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ABSTRACT

Alveolar echinococcosis (AE), caused by the larval (metacestode) stage of *Echinococcus multilocularis*, is considered one of the most serious parasitic zoonoses in Central and Eastern Europe and is emerging also in large parts of Asia and in North America. The red fox represents the main definitive host of *E. multilocularis* in Europe, but the raccoon dog, the domestic dog and to a much lesser extent the domestic cat also represent potential definitive hosts. The natural intermediate hosts of *E. multilocularis* are mainly voles. The spectrum of accidental hosts is broad and includes many species of monkeys, pigs, dogs and humans which get infected by oral uptake of the viable eggs. Yet, human AE is a very rare disease in Europe; incidences have increased in recent years, while the infection is widely distributed in foxes with high prevalences reaching up to 70% in some areas. Generally, infected foxes represent a zoonotic risk, which may be particularly relevant in urban areas. Furthermore, there is concern that the risk for humans to acquire AE may rise due to the suspected geographical spread of the parasite as assessed by infections in its definitive hosts and the high prevalences in some regions. Monitoring and surveillance activities have therefore been initiated in a few European countries. Several diagnostic strategies have been developed and validated in recent years, applying classical worm detection by microscopy, but also immunological (ELISA for coproantigen detection) and molecular tests (copro-DNA detection by PCR). However, there is an urgent need for defining minimal requirements and harmonised approaches for these activities to allow for a reliable assessment of the epidemiological situation in Europe and comparable results from different countries.

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1. Introduction

Alveolar echinococcosis (AE), caused by infections with the larval (metacestode) stage of *Echinococcus multilocularis*, has been considered one of the most dangerous helminthic zoonoses in the northern hemisphere (Eckert et al., 2011). The estimated number of new AE cases in Western and Central Europe (including the Baltic countries and Poland) is in the range of 170–200 per year, with the highest numbers in France, Germany, Switzerland, Lithuania and Poland, but case numbers from Eastern Europe are hardly available. Although human AE is a very rare disease in Europe, there are reports of increasing incidences from Switzerland (Schweiger et al., 2007), Lithuania (Bružinskaitė et al., 2007) and Austria (Schneider et al., 2013).

The red fox (*Vulpes vulpes*) represents the main definitive host of *E. multilocularis* in Europe, but the raccoon dog (*Nyctereutes procyonoides*) is also highly susceptible for patent infections (Kapel et al., 2006). Raccoon dogs have been found infected with *E. multilocularis* in regions where also a substantial proportion of foxes was infected, but the role of the raccoon dog in the wild life cycle of the parasite is still under discussion (Schwarz et al., 2011; Bružinskaitė-Schmidhalter et al., 2011). Dogs and to a much lesser extent cats are possible sources of infections for humans in Europe (Deplazes et al., 2011; Hegglin and Deplazes, 2013). In contrast, dogs are more important than foxes for AE transmission in certain Asian endemic areas (Raoul et al., 2015). The key intermediate hosts of *E. multilocularis* are voles (e.g. *Microtus*, *Arvicola* and *Myodes* spp.), but other small mammals may play an important role in the life cycle in certain epidemiological situations. Furthermore, the spectrum of accidental "intermediate" hosts is broad. Infections of some species are of emerging veterinary importance such as AE in dogs and primates (Deplazes and Eckert, 2001; Rehmann et al., 2005; Scharf et al., 2004; Wenker and Hoby, 2011). In other species such as pigs

and wild boars, *E. multilocularis* has an incomplete development (Deplazes et al., 2005) and therefore, pigs are not involved in the life cycle of *E. multilocularis*. Infections in pigs or wild boars as aberrant hosts can nevertheless serve as an indication of environmental contamination with *E. multilocularis* eggs (Sydler et al., 1998; Böttcher et al., 2013).

The known spatial distribution of *E. multilocularis* in Europe is mainly based on fox investigations. Attempts have been made to assess the approximate current area of distribution and to identify high endemic areas, either by searching for high or increasing prevalences in foxes or substantial or increasing numbers of human AE cases (Davidson et al., 2012). Moreover, the occurrence of *E. multilocularis* has been reported as being focal (Enemark et al., 2013; Combes et al., 2012; Guerra et al., 2014; Osterman Lind et al., 2011; Tackmann et al., 1998; Umhang et al., 2014; Wahlström et al., 2012). Considering this, targeted approaches in the design of epidemiological studies are required to assess the dynamics of the infection in an area, such as studies to determine the size of a focus or to estimate the speed and direction of spread as described by Tackmann et al. (1998) and Denzin et al. (2014), also using molecular markers (Umhang et al., 2014).

2. E. multilocularis diagnosis in definitive hosts

Diagnosis of intestinal *E. multilocularis* infections is based on the direct identification of the parasite by morphological, immunological or molecular techniques (Tables 1 and 2). In principal, these techniques can be applied for all possible definitive hosts; however, the diagnostic sensitivity of the methods can strongly depend on the stage of infection (prepatent or patent period, worm burden, variation of worm development within the same species or between different species). Therefore, test parameters for one species cannot be used for other species and parameters determined with populations from high endemic areas are not fully adequate for low endemic situations without critical consideration. It is important that safety precautions are adopted during sample collection and diagnostic investigation to avoid a contamination with *E. multilocularis* eggs. Detailed information on the diagnosis of *E. multilocularis* in definitive hosts has been published (Craig et al., 2003; Eckert et al., 2001; Deplazes et al., 2003; Mathis and Deplazes, 2006). Furthermore, genetic analyses including microsatellite analyses with worm tissue or eggs may open new insights into the spatial and temporal genetic diversity of parasite populations (Knapp et al., 2015).

Recent progress in developing diagnostic tools (e.g. copro-PCR and coproantigen ELISA) makes it possible to investigate samples collected in the environment (e.g. faecal material or soil) for the presence of *E. multilocularis* and other taeniids (for *E. granulosus* s.l. reviewed in Craig et al., 2015). DNA isolation and PCR enable determination of patent and with lower sensitivity pre- or late-patent infections (without or with only very low egg production) (Al-Sabi et al., 2007) as well as host species from the same sample simultaneously (Dinkel et al., 2011; Nonaka et al., 2009; Laurimaa et al., 2015). In principle, even identification of individual animals is feasible which might help to analyse the temporal and spatial distribution of parasite shed by individual definitive hosts (Galaverni et al., 2012).

2.1. Necropsy techniques

Two major diagnostic procedures, the sedimentation and counting technique (SCT) and the intestinal scraping technique (IST) have been developed and further modified for morphological identification of intestinal stages of *E. multilocularis*. These methods are polyspecific, allowing for an accurate quantitative analysis of all intestinal helminths and to determine their developmental stages (e.g. premature, mature, gravid stages). The specificity for *E. multilocularis* is nearly 100%, only in areas co-endemic for *E. granulosus* s.l. mixed infections or early infections in the pre-patent period could be misdiagnosed. The sensitivity of these techniques was estimated to be very high (for details see below and in Table 1), but autolysis of the intestines and even deep freezing which is required for safety reasons can reduce the sensitivity. The obvious disadvantages of the necropsy techniques are the high logistical requirements to obtain a geographically representative sample distribution as carcasses must be recovered quickly to avoid decomposition. The methods are also time consuming and require special safety precautions due to the infection risk for the investigator. The fact that the necropsy methods can be applied to dead animals renders these methods unsuitable for diagnosis of representative pet animal populations. Data collection by these strategies is strongly influenced by reliance on material obtained from accidents or hunters, and an increased hunting pressure can influence the structure of wild animal populations.

