RIVM report 330040001/2004

The prevalence of *Echinococcus multilocularis* in foxes in Limburg 2002-2003

J.W.B. van der Giessen, A. de Vries, M.L. Chu, V. Storteldert[1] J.L. Mulder[2], C. de Lezenne Coulander, P. Teunis

1. Food and Consumer Product Safety Authority, the Inspectorate for Health Protection and Veterinary Public Health, Division Zuid, Eindhoven.
2. Alterra, Wageningen UR, Wageningen and Mulder-natuurlijk, De Bilt

This investigation has been performed by order and for the account of the Inspectorate of Health Protection and Veterinary Public Health, within the framework of project 330040 Parasitic Zoonoses and by financial support of EU project QLK2-CT-2001-01995, Echinorisk.

RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71

Abstract

This report describes a survey carried out between January 2002 and March 2003 to determine the prevalence of *Echinococcus multilocularis* in the red fox (*Vulpes vulpes*) in the province of Limburg, the Netherlands. *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis, a very serious life threatening disease in humans. Baseline prevalence data are important for obtaining better insight into the possible spread of the parasite *Echinococcus multiocularis* in time and into the potential risk to public health. *Echinococcus multilocularis* was detected in foxes for the first time in Limburg, as recorded in a previous study carried out from 1996 to 1998. In the survey documented here, carried out between January 2002 and March 2003, 196 foxes from the southern part of Limburg were tested using microscopy and PCR. The prevalence of the parasite was estimated at 12.6% (7.6-16.6% with a 95% confidence interval). Results confirm the previous findings that *Echinococcus multilocularis* was detected in foxes in the same geographical area, but now the parasite is also detected in the northernmost border of the tested area. Spatial analysis shows a decreasing prevalence from south to north. Furthermore, the number of adult parasites per fox were higher compared to the previous study. This indicates that the parasite may be become an increasing problem in Limburg.

Contents

Samenvatting

Echinococcus multilocularis is de kleine lintworm van de vos (*Vulpes vulpes*), welke een ernstige ziekte bij de mens, alveolaire echinococcose, kan veroorzaken. Mensen raken besmet door orale opname van de eieren van *E. multilocularis*, die met de vossenfaeces in het milieu terecht komen. De incubatietijd van alveolaire echinococcose is lang, 10 tot 15 jaar, en de klinische verschijnselen zijn weinig typisch. Als de ziekte zich openbaart kunnen pathologische veranderingen al zo ernstig zijn, dat behandeling zeer moeilijk is. Daarom is de preventie van een infectie bij mensen zeer belangrijk. In Europa is de vos het belangrijkste reservoir van *E. multilocularis* en het bekende endemische gebied is gelokaliseerd in Duitsland, Zwitserland, Oostenrijk en Frankrijk. Een uitbreiding van het verspreidingsgebied van deze parasiet naar andere gebieden in Europa wordt echter verondersteld. In Nederland zijn bij een inventariserende studie, uitgevoerd tussen 1996 en 1998, naar het voorkomen van *E. multilocularis*, twee besmette vossen gevonden in een gebied in Groningen en drie besmette vossen gevonden in Zuid-Limburg. Omdat het aantal onderzochte vossen in deze gebieden vrij beperkt was, is in 1998 een prevalentieonderzoek uitgevoerd in Groningen. De prevalentie werd bepaald tussen 1998 en 2000 en bedroeg 9,4% (95% betrouwbaarheidsinterval 5,2-16,5%).
Eenzelfde studie is vanaf 2002 uitgevoerd in Zuid-Limburg. Hier zijn in februari 2002 en vervolgens van november 2002 tot maart 2003 in totaal 196 vossen onderzocht met microscopisch onderzoek en DNA technieken. In totaal werden 25 dieren positief bevonden. De prevalentie in Zuid -imburg was 12,6% (95% betrouwbaarheidsinterval 7,6-16,7%). Dit betekent dat *E. multilocularis* wederom is gevonden en gezien het grotere aantal onderzochte dieren lijkt het of in Zuid-Limburg het aantal gevonden *E. multilocularis* parasieten per vos is toegenomen ten opzichte van het eerste onderzoek. In vergelijking met de eerdere bevindingen in Zuid-Limburg, waarbij alleen positieve vossen rond de zuidgrens met België werden gevonden, worden nu ook positieve vossen rond Sittard aangetroffen. Wat dit betekent voor het risico van overdracht naar de mens is nog vrij onduidelijk. Het is niet bekend welk verband er bestaat tussen de prevalentie van *E. multilocularis* bij vossen en het infectierisico voor mensen. Ook is niet bekend of een toegenomen prevalentie bij vossen leidt tot een verhoogd risico voor mensen, omdat betrouwbare epidemiologische data niet beschikbaar zijn in veel delen van Europa. Dit komt mede door de lange incubatietijd van alveolaire echinococcose. Bij recente introductie van de parasiet, kan het nog jaren

duren voor de ziekte zich voor het eerst openbaart bij mensen, als er zich infecties hebben voorgedaan. Gezien de ernst van de ziekte bij mensen is het daarom van belang om zeer alert te zijn op mogelijke humane gevallen. Tot nu toe zijn er in Groningen en Limburg geen humane gevallen van alveolaire echinococcose voorgekomen, maar alertheid blijft belangrijk. Verder is het van belang om de epidemiologische ontwikkelingen bij vossen te volgen om een mogelijk toenemend humaan infectierisico vast te stellen.

