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Simultaneous detection of the protozoan parasites *Toxoplasma*, *Cryptosporidium* and *Giardia* in food matrices and their persistence on basil leaves

Jeanne Hohweyer [a], Catherine Cazeaux [b], Emmanuelle Travaillé [c], Emilie Languet [b], Aurélien Dumètre [d], Dominique Aubert [a], Christine Terryn [e], Jitender P. Dubey [f], Nadine Azas [d], Maryline Houssin [c], Favennec Loïc [g], Isabelle Villena [a], Stéphanie La Carbona [b, *]

[a] *EA 3800, Protozooses transmises par l'alimentation, Laboratoire de Parasitologie Mycologie, Université de Reims Champagne Ardenne, Faculté de Médecine, SFR Cap Santé Fed 4231, 51 rue Cognacq Jay, 51096 Reims, France*
[b] *ACTALIA Food Safety Department, 310 rue Popielujko, 50000 Saint-Lô, France*
[c] *Labéo Frank Duncombe, 1 route de Rosel, Saint Contest, 14053 Caen Cedex 4, France*
[d] *Infections Parasitaires, Transmission, Physiopathologie et Thérapeutique, UMR MD3, Faculté de Pharmacie, Aix Marseille Université, 27 Boulevard Jean Moulin, CS 30064, 13385 Marseille Cedex 05, France*
[e] *Plateforme Imagerie Cellulaire et Tissulaire, Université de Reims Champagne Ardenne, 51 rue Cognacq Jay, 51095 Reims, France*
[f] *United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705 2350, USA*
[g] *EA 3800, Protozooses transmises par l'alimentation Laboratoire de Parasitologie Mycologie, Université de Rouen, 76183 Rouen Cedex, France*

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ABSTRACT

Toxoplasma gondii, *Cryptosporidium* spp. and *Giardia intestinalis* are emerging pathogen parasites in the food domain. However, without standardized methods for their detection in food matrices, parasitic foodborne outbreaks remain neglected. In this study, a new immunomagnetic separation assay (IMS Toxo) targeting the oocyst's wall of *T. gondii* was developed using a specific purified monoclonal antibody. Performance of this IMS Toxo coupled to microscopic and qPCR analyses was evaluated in terms of limit of detection (LOD) and recovery rate (RR) on: i) simple matrix (LOD = 5 oocysts; RR between 5 and 56%); ii) raspberries and basil (LOD = 33 oocysts/g; RR between 0.2 and 35%). Finally, to simultaneously extract the three protozoa from these food matrices, *T. gondii* oocysts were directly concentrated (without IMS Toxo) from the supernatant of the IMS of *Cryptosporidium* and *Giardia* (oo)cysts. This strategy associated to qPCR detection led to LOD <1 to 3 (oo)cysts/g and RR between 2 and 35%. This procedure was coupled to RT-qPCR analyses and showed that the three protozoa persisted on the leaves of basil and remained viable following storage at 4 °C for 8 days. These data strengthen the need to consider these protozoa in food safety.

1. Introduction

Toxoplasma gondii, *Cryptosporidium* spp., and *Giardia intestinalis* are protozoan parasites, which have been recently highlighted as emerging foodborne pathogens by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2014). Their emergence is favored among other, by changing in eating habits (i.e. preference for fresh or minimally processed foods), the global trade of foodstuff, changes in food production systems and the increased number of sensitive people (Broglia and Kapel, 2011). Contamination by pathogens can occur at any time of the food processing from primary production to consumers through food processing (Newell et al., 2010). In a context of food safety growing concerns, this implies that the food industry has to constantly monitor and control its production chain from the field to the fork.

* Corresponding author.
E-mail addresses: jeanne.hohweyer@gmail.com (J. Hohweyer), s.lacarbona@actalia.eu (S. La Carbona).

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Protozoa are excreted in high quantities in the environment by infected hosts under an environmentally resistant form, i.e. the oocyst for *T. gondii* and *Cryptosporidium* spp., and the cyst for *G. intestinalis*, that can contaminate waters and soils (Palos Ladeiro et al., 2013;'Smith et al., 2014). Consequently, these three parasites are associated with many waterborne outbreaks with more than 30 000 human cases over the world these last 15 years and *Cryptosporidium* was involved in 60% of them between 2004 and 2010 (Baldursson and Karanis, 2011; ECDC, 2014). Fecally-contaminated waters can also represent a risk of contamination for vegetables and mollusks during primary production (Armon et al., 2002; Chaidez et al., 2005; Li et al., 2006; Ma et al., 2014; Robertson, 2007). Consistent with this, some studies described the presence of *Cryptosporidium* oocysts and *Giardia* cysts in vegetables (Bohaychuk et al., 2009; Ferrer et al., 2012; Rzezutka et al., 2010; Shahnazi and Jafari-Sabet, 2010) and red berries (Calvo et al., 2004; Robertson and Gjerde, 2001). During the last five years, occurrence between 3.3 and 77.8%, and 4.3 and 75% were described respectively for *Cryptosporidium* and *Giardia* (oo)cysts on different leafy green vegetables (Amorós et al., 2010; Colli et al., 2015; Duedu et al., 2014; Eraky et al., 2014; Hong et al., 2014; Ranjbar-Bahadori et al., 2013). At the moment, only one study showed the presence of *T. gondii* oocysts in vegetables (Lass et al., 2012). Conventional decontamination treatments applied in minimally processed vegetables (MPV) industries are known to be inefficient on protozoan (oo)cysts (Erickson and Ortega, 2006; Wainwright et al., 2007). Indeed, *Cryptosporidium*and *Giardia* (oo)cysts were recently identified respectively in 5.9% and 1.8% of ready-to-eat leafy greens samples in Canada (Dixon et al., 2013).