The sedimentation and counting technique (SCT) has been proposed as the 'gold standard' for *E. multilocularis* detection at necropsy (Eckert et al., 2001). A modification of the SCT, the "shaking in a vessel" technique (SVT) was described (Duscher et al., 2005). A further modification is the segmental sedimentation and counting technique (SSCT) (Umhang et al., 2011), aiming to reduce the time of investigation. SSCT focuses on the investigation of the posterior part of the small intestine (segment 4 of 5 of the entire intestine) in combination with S1 or S2 of the anterior part. By applying this strategy, only a minimal reduction of sensitivity of around 2% as compared with the SCT was observed. A disadvantage of the SSCT is the loss of accurate quantitative estimation of the worm burdens.

The determination of the analytical sensitivity and the detection limit of the SCT was experimentally approached with samples that were spiked with worms (Karamon et al., 2010). However, the results of this study are of limited value, because fixed (70% ethanol) *E. multilocularis* worms were used, which differ in their physical properties from native worms.

A recent comparative study with a highly specific copro-PCR detection based on DNA extracted with magnetic capture probes (Table 2; Isaksson et al., 2014), revealed that the SCT was negative in 18% of the animals with positive PCR results. Assuming that most of these animals were indeed infected with *E. multilocularis*, sensitivity of the SCT, proposed as the "gold standard test", has to be critically readjusted. This fact has to be taken into consideration for all other test values which have been determined with material characterised with the SCT test (see Tables 1 and 2).

The intestinal scraping technique (IST) is somewhat less laborious than the SCT and is used in several modifications. Deep mucosal scrapings (total 15–24 per intestine) using microscope slides are squashed to a thin layer and examined microscopically. At least a semi-quantitative estimation of the worm burdens is possible. The polyspecificity of the IST is comparable to the SCT. The sensitivity of the IST was estimated to be 78% and 73% as compared to the SCT and SVT, respectively (Hofer et al., 2000; Duscher et al., 2005), and 76% as compared with a copro-PCR approach (Dinkel et al., 1998). IST sensitivity can be considerably improved by using up to 24 slides and covering around 50 to nearly 100% of the mucosa surface (Tackmann et al., 2006). The IST procedures have been widely used for mass screening of foxes for *E. multilocularis* in Europe, and this method can easily be integrated into a general necropsy protocol addressing further ecological or infectiological issues.

Table 1
Characteristics of test systems for morphological and immunological diagnosis of *Echinococcus multilocularis* in definitive hosts (if not noted parameters have been determined in high endemic situations with prevalences above 5%, low endemic situation (LES) with <1% prevalence are mentioned).

Test system	Test characteristics SE^a: sensitivity for *E. multilocularis*, SP^a: specificity for *E. multilocularis* Other parameters	Approx. number of animals/samples investigated per trained person and 5 working days
Arecoline purgation	SE_{dog} 21% (latent-class evaluation, as compared with *E. multilocularis* eggs detection by PCR and setting the specificity at 100% (Ziadinov et al., 2008) SE_{dog} 75.8%, SP_{dog} 100% (latent class analysis including coproantigen and copro-DNA detection, Hartnack et al., 2013) Polyspecific for intestinal helminths	Only few per day
Sedimentation and counting technique (SCT) (Eckert et al., 2001)	SE_{fox} 83.8% (setting SP of the molecular analyses to 100%, Isaksson et al., 2014), SP_{fox} ≈100% Polyspecific for intestinal helminths, allows precise quantification	50–100 depending on worm burdens and quantification (necropsy included)
Segmental SCT (SSCT) (Umhang et al., 2011)	SE_{fox} 98.3% as compared with SCT, SP_{fox} ≈100% Polyspecific for intestinal helminths	50–100, see SCT
Intestinal scraping technique (IST) (Hofer et al., 2000)	SE_{fox} 78% (compared with SCT); SP_{fox} ≈100% SE can be improved by testing nearly the entire mucosa of the large intestine (Tackmann et al., 2006). Application at necropsy, laborious; polyspecific for intestinal helminths	100–150 depending on worm burdens (necropsy included)
Shaking in a vessel technique (SVT) (Duscher et al., 2005)	SE_{fox} 96.2% (based on 26 foxes positive with IST and SVT); SP_{fox} ≈100% Polyspecific for intestinal helminths, allows precise quantification	100 see SCT (necropsy included)
Coproantigen ELISA (Deplazes et al., 1999)	SE_{fox} ≈80% (compared with SCT); SP_{fox} 95-99%; SP_{dog} 99.5% (determined in a LES) Allows in vivo and post mortem diagnosis and testing of field faecal samples, rapid and easy test, infection detectable in prepatent stage	500–800 samples
Coproantigen ELISA (Sakai et al., 1998)	SE_{fox}: ≈ 87% (compared with SCT), SP_{fox} ≈70% On genus level	500–800 samples
Coproantigen ELISA (Allan et al., 1992; Craig et al., 1995)	SE_{dog}: 55%, SP_{dog} 70.6% (latent class analysis including arecoline purgation and copro-DNA detection, Hartnack et al., 2013)	500–800 samples

[a] Animal species used for the validation.

2.2. Coproscopy for taeniid egg detection

The microscopical detection of proglottids and worm eggs in faecal samples after concentration by classical routine diagnostic methods is claimed to suffer from a low sensitivity. Furthermore, eggs of *E. multilocularis* cannot be differentiated morphologically from those of other taeniids. As outlined below, the efficient enrichment of taeniid eggs and their subsequent analysis by PCR can overcome this limitation and open new diagnostic strategies. An efficient enrichment of taeniid eggs was achieved by a combination of sequential sieving and flotation in zinc chloride solution (F/Si-method) (Mathis et al., 1996).

The sensitivities of commonly used flotation or sedimentation/flotation tests for detection of helminth eggs in dogs or foxes have not been validated for patent *Echinococcus* infections. In an experimental study with foxes, the sensitivities of the F/Si-method and of a modified McMaster method for quantitative egg estimation were 89% and 5%, respectively, with 19 samples from the late patent period 81–90 days post inoculation from animals with a mean worm burden of 134 worms per animal and low egg excretion (Al-Sabi et al., 2007). In a field study in Lithuania, significantly more dogs excreting taeniid eggs were diagnosed with the F/Si-method (34 of 240 dogs investigated) as compared to 12 positive animals identified with the modified McMaster method. A multiplex PCR performed on the 34 egg sediments identified by the F/Si method revealed 9 *E. granulosus* and 2 *E. multilocularis* infections, but only one of these *Echinococcus*-positive animals was identified when using the McMaster method as screening test (Bružinskaitė et al., 2009). Improvement and standardisation of the routinely used coproscopical methods for screening large dog populations for helminthic infections as well as simple tests to specifically identity *E. multilocularis* eggs could significantly improve the diagnostic values of such widely used routine procedures.

2.3. Arecoline purgation

Oral administration of arecoline hydrobromide to dogs results in the purgation of intestinal contents after 30–60 min. This material can be examined for the presence of intestinal helminths by washing through sieves, by sedimentation of the worms (Eckert et al., 2001) or by direct examination in the field with a hand held magnifying glass and subsequent DNA analyses (Van Kesteren et al., 2013). Arecoline purgation was used for mass surveillance

Table 2
Characteristics of test systems for molecular diagnosis of *Echinococcus multilocularis* in definitive hosts (if not noted parameters have been determined in high endemic situations with prevalences above 5%, low endemic situation (LES) with <1% prevalence are mentioned).

