Prevalence of *Echinococcus multilocularis* in red foxes in Groningen and Drenthe

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January 2018

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Abstract
The prevalence of the tapeworm *Echinococcus multilocularis* in red foxes in the provinces of Groningen and Drenthe was determined. The eastern part of the province of Groningen was identified to be endemic for the parasite in previous studies in 1997 and 2000. The prevalence was estimated at 2.1% (95% CI 0.6-7.4%; 2/95 examined). This prevalence was lower than the results of the previous study, where a baseline prevalence of 9.4% (95% CI 5.2-16.5%) was found. In the current study, both positive foxes were hunted in the previous described endemic area, studied in 1998-2000. The prevalence in this restricted study area was estimated at 5.9% (95% CI 1.6-19.1%; 2/34 examined). The results suggest that the *E. multilocularis* has spread only little, however, other studies suggests that the parasite has spread more drastically. Studies in Belgium and Germany also suggest that the parasite is still spreading. This emphasizes that importance of monitoring because of the public health risk of the parasite.

Keywords
Belgium, Drenthe, '*Echinococcus multilocularis*', epidemiology, Germany, Groningen, 'intestinal scraping technique', prevalence, qPCR, 'red fox' and spread

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1. Introduction

Echinococcus multilocularis is a tapeworm with a serious zoonotic risk. The parasite has a two-host lifecycle. The main definitive host in Europe and also in the Netherlands is the red fox (*Vulpes vulpes*) and the main intermediate hosts are small rodents. Eggs of the tapeworm are shed by the red fox and develop, after ingestion by small rodents, to larval or metacestode stages (Van Der Giessen, Rombout et al. 1999). Humans can become infected when they ingest eggs of *E. multilocularis*, e.g. by contact with infected definitive hosts or by oral uptake of contaminated food and water (Deplazes, van Knapen et al. 2011). Infection in humans may lead to alveolar echinococcosis (AE), of which symptoms develop after 5-15 years and which can be lethal if left untreated (Deplazes, van Knapen et al. 2011). AE is considered to be the most serious helminthic zoonotic disease in the northern hemisphere (Conraths, Deplazes 2015).

In the 1990s, only regions in Germany, France, Austria, and Switzerland were known to be endemic for the *E. multilocularis*. Nowadays, the range of the parasite has expanded and it has become endemic in at least 21 countries of Europe, including Germany, Belgium, and the Netherlands (Oksanen, Siles-Lucas et al. 2016) (figure 1). Research performed in several European countries showed that the prevalence of *E. multilocularis* increased significantly between 1990 and 2005 (Berke, Romig et al. 2008). Furthermore, a continuous extension of the range of the parasite is suspected (Takumi, de Vries et al. 2008, Denzin, Schliephake et al. 2014, Vervaeke, van der Giessen et al. 2006).

The first cases of *E. multilocularis* in red foxes in the Netherlands were detected in a study in the winter of 1996-1997. In the southern region of Limburg and the eastern region of Groningen, prevalences of respectively 13.6% (99% confidence interval [CI] 4.8-22.3%) and 5.5% (99% CI 0-15.9%) were found (Van Der Giessen, Rombout et al. 1999). Subsequent studies estimated that the

prevalence in Groningen was 9.4% (95% CI 5.2–16.5%) in 1998-2000 (Van Der Giessen, Rombout et al. 2004) and the prevalence in Limburg was 12.8% (95% CI 9.4-17.2%) in 2002-2003 and 11% (95% CI 6.7-18.4%) in 2005-2006 (Takumi, de Vries et al. 2008). However, in a more recent study in Limburg between October 2012 and March 2013, a significantly increased prevalence of 59% (95% CI 43-74%) was found (Maas, Dam-Deisz et al. 2014). A mathematical model, based on the results from 1996 to 2006, shows that the parasite spreads in Limburg with a speed of 2.7 km per year (Takumi, de Vries et al. 2008). The increased prevalence and expanded range suggest that the number of infected foxes increases. However, so far this has not resulted in an increased risk of human alveolar echinococcosis, because the observed increase falls within the predicted range of increase that was made previously (Schweiger, Ammann et al. 2007, Maas, Dam-Deisz et al. 2014).

