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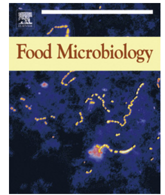

Loop-Mediated Isothermal Amplification-Lateral-Flow Dipstick (LAMP-LFD) to detect *Toxoplasma gondii* oocyst in ready-to-eat salad

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ABSTRACT

The apicomplexan parasite *Toxoplasma gondii* is the causative agent of toxoplasmosis, a foodborne zoonosis with a global distribution and estimated to cause up to 20% of the total foodborne disease burden in Europe. Association between *T. gondii* infection and the consumption of unwashed raw fruits and vegetables contaminated with oocysts has been reported and the increasing habit to eat pre-washed ready-to-eat salads poses a new potential risk for consumers. It is therefore important to trace the occurrence of potential contamination with this parasite to guarantee the safety of ready-to-eat vegetables. Detection of *T. gondii* in vegetables by molecular techniques has been achieved but low sensitivity (PCR) or expensive equipments (qPCR) limit routine applicability. Here, we describe the development and validation of a sensitive and robust method relying on a LAMP assay, targeting the 529 bp locus, to detect *T. gondii* oocysts down to 25 oocysts/50 g in ready-to-eat baby lettuce. The LAMP has been also adapted for a faster visualization of the result by a lateral flow dipstick chromatographic detection method.

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1. Introduction

Theapicomplexan parasite *Toxoplasma gondii* is the causative agent of toxoplasmosis, a foodborne zoonosis with a global distribution (Dubey, 2010). Toxoplasmosis ranked third in Europe among foodborne parasites and it is estimated to cause up to 20% of the total foodborne disease burden (FAO/WHO, 2014; Torgerson et al., 2015). This parasitic disease is usually asymptomatic, but serious outcomes may occur in immunocompromised persons. If acquired during pregnancy, infection can be eventually transmitted from mother to fetus resulting in serious fetal diseases, abortion or permanent damages that can arise later at the adult age (Saadatnia and Golkar, 2012).

Humans can acquire toxoplasmosis either by the consumption of raw/undercooked meat containing *T. gondii* tissue cysts or by the ingestion of oocysts present in soil, water or in contaminated fruits and vegetables (Pereira et al., 2010). An association between *T. gondii* infection and the consumption of unwashed raw fruits and vegetables contaminated with oocysts has been reported (Kapperud et al., 1996; Baril et al., 1999; Fallah et al., 2008; Studenicová et al., 2006; Liu et al., 2009; Pereira et al., 2010). However, the increasing habit to eat pre-washed ready-to-eat salads poses a new potential risk for consumers. Vegetable contamination can occur throughout the entire production process (by means of water irrigation, fecal contaminated soil, mechanical transmission by insects, pre-washing processes and marketing). To guarantee the safety of ready-to-eat vegetables is therefore important to trace the occurrence of potential contaminations. Detection of *T. gondii* in vegetables with conventional PCR or qPCR has been described but is challenged by a low sensitivity (i.e. PCR), due to the low number of oocysts often present in vegetable samples, or by expensive equipment requirements (i.e. qPCR) (Lass et al., 2012; Marchioro et al., 2016; Hohweyer et al., 2016). Loop-mediated isothermal amplification (LAMP) is a relatively novel and easy molecular technique successfully used to monitor microbial pathogens (Karanis and Ongerth, 2009). LAMP relies on *Bst* DNA polymerase large fragment with both high strand displacement and replication activities, which allows the amplification of small traces of nucleic acid under isothermal conditions (60–65 °C). A set of at least four primers (two inner and two outer) annealing with six regions of the target locus highly increases the

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specificity of the method (Notomi et al., 2000). The additional use of two further primers (called loop-primers) can reduce reaction time and improve sensitivity (Nagamine et al., 2002). The final LAMP products are stem–loop DNA molecules with cauliflower-like structures. LAMP has higher specificity, efficiency and rapidity compared to conventional PCR and can reach a sensitivity comparable to qPCR. LAMP has been successfully used to detect *T. gondii* DNA in water and soil as well as in human and animal biological samples (lymph nodes, blood and meat). The targets were either single-copy genes (TgOWP, MIC3, SAG1, SAG2 and GRA1), the multicopy gene B1 or the 529 bp repeated element (Homan et al., 2000; Sotiriadou and Karanis, 2008; Krasteva et al., 2009; Lau et al., 2010; Zhang et al., 2009; Lin et al., 2012; Du et al., 2012; Cao et al., 2014; Trisciuoglio et al., 2015; Sun et al., 2017). To avoid visualization of LAMP products by electrophoresis, chromatographic lateral flow dipstick (LFD) format has been also applied to reveal LAMP products in a simpler and faster way (Kiatpathomchai et al., 2008; Yongkiettrakul et al., 2014; Yang et al., 2016).

Here, we describe the development and validation of a sensitive and robust method applying LAMP to detect *T. gondii* oocysts in leafy green vegetables by targeting the 529 bp locus with the possibility of combination with LFD to visualize the results.

2. Materials and methods

2.1. *T. gondii* oocysts and genomic DNA

Toxoplasma gondii oocysts of the VEG strain (Dubey et al., 1996) were obtained from the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland, USA and transported by air to Rome as in previous investigations (Possenti et al., 2013). Genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue Kit (Qiagen srl, Italy) from tachyzoites of the RH stain (provided by Dr. Furio Spano, Istituto Superiore di Sanità) grown *in vitro* on HFF cell line and quantified spectrophotometrically on a BioSpectrometer (Eppendorf, Germany).