Test system	Test characteristics SE*: sensitivity for *E. multilocularis*, SP*: specificity for *E. multilocularis* Other parameters	Approx. number of animals/samples investigated per trained person and 5 working days
Combined egg isolation/PCR[a] (Mathis et al., 1996)	SE_{fox} 94% (compared with SCT), SP_{fox} 100% Laborious, in the first step (microscopy) polyspecific for helminth eggs, PCR detects patent infection. PCR inhibition not observed	40–80 samples (dependent on the taeniid prevalence, as only egg positive samples are further processed)
Nested-PCR[a] (Monnier et al., 1996)	SE_{fox} 82% (compared with SCT), SP_{fox} 96% Total DNA isolation from faeces allows eggs and parasite tissue detection. PCR inhibition in 11,8% of samples.	70 samples
Nested-PCR[b] (Dinkel et al., 1998)	SE_{fox} 89% (compared with IST), SP_{fox} 100% SE_{dog} 89.2%, SP_{dog} 92.8% (latent class analysis including arecoline purgation and copro-antigen detection, Hartnack et al., 2013) Total DNA isolation from faeces allows eggs and parasite tissue detection. PCR inhibition in 3,6% of samples	70 samples
Combined egg isolation (Mathis et al., 1996) Multiplex-PCR[c] for *E. multilocularis* (Trachsel et al., 2007)	SE_{dog} 50% (latent-class evaluation, as compared with arecoline purgation setting its specificity at 100%) (Ziadinov et al., 2008) *Highly specific for E. granulosus* s.l., *E. multilocularis* and *Taenia* spp. (*T. hydatigena, T. ovis, T. taeniaeformis, T. pisiformis, T. polyacantha, T. serialis/multiceps/krabbei* complex after sequencing). Laborious, in the first step (microscopy) polyspecific for helminth eggs, PCR detects patent infection. PCR inhibition not observed	50–100 samples (dependent on the taeniid prevalence, as only egg positive samples are further processed)
Single tube nested - PCR[b] (Van der Giessen et al., 1999)	SE not evaluated, SP_{fox} 100% Total DNA isolation from faeces allows detecting eggs and parasite tissue. PCR inhibition not observed	70 samples
PCR[c] (Boufana et al., 2013)	SE_{fox} 69% (compared with worm burden at necropsy), SP_{fox} 100% Total DNA isolation from faeces allows detecting eggs and parasite tissue. PCR inhibition observed; avoided with ethanol precipitation of copro-DNA and dilution of the samples	70 samples
Real Time-PCR[d] (Knapp et al., 2014)	SE_{fox} 89% (compared with SSCT), SP_{fox} 100% (if used for foxes samples, but cross-reacting with other canid parasites) Total DNA isolation from faeces allows detecting eggs and parasite tissue. PCR inhibition observed; complete inhibition overcame with dilution in 5/7 cases, partial normalised with an internal control	70 samples
Magnetic Capture – PCR[b] (Isaksson et al., 2014)	SE_{fox} 88% (compared with the SCT), SP_{fox} 99.9% as tested with 2158 foxes in a LES samples Total DNA isolation from faeces allows detecting eggs and parasite tissue. PCR inhibition not observed	240 samples

[a] Target: U1 sn RNA gene fragment.
[b] Target: mt 12S rRNA gene fragment.
[c] Target: nad1 gene fragment.
[d] Target: rrnL gene fragment.

in *E. granulosus* control programs worldwide (Craig et al., 2015) but its sensitivity for detecting *E. multilocularis* infections has not systematically been investigated. A recent field study with dogs in Kyrgyzstan including a latent-class evaluation (setting the specificity to 100% and using *E. multilocularis* egg detection in faeces) calculated a sensitivity of arecoline purgation of 21% for *E. multilocularis* (Ziadinov et al., 2008). Another study, again using a latent-class evaluation (including coproantigen detection and copro-PCR), revealed a much higher sensitivity of 75.8% for arecoline purgation (Hartnack et al., 2013). Safety precautions during field work and parasite identification in the laboratory are essential

and time consuming. Arecoline can also cause serious adverse reactions in dogs requiring strict veterinary supervision, and arecoline hydrobromide is not approved for use in dogs as an anthelminthic compound in most countries.

2.4. Copro-DNA and coproantigen detection

2.4.1. Detection of coproantigen

Tests originally developed for the diagnosis of *E. granulosus* showed cross-reactivity with *E. multilocularis* (Allan et al., 1992; Deplazes et al., 1992). ELISAs using polyclonal chicken and rabbit

or mouse monoclonal antibodies produced against *E. multilocularis* E/S or integument antigens improved the sensitivity (Table 1), but remained *Echinococcus*-genus specific. Presently, no test utilises highly genus-specific monoclonal antibodies or polyclonal antibodies directed to defined antigen fractions, rendering all these tests difficult to reproduce on a large scale and over time. One commercialised ELISA kit includes a rapid test for the detection of *E. multilocularis* coproantigens (EKITTO®, In-Vio Science Inc., Tokyo, Japan), but this test may not be specific in areas with high *Taenia* spp. prevalences. Furthermore, three *Echinococcus*-specific coproantigen tests have been commercialised in China (Huang et al., 2013), but no evaluation for *E. multilocularis* infections is available.

Echinococcus multilocularis coproantigens have shown to be highly resistant to degradation in the environment (Stieger et al., 2002) and some are heat resistant (Nonaka et al., 1996). Similar chemical properties have been described for *E. granulosus* coproantigens (Craig et al., 2015). A recent characterisation of a major *E. multilocularis* coproantigen isolated by the monoclonal MAbA9 (Sakai et al., 1998) revealed an integumental glycoprotein with unique O-glycosylation expressed in experimentally activated protoscoleces and in adult worms from intestinal origin (Hulsmeier et al., 2010). *E. multilocularis* coproantigens are detectable during both the prepatent and the patent periods in dogs, foxes, raccoon dogs and cats, and they disappear within a few days after the elimination of *E. multilocularis* from the host (Sakai et al., 1998; Deplazes et al., 1992; Deplazes et al., 1999; Kapel et al., 2006; Al-Sabi et al., 2007).

The sensitivity for coproantigen detection in an *E. multilocularis* high endemic area was 83.6% in 55 foxes with worm burdens of 4–60,000 as determined by the SCT, but reached 93.3% in the 45 foxes harbouring more than 20 worms. Thus, this test identified those animals harbouring approximately 99.6% of the total number of adult *E. multilocularis* in the fox population investigated (Deplazes et al., 1999). As outlined above, the SCT misses around 20% of infected animals, mainly those with low worm infections. Therefore, the sensitivity of the coproantigen ELISA can realistically be estimated at around 60% and is strongly dependent on the distribution of the worm burden in the fox populations. The sensitivity of the same coproantigen ELISA for patent *E. multilocularis* infections, as determined by PCR from 17 environmental fox faecal samples, was 88% (Stieger et al., 2002).

2.4.2. Detection of E. multilocularis copro- or egg DNA

Only a few *E. multilocularis* genes have so far been targeted in diagnostic PCRs for the detection of intestinal *E. multilocularis* infections in faecal samples of foxes (U1 snRNA gene, mt 12S rRNA gene, rrnL gene, nad1 gene). Diagnostic parameters on several *E. multilocularis* PCR tests are summarised in Table 2. Parasite DNA excreted with eggs, proglottids or parasitic cells can be detected from faeces after amplification by PCR. DNA isolation from faeces was either based on an alkaline lysis step (Bretagne et al., 1993) or on boiling the samples in 0.5% SDS and proteinase K digestion (Van der Giessen et al., 1999) and was later replaced by commercial DNA isolation kits. Due to the presence of substances that are inhibitory for DNA amplification, only a limited amount of material can be processed (0.5–4 g) with these methods. Several groups have reported inhibitory effects on DNA amplification (Table 1), even after following extensive purification steps. A further limitation of copro-PCR is that formalin-fixed faecal material is not suitable due to DNA degradation, but samples stored in 70% ethanol or at −20 °C or −80 °C can be examined (Al-Sabi et al., 2007).

