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Risk factors for acute toxoplasmosis in the Netherlands

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Summary

Toxoplasmosis caused by the protozoan parasite *Toxoplasma (T.) gondii,* occurs worldwide. Infections range from asymptomatic to life-threatening disease. *T. gondii* infection is acquired either via bradyzoites in meat or via oocysts in the environment, but the relative importance of these routes and the different sources remains unclear. This study examined possible risk factors for toxoplasmosis in the Netherlands. A case-control study was conducted including persons with a recent infection and individuals with a negative test result for IgM and IgG to *T. gondii* between July 2016 and April 2021. Forty-eight cases and 50 controls completed the questionnaire. Food history and environmental exposures were compared using logistic regression. Consumption of different meat types was found to be related to recent infection. In the multivariable model, adjusted for age, gender and pregnancy, consumption of meat of large game animals (adjusted odds ratio (aOR) 8.2, 95% confidence interval 1.6-41.9) and sometimes (aOR 4.1, 1.1-15.3) or never (aOR 15.9, 2.2-115.5) washing hands before preparing food remained. These results emphasize the value of the advice to be careful with consumption of raw and undercooked meat. Good hand hygiene also could be promoted in the prevention of *T. gondii* infection.

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Introduction

The protozoan parasite *Toxoplasma (T.) gondii* is the cause of toxoplasmosis in humans. Both the parasite and the disease are spread worldwide. About one third of the human population has been infected with the parasite, whereby the majority of the infections is asymptomatic or otherwise mild and self-limited [1, 2]. Congenital infected children and immunocompromised people are at highest risk of severe clinical manifestations. Primary infection during pregnancy can cause congenital infection which may lead to miscarriage, stillbirth or prematurity, and a wide spectrum of clinical manifestations in the newborn [2]. In Europe, where *T. gondii* genotype II is predominant, the disease is subclinical at birth in 75% of congenital infected children, but untreated these children might develop symptoms later on in life [3]. At birth a minority of the congenital infected children has manifestations, such as chorioretinitis, intracranial calcifications, or hydrocephalus [2, 3]. Severe toxoplasmosis in immunocompromised children and adults mainly manifests as central nervous system disease, myocarditis or pneumonitis [2, 4].

Cats, as definitive hosts, are essential within the life cycle of *T. gondii* as they shed oocysts in the environment. Ingestion of tissue cysts or oocysts by a cat can lead to the subsequent excretion of millions of oocysts [5]. Sporulated oocysts can survive for long periods in the environment and can end up in water, on fresh produce, or are ingested by wildlife or livestock [5, 6]. Homeothermic vertebrates, other than felines, do not shed oocysts, but in these intermediate hosts intracellular tissue cysts develop and may remain in the tissue for life. These tissue cysts are also infective for definitive and intermediate hosts, for example via ingestion of raw or undercooked meat of *T. gondii* infected animals. Transmission of *T. gondii* to humans may occur via ingestion of sporulated oocysts in soil, food or water or direct contact with faeces of infected cats excreting oocysts, or via consumption of undercooked or raw meat containing tissue cysts [4, 5]. Moreover, *T. gondii* can be transmitted vertically during pregnancy, or via blood transfusions or organ transplantations [4, 5].

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Cultural habits, such as eating and hygiene habits, influence the relative contribution of the different routes of transmission on the incidence of toxoplasmosis.