Summary

Echinococcus multilocularis is the causative agent of alveolar echinococcosis, a very serious life threatening disease in humans. Accidental oral uptake of the eggs may be the infection route to humans. The incubation time of this disease can be as long as 10 to 15 years and even then clinical signs are not typically related to the disease. At that time, infestation of the liver and even metastasis in the body can be so serious that treatment is difficult or even impossible and consequently the disease becomes fatal. Hence, prevention of this life-threatening infection in humans is of major importance. In Europe, foxes (*Vulpes vulpes*) are the main reservoir of the parasite, and an endemic area is located in Germany, Switzerland, Austria and France. In the Netherlands, between 1996 and 1998, two *E. multilocularis* positive foxes were detected in one region in the province of Groningen. In Groningen, the base line prevalence was further determined from 1998 to 2000. The prevalence in Groningen was estimated at 9.4% (95% CI: 5.2-16.5%).

In the present study, in February 2002 and from November 2002 until March 2003, 196 foxes in the southern part of Limburg were tested by microscopy and DNA techniques to determine the base line prevalence. The prevalence was 12.6% (95% confidence interval 7.6-16.7%). Although the prevalence was similar as in the previous study carried out from 1996-1998, it was noticed that the number of adult *E. multilocularis* parasites in positive foxes was higher in this study. This indicates a possible increased amount of eggs shed in the environment, although this is not significant. Furthermore, *E. multilocularis* positive foxes are also located even as far north as the surroundings of Sittard. Possible further spreading of the parasite in northern part of Limburg can not be excluded.

Base line data are important to monitor a possible increase of the prevalence and possible spread of the parasite. The occurrence of *E. multilocularis* in foxes is an indication for a potential infection risk of humans. This risk may actually exist in all endemic regions in Europe. However, it is not known, whether an increased prevalence of vulpine echinococcosis directly leads to a higher infection risk in humans, because of the lack of reliable epidemiological data in many regions of Europe. Until now, no human cases have been reported in Groningen and Limburg, but because of the long incubation time, the first human case may only be recognized years after the first introduction of the parasite. In addition, rare cases may be overlooked or misdiagnosed in countries with a lack of medical awareness of this disease. Therefore, it is important to monitor the prevalence of

E. multilocularis in foxes to identify a potential increasing infection risk to humans.

Introduction

Alveolar echinococcosis, caused by the larval stage of *Echinococcus multilocularis*, is a serious parasitic zoonosis. The life cycle of this tapeworm is mainly sylvatic. Eggs shed by the canid definite host, mainly the red fox (V*ulpes vulpes*) in Europe, develop to the larval or metacestode stage after uptake by arvicolid rodents that serve as intermediate host (Eckert, 1989). Humans may get infected by uptake of eggs and this may lead to a very serious disease in humans, called alveolar echinococcosis (Ammann and Eckert, 1996). Treatment can significantly prolong the patients survival time especially when the infection is detected in an early stage. Treatment should include the combination of surgery and chemotherapy. However, treatment is life long and is very expensive. Prevention of human infection is of major importance, although the transmission routes to humans are not clearly understood and the susceptibility for humans of this parasite is assumed to be limited (Tackman *et al.,* 1998). The determination of the prevalence of *E. multilocularis* in definite hosts is an important parameter to estimate the potential infection risk for humans (Deplazes and Eckert, 1996). This risk may actually exist in all endemic areas in Europe as rare cases may be overlooked or misdiagnosed in countries with a lack of medical awareness of the disease (Eckert *et al.,* 2000). However, it may also reflect the recent spread of *E. multilocularis* to newly recognized areas. Important factors with the potential to enhance the infection risk for humans in the future include an increasing prevalence of *E. multilocularis* in foxes. Within some endemic areas in southern Germany it is believed that the prevalence of *E. multilocularis* in foxes increased over the past 15 years (Romig *et al.,* 1999a). Also, the absolute number of foxes in many parts of Europe may have increased the last decades probably due to the successful rabies control campaigns resulting in reduced mortality. However, it should be noticed that increasing fox densities are difficult to determine because of the lack of exact methods to determine. An increase of the prevalence of *E. multilocularis* in foxes is difficult to determine, despite sometimes large data sets from various parts of Europe. In fact, there are only few regions where appropriate sample sizes in time were taken, which allow the comparison of prevalence in time.

In 1996, a survey was undertaken in the Netherlands to detect the presence of *E. multilocularis* at the border area with Germany and Belgium, since in some parts of Germany adjacent to the Netherlands, prevalence rates of 17% upto 33% were reported (Lucius and Bilger, 1995). *E. multilocularis* was found in 5 out of 272 foxes from the border

area in the Netherlands (Van der Giessen *et al.,* 1999). Two distinct areas were positive for *E. multilocularis,* Groningen and the south of Limburg. The base line prevalence in Groningen was determined in a study carried out between 1998 and 2000 and was 9.4% (5.2-16.4%) (Van der Giessen *et al.,* 2000).

In order to determine an increasing prevalence and spread of the parasite in the Netherlands in the future and to get a better insight in the risk for humans, it is important to determine the base line prevalence not only in Groningen but also in Limburg.

In this report, a survey in Limburg is described with the aim to determine the base line prevalence of *E. multilocularis.* The relevance of these findings and the possible consequences for humans are discussed.

2. Material and Methods

2.1 Study area

A region in the eastern and southern part of the province of Limburg, where *E. multilocularis* was detected previously (Van der Giessen *et al.,* 1999) was investigated. This region consists of 12 different hunting areas (Addendum). The size of this region was 800 km^2. The average prebreeding fox population size in this region was estimated to be around 2000 foxes.