As these protozoa are infective at low dose (ID50 = 1 to 10 oocysts of *T. gondii* in mouse), ingestion of raw or MPV, which are likely contaminated by few viable (oo)cysts can lead to human infections. Indeed, epidemiological analyses showed that consumption of raw or unwashed vegetables was a significant risk factor for toxoplasmosis (Baril et al., 1999; Kapperud et al., 1996). Moreover, links between protozoa and food of non animal origin were identified in Europe (EFSA and ECDC, 2014; Lopes et al., 2012) and a large outbreak linked to consumption of frisée salad in Finland was recently published (Åberg et al., 2015). In USA, the cost of these parasitic diseases was estimated at $35 (*Giardia*), $45 (*Cryptosporidium*) and $398 (*Toxoplasma*) million per year (Collier et al., 2012; Karanis et al., 2007). Despite of this, foodborne diseases due to these protozoa remain neglected for three main reasons: i) notifications to public health authorities are not mandatory and official reports do not reflect the real occurrence and incidence of diseases; ii) incubation periods are quite long and association between food and disease is not always possible; iii) although some detection methods exist, they are not easy to implement, they display low sensitivity and require specific skills restricted to specialized laboratories.

A method to detect (oo)cysts of *Cryptosporidium* spp. and *G. intestinalis* by microscopy in lettuce was proposed by Cook and collaborators (Cook et al., 2006a, 2006b, 2007). Detection by PCR was also described, displaying higher specificity and sensitivity, and allowing to get faster results for routine implementation (Frazar and Orlandi, 2007; Ramirez-Martinez et al., 2015). However, depending on the food matrix, PCR analyses could suffer from the presence of PCR inhibitors leading to a decreased sensitivity or false-negative results (Schrader et al., 2012). For *T. gondii*, recovery of oocysts from fruits and vegetables by flocculation using $CaCO_3$ solution and detection by real time PCR were described (Lass et al., 2012). Methods involving detection by microscopy and PCR in the supernatant of washing solutions were also recently proposed independently for *Cryptosporidium parvum* and *T. gondii* (Chandra et al., 2014). To date, no method allowing the simultaneous analysis of the three protozoa in a same food sample is described. One of the most critical steps when processing food or environmental samples is the separation of the parasites from background organic and inorganic particles that interferes with detection or characterization techniques such as microscopy, PCR, and bioassay (Dumètre and Dardé, 2003). Immunomagnetic separation (IMS) methods are commercially available for *Cryptosporidium* spp. and *G. intestinalis* (oo)cysts, which allow to overcome this drawback by specifically concentrating and purifying them from a complex sample. IMS methods have been developed in order to purify *T. gondii* oocysts by using oocyst specific monoclonal antibodies (mAb 3G4 and 4B6) produced as hybridoma cell culture supernatants (Dumètre and Dardé, 2005, 2007). However, these methods suffered from several limitations depending on the used antibody and antibody bead coupling approaches.

Here, we report the development of a new direct IMS assay targeting the *T. gondii* oocyst (IMS Toxo). Performances of this procedure coupled to detection by microscopy or real time PCR (qPCR) were evaluated on simple (liquid) and vegetable (basil and raspberries) matrices. A strategy to simultaneously analyze food samples for the presence of *T. gondii*, *Cryptosporidium* spp. and *G. intestinalis* was proposed leading to recovery rates from 5 to 26% and to limit of detection below 1 (oo)cyst as determined by qPCR. Finally, using reverse-transcription PCR assays, we showed that the three protozoa remain viable at the surface of leaves following storage at 4 °C for 8 days.

2. Materials and methods

2.1. Parasites

Purified *C. parvum* oocysts and *G. intestinalis* cysts suspensions were purchased from WaterborneTM, Inc. (New Orleans, LA, USA), kept at 4 °C until use and used within a month to guaranty their viability. *T. gondii* oocysts were obtained from the feces of cats experimentally infected by strain ME49 genotype II and sporulated as described previously (Dubey J.P., 2010). Both unsporulated and sporulated oocysts were enumerated microscopically on a Nageotte hemacytometer under bright field and epifluorescence, using a Nikon Eclipse E400 microscope equipped with suitable epifluorescence filters ($\lambda_{excitation}$: 330–380 nm; $\lambda_{emission}$: 420 nm) for detecting the oocyst blue autofluorescence under UV excitation (Dumètre and Dardé, 2004). Oocysts were stored at 4 °C in an aqueous solution containing 2% H_2SO_4 until use. Prior to experiments, *T. gondii* oocyst suspensions were washed three times in sterile distilled water (dH_2O) at 2500 g for 10 min to remove sulfuric acid and adjusted to the desired concentrations in sterile dH_2O. These oocyst suspensions were stored at 4 °C and used for experiments within a week.

2.2. Production, purification of the monoclonal antibody (mAb) 3G4 and immunofluorescence assays

The mAb 3G4 (IgM kappa) was previously shown to react with the *T. gondii* oocyst wall (Dumètre and Dardé, 2005). Hybridoma cells secreting the antibody (Dumètre and Dardé, 2005) were sent to P.A.R.I.S. Biotech (Compiègne, France) for antibody production and purification. The antibody reactivity against *T. gondii* oocysts was screened by an indirect immunofluorescence assay on air dried parasites by using a FITC conjugated secondary antibody as described previously (Dumètre and Dardé, 2005). To further refine the localization of the antibody on the oocyst surface, mAb 3G4-labeled oocysts were examined by laser scanning confocal microscopy (LSCM). The reactivity of the mAb 3G4 to the external surface and the internal content of *T. gondii* oocysts was investigated before

and after sonication by using an indirect immunofluorescence assay and confocal microscopy.