In the Netherlands, the seroprevalence of *T. gondii* in the general population was estimated within the PIENTER-study, serological surveys performed in 1996/1997 (40.5%) [7], 2006/2007 (26.0%) [8] and 2016/2017 (29.9%) [unpublished observations]. Identified risk factors differed between surveys and included increasing age, specific regions in the Netherlands, a relatively low educational level, cat ownership, gardening and raw meat consumption, especially raw/undercooked pork meat. Presence of *T. gondii* DNA in soil was examined by a study performed between August 2018 and November 2019 in the Netherlands [9]. A total of 5 of 148 soil samples (3%, 95% confidence interval (CI): 1.5-7.7%) were positive. Most of the examined samples and all five positively tested samples were from private backyard gardens, the remaining were from playgrounds. The main source of infection for humans remained unclear in the Netherlands: infection by consumption of meat containing bradyzoites or via oral ingestion of sporulated oocysts present in the environment. A meta-analysis of 187 studies worldwide revealed environmental factors, such as contact with soil or cats, as well as consumption of raw or undercooked meat, unwashed vegetables, shellfish and raw milk as significant risk factors for toxoplasmosis [10]. However, it is known that risk factors may differ between countries. Moreover, these are factors related to *T. gondii* infections without taking the moment of infection into account. Since the antibody response usually remains detectable lifelong, the infection may have been acquired long before taking the questionnaire, possibly resulting in misclassification of the exposure. Studies analysing risk factors for recently acquired *T. gondii* infections in the general population are scarce. The main risk factor in those studies was consumption of raw or undercooked meat [11-16]. Consumption of other food products identified as risk factors in those studies were unpasteurized goat's milk, shellfish, and unwashed raw vegetables or fruits. Environmental factors identified in those studies were having a pet cat or kittens, cleaning

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the cat litter box, or contact with soil [12-14, 16]. In the present case-control study, risk factors for recently acquired *T. gondii* infections in the Netherlands were investigated.

Methods

Study population

The study population consisted of persons examined by serological methods for toxoplasmosis between 15 July 2016 and 30 April 2021 at 1 of the 14 participating regional laboratories of medical microbiology throughout the Netherlands. Six laboratories were situated in the west (provinces Noord Holland, Zuid Holland, Utrecht), two in the north (Friesland, Groningen, Drenthe), two in the east (Overijssel, Gelderland) and four in the south (Noord Brabant, Limburg). A case was defined as a patient with acute toxoplasmosis if tested positive for *T. gondii* specific IgM in combination with a low to intermediate avidity (<60%) of *T. gondii* specific IgG antibodies. IgG avidity is a measure of the binding strength of IgG antibodies to an antigen [17]. Avidity increases with time, as prolonged or repeated exposure to the antigen results in production of *T. gondii* specific IgG that binds more tightly to the antigens. The controls in this study were individuals with a negative test result for both IgM and IgG to *T. gondii*.

The regional microbiology laboratories were asked to send a letter to the treating physician with the test result, an explanation of the study and an envelope with study materials for the acute toxoplasmosis case. Subsequently, the physician was requested to forward the study materials (explanation of the study for the case, an informed consent form and a questionnaire) to the case after which the case could send the completed questionnaire with informed consent to the National Institute for Public Health and the Environment (RIVM). Furthermore, the regional laboratories were asked to select three individuals that were examined within the same test period (+/- 2 months) with a negative test result, matched on age (maximum difference ten years) and gender. If the case was pregnant, the selected controls had to be pregnant as well. The controls received their study

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materials the same way as the cases did. Ethical approval to conduct this study was not needed, as decided by the Medical Research Ethics Committee (METC-protocol number 16-384/C).

Questionnaire

The questionnaire (61 questions) covered six topics: A. Personal data: birth date, gender, body length and weight, postal code, country of birth, and educational level (12 questions); B. Health and pregnancy: reasons for testing, pregnancy, medical conditions and diseases, medicine use, symptoms, and traveling abroad (eight questions); C. Food and eating habits: consumption of meat, including per animal species, specific meat products and whether or not eaten raw or undercooked, storage in the freezer, consumption of other animal products (i.e. eggs, dairy (products)) and of raw vegetables, fruit and herbs (17 questions); D. Kitchen hygiene: washing hands, use and cleaning of cutting boards and knives (five questions); E. Contact with cats and cat faeces: owning or caring for a cat, number of cats, indoor/outdoor cats, use and cleaning of litterbox (ten questions); F. Contact with animals, soil, sand and water: profession, contact with animals within profession or volunteer work, visits to agricultural business, outdoor activities, gardening, contact with soil, mud, sand or surface water (nine questions).

