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Research Letter

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Toxoplasma gondii in vegetables from fields and farm storage facilities in the Czech Republic

Michal Slany, Radka Dziedzinska*,†, Vladimir Babak, Petr Kralik, Monika Moravkova and Iva Slana

Department of Food and Feed Safety, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic

*Corresponding author: Department of Food and Feed Safety, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic. Tel: +420-778-734-987; Fax: +420-541-211-229; E-mail: dziedzinska@vri.cz

One sentence summary: Washed vegetables from storage rooms showed higher positivity than vegetables from fields.

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†Radka Dziedzinska, http://orcid.org/0000-0001-7203-5569

ABSTRACT

Infection with *Toxoplasma gondii* has usually been connected with consumption of improperly treated meat. However, contaminated water and products of plant origin have emerged as new sources of infection in the last few years. Here, 292 vegetable samples—carrot, cucumber and lettuce—obtained from nine farms in the Czech Republic were examined using triplex `real time PCR` targeting two specific *T. gondii* sequences. Irrigation water and water used for washing of vegetables were also included. Overall, a positivity rate of 9.6% was found in vegetables. The concentration varied between 1.31×10^0 and 9.00×10^2 oocysts/g of sample. A significant difference was found between the positivity of vegetables collected directly from fields and that of vegetables collected from farm storage rooms (4.4–8.6% vs 10–24.1%, respectively). All samples of irrigation water and water used to rinse vegetables were negative. Genotyping based on `restriction fragment length polymorphism (RFLP)` analysis using seven markers revealed the exclusive presence of genotype II.

Keywords: *Toxoplasma gondii*; vegetable; farm; food safety; qPCR; genotyping

INTRODUCTION

Toxoplasma gondii is a zoonotic parasite with worldwide distribution, and a high percentage of the population is assumed to be seropositive. Primary infection of pregnant women with the parasite causes serious congenital infections of foetuses and neonates. In immunocompromised people (HIV infection, immunosuppressive therapy), *T. gondii* can cause damage to the central nervous system or may appear like pulmonary toxoplasmosis or disseminated infection. Toxoplasmosis is assumed to be asymptomatic in healthy individuals, but cases of ocular infections, mild cervical or axillary lymphadenopathy or appendicitis-like presentations have been described in immunocompetent people. Recent research has hinted at potential links between toxoplasmosis and schizophrenia, suicide, depression and rheumatoid arthritis (Torrey and Yolken 2013).

Toxoplasmosis is a food-borne infection, and humans can be infected mainly by the consumption of raw or improperly cooked meat containing tissue cysts. The number of cases of infected domestic animals, especially ruminants, pigs and birds, is still increasing probably due to the high contamination of the environment by oocysts excreted by domestic or feral cats (Hill and Dubey 2013). Excreted oocysts are highly resistant to abiotic factors and show high persistence in the environment where they retain their activity (Lindsay and Dubey 2009; Lelu *et al.* 2012). Therefore, contaminated environments—water, sand pits, garden soil, vegetables and fruits—represent a second important route of infection for humans (Torrey and Yolken 2013). The

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consumption of plant-based food has been increasingly recognised as a potential health risk with respect to *T. gondii* infection in recent years. Changing eating habits (increased preference for fresh or minimally processed food), global food trade, higher mobility of people and an increased number of immunosuppressed people can all contribute to the higher risk of protozoan, including *T. gondii* infections (Broglia and Kapel 2011). In the future, the milder and wetted winters resulting from climate change could also be expected to contribute to an increasing incidence of *T. gondii* in intermediate and final hosts in Europe (Meerburg and Kijlstra 2009).

The detection of *T. gondii* on vegetables and fruits has already been described in spinach imported to Canada (Lalonde and Gajadhar 2016), radishes, carrots and lettuces bought at Polish markets (Lass *et al.* 2012) and ready-to-eat packed salads on sale in Italy (Caradonna *et al.* 2017). However, food-borne diseases caused by *T. gondii*, and indeed all protozoa, remain neglected due to (i) the unspecific clinical symptoms and long incubation period that makes it difficult to establish the connection between the infection and the consumed food, (ii) the fact that notifying public health authorities of the disease is not mandatory and (iii) the lack of validated sensitive and specific detection methods (Hohweyer *et al.* 2016).

