decay. Another reason for the overall weak (methodically and  
205 diagnostically) humoral immune response detected in beavers with alveolar echinococcosis may be  
206 associated to a high susceptibility to infection of this animal species, i.e., infection and resulting organ  
207 lesions may occur in a way that the host cannot mount an appropriate humoral immune response, as  
208 compared to other intermediate hosts such as humans who react strongly by the humoral pathway of  
209 immunity. Nevertheless, this explanation is unlikely as we know from observations in laboratory rodents  
210 that antibody-deficient animals such as the  $\mu$ MT mouse do not show an increased susceptibility (Dai et  
211 al., 2004). However, a weak humoral immune response may be associated to a weak cellular immune  
212 response, and it is known from murine and human alveolar echinococcosis in immunosuppressed  
213 individuals that a weak cellular immune response markedly favours metacestode proliferation (Vuitton  
214 and Gottstein, 2010). Referring to beavers, this weak immune response is likely not a particularity of  
215 some putatively immunosuppressed individuals but rather a characteristic of the species itself. Indeed, if  
216 some beavers would develop a strong antibody reaction (without subsequent lesions), it is probable that  
217 part of the beavers from the “negative” group (i.e., animals without lesions but from endemic areas)  
218 would have been seropositive. Yet, our sample size was limited and serological investigations of a larger  
219 number of beavers originating from endemic areas and submitted to necropsy are necessary to further  
220 address this question. Furthermore, as in alveolar echinococcosis susceptibility to disease is usually  
221 associated to metacestode fertility, we invite wildlife pathologists to document the frequency of related  
222 findings, e.g. protoscolex formation within the parasite tissue, and to carefully record the features of  
223 periparasitic inflammatory and immune-mediated processes that may contribute to either accelerated  
224 or delayed metacestode proliferation and maturation (Vuitton and Gottstein, 2010).

225

226

226 Conclusions

227 Serodiagnosis of *E. multilocularis* infection in beavers is now possible, and so far the best methodical  
228 approach consists in performing immunoblotting based on the detection of anti-Mr21-band-binding  
229 activity. Cross- or non-specific reactions did not occur in our study (100% specificity), and the diagnostic  
230 sensitivity amounts to 85%. With these diagnostic sensitivity and specificity and a putative prevalence  
231 estimated in a future study area, positive and negative predictive values could now be determined. We  
232 encourage wildlife health scientists to make use of this tool in order to support further assessment of  
233 factors that will help to better interpret serological results.

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318 Table 1: Serological investigation of 13 beavers with hepatic lesions associated with *E. multilocularis*  
 319 infection, as evidenced by histology and PCR. Abbreviations: CH: Switzerland; A: Austria; WB:  
 320 immunoblotting.

321

| diagnosis                 | origin   | Diagn. no. | Em2_ELISA   | Em18_ELISA  | WB           |
|---------------------------|----------|------------|-------------|-------------|--------------|
| AE                        | CH       | 12S751     | neg         | neg         | neg          |
| AE                        | CH       | 12S752     | neg         | neg         | <b>pos</b>   |
| AE                        | CH       | W07/0871   | <b>pos</b>  | neg         | <b>pos</b>   |
| AE                        | CH       | W08/0973   | <b>pos</b>  | neg         | <b>pos</b>   |
| AE                        | CH       | W07/4586   | <b>pos</b>  | neg         | <b>pos</b>   |
| AE                        | CH       | W08/973    | neg         | neg         | <b>pos</b>   |
| AE                        | CH       | W07/1152   | neg         | neg         | <b>pos</b>   |
| AE                        | CH       | 12S759     | neg         | neg         | <b>pos</b>   |
| AE                        | CH       | W09/1428   | <b>pos</b>  | neg         | <b>pos</b>   |
| AE                        | A        | 12S751     | neg         | neg         | neg          |
| AE                        | A        | 12S752     | neg         | neg         | <b>pos</b>   |
| AE                        | A        | 12D2967    | <b>pos</b>  | neg         | <b>pos</b>   |
| AE                        | A        | 12D2968    | <b>pos</b>  | neg         | <b>pos</b>   |
| <i>pos/tot</i>            |          |            | <i>6/13</i> | <i>0/13</i> | <i>11/13</i> |
| <b>diagn. sensitivity</b> |          |            | <b>46%</b>  | <b>0%</b>   | <b>85%</b>   |
| "negative"*               | Scotland | n = 27     | n.d.        | n.d.        | 0/27         |
| "negative"                | CH       | n = 25     | n.d.        | n.d.        | 0/25         |
| "negative"                | Austria  | n = 4      | n.d.        | n.d.        | 0/4          |
| <i>neg/tot</i>            |          |            |             |             | <i>0/60</i>  |
| <b>diagn. specificity</b> |          |            |             |             | <b>100%</b>  |