The reference period for the questions was the 9 months before testing, except for the questions about personal data, and reasons for testing. Although high IgG avidity is considered indicative of an infection that has occurred at least 4 months ago, antibody maturation is variable and persistent low avidity results have been reported [17]. Since it was our aim to capture all behaviour that may have resulted in the infections identified based on presence of IgM and low or intermediate avidity IgG in the current study, it was decided to use a longer reference period compared to the 4 months associated with high avidity.

Serological examination

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The laboratories were asked to send in the serum of cases to RIVM for serological examination to confirm that the case was fulfilling the criteria of acute toxoplasmosis and to exclude variation in results caused by different serological methods. IgG antibodies to *T. gondii* were determined by an in-house sandwich ELISA in a serum dilution of 1: 20 (adapted from the method described by Ruitenberg and van Knapen [18]). In short, the antigen used in this ELISA was a saponin-octylglucoside solubilized fraction prepared from in vitro cultured *T. gondii*-RH tachyzoites and the conjugate was a commercially available peroxidase-labelled antihuman IgG conjugate (Dako, Glostrup, Denmark). Patient sera were examined in in a dilution series starting at 1:20 and compared to a cut-off pooled serum standard that was prepared and lyophilised in 1978 and used ever since. For this study only the qualitative results of the ELISA were used (positive or negative in case of an optical density result higher or lower than the cut off serum standard, respectively). IgM antibodies to *T. gondii* were determined by a commercial agglutination assay (Toxo ISAGA IgM kit bioMérieux 75361) [19, 20]. The avidity was determined by a commercial ELISA (*Toxoplasma gondii* avidity determination, Euroimmun EL2410-9601-1G) [17].

Data analyses

Data were analysed with SAS software, version 9.4 M7 (SAS Institute Inc., USA). Demographic characteristics between responding and non-responding cases were checked using chi-square tests, as were demographic characteristics, reason of testing and symptoms between participating cases and controls. Exposures mentioned by at least 20% of the cases were analysed using logistic regression. First, univariable analysis was performed including calculation of odds ratios (ORs) and 95% confidence intervals (95% CI). Factors with $P < 0.15$ in the univariable analysis were included in the multivariable model, and adjusted for age (18-29, 30-39, 40-49, 50+ years), gender and pregnancy. Due to the relatively low number of participants and as consumption of meat and consumption of raw or undercooked meat partly overlap, not all identified variables could be included in one multivariable model. Calculation of correlations between the variables showed

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correlation coefficients higher than 0.60 only between meat products. Therefore, beef and pork were only included as consumption of any raw or undercooked beef and pork, respectively, instead of the different specific meat products. A final model was determined by stepwise backward elimination of variables, but always adjusted for age, gender and pregnancy. For each step, the least significant variable was removed from the model, until all variables in the model reached significance ($P < 0.05$) and the model was significant.

Results

A total of 134 case sera were sent in by twelve laboratories during the inclusion period. From two laboratories, no sera were received. High avidity was the main reason of exclusion (34/38; 90%), in the other four cases IgM positivity could not be confirmed. Sera of 96 cases fulfilled the inclusion criteria for acute disease, of which 26 cases were from the north of the Netherlands, 12 cases from the eastern part, 20 from the western part, and 38 from the southern part. A questionnaire was available for 48 cases. Men completed the questionnaire less often (33%; 13/40) than women (66%; 35/53; p=0.001), gender was unknown for three cases without questionnaire. Median age of non-respondents was significantly lower with 28 years (4-62 years) compared to 37.5 years (7-69 years) of respondents (p=0.013). Completion of the questionnaire was unrelated to year of inclusion (p=0.74). In addition to the 48 cases, questionnaires were also received from 50 controls. Ninety percent of the cases versus 72% of the controls were tested because of health complaints or symptoms (Table 1). Other reasons for testing were general medical examination/on request of a doctor, pregnancy without health complaints, and previous or work-related contact with cats. Most reported symptoms in the 9 months before testing were tiredness and swollen lymph nodes in both cases (73% and 71%, respectively) and controls (64% and 54%, respectively). The only significant differences in symptoms before testing, were stomach ache and skin rash, which were both reported more often by the controls.