2.2. Samples preparation

Packages (200 g each) of ready-to-eat baby lettuce were purchased from a local supermarket and stored at 4 °C for a maximum of 3 days prior to contamination. Aliquots of baby lettuce (50 ± 2 g) were transferred in paddle-filtered bags (Stomacher 400 Classic Bags, Seward, UK) and used as matrix. Only lettuce packages that tested negative by LAMP for *T. gondii* were used in the following seeded experiments. Samples (50 ± 2 g) were individually contaminated with 100 µl of molecular grade water containing different numbers of *T. gondii* oocysts (25 ± 5, 50 ± 5, 100 ± 10). Oocysts were randomly pipetted on the leaves and samples stored overnight at 4 °C. Alternatively, 800 µl of vegetable pellet suspensions, prepared as reported below, were contaminated with 100 µl of molecular grade water containing different amount of *T. gondii* oocysts (5 ± 2, 10 ± 2, 50 ± 5) and stored at −20 °C until DNA extraction. Contaminations were done in triplicate for each amount of oocysts and repeated twice.

2.3. *T. gondii* oocysts recovery from vegetables and DNA extraction

T. gondii oocysts were recovered from baby lettuce as described by Lalonde and Gajadhar (2016) with modifications. Washing buffer (WB, 200 mL, glycine 1 M, pH 5.5) was added to paddle-filtered bag containing the baby lettuce and sample mixed for 30 s at 300 rpm twice using a paddle blender (Star-Blender Digital, VWR, Italy). Eluate was collected, and centrifuged at 2500×g for 10 min at 4 °C. Bag was further washed twice with 10 mL of WB by gentle manipulation and the eluate was centrifuged at 2500×g for 10 min at 4 °C. Supernatants were discarded, pellets were combined and washed once with 50 mL of molecular grade water. Vegetable pellet was finally resuspended in 800 µl of sodium phosphate buffer and stored at −20 °C until DNA extraction. The vegetable pellet suspension was thawed, supplemented with 120 µl of MT buffer (FastDNA-SPIN Kit for Soil, MP Biomedicals), transferred in Lysing Matrix E (FastDNA-SPIN Kit for Soil, MP Biomedicals) and lysed by two rounds of homogenization at 6.0 of amplitude for 40 s with FastPrep-24 5G instrument (MP Biomedicals). DNA was then extracted using the FastDNA-SPIN Kit for Soil (MP Biomedicals) according to manufacturer's instruction and eluted in 100 µl of reagent grade water.

2.4. LAMP and Bio-LAMP amplification

LAMP amplification of the 529 bp repetitive DNA fragment from *T. gondii* genome (GenBank: AF146527.1) was performed using a combination of six oligonucleotides as previously described (Zhang et al., 2009). LAMP reaction was performed using 4 µl of sample DNA in a final volume of 25 µl containing: 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 20 pmol each of LF and LB, 1 µl of Bst DNA polymerase Large Fragment (8U/µL) (New England Biolabs, UK), 1.4 mM of dNTPs mix (New England Biolabs), and 12.5 µL of 2× reaction buffer (1.6 M betaine, 40 mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM $(NH_4)_2SO_4$, 12 mM $MgSO_4$, and 0.2% Tween 20). The reaction mixture was incubated at 63 °C for the indicated time in a Veriti Thermal Cycler (ThermoFisher Scientific, Waltham, USA) following heat inactivation at 80 °C for 2 min. At the end of the reaction, and where indicated, 10 µL of SYBR Green I solution (1:100) (Roche Molecular Systems, Inc.) were added to the vial to visualize the reaction under UV-light (Parida et al., 2005; Hill et al., 2008). LAMP products were separated on 1.5% agarose gel electrophoresis. For the BIO-LAMP reactions, the BIP primer was substituted with a 5′-biotinylated BIP primer at the same concentration and the reaction incubated at 63 °C for 2 h without the final heat inactivation step.

2.5. Chromatographic Lateral-Flow Dipstick (LFD assay)

To detect the LAMP-BIO products by LFD strips, a universal rapid test for the detection of biotinylated and fluorescein isothiocyanate (FITC)-labeled genomic amplicons, was used (Milenia® GenLine HybriDetect, GieBen, Germany). To this aim, a 5′-FITC labeled single strand DNA probe was designed (FITC-5′-TGAGGGGGTGGCG-3′) to hybridize between the FIP and BIP region. After LAMP-BIO amplification, 20 pmol of the DNA probe were added to the reaction and hybridized at 63 °C for 5 min (Khunthong et al., 2013; Yongkiettrakul et al., 2014). Eight µl of the hybridized product were transferred in a new tube with 150 µL of assay buffer (Milenia® GenLine HybriDetect). The LFD strip was immersed into the mixture and the result read after 5–10 min, according to manufacturer's instruction.

2.6. qPCR

PCR amplification was performed in 96-wells plate using a LightCycler480 thermal-cycler instrument (Roche, Almere, The Netherlands), as previous described (Opsteegh et al., 2010), targeting the 529 bp repeat elements. The following primers were used: Tox-9F 5′-AGGAGAGATATCAGGACTGTAG-3′; Tox-11R 5′-GCGTCGTCTCGTCTAGATCG-3′; Tox-TP1 5′-CCGGCTTGGCTGCTTTTCCT-3′: Briefly, the reaction mixture (30 µl) consisted of 15 µl 2 × concentrated LC 480 Probes Master (Roche), 0.7 µM of each primer (Tox-9F and Tox-11R), 0.1 µM of Tox-TP1 and

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