2.2. Animals and sample size

Red foxes shot by hunters in February 2002 and from November 2002 until March 2003, were frozen at -20^{0}C within 24 h after shooting and subsequently sent to the RIVM, carcasses were frozen at -80^{0}C for 1 week, prior to necropsy. At necropsy, small intestines and content of colon were removed. Strict safety precautions were taken during handling of the animals, necropsy and parasitological examinations to avoid or exclude infection risk (Eckert and Deplazes, 1996). Based on an estimated prevalence of *E. multilocularis* ranging from 5 to 10%, adopting an error of 5% and a 95% confidence interval (C.I.), the sample size needed for prevalence determination ranged from 150 to 200 foxes (Episcope 3.0).

2.3 DNA isolation from fecal colon content, intestinal mucosal material and PCR

One gram of fecal colon content of foxes was further analyzed using a nested PCR based method in a single tube. If colon content was not available, DNA was extracted from 0.02 g of mucosal scraping material using the animal tissue protocol of the PureGene®, DNA isolation kit (Gentra systems Minneapolis, USA). Of the isolated DNA, one µl was used in the nested PCR as described before (Van der Giessen et al, 1999). For the detection, 10 µl of PCR product was run on 1.5% agarose gels in ethidium bromide as described previously (Sambrook *et al.,* 1989).

2.4 Southern blot hybridization

After agarose gel electrophoresis in 1x TAE, the gel was denaturated in 0.5M NaOH for

15 min. The gel was blotted on positively charged nylon membrane (Boeringer) by using a vacuum-blotting unit (LKB, Bromma). After blotting the membrane was denatured in 0.5M NaOH and washed in 5x SSC. After prehybridisation for 30 min in 20 ml 2x SSPE, 0.1% SDS at 42 ^{0}C, the membrane was hybridized for 45 min at 42 ^{0}C by adding 20 pmol biotin-labeled oligonucleotide internal probe as described by Dinkel et al. (1998). The PCR products were detected by ECL (Amersham).

2.5 Parasitological examination

Parasitological examination of the small intestines was carried out according to the methods recommended by the WHO Collaborating Center for Parasitic Zoonoses in Zurich (Deplazes and Eckert, 1996) and as described before (Van der Giessen *et al.,* 1999). The age of the foxes was determined ('estimated age') as adult (born previous year) or subadult (born in current year) according to Wagenknecht (1979). In addition, age of 123 foxes was also determined more accurately ('exact age') by cementum layer counting of the canine teeth (Grue and Jensen, 1979).

2.6 Statistical Analysis

Estimation of the prevalence

The prevalence was determined from the numbers of positive foxes (s) and the total number examined (n) as a binomial proportion, with a uniform Beta (1,1) distributed prior. The posterior then is Beta (s+1, n-s+1) distributed. The mode of the Beta distribution was used as point estimate of the prevalence and quantiles were used to define a 95% predictive interval (PI). The significance of differences in prevalence was tested with a likelihood ratio test, comparing the hypotheses of prevalence being equal and not equal.
Possible age or sex dependency was investigated by multivariate logistic regression (using the proc Logistic SAS System version 8.01).

Spatial gradient

The positions of all foxes were recorded in standard co-ordinates, allowing a logistic regression with the position vector $\vec{x} = (X, Y)$ as covariable:

$$\text{logit}(p) = \frac{e^p}{1+e^p} = a + \vec{b} \cdot \vec{x}$$

Using uninformed lognormal priors, posterior mode estimates were obtained for the parameters a and $\vec{b} = (b_x, b_y)$, and a 95% predictive interval was constructed for the prevalence using Markov Chain Monte Carlo sampling employing the algorithm of Metropolis and Hastings (Carlin and Louis, 1996).

3. Results

3.1 *E. multilocularis* in foxes

A total of 207 foxes were sent to the RIVM. Of these, 196 foxes, 106 males and 90 females, were tested for *E. multilocularis.* Of the other 11 foxes, either intestinal tissue and colon content was lacking or the foxes were excluded because they were too young to become infected (< 6 weeks of age).

Of the 196 foxes tested, 25 foxes were positive for *E. multilocularis.* Of these 25 positive foxes, 18 were males of which 7 were adults. Of the 15 positive foxes as determined by microscopy, either the PCR and/or the blot hybridization results were positive. The blot hybridization of the PCR products was carried out to confirm if the amplicons were specific for *E. multilocularis* as detected by agarose gel electrophoresis. The blot hybridization detected another 10 positive foxes, which were not detected by microscopy and PCR after using agarose gel electrophoresis alone (Table 1).

Furthermore, it was noticed that *E.multilocularis* infected foxes were detected most frequently in January and February (Table 2).

Table 2. Month and year of E. multilocularis positive fox detection

Time of infection	*Em*. positives	*Em* negatives	% *Em* positives	Total number
February 2002	3	22	12.0	25
November 2002	3	35	7,9	38
December 2002	3	26	10,3	29
January 2003	7	36	16.3	43
February 2003	12	66	15,4	78

Table 1. Results of the E. multilocularis positive foxes in Limburg (nd=not done).