One approach to overcome the limitations of restricted specimen volume and PCR inhibition is to first concentrate taeniid eggs (e.g. with the F/Si-method). Helminth eggs, which are highly resistant in the environment, can be concentrated from large sample volumes in a few microlitres of fluid and detected by means of an inverted microscope in a closed tube. As microscopic egg detection using this approach was shown to be very sensitive (one egg per 4 g faeces could be detected; Mathis et al., 1996), only samples containing taeniid eggs need to be further investigated by PCR. DNA isolation from these egg-containing samples was achieved using a simplified protocol. Obviously, this approach is suitable for the diagnosis of patent infections with eggs being present in the faeces, however, worm material was retained in the filters in some samples from prepatent infections resulting in positive PCR results (Al-Sabi et al., 2007). Based on egg isolation, a multiplex PCR based on targets in mitochondrial genes, which allows the differentiation among *E. multilocularis*, *E. granulosus sensu lato* (all genetic variants) and *Taenia* spp. infections (Trachsel et al., 2007) has been used in several epidemiological studies (Bružinskaitė et al., 2009; Ziadinov et al., 2008; Guerra et al., 2014). Sequence analyses of the amplicons allow identification of some *Taenia* species (*T. hydatigena*, *T. ovis*, *T. taeniaeformis*, *T. polyacantha*, but cannot clearly differentiate between *T. multiceps*/*T. serialis*/ *T. krabbei*). Identification of *Taenia* spp. can be of value in *Echinococcus* or *Taenia* control programs or in very low endemic areas to trace back *Taenia* infections typically originating from farm animals (*T. hydatigena*, *T. ovis*, *T. multiceps*) or from rodent intermediate hosts (*T. crassiceps*, *T. polyacantha*, *T. taeniaeformis*) (Jenkins et al., 2014; Eichenberger et al., 2011).

A recent approach based on a semiautomatic magnetic capture probe DNA extraction method combined with a real time PCR assay (MC-PCR) for the detection of *E. multilocularis* in fox scats ensures low PCR inhibition (Isaksson et al., 2014). The sensitivity was determined as compared with the SCT on faecal samples from foxes of a highly endemic area. Of 93 foxes samples that were positive in the SCT, 82 (88%) were positive in the MC-PCR. The specificity was evaluated with 2158 fox scats collected in Sweden, a known low endemic area, resulting in only two positive reactions resulting in a specificity of at least 99.9%. This test represents a new, but rather expensive, alternative to the other diagnostic methods for mass screening and has so far been used in studies in Sweden (Isaksson et al., 2014).

The real-time PCR technology (Knapp et al., 2014; Isaksson et al., 2014) offers the possibility to quantify *E. multilocularis* DNA in faeces. However, though such approaches are technically feasible, their value must be critically evaluated as wild carnivores excrete variable quantities of faeces dependent on food supply and quality. Moreover, single eliminated worms that are present in the sample can, without relation to the general worm burden, influence the DNA amount in the samples. Therefore, quantitative data with even information of the developmental stage as determined by the SCT might be more reliable to estimate the parasite reproduction than excreted DNA concentrations. On the other hand, a determination of *E. multilocularis* egg numbers (which is not possible by the McMaster method because of indistinguishable *Taenia* eggs in many samples) appears feasible. In this case, the irregular shedding of eggs (Kapel et al., 2006) has to be taken into account. However, a quantitative approach might contribute to transmission studies or epidemiological assessments when employed on a larger scale in populations of definitive hosts over a prolonged period of time (Mathis and Deplazes, 2006).

2.5. Detection of E. multilocularis in the environment

As outlined above, investigations of collected faecal samples in the environment with methods detecting coproantigen or copro-DNA by PCR allows an estimate of the environmental contamination with the parasite. As this procedure cannot establish whether multiple samples from the same individual have been collected, the results should be expressed as a "contamination index" and should not be used to estimate prevalences. This

strategy was applied in the monitoring of the infection pressure in endemic areas (Stieger et al., 2002; Raoul et al., 2003) or during baiting campaigns, for epidemiological investigations (see Hegglin and Deplazes, 2013).

Only few studies have addressed the environmental contamination with *Echinococcus* eggs beyond carnivore faecal samples. A pioneer approach was based on monoclonal antibodies for the detection of *E. granulosus* eggs in environmental contamination sites in settlements in Turkana (Kenya) (Craig et al., 1988); however subsequently, this approach was not further exploited in other studies. In another environmental study, Shaikenov et al. (2004) investigated 120 soil samples using a modified flotation method (O'Lorcain, 1994) followed by PCR identification of *E. granulosus* eggs. Recently, Szostakowska et al. (2014) have analysed soil samples for the presence of *E. multilocularis*. They subjected samples (40 g, air-dried) to sedimentation/flotation (saturated $ZnCl_2$) and isolated DNA by repeatedly freezing-thawing and applying a commercial kit. (Semi-) nested PCRs, which are prone to cross-contamination, were indicative for *E. multilocularis* in 7/62 soil samples; sequencing of 3 amplicons confirmed the diagnosis (but it is not comprehensible that the essays were applied as described).

The F/Si-method can be individually modified by using much larger sieves. For example, with such an adapted sieving system, detection of *E. multilocularis*, *Taenia saginata* and *Diphyllobothrium latum* eggs from large volumes of purified wastewater of a water purification station was achieved before the last step of filtration (Deplazes P., unpublished data). Furthermore, such an egg isolation approach was useful for the isolation for subsequent PCR and sequence analyses (Trachsel et al., 2007) of taeniid eggs (*E. granulosus s.l.*, *T. saginata*, *T. taeniaeformis*, *T. hydatigena* and *T. ovis*) from the washing water of heads of lettuces produced in different European countries in an ongoing study (Federer K. and Deplazes P., unpublished data). Using the newly available tools for egg or DNA isolation from a variety of materials and applying the highly sensitive molecular tools (Table 2), environmental investigations aiming to address ways of egg transmission to humans now seem feasible.

2.6. Serology

Serological screening using crude parasite antigens or affinity-purified Em2 antigen has been considered unsuitable for a reliable diagnosis of intestinal *E. multilocularis* infections because of the poor correlation between the presence of antibodies in the serum and worms in the intestine. Furthermore, for example in dogs, such tests were not able to differentiate between intestinal *E. multilocularis* infections and AE (Staebler et al., 2006).

3. Diagnostic strategies and data quality for diagnosis of intestinal infections

The choice of the diagnostic strategy is based on the scientific approach or diagnostic requirement and has to consider economics, methodology and logistics (e.g. storage and stability of material, transport, laboratory equipment and education of the laboratory personnel). In individual cases, for example if a child was orally exposed to a putative fox faecal sample, direct taeniid egg isolation of the complete sample by the highly sensitive F/Si method and subsequent taeniid egg identification by PCR is recommended to demonstrate or exclude as far as possible an *E. multilocularis* egg exposure. In such cases, high predictive values of the diagnostic strategy for a patent infection are required as basis for further individual recommendations to the exposed persons.

If small numbers of dead definitive hosts are available, or if quantitative investigations of a variety of possible intestinal helminths are investigated, the laborious SCT or IST are the most reliable tests. Screening tests should be highly sensitive, fast and cheap. Furthermore, a wide distribution of the test methodology in diagnostic laboratories enables multicentre studies covering large areas. For example, the IST and more recently the SSCT have been used in many laboratories to investigate more than ten thousand foxes for *E. multilocularis* infections in Central Europe, and regular proficiency testing using the SCT has increased confidence in the reliability and comparability of test results in the European Union.

For epidemiological investigations or for the surveillance in control programs, well-designed studies based on faecal samples of domestic or wild definitive hosts have many advantages. Several diagnostic strategies have recently been developed for mass screening of faecal samples of definitive hosts (Tables 1 and 2). Multiple diagnostic tests are often used in population studies. This may include a screening test of high sensitivity and a highly dependent confirmatory test of high specificity. This situation is given for the detection of intestinal infections by egg detection, copro-antigen detection or copro-PCR, all directly detecting the presence of the parasite. Parallel screening using two (or more) tests on the whole population are another option (Torgerson and Deplazes, 2009).