Slides were observed with a Zeiss 710 NLO confocal microscope equipped with a titanium sapphire laser (Chameleon, Coherent Laser Group, Santa Clara, CA, USA) and an ×63 1.4 numerical aperture (NA) oil immersion lens. LSCM images were recorded for FITC (490–565 nm) and oocyst autofluorescence (370–450 nm) emission by using laser excitation at 800 nm and 740 nm respectively. Images were acquired as Z stacks with a 0.25 μm Z step size and an ×3 numerical zoom.

2.3. IMS Toxo procedure on simple matrix

Superparamagnetic beads (Activ MasterBeads® #02650) were obtained from Ademtech (Pessac, France). Neodymium iron boron permanent magnets (MPC-S/L) were purchased from Dynal Biotech (Compiègne, France). Ten microliters of magnetic beads (i.e. ~20 mg of magnetic beads) were washed twice in 1 mL activation buffer (Adem buffer® #10101, Ademtech) according to the manufacturer's recommendations, and incubated with 5 μg of purified mAb 3G4 for 24 h at 37 °C in a rotating shaker at 20 rpm. Then, the beads were washed twice with storage buffer (Adem buffer® #10201, Ademtech) and suspended in 100 μL of storage buffer. Antibody coated magnetic beads were stored at 4 °C for 24 h maximum. IMS Toxo was validated on simple matrix as followed: 100 μL of the desired quantity of oocysts were spiked in 8 mL of IMS Toxo buffer (PBS pH 7.4 BSA 1%) in glass Leighton tubes (8 mL) supplemented by 100 μL of 3G4 coated beads. Leighton tubes were then incubated for 30 min at 37 °C in a rotating shaker at 40 rpm and, in order to retain oocyst bead complexes, tubes were placed in a magnetic particle concentrator (MPC magnet) for 15 min motionless, 5 min with gently rock (tilt of the tube at 90°) and 10 min more motionless. Supernatant was removed and oocyst bead complexes were further concentrated in 1 mL of IMS Toxo buffer and transferred into a 1.5 mL microtube. After another static magnetization for 5 min, the supernatant was discarded and 100 μL of 0.1 N HCl were added to dissociate the oocyst bead complexes. Tube was mixed thoroughly for 3 min by vortexing at full speed, left in vertical position for 10 min at room temperature (RT, 20–22 °C) and then mixed again for 3 min. Following a final magnetization step of 2 min, the supernatant was recovered and neutralized by adding 10 μL of NaOH 1 M. The final supernatant was used for oocyst quantification by fluorescence microscopy and/or qPCR.

2.4. Recovery of T. Gondii, C. Parvum and G. intestinalis (oo)cysts from experimentally contaminated fruit and vegetable samples

Basil and raspberries from various origins were purchased in local supermarkets and processed within 24 h maximum. For basil, spoiled leaves and stems were eliminated, and remaining leaves were mixed. The size of food sample was harmonized at 30 g in sterile filtered stomacher bags (Interscience, France) in order to standardize the procedure. Different doses of each parasite (from 5 to 10000 depending on the experiments) were extemporaneously mixed together in a final volume of 100 μL sterile dH_2O. To mimic how (oo)cysts adhere to vegetable's surfaces in field setting, each sample was artificially contaminated by spotting 10 μL volumes on vegetable's surfaces at different areas. Based on previous studies (Cook et al., 2006a, 2006b), following incubation at room temperature for 2 h, the food matrix was suspended in 200 mL of glycine buffer (1 M, pH 5.5) and carefully agitated 1 min on a shaking table (80 rpm). The filtrated sample was recovered and the stomacher bag was rinsed with additional 25 mL of glycine buffer. Then, the filtrate was centrifuged at 2500 g for 30 min (at 15 °C for basil and 4 °C for raspberries) and the supernatant was discarded. When conducting IMS Toxo assays, the pellet was resuspended in 8 mL of IMS Toxo buffer and processed for IMS Toxo as described above (see Section 2.3). Assays without IMS Toxo were performed as followed: food matrix pellet was resuspended in 10 mL of sterile dH_2O and transferred into a Leighton tube to proceed to IMS targeting the *Cryptosporidium* and *Giardia* (oo)cysts (IMS-GC) using Dynabeads GC Combo IMS kit (Invitrogen Dynal AS, Norway). After the first magnetic particle concentrator step of the IMS-GC, the supernatant was recovered to concentrate *T. gondii* oocysts by centrifugation (10 min at 3000 g) and beads were further processed for IMS-GC according to manufacturer's instructions. *T. gondii* pellet was finally resuspended in sterile dH_2O in a maximum of 300 μL (depending on the pellet's size) for PCR assays.

2.5. T. Gondii oocyst recovery assessed by fluorescence microscopy

Oocysts recovered by IMS Toxo were loaded onto Nageotte hemacytometers and examined microscopically at ×40 under bright field and epifluorescence for oocyst autofluorescence as described above. Both unsporulated and sporulated oocysts were quantified based on their morphology and summed to determine the total number of oocysts. The oocyst recovery rate was calculated as followed: (N_0/N) x 100 with N and N_0, the total number of oocysts recovered after IMS and initially spiked respectively. The limit of detection was defined as the last dose for which 95% of the positive samples are detected (at least one sporulated or unsporulated oocysts).