Examination of plant products for parasitic oocysts is mainly carried out using real-time PCR (qPCR) due to its high sensitivity and the generally low concentration of oocysts on vegetable/fruit. Application of qPCR must be preceded by release of DNA, which is commonly difficult in *T. gondii* due to the robustness of the oocyst wall. DNA can be obtained, however, either by excystation or by destroying the oocyst itself (Dumetre and Darde 2003). Microscopy can also be used; however, its sensitivity is lower compared to qPCR (Caradonna *et al.* 2017). Furthermore, it is time consuming and there is a risk of confusing *T. gondii* with other related coccidia. One limitation of PCR methods is the inability to assess the viability of oocysts. However, due to the persistence of oocysts (Hohweyer *et al.* 2016) and the strong correlation between qPCR results and the dose infecting 50% of mice (Lelu *et al.* 2012), any finding should be considered as a potential risk for consumers (Caradonna *et al.* 2017).

Within this study, vegetables grown and stored on several farms located in the Czech Republic, as well as water intended for irrigation and water used for the rinsing of vegetables, were examined for the presence of *T. gondii* contamination. The detection and quantification were carried out using a triplex qPCR assay targeting two specific sequences in the *T. gondii* genome for higher specificity. *T. gondii* isolates were genotyped using nested PCR followed by RFLP analysis using multiple markers.

MATERIALS AND METHODS

Sample collection

Samples of vegetables—carrots, cucumbers and salads—were collected during one harvesting period from nine farms within the Czech Republic ($n = 292$). Samples included carrots (carrots with or without tops), cucumbers (slicing cucumbers) and salads (butterhead lettuce, iceberg lettuce, little gem and lollo lettuce). Vegetables were obtained directly from fields ($n = 218$) or from farm storage rooms ($n = 74$). Vegetables harvested directly from fields contained vestigial amounts of soil. Before storage, carrots and cucumbers were washed using vegetable washing machines. For salads, bottom leaves were removed, and salads were either packed directly in the field or left unpacked (depending on variety). Apart from vegetables, one sample of irrigation water and one sample of water intended for the washing of vegetables were obtained from each farm. Water from nearby sources, such as rivers, lakes or wells, was used for irrigation; water from drinking water distribution system or wells was used for washing. Samples were transported to the laboratory at 4°C, and processed within 24 h after collection. Isolated DNA was stored at −80°C.

Sample processing

Trizma base–glycine beef extract (230 ml; pH 9.5, in-house) was added to 100 g of each vegetable sample. Samples were shaken (Biosan, Riga, Latvia) for 20 min to ensure proper release of oocysts from the surface of vegetable. The obtained suspension was transferred to a centrifuge tube and then centrifuged at 8000 × *g* for 20 min (4°C). The supernatant was discarded and the pellet was immediately resuspended in 10 ml of buffered peptone water (Oxoid, UK). An aliquot of 2 ml (representing 20 g of sample) was centrifuged at 5000 × *g* for 5 min to obtain the pellet. The pellet was stored at −80°C and subsequently used for DNA isolation.

Water samples (10 l) were filtered as described previously (Kaevska *et al.* 2016). Briefly, the filtration was performed using a 0.22-μm Millipore nitrocellulose filter (Merck, Darmstadt, Germany) in a stainless steel filter holder. The filter was cut into small pieces and transferred to a 50-ml tube containing 10 ml of phosphate-buffered saline supplemented with 0.05% Tween 80 and 3.5-mm glass beads (Biospec, Bartlesville, OK, USA) and vortexed for 5 min. The suspension was transferred to a new tube and centrifuged at 7000 × *g* for 5 min to obtain the pellet, which was subsequently used for DNA isolation.

Molecular detection and characterisation of *T. gondii*

DNA was isolated from the prepared pellets using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA, USA). The manufacturer's protocol was slightly modified to include mechanical homogenisation with 350 mg of 0.1-mm zirconia/silica beads (Biospec, Bartlesville, OK, USA) in a MagNA Lyser instrument (Roche, Mannheim, Germany) at 6400 rpm for 60 s. The detection and quantification of *T. gondii* was performed using a triplex qPCR assay. It included the amplification of the species-specific *B1* and *529rep* loci (Slany *et al.* 2016) and amplification of an internal amplification control to aid the identification of false negative results (Vojkovska, Kubikova and Kralik 2015). The qPCR reaction contained 1× DyNAmo Flash Probe qPCR Kit (Finnzyme, Espoo, Finland), 0.05 μl of each primer (100 pmol/μl), 0.15 μl of Cy5 (internal amplification control) and HEX (*529rep* locus) probes (20 pmol/μl) and 0.10 μl of FAM (*B1*) probe (20 pmol/μl), 0.2 U of uracil DNA glycosylase (Sigma, St Louis, MO, USA) and 5 μl of DNA in a total reaction volume of 20 μl. Amplification and fluorescence detection were performed on a LightCycler 480 Real-Time PCR instrument (Roche, Mannheim, Germany) using 96-well PCR plates under the following conditions: initial denaturation at 95°C for 7 min, followed by 47 cycles of 95°C for 5 s and 60°C for 40 s. Quantification was performed according to a plasmid gradient with a known number of copies of *B1* and *529rep* loci (10^5–10^0). Subsequent analysis was carried out using the 'Fit point analysis' option of the LightCycler 480 software (version 1.5.0.39). Each sample was tested in duplicate.