Fox number	Sex m:male f:female	Estimated age adult or subadult	Microscopical positive	Em PCR positive	Em blot positive	Em PCR mucosal tissue positive	Exact age in months
605	m	a	5	+	+	nd	nd
610	m	a	nd	+	+	nd	nd
619	m	a	10	+	+	nd	nd
642	m	s	5	-	-	+	nd
664	m	a	1	-	-	+	nd
669	m	a	-	+	+	nd	nd
673	m	s	1000	+	+	nd	nd
686	m	s	300	+	+	nd	10
705	f	a	-	-	-	+	10
715	m	a	25	-	-	+	10
718	f	a	-	-	-	+	11
720	f	s	-	nd	nd	+	10
723	f	a	-	nd	nd	+	10
725	m	s	50	+	+	nd	10
736	f	a	50	+	+	nd	10
747	m	a	-	+	+	nd	35
749	f	a	-	nd	nd	+	21
753	f	s	200	+	+	nd	11
766	m	a	1000	+	+	nd	11
767	m	a	6	nd	nd	+	11
781	m	a	1	+	+	nd	10
790	m	s	100	+	+	nd	11
797	m	a	-	+	+	nd	59
804	m	s	500	+	+	nd	11
807	m	s	nd	+	+	nd	11

3.2 The prevalence of *E. multilocularis* in Limburg

The estimated prevalence in this study was 12.6% (95% CI: 7.6-16.8%). This prevalence is in the same range compared to the prevalence found in the study in 1996-1998 (13.6%; 99% CI: 4.8 to 22.3%) (Van der Giessen *et al.,* 1998). It should be noted that the study in 1996-1998 was only designed to detect the presence of the parasite and the sample size in that study (n=22) was not sufficiently high to determine the prevalence of *E. multilocularis*. In addition, in this study, the PCR results were confirmed by southern blot. Southern blot was not carried out in the 1996-1998 study.

3.3 Spatial distribution of *E. multilocularis* in foxes in Limburg

The *E. multilocularis* positive foxes were randomly distributed within the region, although in the southern part positive foxes were detected more often at the border to Belgium (Fig. 1). In addition, we were able to count the worm burdens of the microscopical positive foxes and we recognized that foxes with high worm burdens were detected especially in the border area with Belgium (Fig. 2). In 1996-1998, 3 positive foxes were detected in Limburg. Of these, 1 fox was microscopically positive with a worm burden of 100. In the present study, the number of adult parasites per fox were higher compared to a previous study. In addition, spatial analyses of the prevalence using microscopical results showed that there was a tendency that the prevalence was highest in the border area with Belgium (12%) and decreased from southwest to northeast direction (4% around Sittard), although this was not significant at the 95% level (likelihood ratio test against uniform prevalence) The best fitting gradient is shown in Fig. 3.

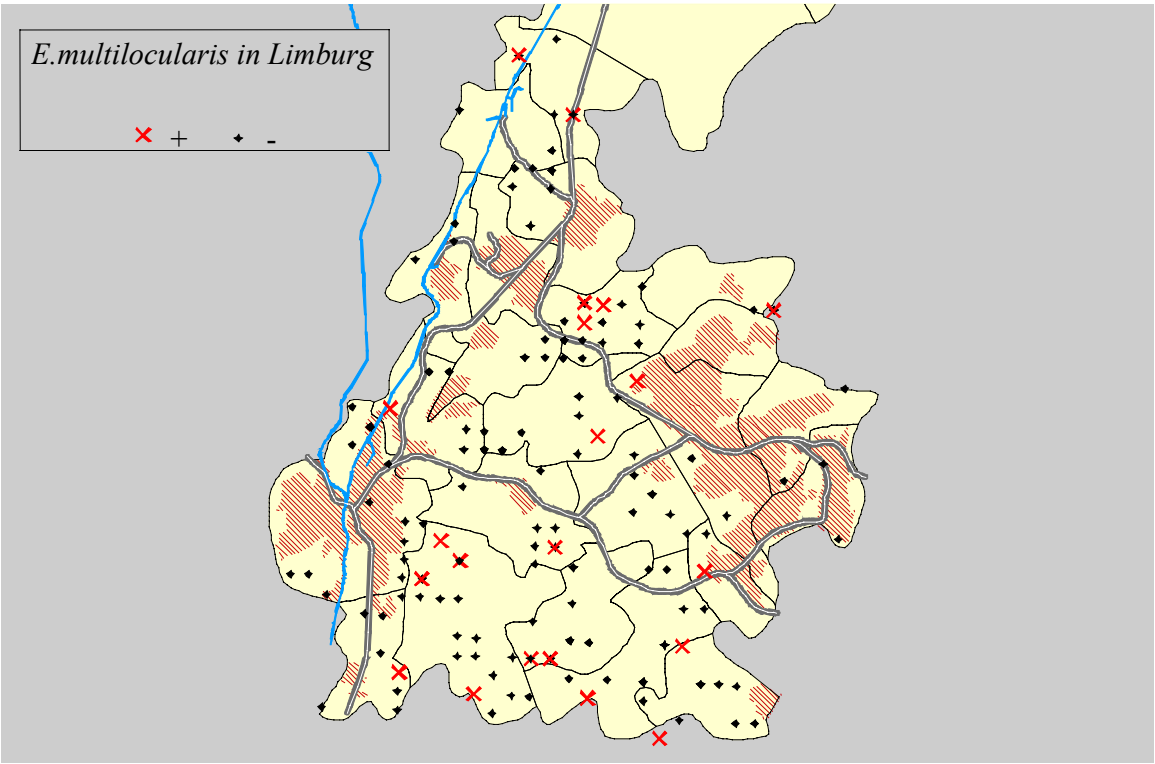


Figure 1. Geographical distribution of E. multilocularis PCR positive (x) and E. multilocularis negative (♦) foxes in the Netherlands as was studied in the province of Limburg.