2.6. (Oo)cysts recovery assessed by qPCR

Before DNA extraction, recovered (oo)cysts were submitted to six heat shock cycles consisting of freezing at −80 °C for 5 min and thawing at 95 °C for 5 min followed by 1 min of ultrasonic treatment (37 Hz). DNA was then extracted and purified by adding one volume of InstaGene Matrix from the InstaGene Matrix kit and following the manufacturer's instructions (Bio Rad, Marnes la Coquette, France). The final supernatant was carefully transferred to a new tube and stored at −20 °C until detection by qPCR. Specific DNA amplifications of *T. gondii*, *C. parvum* and *G. intestinalis* were performed as previously described (Palos Ladeiro et al., 2014) on the Bio rad CFX96 Touch instrument. Inhibition was systematically evaluated by serial dilutions of DNA and each PCR reaction was performed in duplicates, and Cq (quantification cycle) values were averaged. The Cq value corresponds to the cycle number at which the fluorescence excesses a fixed threshold and allows the quantification of the amount of the target DNA. However, the number of specific targets per parasite is variable depending on the parasite and the ploidy of the (oo)cyst's stage (4 for *C. parvum* oocyst, 64 to 520 for *Giardia duodenalis* cyst and 800 to 2400 for *T. gondii* oocyst). To overcome these variations, DNA extracted from known quantity of (oo)cysts were serially ten fold diluted (5 $\log_{10}$ concentrations) and used to establish calibration curves where the $\log_{10}$ number of (oo)cyst per reaction was plotted against Cq values obtained from each dilution. Quantification results were then expressed as (oo) cyst equivalents to take into account this variability. Recovery rates N/N_0 were further determined, with N the (oo)cyst equivalents recovered at the end of extraction/purification and N_0, the (oo)cyst equivalents initially inoculated to the matrix. Student's *t* test was used for statistical comparison and was considered as 'significant' when P values < 0.05. The limit of detection was defined as the lowest concentration at which 95% of the positive samples were detected (Bustin et al., 2009).

2.7. RT-qPCR assays

Food matrix pellets (Section 2.4) were processed as previously described (Travaillé et al., 2016). Briefly, after mRNA expression induction at 45 °C for 20 min, pellets were resuspended in 200 μL of lysis binding buffer, and (oo)cysts were disrupted by 6 freezing/thawing cycles. mRNA were then extracted using the Dynabeads® mRNA DIRECT™ Kit and RT-qPCR was performed using the OneStep RT-PCR Kit (Qiagen, Courtaboeuf, France) on 2 μL of mRNA, using primers and probes previously described and targeting *SporoSAG (*sporozoite specific SAG protein) for *T. gondii*, *hsp70* (heat shock protein 70) for *C. parvum* (after heat shock induction) and *beta giardin* for *G. intestinalis*. As described in Section 2.6, calibration curves were established using known quantities of viable (oo)cysts and allowed to determine the viable (oo)cyst equivalents in each sample.

2.8. Persistence and viability assays

Viability of (oo)cysts suspensions was determined by RT-qPCR before each experiment. For each experiment, six samples of 30 g of basil were prepared and inoculated as previously described (Section 2.4) with 340 oocysts/g of *T. gondii* and 4350 (oo)cysts/g of *C. parvum* and *G. intestinalis.* qPCR and RT-qPCR analyses were performed the day of inoculation (Day 0) and after two times of storage at 4 °C at Day 4 and Day 8. At each time, two samples were treated leading to two food matrix pellets: one pellet was processed to extract DNA from the three protozoa and analyzed by qPCR to determine the total (oo)cyst equivalents persisting on the matrix (see Sections 2.4 and 2.6); the second pellet was processed to recover mRNAs to determine the viable (oo)cyst equivalents on the matrix by RT-qPCR (Section 2.7). Four independent assays were performed.

3. Results

3.1. MAb 3G4 mainly binds to the external surface of the T. Gondii oocyst

The antibody bounded to the external surface of the wall of both non sonicated, and apparently intact, unsporulated and sporulated oocysts (Fig. 1 A, B, and C), but not to the sporocysts (Fig. 1 D, E, and F). We further investigated if mAb 3G4 was able to recognize the sporocyst in the case of infiltration through an injured oocyst wall. For this, oocysts were mechanically damaged by sonication prior to be in contact with the antibody. In this case, the antibody bounded again to the entire external surface of the oocysts but not to the sporocysts, even after inducing large openings in the oocyst wall (Fig. 1 G, H, and I).

3.2. Performances of the new IMS Toxo assay in simple matrix

The purified mAb 3G4 was tested in an IMS procedure. Oocysts mean recovery rates at different spiking doses (between ~5 and 220 parasites in 100 μL) ranged from 32 to 56% by fluorescence microscopy and from 5 to 20% by qPCR (Table 1). Oocysts were detected in all samples at each tested dose and no statistical difference was observed in overall recovery rates between the five doses (p *values* > 0.05). Limit of detection (LOD) of the technique was set at 5 oocysts by microscopy and qPCR with 100% of positive samples detected (Table 1). When using different batches of 3G4 coated magnetic beads prepared the same day or different days, recovery rates were similar suggesting that coupling was repeatable and reproducible (Table S1, supplementary data).