Up-to-date information about the prevalence is needed to estimate the public health risk of the parasite. Current data about the prevalence and the spread of *E. multilocularis* in Groningen is not present. However, the increased prevalence in foxes in Limburg in 2013 and epidemiological situation in adjacent countries in the precious decades, suggests that the parasite in Groningen most likely also expanded its range and may have increased in prevalence since the last performed study. For this reason, this study was designed to estimate the prevalence of *E. multilocularis* in Groningen and Drenthe and to determine the current spread. This information can be used to predict potential cases of human alveolar echinococcosis and to target public education towards risk areas. The correlation in foxes between infection with *E. multilocularis* and gender and age was also studied, to get a better insight in the epidemiology of the parasite in red foxes in this part of the Netherlands.

Figure 1: *Distribution of Echinococcus multilocularis in Europe in 2012. Reprinted from: Davidson RK, Romig T, Jenkins E, Tryland M, Robertson LJ. The impact of globalisation on the distribution of Echinococcus multilocularis. Trends Parasitol. 2012 Jun;28(6):239-47*

2. Materials and methods

2.1. Red foxes

Foxes were collected in the provinces Groningen and Drenthe. The land area of these provinces is about 2.333.28 km^2 (Groningen) and 2.641.09 km^2 (Drenthe). The fox population density in this study area is estimated to be 4-5 foxes per km^2 at the start of the winter and 2 foxes per km^2 at the end of the winter (pers. communication Jaap Mulder, Bureau Mulder Natuurlijk). Based on these densities, fox population sizes of 4667 - 9333 and 5282 - 10564 foxes were calculated for respectively Groningen and Drenthe. Assuming a prevalence of *E. multilocularis* of 10% in Groningen and 5% in Drenthe, sample sizes of 151-153 and 88-89 foxes respectively were calculated (EpiTools epidemiological calculators), with a confidence level of 95% and an assumed sensitivity and sensitivity of 99%. The red foxes were shot by hunters between 1 October 2016 and 31 March 2017 and were sent to the National Institute of Public Health and Environment (RIVM).

2.2. Diagnostic techniques

Necropsy and intestinal scraping technique (IST)

To kill any potential infectious eggs, the foxes were frozen for six days at -80^{o}C prior to necropsy. Thereafter, dissection was done and the small intestine and colon content of each fox were collected for parasitological examination and molecular diagnostics. At dissection, age and gender of the foxes were estimated, to study the correlation between

age or gender and infection with *E. multilocularis*. The age of the foxes was determined based on tooth wear and was classified as juvenile (born in the current year) or adult (born in a previous year).
To determine the prevalence of *E. multilocularis*, the mucosal content was screened for *E. multilocularis*, using the intestinal scraping technique (IST). The small intestine was divided in six parts. These parts were slit open longitudinally with scissors. Coarse material and intestinal content were removed and three deep intestinal scrapings were made of each part of the small intestine, using microscope slides. The slides were put in a plastic Petri dish and examined microscopically to detect *E. multilocularis* and to estimate the worm burdens of the parasite (Eckert, Deplazes et al. 2001, Vervaeke, Dorny et al. 2003).