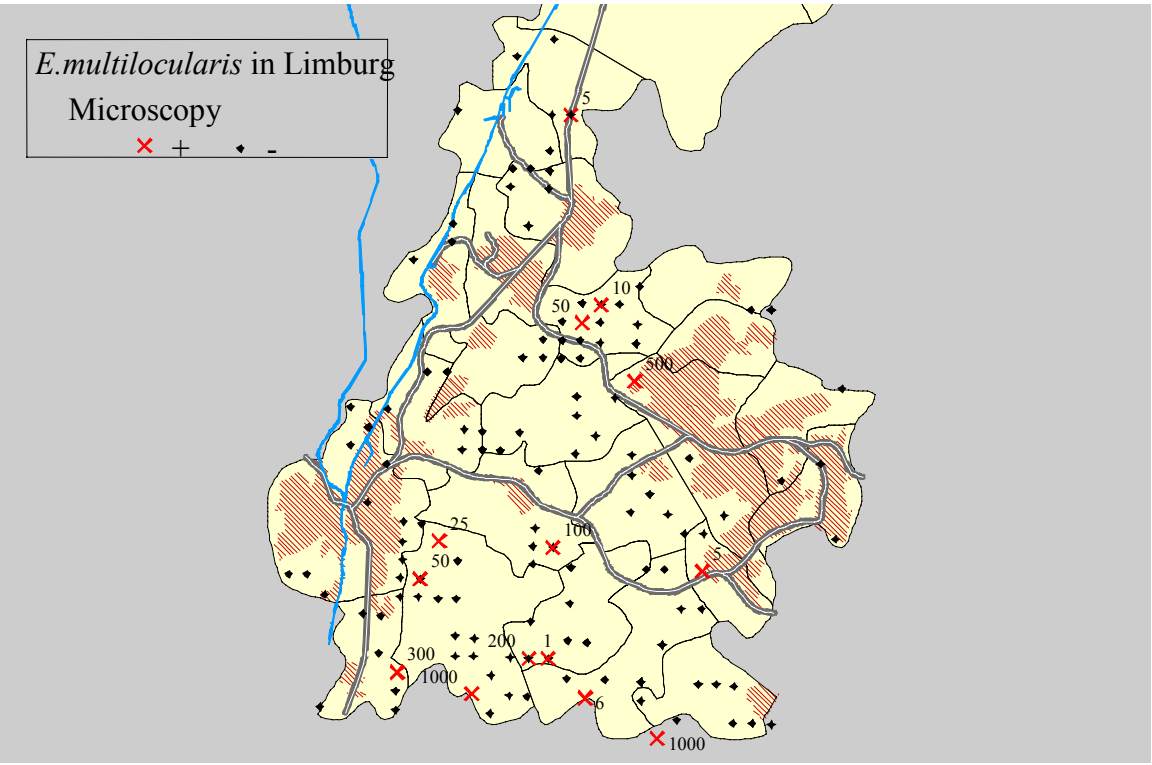


Figure 2. Geographical distribution of E. multilocularis microscopical positive (x) and E.multilocularis negative (♦) foxes in the province of Limburg. The worm burdens of positive foxes is indicated.