3.3. Evaluation of IMS Toxo assay for detection of T. Gondii in food matrices

Considering the promising results obtained on simple matrix, the potential of the developed IMS Toxo was evaluated to extract *T. gondii* oocysts from food matrices. Two different matrices, including fresh green leafy vegetable (basil) and berry fruit (raspberries), were inoculated at 33 oocysts/g to determine recovery rates by microscopy and qPCR. By qPCR assays, mean rates of 35% and 29% were reached on basil and raspberries respectively (Table 2). Large variations were observed among the experiments especially for raspberries (1–58%), which might be due to interferences of the beads with the matrix. By microscopic analyses, rates dropped to less than 2%. Hence, contrarily to simple matrix, IMS Toxo coupled to qPCR assays produced higher recovery rates than when associated with microscopy. LOD was set at 33 oocysts/g by qPCR and microscopy (Table 2).

3.4. Procedure for simultaneous analysis of T. Gondii, C. Parvum and G. intestinalis (oo)cysts in vegetables

The variation of recovery rates observed on raspberries and the relatively high LOD (33 oocysts/g) obtained with IMS Toxo could be limiting factors when working on naturally contaminated matrices. To circumvent this inconvenience, extraction of *T. gondii* was performed without IMS Toxo purification step by directly concentrating oocysts from the supernatant of the IMS of *Cryptosporidium* and *Giardia* (IMS-GC) (see Material and Methods). Food residues prevented microscopic analyses for *T. gondii* oocysts and consequently recovery rates were determined only by qPCR assays for each parasite (Table 3). When applied to basil, the procedure produced recovery rates of 35, 11 and 2% for *T. gondii*, *C. parvum* and *G. intestinalis* respectively and led to LOD of 3 (oo)cysts/g for both *Cryptosporidium* and *Giardia*, and below 1 oocyst/g for *Toxoplasma*. On raspberries, LOD <1 (oo)cyst/g was reached for all parasites and mean rates of 2.5, 14 and 21% were obtained for *T. gondii, C. parvum* and *Giardia* (oo)cysts respectively (Table 3). For *T. gondii* and *G. intestinalis*, recovery rates were statistically different (p *values* < 0.05) depending on the food matrix.

3.5. Application of the procedure to evaluate persistence and viability of protozoa at the surface of vegetables

The developed procedure was then implemented to estimate the persistence of the three protozoa on the surface of basil's leaves during storage at 4 °C. Basil was inoculated with 340 oocyst/g of *T. gondii* and 4350 (oo)cysts/g of *C. parvum* and *G. intestinalis*. The evolution of total (oo)cyst equivalents/g of basil was followed during 8 days and qPCR analyses showed that the three parasites are still detected at the end of storage (Fig. 2A), roughly at the same concentrations. RT-qPCR assays showed that the viability of protozoa was not affected by storage at 4 °C for 8 days (Fig. 2B). Moreover, we found that contrary to *Giardia* cysts and *C. parvum* oocysts calibrated suspensions, only 1% of *T. gondii* oocysts were viable in the used suspension, as determined by RT-qPCR assays. Consistent with this, each newly produced *T. gondii* oocyst suspension should be characterized.

4. Discussion

In the last 15 years, immunomagnetic separation (IMS) methods have greatly contributed to improve the specific detection of microbial pathogens in food and water samples, especially of the protozoan parasites *Cryptosporidium* spp. and *G. intestinalis* (Safarik et al., 2012). However, detecting *T. gondii* oocysts in the same types