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Four cases and seven controls were younger than 18 years. These low numbers did not allow separate analyses of children. However, inclusion of the children in the analyses of the adults was also not opportune due to expected differences in eating habits, such as consumption of raw or undercooked meat, and hygiene habits in especially the younger children. This left 44 cases in the age of 18-69 years and 43 controls in the age of 19-70 years, of whom 13 women (three cases and ten controls) were pregnant. Table 2 shows the risk factors identified in the univariable analysis. Beef and veal were eaten more often by cases than controls, both in general and in case of raw/undercooked beef. If split into consumption of specific beef products, beef prepared undercooked (for example steak), steak tartare and roast beef were risk factors for acquiring toxoplasmosis. Consumption of meat from lamb, duck/goose, and large game animals was also more often reported by cases. Consumption of pork meat was only a risk factor for toxoplasmosis if prepared undercooked, or as raw bacon, spreadable pork sausages or toppings of raw pork. Furthermore, consumption of raw or undercooked crustaceans or shellfish was also reported more often by cases. Finally, sometimes or never washing hands before preparing food was found to be a risk factor. Recreation in wooded areas was reported more often by controls.

After adjustment for age, gender and pregnancy, two factors remained as risk factor in the final model: consumption of meat of large game animals (adjusted OR: 8.2; 95%CI 1.6-41.9) and sometimes or never washing hands before preparing food (4.1; 1.1-15.3 and 15.9; 2.2-115.5, respectively). Recreation in wooded areas remained to be associated with a lower risk of being infected by *T. gondii* (0.1; 0.03-0.6).

Discussion

Risk factors for *T. gondii* infection are mainly studied in retrospective studies based upon IgG seropositivity. In the Netherlands, the three serosurveys done in in 1996/1997 [7], 2006/2007 [8] and 2016/2017 [unpublished observations] were used to also analyse risk factors for anti-*T. gondii*

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IgG antibodies in the general population. In such serosurveys, the moment of infection cannot be taken into account as this is unknown, and associations between exposure and infection do not necessarily indicate a causal relationship. Consumption of various meat products made up the largest group of risk factors for a recent *T. gondii* infection in the current univariable analyses, of which consumption of large game, such as roe deer and boar, remained a risk factor in the multivariable model. The other risk factor in the multivariable model was insufficient washing of hands before preparing food. Consumption of meat products has been described as source of outbreaks [6] and as risk factor in other case-control studies [11-16]. In a study in England and Wales, consumption of beef, especially if eaten raw or undercooked, had the strongest association with a recent *T. gondii* infection [11]. Consumption of raw ground beef, rare lamb, or locally produced cured, dried or smoked meat remained the meat products associated with recent acquired toxoplasmosis in the multivariable analysis of a study in the United States [12]. Meat products previously reported to be associated with recent infections in pregnant women were raw or undercooked lamb, mutton, beef, pork, and game, raw or undercooked minced meat products, and cured pork [13-16]. As cats are essential within the life cycle of *T. gondii* as they shed oocysts in the environment, it was hypothesized that having a cat or have contact with cats or its excrements are risk factors. Yet, only few studies reported cat related risk factors: having three or more kittens [12], having a pet cat [13], or cleaning the cat litter box [16]. In our study none of the variables about cats or its excrements appeared to be associated with a recent *T. gondii* infection. Possibly, since cats shed oocysts only for 2 to 3 weeks upon primary infection whereas oocysts can survive for months in the environment, other routes of exposure to oocysts could be more important than direct contact with cats. Hand hygiene was included in two studies, in which this topic was inquired as “wash hands after food preparation and/or wash hands before meals” [13] or “wash hands after handling raw meat” [12]. In both studies these factors did not reach significance. However, in our study, washing hands before food preparation was associated with toxoplasmosis, whereas washing hands after preparing or cutting raw meat or after contact with soil or sand were not. This suggest that infection

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could also occur via cross-contamination of food via sources other than food. Other risk factors identified in the previous studies, but not in our study were working with meat, drinking unpasteurized goat's milk, eating raw oysters, clams or mussels [12], consumption of raw vegetables eaten outside home [13], contact with soil, travel outside Europe and the United States [14], eating unwashed raw vegetables or fruits, and washing knives infrequently after preparation of raw meat prior to handling of another food item [16]. Milk and dairy products, water, and raw vegetables have also been reported as sources of toxoplasmosis outbreaks [6]. Recreation in wooded areas showed a lower chance of having a recent infection in our study. The potential underlying mechanism is unclear.