In the past, the use of classical coproscopical methods for parasite egg detection was limited due to the undistinguishable morphology of taeniid eggs. This issue can be overcome by a PCR investigation of all samples containing taeniid eggs. Recently, 21,588 faecal samples of dogs and 10,650 of cats routinely submitted to a private veterinary laboratory were examined with a $ZnSO_4$-NaCl flotation method, and 54 dog and 37 cat samples containing taeniid eggs were further investigated by PCR for *E. multilocularis* infections. This study underestimated the true prevalence in the dog and cat population because it could not detect prepatent infections combined with the low sensitivity of egg detection. However, the study convincingly documented patent *E. multilocularis* infections in pet animals presented to veterinarians in several parts of Central Europe (Dyachenko et al., 2008).

A field study in Kyrgyzstan suggested that the sensitivity of egg isolation (F/Si method) followed by PCR is 78% (95% CI 57–87%) for *E. granulosus* and 50% (95% CI 20–72%) for *E. multilocularis* infections in dogs proven to be infected by arecoline purgation (Ziadinov et al., 2008). In an experimental setting, egg isolation by the F/Si method detected all (95% CI 74–100%) samples during the high patent period and 77% (95% CI 58–90%) during the late patent period (Al-Sabi et al., 2007). Attempts to improve the sensitivity could include repeated faecal sampling and/or using techniques that do not rely on egg isolation, thus detecting prepatent infections by copro-DNA or coproantigen detection.

However, the dynamics of copro-DNA excretion during prepatency is dependent on the excretion of parasite stages (protoscoleces during the first days of infection and non-gravid stages later on), whereas coproantigen concentrations are related to the metabolic activity of parasites (Al-Sabi et al., 2007; Deplazes et al., 2003). Comparing these two approaches, a significantly higher sensitivity for coproantigen detection during prepatency (63% compared to 16%) was found in foxes experimentally infected with *E. multilocularis*, but no significant differences were found during the patent period (Al-Sabi et al., 2007). However, due to the high specificity of copro-DNA detection, this method has a high potential to become a routine procedure if PCR-inhibition problems can be overcome with reasonable efforts.

Coproantigen detection by ELISA has been shown to be useful for large-scale investigations especially for the monitoring in control programs assessing the environmental contamination (Comte et al., 2013; Hegglin and Deplazes, 2008). However, in areas of low endemicity of *E. multilocularis*, in dog and cat populations with a low prevalence, or in fox populations after long-lasting baiting interventions, ELISA results have a very high negative but a low pos-

itive predictive value. Therefore, positive ELISA results need further confirmation with PCR, a strategy used in several studies (Antolova et al., 2009; Deplazes et al., 1999; Gottstein et al., 2001; Stieger et al., 2002).

4. Diagnosis and detection in intermediate, accidental and aberrant animal hosts

The diagnosis of *E. multilocularis* metacestode infections is based on pathognomonic macroscopic, microscopic, histological and immunohistological (HE- and PAS-stain) findings and on molecular analyses (Deplazes et al., 2003). *E. multilocularis* metacestode samples fixed over 60 years in formalin were immunohistochemically confirmed (Barth et al., 2012), and this method can be used for retrospective investigations on preserved material. Furthermore, formalin fixed, paraffin embedded tissue samples are a reliable source of DNA even after years of storage. Sequence and microsatellite analyses of parasite material may provide more detailed information on the spatial origin of the parasite (Knapp et al., 2015).

Small, atypical or calcified liver lesions are recalcitrant to morphology-based methods. Specific metacestode antigen (Em2) can be detected immunohistochemically (Barth et al., 2012) but this method is only established in a few specialised laboratories. The method of choice for identifying *E. multilocularis* from small non-fertile or calcified lesions is PCR. In recent studies, a nested PCR (Dinkel et al., 1998) or a single PCR with the slightly modified inner primer pair (Stieger et al., 2002) was used for screening rodent populations. In *Arvicola scherman*, the amount of immature or non-fertile *E. multilocularis* lesions only diagnosable by PCR ranged between 68% and 94% in several studies (Dinkel et al., 1998; Reperant et al., 2009; Stieger et al., 2002).

It has been shown that the number of protoscoleces in *E. multilocularis* metacestodes is highly over-dispersed. Single animals with loads of more than 200,000 protoscoleces can be found (Stieger et al., 2002). Therefore, studies investigating intermediate hosts should record age and numbers of protoscoleces in infected animals (Burlet et al., 2011). This can be done by cutting the metacestodes into small pieces, washing them with PBS through a sieve and counting the protoscoleces. High numbers can be calculated by counting several subsamples of sieved material. For ecological studies, the proportion of protoscolex-containing animals and protoscolex numbers are important for the estimation of parasite reproduction in a given intermediate host population. The total number of infected animals reflects the infection pressure in a rodent habitat. To assess the significance of different intermediate host species in the life cycle, the preferences of final hosts for potential intermediate hosts are also of importance. Monitoring the prevalence in intermediate hosts has been performed aimed at determining spatial and temporal transmission of the parasite (Burlet et al., 2011; Delattre et al., 1985; Gottstein et al., 2001; Reperant et al., 2009; Stieger et al., 2002) or at studying the effects of long-term anthelminthic baiting of foxes with praziquantel (Tsukada et al., 2002; Hegglin and Deplazes, 2008).

Increasing numbers of AE in accidental or aberrant hosts such as domestic dogs and zoo animals can be observed in endemic areas as an effect of the high infection pressure. In these cases including wild beavers, diagnosis in the individual live animal is important. Imaging and serological techniques have been developed which are similar to those used in humans (Scharf et al., 2004; Staebler et al., 2006; Gottstein et al., 2014). However, as dogs can be both a definitive and an aberrant intermediate host, serology combined with coproantigen or copro-DNA tests will indicate the presence or absence of intestinal co-infections (Staebler et al., 2006). Furthermore, biopsy material can be investigated morphologically, but preferentially by histology, immunohistochemically or by PCR (as outlined above for rodents or pigs).

Epidemiological studies in aberrant hosts such as pigs or wild boars are of interest as these animals may serve as markers for environmental contamination with *E. multilocularis* eggs. Generally, in pigs only small, calcified "died out" lesions are found, but in a few cases viability of parasites originating from pigs was demonstrated. Confirmation of diagnosis can be achieved by histology demonstrating typical alveolar structures or laminated layer fragments in calcified lesions with Periodic acid-Schiff (PSA) (Böttcher et al., 2013; Deplazes et al., 2005) or immunohistochemically (Barth et al., 2012). Furthermore, PCR confirmation is also possible in the majority of "died out" lesions.

5. Monitoring definitive host populations

The definitive hosts of *E. multilocularis* include domestic and wild carnivores. The main definitive hosts in wildlife are foxes, but the raccoon dog, which represents an invasive carnivore species in Europe, can be highly infected in some areas (Schwarz et al., 2011; Bružinskaitė-Schmidhalter et al., 2011). Although the epidemiological role of raccoon dogs for the life cycle of *E. multilocularis* is not well understood, the prevalence and abundance of *E. multilocularis* in this species represents an indicator of the regional infection pressure. While monitoring of domestic carnivores (dogs and cats) is often confined to convenience samplings ('test what you get'), wild carnivores can be monitored for infection with *E. multilocularis* using sampling strategies that come close to random sampling which helps to avoid biased samples influenced by factors that are difficult to quantify and sometimes even to identify. Normally, monitoring definitive hosts for *E. multilocularis* aims at analysing changes of periodic prevalences in space and time or at verifying that a particular country or area is free from the parasite.