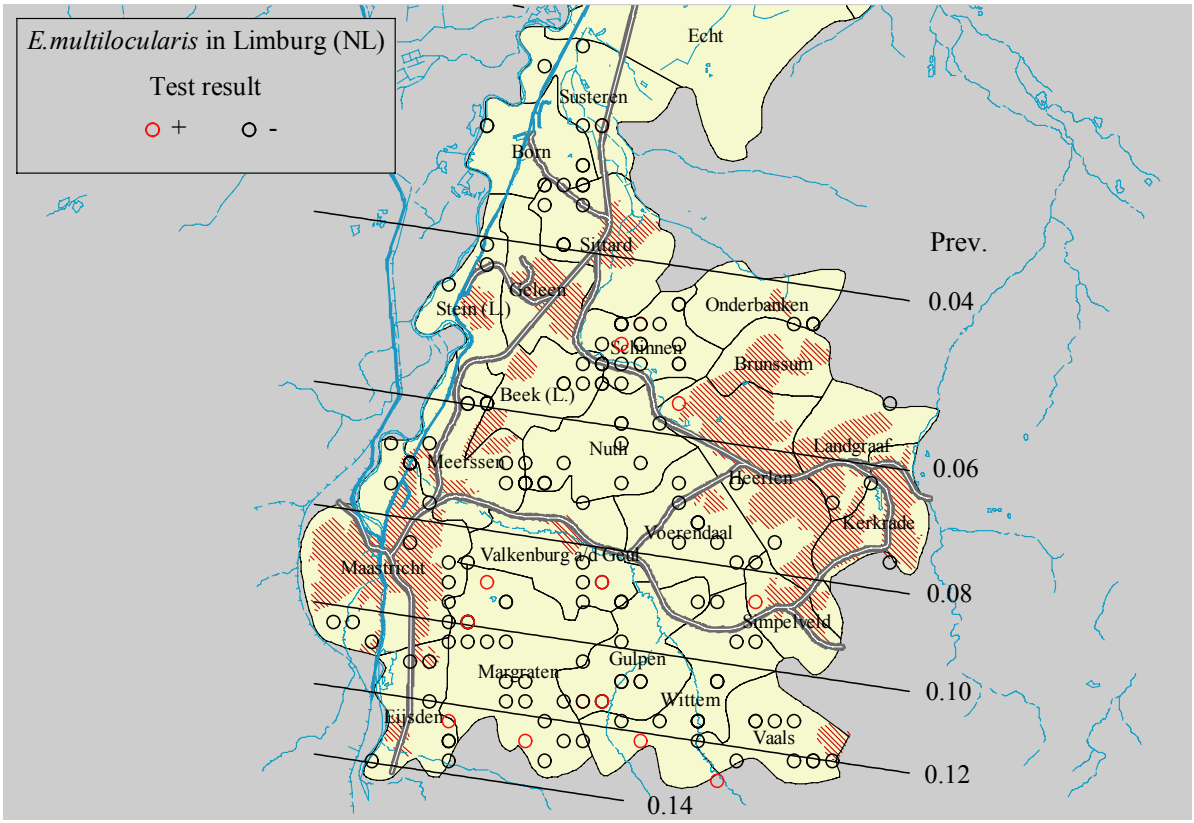


Figure 3. Map of the studied region (province of Limburg) with positions of foxes. Also shown are contours of the prevalence gradient, showing increasing values in North-Eastern direction.

3.4 Analysis of age and sex dependancy

The number of *E. multilocularis* positive (infected) and negative (non-infected) adult and subadult foxes using the estimated age determination are shown in Table 3. This method was also used in previous studies (Van der Giessen *et al.,* 1998 and 2000). In this study, an additional age determination method was used. To results of the exact age determinations method is shown in Table 4. For the exact age determination, 123 of the 195 foxes were used. Age dependency was not significant correlated with *E. multilocularis* positivity. Odds Ratio (OR) after uni and bivariate analysis was 0.78 (95% CI: 0.33-1.84, P value 0.57) using the estimated age determination. When the exact age was analysed, subadult animals were slightly more positive, but this was not a significant difference OR: 0.55 (0.15-2.04; P value 0.37).

Table 3. The number of foxes positive for E. multilocularis (infected) and negative for E. multilocularis (non-infected) divided into estimated age categories (adult>12 months and subadult < 12 months of age).

	Infected	Non-Infected	Total
Adult	15	112	127
Subadult	10	58	68
Total	25	170	195*

*: 1 fox no age determined

Table 4. The number of foxes positive for E. multilocularis (infected) and negative for E. multilocularis (non-infected) divided into exact age categories (adult>12 months and subadult < 12 months of age).

	Infected	Non-Infected	Total
Adult	3	28	31
Subadult	15	77	92
Total	18	105	123#

#: Exact age was determined in 123 foxes

Highest worm burdens were found in subadult *E. multilocularis* positive foxes. This was independent from the age determination method used (Table 5).

Table 5. Comparison of estimated and calculated ages of the E. multilocularis positive foxes.

Foxes	Number (estim. age)	Microscopical (worm burden	Number (exact age)*	Microscopical positive (worm burden)
Adult	16	2 (1-10)	3	0
Subadult	9	13 (1-1000)	15	10 (1-1000)

*: Exact age in months was determined from 123 foxes. Adult >12 months, subadult <12 months of age.

In Table 6, the results of the *E. multilocularis* positive foxes (infected) and negative (non-infected) are compared by sex. Uni-and bivariate analysis of sex and *E. multilocularis* positive foxes showed that female animals were slightly less often positive OR: 0.41 (95% CI: 0.15-2.04) but this was not significant at the 95% level.

Table 6. The number of foxes positive for E. multilocularis (infected) and negative for E. multilocularis (non-infected) by sex.

	Infected	Non-Infected	Total
Male	18	88	106
Female	7	83	90
Total	25	171	196

4. Discussion

In this report, the base line prevalence of *E. multilocularis* in southern Limburg was determined. The prevalence was 12.6% (95% CI: 7.6-16.8%). This is within the same range as was found 4 years ago in the same region, although the sample size in this study was larger. Remarkable in the present study was the recognition of 15 microscopical positive foxes with worm burdens per infected fox ranging form 1-1000 parasites. In the previous study of 4 years ago, 3 infected foxes were detected out of 22 foxes examined in the same region. In that study, only one fox was positive by microscopy with a worm burden of 100 parasites. The other two were only PCR positive. This suggests an increasing worm burden per animal and thus an increasing number of eggs shed in the environment in a short time interval, although the number of data of the first study was too low to show any significant difference. In the previous study, positive foxes were only detected around the border with Belgium. In the recent study, positive foxes were also found around Sittard. This means that it can not be excluded that *E. multilocularis* occurs even in the northern part of Limburg. Remarkable was the different outcome of the two age calculation methods. Only 67% of the results were the same. When we assume, that the exact age determination is the gold standard method, the results indicate that the estimated age determination is not very reliable. However, the analysis of age and *E. multilocularis* positivity was not significantly different. Using the exact age determination, subadult foxes were slightly more positive despite the smaller sample size compared to the estimated age determination. This suggests that subadult foxes are more likely to become infected in Limburg and in addition carried higher worm burdens.We also noticed that the majority of *E. multilocularis* positive foxes were found in January and February. Sofar, we have no explanation for this result. Contamination of the environment by shedded eggs originating from definite hosts is one of the potential routes of infection of humans.