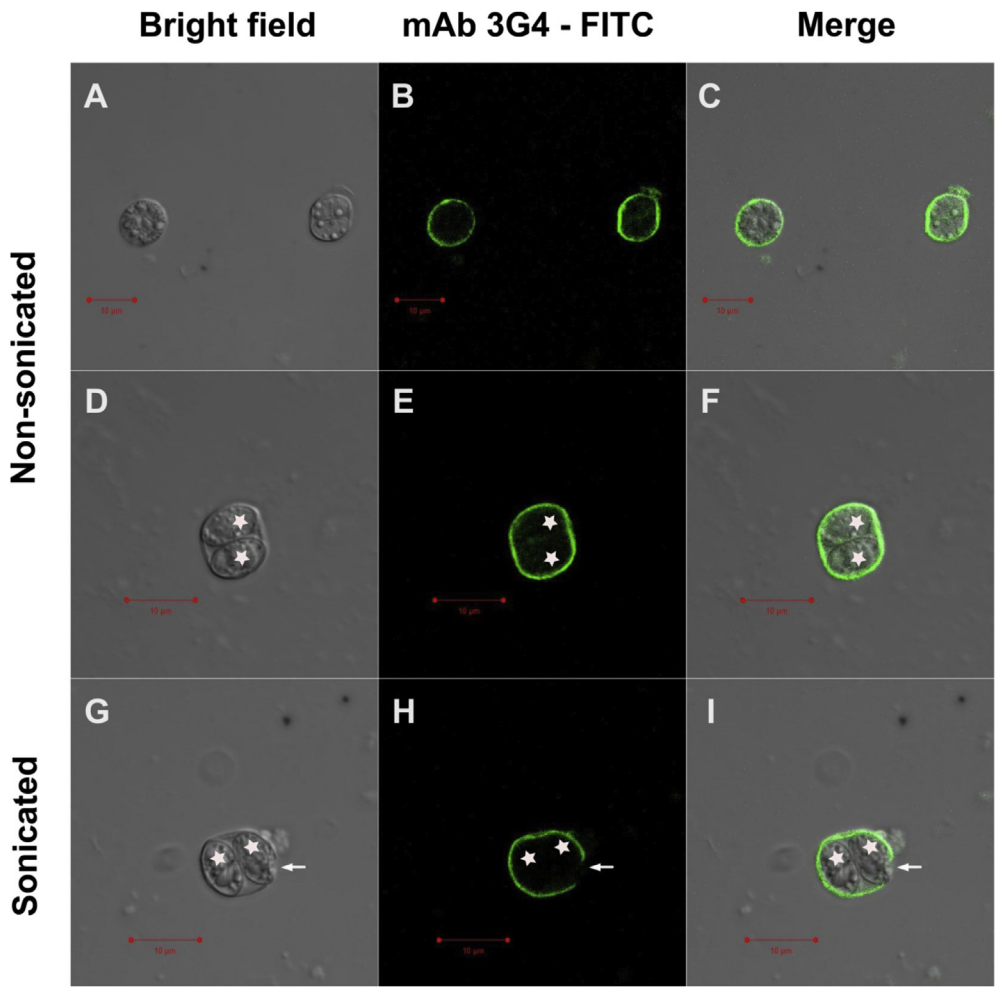

Fig. 1. Fluorescence pattern of the wall of *T. gondii* oocysts following incubation with the mAb 3G4. Non sonicated unsporulated (left) and sporulated (right) oocysts (A, B, C). Zoom on a sporulated oocyst exhibiting distinctly its two sporocysts (stars) (D, E, F). One sporulated oocyst following sonication showing an opening in its oocyst wall (arrow) and intact sporocysts (stars) (G, H, I). Scale bars = 10 μm.

Table 1
Performances of the IMS Toxo in simple matrix determined by fluorescence microscopy and qPCR.

Fluorescence microscopy			qPCR		
No. of spiked oocysts	No. of recovered oocysts	Recovery rate[b] [min −max]	No. of spiked oocysts (Cq values)	No. of recovered oocysts (Cq values)	Recovery rate[a,b] [min −max]
211.3 ± 16.3	92.3 ± 13.4	44% [40–51%][c]	24.2 ± 0.11	29.57 ± 0.29	5% [4–7%][c]
179.3 ± 1.2	100.0 ± 35.0	56% [35–73%][c]	24.9 ± 0.01	29.37 ± 0.4	10% [7–14%][c]
49.3 ± 8.7	25.0 ± 6.1	51% [37–59%][c]	26.4 ± 0.26	30.4 ± 0.45	13% [8–18%][c]
14.7 ± 4.7	4.7 ± 0.6	32% [27–34%][c]	28.5 ± 0.48	34.3 ± 1.98	7% [1–17%][c]
5.0 ± 1.7	2 ± 1	40% [20–60%][c]	29.2 ± 0.52	32.73 ± 0.85	20% [8–31%][c]

[a] The numbers of spiked and recovered oocysts were quantified by qPCR and Cq values were expressed as eq. numbers of (oo)cysts using standards to determine recovery rates.
[b] Mean recovery rates of 3 independent experiments are indicated. [Min-max]: minimal and maximal recovery rates.
[c] 100% of positive samples are detected.

of matrices still remains very challenging, mainly due to the lack of specific separation methods such as IMS (Dumètre and Dardé, 2003; Jones and Dubey, 2010). We previously described an indirect IMS assay targeting *T. gondii* oocysts using unpurified 3G4 antibody (Dumètre and Dardé, 2005) and a direct IMS assay targeting the sporocysts that required a sonication step (Dumètre and Dardé, 2007). However these methods suffered from a lower sensitivity and efficiency in turbid water samples. The new IMS method described in this study targeted the whole oocysts of *T. gondii* and included antibody purification, covalent coupling of

Table 2
Performances of the IMS Toxo applied to basil and raspberries experimentally contaminated with *T. gondii* oocysts.

Food matrices	Fluorescence microscopy		qPCR	
	Recovery rate[a] [min–max]	Limit of detection[b,c]	Recovery rate[a] [min–max]	Limit of detection[b,d]
Basil	0.2% (n = 3) [0.1–0.3%]	33 oocysts/g	35% (n = 5) [16–48%]	33 oocyst/g
Raspberries	2.0% (n = 3) [0.3–3.0%]	33 oocysts/g	29% (n = 7) [1–58%]	33 oocyst/g

[a] Food matrices were artificially contaminated with a dose of 33 oocysts/g and (oo)cysts were quantified by fluorescence microscopy and qPCR. Mean recovery rates of n independent experiments are indicated. [Min-max]: minimal and maximal recovery rates.
[b] Food matrices were artificially contaminated with doses ranging from 33 to 0.3 oocysts/g. The limit of detection corresponds to the lowest concentration at which ≥95% of the positive samples were detected.
[c] One sample is positive if one oocyst is detected (n = 3).
[d] One PCR replicate is positive if Cq < 40 (n = 6).

Table 3
Performances of the procedure for simultaneous extraction of *T. gondii*, *C. parvum* and *G. intestinalis* (oo)cysts from experimentally contaminated basil and raspberries.

Food matrices	Parasites	qPCR	
		Recovery rate[a] [min–max]	Limit of detection[b]
Basil	*T. gondii*	35% [23–61%]	<1 oocyst/g
	C. parvum	11% [6–23%]	3 oocysts/g
	G. intestinalis	2% [1–3%]	3 cysts/g
Raspberries	*T. gondii*	2.5% [0.2–7%]	<1 oocyst/g
	C. parvum	14% [1–45%]	<1 oocyst/g
	G. intestinalis	21% [6–51%]	<1 cyst/g

[a] Food matrices were artificially contaminated with a mean dose of 408 (oo)cysts/g and (oo)cysts were quantified by qPCR. Mean recovery rates of two to three independent experiments are indicated. [Min-max]: minimal and maximal recovery rates.
[b] Food matrices were artificially contaminated with doses ranging from 333 to 0.3 oocysts/g. The limit of detection corresponds to the lowest concentration at which 95% of the positive samples were detected (n = 6).