Current concepts of prevalence estimates or demonstration of the freedom of an area from infection are usually based on sampling a fraction of the total population and to estimate the prevalence in the population from the proportion of infected individuals in the sampled fraction. This extrapolation is only justified if the tested sample is randomly selected, i.e. each member of the sample must have the same chance to enter the sample (random sample). If it is planned to stratify the sample, e.g. for age, the members of the strata must also be randomly selected and the number of sampled individuals must be sufficient to come to statistically valid conclusions.

5.1. Monitoring wild carnivore populations

For several reasons, field studies investigating the distribution of *E. multilocularis* in wild carnivores may fail to fulfil the strict requirements of random sampling (Conraths et al., 2003). Hunting foxes is not a random selection process as hunters have objectives (e.g. reducing predator or scavenger populations in their hunting district) and follow certain rules (e.g. respecting close hunting seasons) when shooting foxes. However, at least as far as the infection status of individual foxes with *E. multilocularis* is concerned, infected and uninfected animals seem to have the same chance to enter a sample because the infection status of a definitive host has no known impact on its fitness, clinical condition, behaviour etc., while young age may be associated with an increased risk of infection.

With respect to regional origin, samples obtained by hunting are usually heterogeneous, because the places where the animals were shot are not randomly distributed but follow rules. This may lead to an under-representation of parts of the study area while other regions may be over-represented at the same time. This type

of bias might be of major concern in control programs, if hunters are involved in the distribution of anthelminthic baits and at the same time in collecting faecal samples or foxes in the same area for monitoring the infection pressure.

Social structures in the host population may also result in bias if, for instance, entire fox families are sampled. Samples obtained from different members of a family may not be independent because a familiar exposure to infection is possible if a bitch feeds infected intermediate hosts to her offspring. By contrast, unweaned cubs are not exposed to *E. multilocularis* infection, even if their mother is. Finally, the spatial distribution of *E. multilocularis*-infected foxes is often heterogeneous at the population level, and the samples obtained in such a scenario may also be heterogeneously distributed in space (Tackmann et al., 1998).

Generally, the spatial representation of a sample has to be taken into account in the interpretation of the data. A reliable prevalence estimate can only be obtained for a spatial unit if the entire sample and the infected animals are nearly homogeneously distributed. In all other cases, endemic foci may be overlooked or assumed prevalence changes may be caused by a spatial bias in the tested sample. The average home range of wild carnivores that are definitive hosts of *E. multilocularis* should also be taken into account (Tackmann et al., 1998; Stiebling, 2000; Deplazes et al., 2004). Moreover, habitat factors which may have a limiting influence on the life cycle of the parasite also seem to be effective at the local level (Staubach et al., 2001). Therefore, temporarily stable endemic foci of less than 400 square km are possible (Tackmann et al., 1998).

Different age intervals may correspond to different cumulative periods of exposure. Unweaned fox cubs are obviously not exposed to infection with *E. multilocularis* while the exposure of older animals may depend on their age. As the age of a definitive host is related to its risk of contracting an infection with *E. multilocularis*, the age structure of the sample can influence the result of the analysis. In some studies it has been observed that in moderate or high endemic areas juvenile foxes were more frequently infected than adults. Therefore, an overrepresentation of juvenile foxes in the sample compared with the original population would inevitably lead to an overestimation of the prevalence. Furthermore, differences in the age structure of the sample can bring about spatial and temporal changes of the prevalence.

Since the number of animals that can be sampled is limit sired sample sizes can often only be achieved over an extended period of time. Prevalence changes occurring during these intervals can therefore not be recorded. It should also be noted that, strictly speaking, an unbiased observation of the population over time is not possible, if infections with *E. multilocularis* are diagnosed post mortem, i.e. the animals are irreversibly removed from the population and therefore also from the life cycle of the parasite. On the other hand, post mortem investigations make sure that a single animal can enter the sample only once, thus avoiding multiple reporting of the same infection of an individual animal. Due to hunting habits etc. sampling is also discontinuous: samples are not evenly distributed over the period for which the prevalence is estimated. Therefore, particular attention should be paid to possible seasonal influences which may otherwise be overlooked. To avoid problems with heterogeneity in time, the sampling intervals should be kept as short as possible.

In the epidemiological analysis of monitoring or surveillance (M/S) data, the variables 'space', 'time' of sampling, the 'age' of the animal and its infection status regarding *E. multilocularis* must be taken into account. Therefore, the parameters place of origin (e.g. municipality or geographic coordinates), age (juvenile vs. adult), time (day, month and year of sampling), and the infection status with regard to *E. multilocularis* (yes/no; if possible, also the intensity of infection, expressed by the counted or estimated number of parasites per animal) should be recorded. If a heterogeneous

Table 3

Tabulation of minimum sample sizes for given prevalence thresholds (horizontal) and populations sizes (vertical) at the 95% confidence level. The tabulated sample sizes represent the minimal number of animals that need to be examined to find at least one positive animal, if the proportion of infected animals is above the chosen threshold at the given population size (Cannon and Roe, 1982; Conraths et al., 2003).

N	50%	40%	30%	25%	20%	15%	10%	5%	2%	1%	0,5%	0,1%
10	4	5	6	7	8	9	10	10	10	10	10	10
20	5	6	7	9	10	13	16	19	20	20	20	20
30	5	6	8	9	11	14	19	26	30	30	30	30
40	5	6	8	10	12	15	21	31	40	40	40	40
50	5	6	8	10	12	16	22	35	48	50	50	50
60	5	6	8	10	13	16	23	38	55	60	60	60
70	5	6	8	10	13	17	24	40	62	70	70	70
80	5	6	9	10	13	17	24	42	68	79	80	80
90	5	6	9	10	13	17	25	43	73	87	90	90
100	5	6	9	10	13	17	25	45	78	95	100	100
120	5	6	9	11	13	18	26	47	86	111	120	120
140	5	6	9	11	13	18	26	48	92	124	139	140
160	5	6	9	11	13	18	27	49	97	136	157	160
180	5	6	9	11	13	18	27	50	101	146	174	180
200	5	6	9	11	14	18	27	51	105	155	190	200
250	5	6	9	11	14	18	27	53	112	175	228	250
300	5	6	9	11	14	18	28	54	117	189	260	300
350	5	6	9	11	14	18	28	54	121	201	287	350
400	5	6	9	11	14	19	28	55	124	211	311	400
450	5	6	9	11	14	19	28	55	127	218	331	450
500	5	6	9	11	14	19	28	56	129	225	349	499
600	5	6	9	11	14	19	28	56	132	235	379	597
700	5	6	9	11	14	19	28	57	134	243	402	691
800	5	6	9	11	14	19	28	57	136	249	421	782
900	5	6	9	11	14	19	29	57	137	254	437	868
1000	5	6	9	11	14	19	29	57	138	258	450	950
1200	5	6	9	11	14	19	29	58	140	264	471	1102
1400	5	6	9	11	14	19	29	58	141	269	487	1236
1600	5	6	9	11	14	19	29	58	142	272	499	1354
1800	5	6	9	11	14	19	29	58	143	275	509	1459
2000	5	6	9	11	14	19	29	58	143	277	517	1553
3000	5	6	9	11	14	19	29	58	145	284	542	1895
4000	5	6	9	11	14	19	29	58	146	288	556	2108
5000	5	6	9	11	14	19	29	59	147	290	564	2253
6000	5	6	9	11	14	19	29	59	147	291	569	2358
7000	5	6	9	11	14	19	29	59	147	292	573	2437
8000	5	6	9	11	14	19	29	59	147	293	576	2498
9000	5	6	9	11	14	19	29	59	148	294	579	2548
10000	5	6	9	11	14	19	29	59	148	294	581	2588
∞	5	6	9	11	14	19	29	59	149	299	598	2995

distribution of data regarding the first three variables is expected or registered, care should be taken that these data can be analysed in the respective intervals or strata, in which they were collected (e.g. by stratifying the random sample for the variables "month" or "quarter of year", "municipality", "juvenile" vs. "adult" etc.).