Magnetic capture DNA extraction

Colon content of the foxes was used for molecular testing. DNA was extracted by magnetic capture DNA extraction (Opsteegh, Langelaar et al. 2010, Maas, van Roon et al. 2016). 1 gram fecal colon content was used, and 12 ml cell lysis buffer (100 mM Tris HCl pH 8.0; 5 mM EDTA; 0.2% SDS; 200 mM NaCl) was added. The suspension was incubated at 100°C for 10 minutes. The samples were cooled down and centrifuged. Afterwards, 50 µl proteinase K (Qiagen 20 mg/ml) was added, followed by incubation at 56 °C for two hours and centrifuging for 45 minutes at 3500 x g. The supernatant was incubated at 100°C for 10 minutes. In this way, the proteinase K was inactivated. Streptavidine sepharosa, washed in phosphate buffered saline, was added to each sample and incubated at room temperature for 45 minutes, followed by centrifuging. The supernatant was transferred to a tube, and capture-oligonucleotides CapF and CapR were added. This capture-oligonucleotides were labelled with biotin-triethylene-glycol. To denature the DNA, the supernatants were heated for 15 minutes at 100°C. The tubes were put into a water bath of 55°C to allow for hybridisation of the DNA of *E. multilocularis* and the capture-oligonucleotides. Thereafter, the tubes were cooled down. Per sample, M-270 Streptavidin Dynabeads, washed in Binding & Washing buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 M NaCl) and 2 ml 5 M NaCl was added. After incubation at room temperature for 60 minutes, a complex of M-270 Streptavidin Dynabeads and the labelled capture-oligonucleotides with hybridised *E. multilocularis* DNA was generated. The tubes were transferred into a Dynal MPC-1 magnet and the complexes were isolated. The supernatant was removed with a pipette. The tubes were washed, and re-suspended with 100 µl distilled water. Afterwards, the tubes were heated at 100°C to remove the beads from the DNA. DNA was stored at -20°C until further processing.

Real-time quantitative PCR (qPCR)

After magnetic capture DNA extraction, real-time quantitative PCR was performed with a Lightcycler® 480 multiwell Plate 96 (Roche), using the IQ powermix (Biorad). The reaction mixture consisted of 10 ul of IQ powermix (Biorad), 50 pmol of primer EM-3 [100 µM] and primer EM-4 [100 µM], 2 µl of Em-probe-3LNA [10 µM] and 2.5 µl of template DNA. This mixture was incubated for 10 minutes at 95°C to activate the DNA polymerase. Thereafter 50 cycles were carried out, each consisting of a denaturation step (10 seconds at 95°C), an annealing step (22 seconds at 55°C) and extension (20 seconds at 72°C). The samples were cooled to 40°C for 5 seconds. At the end of each extension step, fluorescence at 483-533 nm was measured. To estimate the PCR efficiency, standard series of *E. multilocuaris* DNA were included. A fluorescence-by-cycle-curve was made for each sample. The quantification cycle was estimated at < 40. Samples with a Cq lower than 40, were considered to be positive for *E. multilocularis* (Opsteegh, Langelaar et al. 2010, Maas, van Roon et al. 2016).

2.3. Literature study

A literature study was performed to analyse the spread and prevalence of *E. multilocularis*

in Belgium and Germany, to get a better view of the situation and progression of the parasite in other parts of Europe located close to the Netherlands. In the search strategy, the following databases were used: PubMed, SCOPUS and Google-Scholar. The articles were selected on publication date and relevance. To find relevant articles, the following keywords were used: 'alveolar echinococcosis', Belgium, Brussels, distribution, '*Echinococcus multilocularis*', epidemiology, Flanders, fox, Germany, 'intestinal scraping technique', Limburg, 'Lower Saxony', prevalence, qPCR, 'red fox', spread, 'the Netherlands', '*Vulpes vulpes*'.

2.4 Data analysis

R vision 3.0.2 was used to analyze the data of the foxes. The p-value was determined at 0.05 and the 95% confidence interval was calculated. To determine the correlation between infection and age and infection and gender, the Chi-squared test was used. The p-value was determined at 0.05.

3. Results

Between October 2016 and January 2017, a total of 95 red foxes was examined for *E. multilocularis*. Of these foxes, 55 foxes were collected in the province of Drenthe and 40 in the province of Groningen, of which 34 foxes were hunted in the same study area as the previous study (figure 2). 57 male foxes and 38 female foxes were included. Most of the foxes were classified as juvenile (65 juveniles compared to 30 adults). All foxes were examined using the intestinal scraping technique and two foxes tested positive for the parasite. Molecular testing of the colon content using the qPCR was not carried out yet, because of technical difficulties. So, in total, 2 out of 95 foxes were examined positive (2.1%; 95% CI 0.6-7.4%). Of the infected foxes, one was a juveniles (< 1 year) and one an adult (> 1 year). All positive foxes were males.
No correlation was found between gender and infection (chi-squared test, p= 0.24) and age and infection (chi-squared test, p= 0.57).