2010), the oocyst working suspension used in this study contained a high proportion of sporulated stage (88%; Table S1). This could contribute to the lower recovery rates obtained by qPCR since sporulated oocysts require the breakup of the oocyst and sporocyst walls, and the sporozoite membrane to access to the genetic content. Considering that the composition of each layer of the oocyst and sporocyst walls is really different (Bushkin et al., 2013; Dumètre et al., 2013; Samuelson et al., 2013), lyses treatments should be adapted to efficiently access to DNA (Palos-Ladeiro and Cazeaux, personal communication). Consequently, PCR experiments may lead to different results depending on the relative proportions of each maturing oocyst stage. However, as mAb 3G4 also reacts with the oocysts of closely related *Toxoplasma* coccidia (*Hammondia hammondi*, *Neospora caninum*), PCR is required to specifically detect IMS-purified *T. gondii* oocysts from naturally contaminated samples. Moreover, *Toxoplasma* DNA may give valuable information on the genotype of the detected parasites, in

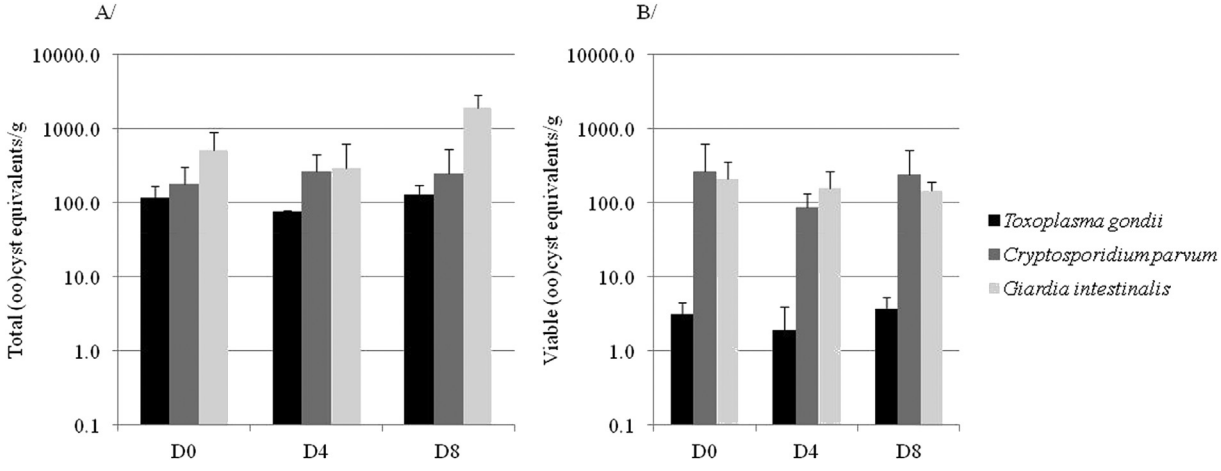

Fig. 2. Persistence of *T. gondii* and *C. parvum* oocysts, and *G. intestinalis* cysts at the surface of basil leaves during storage at 4 °C. A/Total (oo)cysts (i.e. viable and dead). B/Viable (oo)cysts. Total (oo)cysts and viable (oo)cysts equivalents per gram of matrix were quantified by qPCR and RT-qPCR respectively. Means ± standard deviations of four independent experiments are represented in $\log_{10}$ scale. D0: day 0; D4 and D8: days 4 and 8 of storage at 4 °C.

the antibody onto chemical activated beads instead of anti-IgM precoated beads, and direct IMS capture of the whole oocysts. This IMS assay led to good performances in a simple matrix with recovery rates ranging from 32 to 56% as assessed by microscopy and from 5 to 20% by qPCR (Table 1). Both methods detected a dose of 5 oocysts per sample, which is promising to detect *T. gondii* oocysts in naturally contaminated environmental samples (Dumètre and Dardé, 2003). As it was already described (Villegas et al., particular in an epidemic context of waterborne/foodborne toxoplasmosis. Compared to other oocyst extraction techniques (e.g. flotation, flocculation) (Kourenti et al., 2003; Villena et al., 2004), this IMS-PCR is a more rapid and specific technique allowing the analysis of multiple samples within a day or less, and could address many important issues about the prevalence and survival of the *Toxoplasma* parasites throughout the environment.

The potential of IMS assay to extract *T. gondii* from food matrices

was also tested. Basil and raspberries were chosen because of their implication in foodborne outbreaks (Åberg et al., 2015; Gherasim et al., 2012; Lopez et al., 2001; Shahnazi and Jafari-Sabet, 2010). IMS Toxo coupled to microscopic assay was not efficient for food sample analyses (oocyst recovery rates $\leq$ 2%). However, when coupled to qPCR analyses, mean recovery rates of the IMS Toxo were 35 and 29% on basil and raspberries respectively. To our knowledge, no comparative data are available on food matrices but these rates were higher than those determined on soil samples using flotation technique (Lélu et al., 2011). IMS Toxo led to LOD of 33 oocysts/g (~103 oocysts/sample) by qPCR analyses on the two tested matrices. Comparable LODs were already described on radish (102 oocysts/sample) and strawberry samples (104 oocysts/sample) using flocculation method and qPCR targeting the B1 gene for *T. gondii* (Lass et al., 2012).