In the Netherlands, source attribution of *T. gondii* infections is also carried out using quantitative microbial risk assessment (QMRA). Note that sources identified as important on the population level do not necessarily have to be associated with a large odds ratio in a case-control study, as odds ratios do not take into account the prevalence of exposure in the population. Both the original and the updated QMRA for meatborne infections point towards filet americain (a raw beef spread) as the most important source in the Dutch population [21, 22]. Filet americain was not identified as a risk factor in the current study, whereas game meat was not considered in the QMRA model. Although in the QMRA model for *T. gondii* infections many knowledge gaps and uncertainties were identified, the results indicated that transmission via soil may be more important than anticipated [9]. This is in contrast to the current study, as, with the exception of consumption of raw crustaceans and shellfish, mainly factors linked to meat consumption were identified as risk factors in the univariable analyses.

An important limitation of our study is the small study population. The inclusion of both cases and controls was lower than anticipated at the start of the study. The number of cases identified at the laboratories was lower than estimated in advance based on the seroprevalence of *T. gondii*

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antibodies in the Netherlands. The numbers dropped even further during the covid-19 pandemic (12 of the 96 cases were included in 2020-2021; data not shown), indicating that not all cases are identified or included by the laboratories. Among the identified cases with acute toxoplasmosis only fifty percent actively participated by completing the questionnaire. Cases and controls were contacted via two intermediaries: RIVM send the materials to the laboratories, who subsequently asked the physicians to send the materials to the cases and controls. Therefore, it is unclear how much each part of this chain contributed to the non-response rate. Laboratories that contacted submitting physicians in an effort to increase participation occasionally got back that the physician decided not to send the questionnaire to a patient, especially in case of perceived personal suffering of the patient. The study aimed to invite three controls per case to end up with at least one control per case. Although about as much cases as controls were included in the analyses, matching was not possible as a large part of the controls who participated were invited based on acute toxoplasmosis cases who did not respond. The largest impact of the small study size was seen in the children as only four cases and seven controls under the age of 18 years could be included. This made it impossible to analyse potential risk factors for this age group. Separate analyses for pregnant women were also not possible due to the low numbers, but were included in the overall analyses with pregnancy as confounder. Nevertheless, no statements on specific risk factors for pregnant women can be given, which is unfortunate as they are a specific risk group for toxoplasmosis and may have altered their exposure to known risks for *T. gondii* infection.

The risk factors presented are based on habits and preferences in the 9 months before testing rather than specific moments of exposure. The long reference period could have introduced recall bias. However, habits and preferences are generally stable over a period of time, reinforcing the reliability of the answers, especially as it addresses the recent past and refers to the period adjacent to the infection.

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In conclusion, studies about risk factors for recent *T. gondii* infection are scarce, and most point towards consumption of raw or undercooked meat as an important factor. However, types of meat vary between studies. In our study, different meat types came out of the univariable analyses, but only consumption of game meat was found still significant in the multivariable analysis. Vulnerable persons such as pregnant women should therefore keep receiving the recommendation to eat meat only thoroughly cooked [23]. Although the evidence for the effect of washing hands is less clear, it also appears to be prudent, especially before preparing food.

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Conflict of interest

None.

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Data availability statement

The data that support the findings of this study is available from the corresponding author on reasonable request, with exception of data that could violate the privacy of research participants.