All qPCR-positive samples were subjected to genotyping analysis. *Toxoplasma gondii* genotype was determined by nested PCR followed by RFLP analysis using seven genetic markers

Table 1. The presence of *T. gondii* on vegetables from fields and farm storage facilities.

Source	Number of samples	Carrots		Cucumbers		Salads	
		Field	Storage	Field	Storage	Field	Storage
Farm 1	20	0/20					
Farm 2	75	1/20	2/6	4/30	4/19		
Farm 3	20	0/10		0/10			
Farm 4	20	0/10		0/10			
Farm 5	77	2/8	2/19			2/40	1/10
Farm 6	20			0/20			
Farm 7	20			2/10	3/10		
Farm 8	20					3/10	1/10
Farm 9	20					1/20	
Subtotal		3/68	4/25	6/80	7/29	6/70	2/20
Positivity (%)		4.4	16.0	7.5	24.1	8.6	10.0
Total	28/292	7/93		13/109		8/90	
Positivity (%)	9.6	7.5		11.9		8.9	
Genotyping[a]		NA		II (5)		II (2)	

NA: not available for genotyping. Unfilled boxes—not applicable within the farm (not grown).
[a]Genetic markers *BTUB*, c22-8, c29-2, *GRA6*, *L358*, *PK1*, and *SAG3* were analysed (RFLP-positive isolates) with reference to Su, Zhang and Dubey (2006).

(*BTUB*, *c22-8*, *c29-2*, *GRA6*, *L358*, *PK1* and *SAG3*) as described previously (Su *et al.* 2006). DNA from *T. gondii* reference strain genotypes I (RH), II (ME49) and III (NED) was included as positive controls in each nested PCR/RFLP analysis (reference strains for DNA isolation were kindly provided by Dr Daugschies, University of Leipzig).

Statistical analysis

Statistical evaluation of the data was carried out using STATISTICA 13.2 (Dell, Inc., Tulsa, OK, USA). The occurrence of *T. gondii* in vegetable samples taken from fields and storage facilities was analysed using a log-linear model with three categorical variables: (i) vegetable (carrot, cucumber, lettuce), (ii) origin (field, storage room) and (iii) *T. gondii* positivity (yes, no). *P* values <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Twenty-eight out of 292 samples of vegetables (9.6%) showed positivity for *T. gondii* using qPCR. The positive vegetables originated from five farms; products from the remaining four farms were negative. The highest positivity was found in cucumbers (11.9%); carrots and lettuces showed similar prevalence rates (7.5% for carrots and 8.9% for lettuces; Table 1). The differences in overall prevalence rates of *T. gondii* between individual groups of vegetables (carrots, cucumbers, lettuces) were not significant (log-linear model, post-hoc tests; $P > 0.05$). In vegetables collected from Poland, carrots and lettuces showed positivity rates of between 18% and 19.5%. The vegetables from that study were obtained from small greengrocers, and the high positivity was explained by the close contact of felines with small farms and the absence of washing in vegetables supplied directly from producers compared to those bought in shops (Lass *et al.* 2012). The prevalence of *T. gondii* on leafy green vegetables sold in local markets in Saudi Arabia was 6.6% (Al-Megrm 2010). Only three of 1171 samples of vegetables were *T. gondii*-positive in a Canadian retail survey (Lalonde and Gajadhar 2016). Mixed salads supplied by Italian industrial companies and local producers also showed a low prevalence rate (0.8%; Caradonna *et al.* 2017). Within this study, the occurrence of *T. gondii* ranged between 4.4% and 24.1% and the concentration of pathogen varied between 1.31×10^0 and 9.00×10^2 oocysts/g, with a median of 1.02×10^2 oocysts/g. Similarly, the concentration of *T. gondii* oocysts in Italian packed salads ranged from 6.2×10^1 to 5.54×10^2 oocysts/g (Caradonna *et al.* 2017); a lower concentration was found in Polish vegetables with not more than 2×10^1 oocysts per sample (Lass *et al.* 2012).