5.2. Objectives of monitoring

When a M/S program is planned, it must be determined if an estimate of the prevalence with a given accuracy (e.g. the value of the prevalence + 5%) and at a specified level of confidence (e.g. 95%) is required or if it is sufficient to establish whether the prevalence exceeds a specified prevalence threshold (design prevalence, e.g. 5%). The latter approach is often used to demonstrate that an area is free from infection by testing a random sample showing that no animals are infected, and concluding that the true number of infected animals does exceed the chosen design prevalence (e.g. 1%) at a specified level of confidence (e.g. 95%). The sample sizes required for these approaches can differ considerably depending on the chosen prevalence threshold (the lower the threshold, the higher the required number of sampled animals), the true prevalence in the case of prevalence estimates (maximum sample size if the expected true prevalence is 50%), accuracy of prevalence estimates (the higher the chosen accuracy, the higher the sample size)

and the level of confidence (the higher the level of confidence, the higher the sample size).

5.3. Detecting presence or absence above a specified level of prevalence

For detecting if the prevalence exceeds a specified design prevalence on a chosen confidence level (e.g. 95%), the required sample size is relatively small, but at the same time the information resulting from the study is limited. If the population size is known and the design prevalence and confidence level chosen, the required sample size can be read from a table (Table 3) or calculated using epidemiological software packages (e.g. http://epitools. ausvet.com.au). If a perfect diagnostic test (i.e. 100% sensitivity and specificity) is used and if at least one sampled animal is found infected, the true prevalence will be equal or higher than the design prevalence at the selected confidence level. If no infected animal is found in the sample, the true prevalence is lower than the selected prevalence threshold.

To overcome the problem of unknown host population sizes, it is possible to set the population size to infinite when calculating sample sizes. This approach avoids having a sample size that is too small to obtain the required accuracy of a prevalence estimate or to detect infections above a chosen prevalence threshold, but may lead to sample sizes which are larger than needed. If the sensitivity and specificity of the chosen diagnostic test deviate from 100%, the required sample sizes should be adjusted accordingly (Humphry et al., 2004).

5.4. Prevalence estimates

The required sample sizes for prevalence estimates depend on the expected prevalence, the desired precision and the confidence level of the estimate and can be read from Table 4 (Conraths et al., 2003). The largest sample sizes are required for prevalence estimates of 50%, while lower sample sizes are needed for lower and higher prevalence estimates. To minimise risks, one can use the maximal sample size (i.e. for a suspected prevalence of 50%), which is likely to lead to increased study costs. Higher precision (i.e. smaller confidence intervals) and higher confidence levels (90%, 95%, 99%) increase the required sample size.

5.5. Temporal analysis

To improve the confidence in prevalence estimates or their temporal trend, it is also possible to combine data from several years (Murphy et al., 2012; Schwarz et al., 2011; Wahlström et al., 2011) or analyse data over time (Staubach et al., 2011; Staubach et al., 2002) for research purposes. Such approaches can also help to overcome data gaps in time and space, e.g. by using Bayesian models, and to assess spatial and temporal trends. Risk-based sampling and monitoring strategies are increasingly propagated (Frossling et al., 2013; Gonzales et al., 2013; Pozio, 2014; Schuppers et al., 2010; Stark et al., 2006; Wilking et al., 2009) and may be useful if criteria for an increased or reduced risk can be defined, e.g. foci with an increased prevalence in foxes, regions with a dynamic situation, for example increasing prevalence or areas with increased incidence of alveolar echinococcosis in humans. To assess the latter, other study types such as case-control studies are needed to identify potential risk factors for human infection, which may be associated with the spatio-temporal distribution of E. multilocularis in its definitive hosts.

However, if the free status of an area has to be shown every year (e.g. to justify additional guarantees for countries deemed free from E. multilocularis in the European Union; Annex II of the Commission

Table 4
Tabulation of minimum sample sizes for estimating the prevalence for expected prevalence levels (horizontal) and populations sizes (vertical) with 5% accuracy at the 95% confidence level. If no information is available to determine the expected prevalence, it is common practice to select the sample sizes at the 50% prevalence level, where the sample size is maximal, to avoid that the desired accuracy is not reached (Cannon and Roe, 1982; Conraths et al., 2003).

N	Expected prevalence								
	10%	20%	30%	40%	50%	60%	70%	80%	90%
10	10	10	10	10	10	10	10	10	10
20	18	19	19	19	20	19	19	19	18
30	25	27	28	28	28	28	28	27	25
40	32	35	36	37	37	37	36	35	32
50	37	42	44	45	45	45	44	42	37
60	42	49	51	52	52	52	51	49	42
70	47	55	58	59	60	59	58	55	47
80	51	61	65	66	67	66	65	61	51
90	55	66	71	73	73	73	71	66	55
100	59	72	77	79	80	79	77	72	59
120	65	81	88	91	92	91	88	81	65
140	70	90	98	102	103	102	98	90	70
160	75	97	107	112	113	112	107	97	75
180	79	104	116	121	123	121	116	104	79
200	82	111	124	130	132	130	124	111	82
250	90	124	141	149	152	149	141	124	90
300	95	136	156	166	169	166	156	136	95
350	100	145	168	180	184	180	168	145	100
400	103	153	179	192	196	192	179	153	103
450	106	159	188	203	208	203	188	159	106
500	109	165	197	213	218	213	197	165	109
600	113	175	210	229	235	229	210	175	113
700	116	182	221	242	249	242	221	182	116
800	118	189	230	253	260	253	230	189	118
900	120	194	238	262	270	262	238	194	120
1000	122	198	244	270	278	270	244	198	122
1200	125	205	255	283	291	283	255	205	125
1400	126	210	263	292	302	292	263	210	126
1600	128	214	269	300	310	300	269	214	128
1800	129	217	274	307	317	307	274	217	129
2000	130	219	278	312	323	312	278	219	130
3000	133	228	292	329	341	329	292	228	133
4000	134	232	299	338	351	338	299	232	134
5000	135	235	304	344	357	344	304	235	135
6000	136	237	307	348	362	348	307	237	136
7000	136	238	309	351	365	351	309	238	136
8000	136	239	311	353	367	353	311	239	136
9000	137	240	312	355	369	355	312	240	137
10000	137	240	313	356	370	356	313	240	137
∞	139	246	323	369	385	369	323	246	139

Delegated Regulation (EU) No. 1152/2011), methods that combine data from several years cannot be considered.

Monitoring activities and epidemiological studies in regions endemic for E. multilocularis can for example be used to determine the size of an endemic focus (Staubach et al., 2001), to detect prevalence changes in time or the expansion of an endemic area (Staubach et al., 2011) or to assess the speed of dispersal of the parasite into previously unaffected areas (Combes et al., 2012; Denzin et al., 2014; Takumi et al., 2008). However, the costs of continuous monitoring activities may have to be balanced against the expected results.

Any measures to control the infection of animals with E. multilocularis need to be accompanied by appropriate surveillance activities to monitor and adjust the control programme accordingly. Techniques to monitor and – if necessary – refine control activities have been described or proposed in several publications (Atkinson et al., 2013; Comte et al., 2013; Hansen et al., 2003; Hegglin and Deplazes, 2013; Pleydell et al., 2004; Romig et al., 2006; Schelling, 1991; Tackmann et al., 2001).