The positive foxes were hunted in the south-eastern part and the central part of the province of Groningen. No foxes were found positive in the province of Drenthe (figure 1).
The mean worm burden per fox was 25 worms per fox. One fox had a worm burden between 1 and 10 and one foxes between 40 and 50 worms. Table 1 shows the results of the detection of *E. multilocularis* in this study.

4. Discussion

Limburg and Groningen are considered to be the most western border area of *E. multilocularis* in Europe at current (Takumi, de Vries et al. 2008). The aim of this study was to monitor the parasite and to determine the spread and the prevalence of *E. multilocularis* in Groningen and Drenthe.
The average prevalence was estimated at 2.1% (95% confidence interval (CI) 0.6-7.4%). This prevalence is lower than the base line prevalence in the south-eastern region of Groningen, which was estimated at 9.4% (95%

Figure 2: *Geographical distribution of positive and negative tested red foxes for E. multilocularis in Groningen and Drenthe.*

CI 5.2–16.5%) in a study in 1998-2000 (Van Der Giessen, Rombout et al. 2004). This suggests that the prevalence of *E. multilocularis* in this region has decreased since the previous study. The decreasing prevalence in Groningen and Drenthe is in contrast with other recent studies in the Netherlands and Belgium, where the prevalence of *E. multilocularis* has increased significantly. In South Limburg, the prevalence had increased from 11% (95% CI 7–18%) in 2005-2006 to 59% (95% CI 43–74%) in 2012-2013 (Maas, Dam-Deisz et al. 2014, Takumi, Fonville et al. 2007). Also in the Belgian region of Voeren, close to the border with Limburg, an increased prevalence of 62% (13/21 examined) was found in 2012-2015 compared to 1.7% (4/237 examined) in 1996-1999 in northern Belgium (Vervaeke, Dorny et al. 2003). It is noteworthy that the prevalence in Groningen and Drenthe seems to have decreased, because we expected that the prevalence would have increased in the past decade just as the prevalence in South Limburg.

However, there are also other factors which could explain the decreased prevalence. The current study area was much larger than the study area in the previous study in Groningen, which was restricted to south-eastern part of the province. In this region (an area with a size of approximately 865 km^2) *E. multilocularis* was detected previously in a study in 1996-1997 (Van Der Giessen, Rombout et al. 1999). Therefore, the current study area, compared to the study in 1998-2000, included regions where the parasite never has been detected. The inclusion of the province of Drenthe could be an explanation of the lower average prevalence in this study, because no positive foxes were found in this province. So, the lower prevalence in the current study area does not prove that the prevalence in the fox population of the restricted area, studied in 1998-2000, has decreased. In addition, the prevalence of *E. multilocularis* in foxes which came from the same study area as the previous study was higher than the prevalence of the total study area (5.9%; 95% CI 1.6-19.1%; 2/34 examined). The prevalence in this restricted area is not significant lower than the prevalence in the previous study (9.4%; 95% CI 5.2–16.5%) (Van Der Giessen, Rombout et al. 2004). It is an unexpected result that no positive foxes were found thus far from the province of Drenthe (0%; 95% CI 0-6.5%). This suggest that the parasite is not present in the province of Drenthe right now and that the range of *E. multilocularis* has not extended, but is still restricted to the eastern part of Groningen. The current study is in contrast with the study in Limburg, where a northward spread with a speed of 2.7 km per year was detected (Takumi, de Vries et al. 2008). Although no positive foxes were found in Drenthe, it is possible that *E. multilocularis* is already present in this province. In the current study, one positive fox was hunted a few kilometres west of the area where positive foxes were found in previous studies. This could suggest a slow extension of the range of the parasite in westward direction (Van Der Giessen, Rombout et al. 1999, Van Der Giessen, Rombout et al. 2004).