322 \*data used to calculate the negative-positive threshold

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324 Legends to Figures

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326 Figure 1: Quality control of the new anti-beaver-IgG-alkaline-phosphate conjugate. (A) Immunoblotting  
327 approach with 1 or 5  $\mu\text{g}$  of purified beaver-Ig, SDS-PAGE separated and transferred onto nitrocellulose.  
328 Subsequent antibody fragment detection was performed with the new conjugate. (B) Purity status of  
329 the purified beaver-IgG used to generate a polyclonal hyperimmune chicken IgY directed against beaver-  
330 IgG. The left blue lane shows stained Mr markers and corresponding Mr sizes. The estimated relative  
331 molecular mass of the beaver IgG heavy chain is Mr 55 kDa, while that of the light chain appears at  
332 approximately Mr 25 kDa.

333

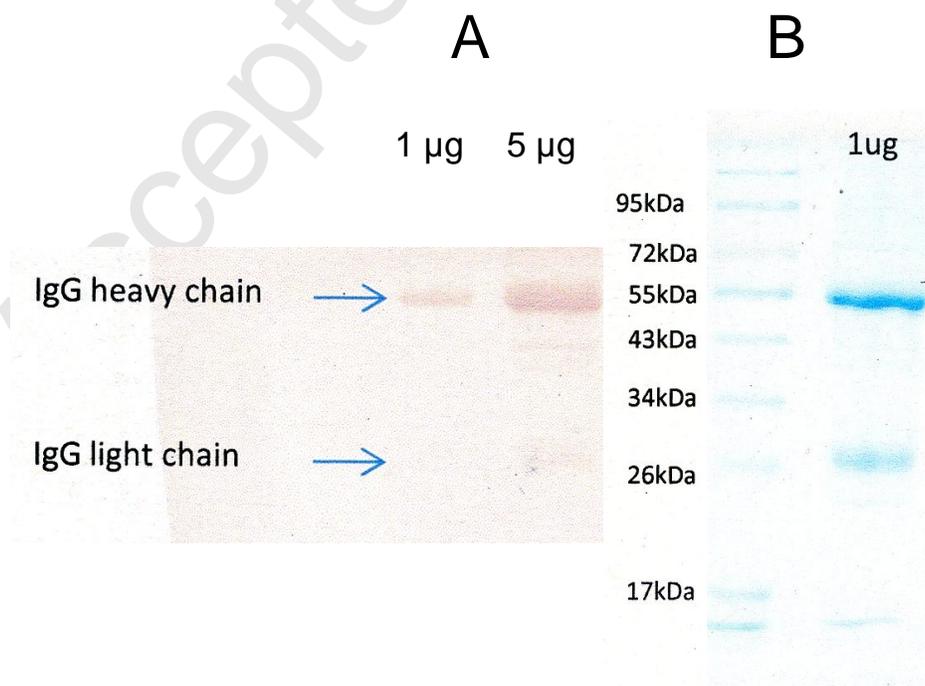
334

335 Figure 1

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338 Legends to Figures

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340 Figure 2: *E. multilocularis* immunoblot analysis with the following sera: H1 – human AE-patient (positive  
341 banding pattern control); B1, B2 - samples from beavers with alveolar echinococcosis (infection  
342 confirmed by histology and PCR); B3-B5 – samples from three beavers showing no macroscopic evidence  
343 for an infection with *E. multilocularis* (= negative animals). Arrow points at the diagnostic major band  
344 at Mr21. Left lane shows stained Mr markers and corresponding Mr sizes.

345

346 Figure 2

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