If monitoring data from several regions are combined for a joint analysis, it is of the utmost importance that data collection and reporting is standardised. It is absolutely necessary to agree at least

on the period of sampling, the size of spatial units where the sampling is performed, the diagnostic techniques including information on their sensitivity and specificity, the number of tested samples and the number of samples in which *E. multilocularis* was detected. In this respect, there is much room for improvement concerning the data currently reported by European countries and evaluated by the European Food Safety Authority for zoonoses trend reporting (EFSA, 2012).

5.6. Spatial analysis

Another important issue is the character and size of the spatial unit for which the disease status or prevalence estimate is obtained. Ideally, these spatial units should be normalised with regard to their area (raster formed by grid cells of equal size) or to the size of the target population. However, this is often not practical as the use of a raster may lead to shared responsibilities of two or more administrative units in coordinating the sampling in a given grid cell. As a consequence, official monitoring programmes often use administrative units (municipalities, districts, countries etc.) as the spatial reference, although these are irregular in shape, differ in size and may harbour target populations of varying size. In the European Union, the NUTS (nomenclature des unités territoriales statistiques) system is frequently used, which attempts to standardise the human population size on the level of administrative units to some extent. It is still disputable, however, whether this system is suitable for designing monitoring programmes for *E. multilocularis* as the size of the units varies considerably between different countries on each NUTS level and because it does not take the population size of the animal populations of interest into account.

Explorative spatial analysis can be done by plotting all examined animals as dots on a map of the study area using the municipalities, where the foxes were shot, as the geographic unit. Different colours are used for infected and uninfected animals. This approach represents an easy descriptive technique, which allows the identification of heterogeneous distribution patterns in the total sample (infected and uninfected animals) and among the infected animals. In this way it is possible to recognise regions in the study area, where the sample may have been too small to come to valid conclusions. Mapping of the results also provides a first impression of regional clusters of infected animals which may indicate endemic foci. It must be emphasised, however, that this method of explorative data analysis only allows building hypotheses, which have to be further evaluated by epidemiological or statistical procedures including mathematical modelling (Berke and von Keyserlingk, 2001; Denzin et al., 2014; Staubach et al., 2001; Staubach et al., 2011; Takumi et al., 2008; Takumi et al., 2012; Takumi and Van der Giessen, 2005). It should be emphasised, however, that mathematical models need to be validated and subjected to a sensitivity analysis if possible.

5.7. Monitoring dogs and cats

The same principles outlined for designing epidemiological studies in wild definitive hosts, in particular foxes, apply also to domestic animals. However, it is usually more difficult to obtain random samples of domestic carnivores. Necropsy studies are biased as animals investigated do not represent the average animal population (e.g. stray cats, hospitalised animals). Furthermore, specimens sent to diagnostic laboratories for example (Dyachenko et al., 2008) have been selected on the basis of clinical symptoms or owners' concerns. Such samples may be considerably biased and even the species from which they were taken may be questionable in some cases. Furthermore, the prevalence of intestinal *E. multilocularis* infections, which can vary between 0 and 7% within the European endemic area (Deplazes et al., 2011) is strongly

dependent on transmission risks such as free access to rodents (e.g. for farm dogs, hunting and stray dogs). Additional sources of bias, in particular selection bias, must therefore been taken into consideration in the epidemiological analysis of the data to avoid substantial over or underestimates of the true prevalence or incidence of *E. multilocularis* infections in dogs and cats.

5.8. Monitoring intermediate hosts

Monitoring small mammals for infection with *E. multilocularis* represent another option to study the spatial and temporal distribution of the parasite in an area. Various approaches have been used and proposed by several groups in attempts to adapt the study protocols to the local situation, e.g. in Austria (Fuhrer et al., 2010), China (Giraudoux et al., 2013), France (Magnaval et al., 2004), Japan (Saitoh and Takahashi, 1998; Takahashi and Nakata, 1995) Svalbard, Norway (Fuglei et al., 2008), Switzerland (Schmitt et al., 1997; Burlet et al., 2011) or in urban situations (Hegglin and Deplazes, 2013) and to the research question, e.g. epidemiologically important intermediate hosts and relationship to the definitive host in a given scenario (Guerra et al., 2014; Guislain et al., 2007; Hansen et al., 2004; Raoul et al., 2003) to study transmission ecology (Wang et al., 2010; Burlet et al., 2011) or as bioindicators for the presence of *E. multilocularis* in known (Reperant et al., 2009) or new endemic areas (Umhang et al., 2013), or during control experiments (Hegglin and Deplazes, 2008).

Caveats for these types of studies include the need to define the epidemiological role of some species, which are readily accessible for epidemiological studies because they are regarded as pests and therefore target of control measures e.g. nutrias (*Myocastor coypus*) or muskrats (*Ondatra zibethicus*), but they can be used to study environmental pollution with the parasite and assess the biomass in the environment as a measure for the risk for human infection. Furthermore, the spatial distribution of the target species and potential habitat influences on their abundance and on the prevalence of *E. multilocularis* have to be considered (Hansen et al., 2004).

6. General considerations and recommendations

For the purpose of M/S, a distinction between countries or regions deemed free from *E. multilocularis* and endemic areas seems appropriate. To demonstrate freedom from *E. multilocularis*, it has to be shown that the prevalence is below a defined threshold (e.g. 1%) by testing an appropriate number of samples (e.g. 300 if the test is assumed to be perfect) from comparable geographic units at least at the 95% confidence level. Such an approach is successfully practiced in Finland, Ireland and the United Kingdom based on Annex II of the Commission Implementing Decision 2011-874-EU of 15 December 2011 (Anonymous, 2013; Murphy et al., 2012; Wahlström et al., 2011). A reduction of sample sizes may be possible by utilising risk-based approaches (Hadorn et al., 2002) and combining the results of surveys performed in several species may also be used to demonstrate freedom from *E. multilocularis* (Wahlström et al., 2011).

To monitor the status in endemic regions, it may be sufficient to conduct surveys in particular regions at regular intervals covering all parts of the country over time during intervals, in which major changes in the prevalence cannot be missed. It seems possible to use data accumulated over time to reduce sample sizes.

Specifically designed studies to monitor the situation after the detection of cases in regions previously deemed free of *E. multilocularis* to assess the size of a new focus or estimate the potential direction and speed of spread are needed. The same applies to areas with a highly dynamic epidemiological situation characterised by

substantial changes in incidence or prevalence, the emergence or an unexpected rise of human AE cases.

It may often be desirable to assess the epidemiological situation regarding *E. multilocularis* in animals across borders. This requires the analysis of data obtained from various sources (e.g. regions or countries). In these cases, a high level of harmonisation or standardisation of data collection, recording and reporting is of utmost importance to avoid to misleading interpretations of joint analysis. This is particularly relevant if the study results are used by decision makers, who may not be experts in parasitology, for implementing monitoring or control measures (including the necessary surveillance). Current standards in study designs, data collection, recording and reporting in the European Union fail to meet the requirements for assessing the epidemiological status in the area in a reliable fashion, although data of high quality are available for some countries.

7. Conclusions

- Reliable data exist on the spatial distribution of *E. multilocularis* in definitive hosts (wild carnivores) in Europe, but historic information needs to be verified. Less monitoring efforts in endemic regions can be justified. The status of countries for *E. multilocularis* needs to be regularly checked, but evidence accumulating over time may be used to reduce sample sizes.
- New highly sensitive and specific diagnostic strategies for the diagnosis of *E. multilocularis* on individual or population level have been developed in recent years. However, there is an urgent need for further harmonisation of the monitoring activities regarding *E. multilocularis* to allow for detailed epidemiological analysis at supranational level.
- Studies are needed to investigate causal relationships (e.g. case/control studies) between infection of definitive hosts, other possible infection risks and human AE (determination of risk factors).

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