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355 Table 1. Demographic characteristics, reason of testing and symptoms, for cases of acute

356 toxoplasmosis and controls with a completed questionnaire

Variables	**Cases**	**Controls**	**p-value***
categories	**n (%)**	**n (%)**	
N	48	50	
Gender			
male	13 (27)	19 (38)	0.25
female	35 (73)	31 (62)	
pregnant	3	10	
Age (years)			
median (range)	37.5 (7-69)	31.5 (2-70)	0.09
< 18	4 (8)	7 (14)	0.36
18-29	12 (25)	13 (26)	
30-39	11 (23)	15 (30)	
40-49	9 (19)	10 (20)	
> 49	12 (25)	5 (10)	
Reason of testing			
health reasons	43 (90)	36 (72)	0.07
other reasons	4 (8)	13 (26)	
medical examination/request of doctor	2	5	
pregnancy without health complaints	1	5	
previous/work-related contact with cats	1	2	
unknown	0	1	
unknown	1 (2)	1 (2)	

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Symptoms in 9 months before testing			
fever	17 (35)	13 (26)	0.31
tiredness	35 (73)	32 (64)	0.34
swollen lymph nodes	34 (71)	27 (54)	0.09
headache	19 (40)	23 (46)	0.52
myalgia	15 (31)	12 (24)	0.42
stomach ache	4 (8)*	18 (36)	0.001
sore throat	7 (15)	11 (22)	0.34
skin rash	4 (8)**	12 (24)	0.04

357 * p-values are from chi-square tests

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358 Table 2. Odds ratios of exposures mentioned by at least 20% of the cases and $P < 0.15$ (univariable
359 logistic analyses) in the case control study on acute toxoplasmosis

Variables	**Cases**	**Controls**	**OR (95% CI)***
categories	**n (%)**	**n (%)**	
N	44	43	
Consumption of meat			
beef	43 (98)	34 (79)	**11.4 (1.4-94.3)**
veal	16 (36)	4 (9)	**5.6 (1.7-18.5)**
lamb	18 (41)	7 (16)	**3.6 (1.3-9.8)**
duck/goose (farm)	12 (27)	3 (7)	**5.0 (1.3-19.3)**
large game animals	14 (32)	3 (7)	**6.2 (1.6-23.6)**
Consumption of raw/undercooked meat			
any raw/undercooked beef	40 (91)	29 (67)	**4.8 (1.4-16.2)**
beef prepared undercooked	31 (70)	16 (37)	**4.0 (1.6-9.9)**
(steak) tartare	18 (41)	9 (21)	**2.6 (1.0-6.8)**
carpaccio	23 (52)	15 (35)	2.0 (0.9-4.8)
roast beef	19 (43)	9 (21)	**2.9 (1.1-7.4)**
smoked beef	16 (36)	9 (21)	2.2 (0.8-5.6)
any raw/undercooked pork	35 (80)	28 (65)	2.1 (0.8-5.5)
pork prepared undercooked	17 (39)	5 (12)	**4.8 (1.6-14.6)**
raw bacon	18 (41)	8 (19)	**3.0 (1.1-8.0)**
spreadable pork sausage	21 (48)	11 (26)	**2.7 (1.1-6.6)**
toppings of raw pork	9 (20)	2 (5)	**5.3 (1.1-26.0)**
dried/smoked sausage	26 (59)	18 (42)	2.0 (0.9-4.7)
Consumption of raw/undercooked crustaceans or shellfish	10 (23)	2 (5)	**6.0 (1.2-29.4)**

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Consumption of fresh fruit or vegetable juice	23 (52)	15 (35)	2.0 (0.9-4.8)
Hand washing before preparing food			
always	15 (34)	28 (65)	1.0
sometimes	19 (43)	13 (30)	**2.7 (1.1-7.0)**
never/not applicable	10 (23)	2 (5)	**9.3 (1.8-48.2)**
Contact with sand/soil			
never	7 (16)	8 (19)	1.0
monthly	19 (43)	28 (65)	0.8 (0.2-2.5)
weekly	18 (41)	7 (16)	2.9 (0.8-11.2)
Animal contact (profession, volunteer work)	13 (30)	7 (16)	2.2 (0.77-6.1)
Recreation in wooded area	28 (64)	38 (88)	**0.2 (0.1-0.7)**

360 * Odds ratios (OR) with 95% confidence interval (95% CI) shown in bold are significant ($P < 0.05$)

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