Cucumbers showed the highest positivity (24.1%) when sampled from storage rooms, while salads showed the highest positivity in the group of field samples (8.6%). A significant association was proven between the prevalence in vegetables sampled directly from fields (4.4–8.6%) and those collected in storage rooms (10–24.1%; Table 1). In the latter, an almost three times higher positivity was observed compared to samples from fields (OR $= 2.88$, $P < 0.05$, Fisher's exact test; Table 1). Since it was not possible to retrospectively re-examine the storage rooms, the reason for the higher positivity of vegetables from store rooms is not clear.

In all cases, stores were closed spaces, so the access of animals (cats) was limited. Before storage, vegetables were mostly washed using water from water distribution systems or local sources (well). All producers assess the microbiological quality of water, but as parasites are not controlled, their presence in well water cannot be excluded. However, the presence of parasites in water was not proven within the study, so this could not be confirmed. It is known that oocysts of *T. gondii* are able to survive in the environment as has been proven in soil (Lelu *et al.* 2012), water (Lindsay and Dubey 2009), sand (Lass *et al.* 2009), air (Lass *et al.* 2017), basil (Hohweyer *et al.* 2016) and soft fruits (Kniel *et al.* 2002). It can be hypothesised that during washing, oocysts from even one or a few pieces could contaminate water used for the washing of an entire bunch of vegetables and that this could result in the contamination of other, previously non-contaminated pieces of vegetable. Oocyst-containing vegetables kept in storages could also contaminate vegetables stored in direct proximity at later dates. The lower number of samples collected from storage rooms compared to those from fields could have also contributed to the differing prevalence rates between the two groups.

All samples of irrigation water and water after rinsing were *T. gondii*-negative. In previous studies performed in three European countries, the positivity of various water resources was below 10% (Villena *et al.* 2004; Gallas-Lindemann *et al.* 2013; Wells *et al.* 2015). For water filtering in laboratories, 10 l is often used; however, higher volumes promise a higher probability of positive results. In all of the mentioned water studies, volumes higher than 10 l were filtered, which could reflect higher parasite positivity. Seasonal variation can also play a role in water positivity. In a study performed in Scotland, water positivity for *T. gondii* increased in autumn when rainfalls and surface runoff occur frequently (Wells *et al.* 2015). In vegetables examined in Italy, assessment of seasonal variation meant that prevalence was highest in summer (Caradonna *et al.* 2017). Samples of vegetables and water included in our study were collected during the harvesting period (summer), so the number of oocysts could be maybe lower compared to the wetter period. Additionally, the number of oocysts in water is generally low. Thus, losses due to the filtering process in the laboratory could also lead to a fall in oocyst number below the detection limit.

The nested RFLP–PCR applied for genotyping of *T. gondii* resulted in lower positivity rates compared to the qPCR assay. This was caused by both the lower sensitivity of the nested PCR (Liu *et al.* 2015; Slany *et al.* 2016) and the very low concentrations of *T. gondii* in the analysed samples. Therefore, the determination of genotype could be done only for seven samples and exclusive presence of genotype II was identified (Table 1; Fig. S1, Supporting Information). In Europe and North America, genotype II is the most prevalent in patients (Ajzenberg *et al.* 2002; Nowakowska *et al.* 2006) as well as in agricultural animals (Sibley *et al.* 2009). In a few studies, *T. gondii* genotypes have been determined in environmental samples, such as soil, sand or water (Lass *et al.* 2009; 2017; Adamska 2018). Determination of *T. gondii* genotypes in vegetables was carried out only in one study, according to our knowledge, and both type I and type II were found (Lass *et al.* 2012). In most of the above-mentioned studies, typing based only on one marker (SAG2) was used, which is, however, burdened by serious limitations. Typing using multiple genes is rather recommended (Su *et al.* 2006), and we adopted this approach in our study.

In conclusion, the presence of *T. gondii* in fresh vegetables intended for direct consumption without any heat treatment was highlighted in this study. In almost 10% of 292 vegetable samples, *T. gondii* was found in concentrations from 10^0 to 10^2 oocysts/g. Significantly higher positivity was found in stored vegetables (after washing) compared to vegetables directly sampled from fields. As protozoan oocysts are resistant to low temperatures, as well as the washing and disinfection procedures used during the minimal processing of vegetables and fruits, these findings could be of concern with respect to food safety. The application of preventive measures in agricultural production should be the basis for minimizing the contamination of products and potential risk for consumers. Genotyping of vegetable samples showed the exclusive presence of *T. gondii* type II. Information on the occurrence of *T. gondii* genotypes in food, especially vegetables and fruits, is missing. Determining the parasite's genotype in vegetables can thus increase our knowledge of its epidemiology and the risk to human health.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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