It is notable that the prevalence of *E. multilocularis* has increased in South Limburg compared to the prevalence in the Groningen and Drenthe in our study. Possibly, the high prevalence, found in South Limburg and the Belgian region of Voeren, is not representative for the whole region, but just for a small hotspot (Maas, Dam-Deisz et al. 2014). Clustering of infection in a small endemic hotspot is also reported in other regions (Eckert, Conraths et al. 2000). In a study performed in Flanders from 2012 to 2015, the prevalence of *E. multilocularis* was increased in the region of Voeren, close to the border of South Limburg. However the prevalence and spread remained unchanged in the other parts of Flanders, suggesting that the increased prevalence in Voeren, is not representative for the whole of Flanders (personal communication Marleen Claes, Institute of Tropical Medicine, Belgium). Likewise, this could explain that the prevalence in Groningen and Drenthe is not increased as in South Limburg.

Another explanation for this difference is the no-endemic area between Groningen and Limburg (figure 1). A spatial analyse, of several studies in Belgium and Limburg suggests that *E. multilocularis* is spreading in northward direction, and that the range of the parasite have expanded from the southern region of

Location	Sex	Age	Total	No. (%) positive	IST (worm count)
Groningen	Male	Juvenile	15	1 (6.7)	44
		Adult	8	1 (12.5)	6
	Female	Juvenile	13	0 (0)	-
		Adult	4	0 (0)	-
Drenthe	Male	Juvenile	25	0 (0)	-
		Adult	9	0 (0)	-
	Female	Juvenile	12	0 (0)	-
		Adult	9	0 (0)	-

Table 1: *Number of foxes positive and negative to infection using the intestinal scraping technique.*

Belgium (Wallonia) to the northern region of Belgium (Flanders) and the Netherlands. However, the analyse showed a decreasing prevalence of 40% in Wallonia to approximately 10% near to the border with the Netherlands. (Vervaeke, van der Giessen et al. 2006). The prevalence in northern regions is suggested to be very low, resulting in an area without *E. multilocularis* between Groningen and Limburg. (Van der Giessen, Rombout et al. 1999). Therefore, it is likely that the parasite has migrated from the fox population in Belgium into Limburg and from the population in Germany into Groningen. This no-endemic area between both endemic spots in Limburg and Groningen also explains that the prevalence in Groningen and Drenthe is not increased as in South Limburg.

Although our study suggests that *E. multilocularis* has only spread little, another current study has identified *E. multilocularis* for the first time outside the previous described endemic region in the Netherlands (Franssen, Nijsse et al. 2014). In another study, a raccoon dog was tested positive for *E. multilocularis* in the province of Flevoland, which is not known to be an endemic region for the parasite (Maas, van den End et al. 2016). This suggests, in contrast to our study, that the parasite has spread more drastically and emphasizes the importance to monitor the spread of the parasite.

The most likely explanation for the different prevalences in Limburg and Groningen is that ecological factors in Limburg make that region a more suitable habitat for intermediate hosts of *E. multilocularis* than Groningen. This might results in a smaller proportion of small rodents in the diet of foxes in Groningen than in Limburg. This may be the result of differences in agriculture, climatic conditions and vegetation between Groningen and Limburg. In Belgium, behavioural, dietary end ecological factors are thought to play a role in the different prevalence in the southern part and the northern part of the country. (Van Gucht, Van Den Berge et al. 2010). Interestingly, the prevalence of *E. multilocularis* in red foxes in Belgium decreased together with the altitude. This may play a role in the lifecycle of the parasite, because of milder climatic conditions in the northern lower part of the country (below 100 meter above sea level) compared to the southern part (maximum altitude of 700 meter above sea level) (Losson, Kervyn et al. 2003a, Vervaeke, Dorny et al. 2003). The difference in altitude in Groningen and Limburg might also play a small role in the different epidemiology in both regions. However, because of the low differences in altitude between Limburg and Groningen, it is more likely that factors like vegetation and agriculture play an important role.

In this study, no significant correlation was found between age and infection with *E. multilocularis* (chi-squared test, p= 0.57). One of the positive foxes was juvenile (< 1 year) and one adult (> 1 year).

However, association between prevalence/worm burden and age has been reported frequently (Otero-Abad, Torgerson 2013). Many studies in high endemic regions have reported a higher prevalence of *E. multilocularis* in juvenile foxes than in adults (Eckert, Conraths et al. 2000, Losson, Kervyn et al. 2003b, Tackmann, Löschner et al. 2001,

Ziadinov, Deplazes et al. 2010, Brossard, Andreutti et al. 2007). However, in some other studies, this difference was not found (Robardet, Giraudoux et al. 2008, Tackmann, Löschner et al. 1998).