The prevalence of 12.6% (95% CI: 7.6-16.8%) in Limburg is the second base line prevalence result in the Netherlands. Until now, no human cases were reported in Limburg but this can be due to several factors, which were described earlier. In a region in southwestern Germany showing a prevalence of *E. multilocularis* in foxes of 15% (95% CI: 10-21%), no human seroreactors were identified (Romig *et al.*, 1999c). This indicates that the human risks of exposure to the parasite are still low. However, this result is based on a limited number of data, so care should be taken by drawing any conclusion. Only a few long term data are available which could reflect changes in the epidemiological

situation. However, it cannot be excluded that increasing fox populations, the invasion of urban areas by foxes, increasing prevalence of *E. multilocularis* in foxes, transmission from the sylvatic to the synanthropic cycle, changes in land use patterns and other factors may influence the transmission patterns and increase the infection risk for humans in the future (Eckert *et al.,* 2000). In this respect, the possible increasing fox population densities in the Netherlands as a consequence of the prohibition of shooting foxes should be carefully studied. Prevention of infection in humans is still one of the key instruments to control the disease and more efforts should be undertaken to monitor the parasite in human and animal populations in endemic areas. Possible control measures in foxes should be analysed. For this purpose, mathematical models describing the consequences of intervention strategies are being developed at the moment and these models will be very helpful. Furthermore, attention should focus on prevention of the transmission of *E. multilocularis* to humans and the registration of human cases in newly recognized areas. The costs to control the parasite in animals, the costs for life long treatment of a human case or the loss after a fatal case and the risk perception of the public should be considered when deciding if intervention is needed in the animal reservoir.

5. Conclusions and Recommendations

- The prevalence of *E. multilocularis* in the current study is 12.6% (95% CI: 7.6-16.8%).

- The worm burden per fox is between 1 and 1000 parasites and highest worm burdens are being found in subadult foxes. This is the first time that worm burdens of around 1000 parasites per fox were detected in the Netherlands. This could implicate a further environmental contamination and thus an increasing human infection risk.

- The parasite is not randomly distributed in the study area, as a higher prevalence is found around the border area with Belgium and a decreasing prevalence to 4% is found around Sittard, the most northern parts of the study area . A wider study area may be needed to establish a geographical gradient, possibly extending into adjacent countries (Germany and Belgium).

- It is unknown if the fox population north of the study area in Limburg and Noord-Brabant is infected with *E. multilocularis.* To get information of the epidemiological situation more studies in other parts of Limburg and Noord-Brabant need to be conducted.

- To get insight in a possible spread of the parasite in the Netherlands and to get insight in the possible human infection risks, future surveillance is needed.

- To study possible control measures which could be undertaken with the aim to decrease the further spread of the parasite, a mathematical model describing the population dynamics of the infection is being developed.

Acknowledgment

The authors thank Margriet Montizaan, Ger van Hout, Sjeng Jehae and Leon Wolfs of the Royal Dutch Shooting Society (KNJV) and participating hunters for their collaboration. Rob van Oosterom and Moniek Aalten of the Inspectorate of Health Protection and Veterinary Public health are acknowledged for their efforts to receive permission for the study in Limburg. Martijn Bauknegt is acknowledged for the multivariate analyses and technical help of Manoj Fonville is appreciated. We also thank Katsuhisa Takumi for critical reading of the manuscript.

References

-Ammann, R.W. and Eckert, J. (1996). Cestodes Echinococcus Gastroenterology Clinics of North America 25:655-689.

-Carlin, B. P., Louis T. A., 1996. Bayes and empirical Bayes methods for data analysis In: Monographs on statistics and applied probability, Volume 69. Chapman and Hall, London.

-Deplazes, P., Eckert, J. (1996). Diagnosis of the *Echinococcus multilocularis* infection in final hosts. Applied Parasitology 37:245-252.

-Deplazes, P., Alther, P., Tanner, I., Thompson, A., Eckert, J. (1999*). Echinoccous multilocularis* coproantigen detection by Enzyme linked immunosorbent assay in fox, dog, and cat populations. J. Parasitol. 115-121.

-Dinkel, A., Van Nickisch-Rosenegk, M., Bilger, B., Merli, M., Lucius, R., Romig, T. (1998). *Echinococcus multilocularis* in the definite host, coprodiagnosis by PCR is an alternative to necropsy. J. of Clin. Microbiol. 36:1871-1876.

-Eckert, J. (1989). Prevalence and geographical distribution of *Echinococcus multilocularis* infection in humans and animals in Europe. WHO informal Consultation on alveolar echinococcosis, 14-16 August 1989, Hohenheim (WHO/VPH/ECHIN.RES./WP/89.4).

-Eckert, J., Desplazes, P. (1996). Methods for surveys on *Echinococcus multilocularis* in final hosts. In: Alveolar Echinococcosis: Strategy for eradication of alveolar echinococcosis of the liver. J. Uchino; N. Sato (eds), Fuji Shoin, pp 151-163.

-Eckert, J., Desplazes, P. (1999). Alveolar echinococcosis in humans: the current situation in central Europe and the need for countermeasures. Parasitology Today 15:315-319.

-Eckert, J., Conraths, F.J., Tackman, K. (2000). Echinococcosis: an emerging or re-emerging zoonosis? Int. J. parasitol. 30: 1283-1294.

-Grue, H., Jensen, B. 1979. Review of the formation of incremental lines in tooth cementum of terrestrial mammals. Danish Review of Game Biology 11(3):1-48.

-Hofer, S., Gloor, S., Muller, U., Mathis, A., Hegglin, D., Desplazes, P. (2000). High prevalence of *E. multilocularis* in urban red fox (*Vulpes vulpes*) and *voles (Arvicola Terrestris*) in the city of Zurich, Switzerland. Parasitology 120:135-142.

-Lucius, R., Bilger, B. (1995). *Echinococcus multilocularis* in Germany: increased awareness or spreading of a parasite? Parasitology today 11: 430-433.