To simultaneously extract *T. gondii*, *C. parvum* and *G. intestinalis*, we finally tested a global strategy including: i) an IMS separation step for *Cryptosporidium* and *Giardia* (oo)cysts as already described (Cook et al., 2006a, 2006b, 2007) combined to a direct analysis for *T. gondii* oocysts; ii) detection of each parasite by qPCR. When applied to the two matrices, this last procedure showed better performances for *T. gondii* than IMS Toxo regarding the LOD (<1 oocyst/g). However, ten times lower recovery rate was obtained on raspberries (2.5 versus 29% with IMS Toxo; p *value* < 0.05) while mean recovery rates with or without IMS Toxo were comparable on basil (35%). It was suggested that *T. gondii* oocysts are entrapped by the surface of raspberries consisting in tiny hairlike projections that interact with oocysts (Kniel et al., 2002). Hence, IMS Toxo could be more suitable for processing this type of food by helping to extract the oocysts from the tangle of the matrix, which could not be efficiently done by direct analysis. Considering *C. parvum* and *G. duodenalis*, our method using qPCR detection produced recovery rates of 2–21% on raspberries and basil (Table 3), which are comparable to recovery rate obtained using a PCR procedure for the detection of *G. intestinalis* cysts in lettuce (Ramirez-Martinez et al., 2015), but are lower than those obtained using microscopy by Cook and collaborators (Cook et al., 2006b, 2007). The use of a distinct IMS kit (Dynabeads versus Crypto Scan®), which are known to not perform equally well depending on the sample turbidity (Bukhari et al., 1998), as well as a lower size of initial sample (30 g versus 60 g), could explain the lower recovery rates obtained on raspberries for *C. parvum*. Moreover, on basil, a significantly lower efficiency was observed for *Giardia* cysts, which might be due to the variability of noncovalent interactions between *G. intestinalis* cysts and various fresh produce types as already observed (Cook et al., 2007). Our procedure using qPCR detection led to limits of detection below 3 (oo)cysts/g, which are suitable for analyses of naturally contaminated samples. Moreover, recent studies demonstrated that detection probability and hence sensitivity are improved by PCR methods (Dixon et al., 2013; Ramirez-Martinez et al., 2015).

Altogether, these data suggest that each food matrix displays specific characteristics that may interfere with protozoa extraction/elution (trapping, adhesion force) and qPCR detection (inhibitors), and hence may require the implementation of different methods depending on the parasite. Each strategy should be qualified in term of recovery rates and LOD, at different contamination levels and the use of a reporter system as already proposed could be useful to control the extraction procedure in microscopy assays (Cook et al., 2007). Although our method using molecular assays displayed slightly lower recovery rates, it is easier to standardize for routine analyses, more rapid in terms of time to result, specific to *T. gondii, C. parvum* and *G. intestinalis*, and does not require expertise of individual microscopists, which could lead to great variations between laboratories (Cook et al., 2007). To increase recovery rates and to standardize the procedure for the three protozoa, further works should focus on elution buffers including Alconox® or HCl pepsin (Chandra et al., 2014; Shields et al., 2012). Other molecular tools such as LAMP-PCR or droplet PCR, which are less sensitive to inhibitors, are also of great interest to gain in sensitivity on complex matrices (Dingle et al., 2013; Du et al., 2012; Nixon et al., 2014; Plutzer and Karanis, 2009; Sotiriadou and Karanis, 2008).

The ability of *T. gondii, C. parvum* and *G. intestinalis* (oo)cysts to persist for long time period at cold temperature in the environment and to retain their infectivity is now established (Dubey, 1998; Lindsay et al., 2002; Robertson et al., 1992; Tonani et al., 2013). Moreover, it was recently shown that organic matter enhanced the survival of *Cryptosporidium* and *Giardia* (oo)cysts (Alum et al., 2014). Therefore, the question of the fate of these (oo)cysts when they get to the surface of vegetables and undergo a cold temperature storage, is crucial in terms of food safety. Our work showed that the number and the viability of (oo)cysts present at the surface of basil's leaves were not affected by low temperature suggesting that the three protozoa may still be infectious after several days in a household refrigerator. This is consistent with a previous study that showed that *T. gondii* can remain infectious on raspberries and blueberries for at least 8 weeks under refrigerator conditions (Kniel et al., 2002). As well, *C. parvum* oocysts were found to attach to apples and to remain infectious after 4 weeks of storage at 6 °C (Macarisin et al., 2010a). The underlying mechanisms for the parasite persistence onto vegetables remain unclear. Internalization at the mesophyll level could occur as described for *C. parvum* on spinach leaves (Macarisin et al., 2010b) or specific attachment tightly linked to the matrix could be involved (Kniel et al., 2002).

In conclusion, our work allowed to develop an IMS Toxo tool coupled to qPCR assay, which is a promising tool to ensure the detection and quantification of *T. gondii* oocysts in environmental samples. In addition, we proposed a standardized method to simultaneously extract and detect by qPCR *T. gondii, C. parvum* and *G. intestinalis* (oo)cysts in food matrices. This procedure reaches performances that are concordant with expected natural levels of contamination and could be useful to better evaluate the risk for humans in foods. Finally, we highlighted that contaminated vegetables can represent a risk for the consumer after refrigerated storage. Considering that protozoan (oo)cysts are resistant to washing and disinfection procedures widely used in minimally processed vegetables industries, these findings raise concerns regarding food safety.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.01.002.

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