The cause of a higher prevalence in juveniles is still unclear. A possible reason is a difference in diet between adults and juveniles. The proportion of rodents might be higher in the diet of juveniles than in the diet of adults who have, as a consequence of more experience, more difficult prey and anthropogenic food in their diet. Inexperienced juveniles might be inclined to prey on and eat more voles who are infected with *E. multilocularis*, if infection adversely affects the voles (Otero-Abad, Rüegg et al. 2017).

In another study, the worm burdens decreased significant with the age of the foxes. Higher worm burdens were found in juvenile foxes: 85% of the total biomass of *E. multilocularis* in the fox population was present in juvenile foxes. However, no significant differences were found in prevalence between juvenile and adult foxes in this population (Hofer, Gloor et al. 2000). This emphasizes the importance to study not just the prevalence of *E. multilocularis*, but also the worm burdens of individual foxes. The epidemiological situation of the parasite is not only determined by the prevalence, but also by the worm burdens of individual foxes, because only a few highly infected foxes can shed thousands of infective eggs into the environment, resulting in more egg contamination of an area than the limited shedding of several low infected foxes (Hofer, Gloor et al. 2000, Otero-Abad, Rüegg et al. 2017).

Besides the dietary factors, the lower worm burdens in adult foxes may also be the result of immunological response of adult foxes, developed after previous infections. Juveniles are more susceptible than adults, because of the lack of this partial immunity. The developed herd immunity in a fox population plays an important role in the epidemiology of *E. multilocularis* in the population (Torgerson 2006).

As a result of the assumed higher worm burdens and/or prevalences, juveniles are thought to play a major role in the transmission of *E. multilocularis*. Furthermore, the dispersion and exploratory behaviour of juvenile foxes during the autumn and winter, contribute to the spatial dynamics and spreading of the parasite (Hegglin, Bontadina et al. 2007).

The two positive tested foxes in this study were both males, however, no significant correlation was found between gender and infection (chi-squared test, p= 0.24).

There is less scientific evidence for an association between gender of the foxes and infection. Only in one study in red foxes in Lithuania, significant higher worm burdens were found in males than in females (Bružinskaitė-schmidhalter, Šarkūnas et al. 2012). However, a study in coyotes (*Canis latrans*), a wild canid that may be a prominent host of *E. multilocularis* in North America, the prevalence in males was significant higher than in females (34.19% (15/34 examined) in males compared to 15.2% (7/46 examined) in females) (Catalano, Lejeune et al. 2012). Male foxes, just like juvenile foxes, are thought to play an important role in the spreading of the parasite, because of their tendency to migrate further and to expand their territories more than age-matched females (Otero-Abad, Torgerson 2013, Hofer, Gloor et al. 2000).

5. Conclusion

In this study, no positive red foxes were found in the province of Drenthe and a lower prevalence of *E. multilocularis* was found in the province of Groningen compared to previous studies. In contrast to other studies, only a little spread of the parasite was detected. However, the firs identification of *E. multilocularis* in recent studies outside the previous described endemic area, emphasizes the importance to follow the spread of the parasite in the future (Franssen, Nijsse et al. 2014, Maas, van den End et al. 2016). Monitoring of the parasite is very important because of the public health risk of the parasite.

It is still unclear what factors plays an important role in the epidemiology of *E. multilocularis*. More research is needed to determine the influence of ecological, dietary and behavioural factors on the epidemiology of the parasite. This may explain the different

epidemiological patterns that are seen within the different endemic regions in the Netherlands.

Acknowledgements

I would like to acknowledge my supervisors dr. M. Maas for her support and dr. E.R. Nijsse and dr. P.A.M. Overgaauw for their support as supervisors. I also would like to to thank A. M. van Roon and dr. J.W.B. van der Giessen from the National Institute for Public Health and the Environment (RIVM) for helping me with necropsies and microscopic examinations.

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