-Romig, T., Bilger, B., Dinkel, A., Merli, M., Mackenstedt, U. (1999a). *Echinococcus multilocularis* in animal hosts: new data from western Europe. Helminthologia 36:185-191.

-Romig, T., Kratzer, W., Kimmig, P., Frosch, M., Gaus, W et al. (1999c). An epidemiologic survey of human alveolar echinococcosis in southwestern Germany. A. J. Trop. Med. Hyg. 6: 566-573.

-Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular cloning, a laboratory manual. second edition. Cold Spring Harbor Laboratory Press.

-Tackman, K., Löschner, U., Mix, H., Staubach, C., Thulke, H.H., Conraths, F.J. (1998). Spatial distribution patterns of *Echinococcus multilocularis* (Leuckart 1863) (Cestoda: Cyclophyllidea: Taeniiidae) among red foxes in an endemic focus in Brandenburg, Germany. Epidemiol. Infect. 120:101-109.

-Van der Giessen, J.W.B., Rombout, Y., Limper, L., Van der Veen, A., Moolenbeek, C., Franchimont, H., Homan, W. (1998). The presence of *Echinococcus multilocularis* in the red fox (*Vulpes vulpes)* in the Netherlands. RIVM report 258725001, RIVM, Bilthoven.

-Van der Giessen, J.W.B, Rombout, Y., Franchimont, H., Limper, L., Homan, W. (1999). Detection of *Echinococcus multilocularis* in foxes in the Netherlands. Veterinary Parasitology 82: 49-57.

-J.W.B. van der Giessen, Rombout, Y., Evers, E.G., Arends, B., Van der Veen, A.(2000). The prevalence of *Echinococcus multilocularis* in foxes in Groningen 1998-2000. RIVM report 258725003, RIVM, Bilthoven.

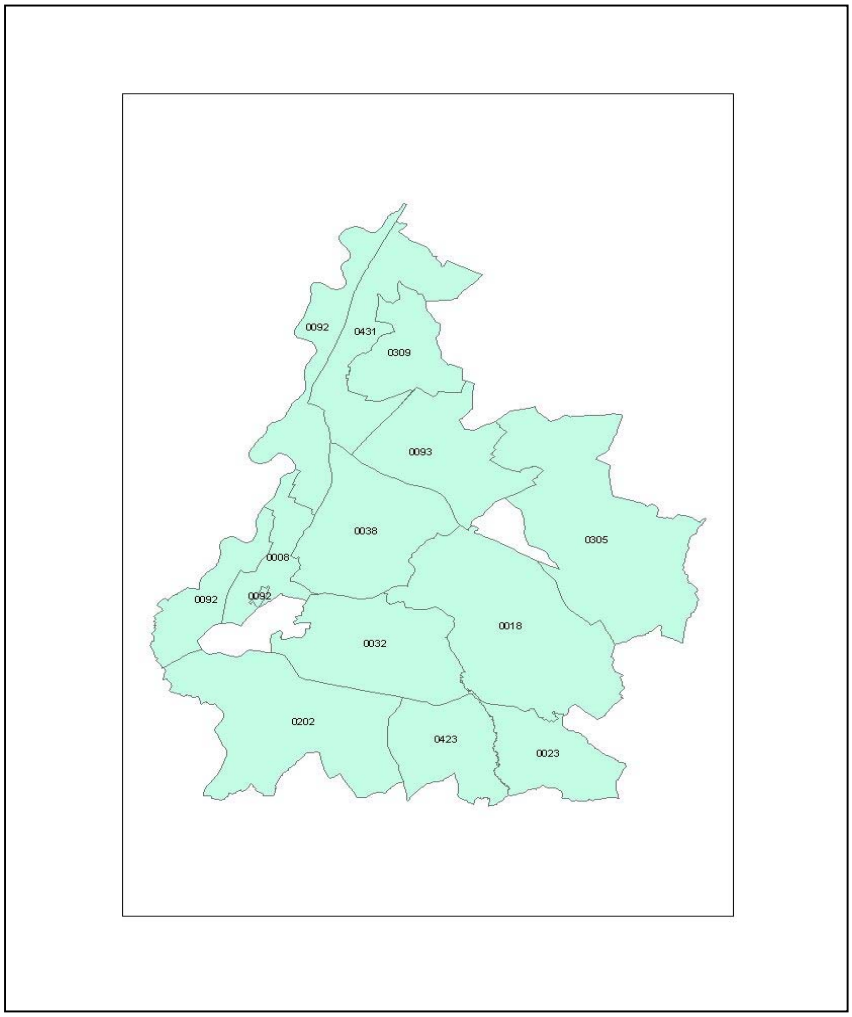
-Van der Giessen, J.W.B., Rombout, Y., Teunis, P. (2004). Spatial distribution of *Echinococcus multilocularis* in a newly recognized border in Europe. Vet. Parasitol. 119:27-35.

-Wagenknecht, E. (1979). Alterbestimmungen des erlegten Wildes, 5th Ed. Melsungen, Berlin.

Addendum

List of Game Management Units (wild beheer eenheden, WBE) in Limburg participating in this study. Between brackets the WBE number and location as listed in the map of southern Limburg.

1. Moorveld (0008)
1. Voerendaal (0018)
2. Grensland Vaals (0023)
3. Geuldal (0032)
4. Beekdal (0038)
5. Maasvallei (0092)
6. Hondskerk (0093)
7. Savelsbos (0202)
8. Brunssummerheide (0305)
9. Swentibold (0309)
10. Heuvelland (0423)
11. Susteren/ Graetheid (0431)



Source: Margriet Montizaan, KNJV