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

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Abstract

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

Keywords

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

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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-](http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase/conjugation.html)
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

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

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

ELISA

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

Immunoblotting

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

Statistical analyses

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

Results

Anti-beaver IgG-conjugate

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

ELISA

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

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

Immunoblotting

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

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

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

Conclusions

Serodiagnosis of *E. multilocularis* infection in beavers is now possible, and so far the best methodical approach consists in performing immunoblotting based on the detection of anti-Mr21-band-binding activity. Cross- or non-specific reactions did not occur in our study (100% specificity), and the diagnostic sensitivity amounts to 85%. With these diagnostic sensitivity and specificity and a putative prevalence estimated in a future study area, positive and negative predictive values could now be determined. We encourage wildlife health scientists to make use of this tool in order to support further assessment of 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* infection, as evidenced by histology and PCR. Abbreviations: CH: Switzerland; A: Austria; WB: immunoblotting.

diagnosis	origin	Diagn. no.	Em2_ELISA	Em18_ELISA	WB
AE	CH	12S751	neg	neg	neg
AE	CH	12S752	neg	neg	pos
AE	CH	W07/0871	pos	neg	pos
AE	CH	W08/0973	pos	neg	pos
AE	CH	W07/4586	pos	neg	pos
AE	CH	W08/973	neg	neg	pos
AE	CH	W07/1152	neg	neg	pos
AE	CH	12S759	neg	neg	pos
AE	CH	W09/1428	pos	neg	pos
AE	A	12S751	neg	neg	neg
AE	A	12S752	neg	neg	pos
AE	A	12D2967	pos	neg	pos
AE	A	12D2968	pos	neg	pos
<i>pos/tot</i>			<i>6/13</i>	<i>0/13</i>	<i>11/13</i>
diagn. sensitivity			46%	0%	85%
"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>
diagn. specificity					100%

*data used to calculate the negative-positive threshold

Legends to Figures

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

Figure 1

Legends to Figures

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

Figure 2