IDENTIFICATION OF DNA *of Toxoplasma gondii* IN FOOD MATRICES (MEAT AND MEAT PRODUCTS) BY LAMP

INDEX

1. Aim and field of application

To identify *Toxoplasma gondii* DNA in food matrices by LAMP (Loop-Mediated Isothermal Amplification). This method can be applied to meat and fresh (not cooked or seasoned) meat products (i.e. sausages, meat balls).

2. Principle of the method

The LAMP is a molecular biology technique that allows the amplification of specific nucleic acid fragments, which initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species has its own characteristic DNA portion, due to its composition, it is possible to design 2 or 3 pairs of oligonucleotides (primers) allowing for its amplification. Combined use of such primers allows the unique amplification of DNA of the target species. The reaction is performed by a particular DNA polymerase having both high strand displacement activity in addition to a replication activity. The reaction is carried out at a constant temperature (62-65°C) with the final products being stem–loop DNAs with cauliflower-like structures. Compare to conventional PCR (Polymerase Chain Reaction), LAMP has an higher specificity, efficiency and rapidity, allowing amplification of small traces of target DNA in the tested matrix.

Toxoplasma gondii is a protozoan parasite able to infect all warm blood animal (mammals or birds) including humans. The sexual stage of the parasite life cycle occurs in felids, the definitive host, and the parasite environmental resistant stage, the oocyst, is shed with the faeces. Following oocyst ingestion by mammals or birds, the intermediate hosts, the parasite invades different tissues of the host (especially muscles and brain), where the asexual stage, the tachyzoite, undergoes multiple replication cycles and finally forms tissue cysts containing tens to thousands parasite, the bradizoytes. The cycle is completed when felids eat meat containing *T. gondii* tissue cysts (or directly oocysts). Human infection, toxoplasmosis, can be acquired by direct ingestion of fruits, vegetables or water contaminated by oocysts or consumption of raw or undercooked meat containing tissue cysts. Usually toxoplasmosis is asymptomatic, but in immunocompromised persons it can cause encephalitis, myocarditis, pneumonia, retinochoroiditis and hepatitis. In case of infection acquired during pregnancy, the parasite can be transmitted to the fetus causing serious fetal diseases, eventually resulting in abortion or to permanent damages that can arise later at adult age.

By the use of LAMP it is possible to detect *T. gondii* DNA in animal tissue by the amplification of specific nucleotide sequence of 529 bp presented only in the genome of *T. gondii* and repeated up to 300 copies. A mx of 6 primers (see Table A) allow the specific amplification of the 529 bp sequence. After LAMP reaction, the presence of the amplification products can be visualized either directly i) by the addition of compound that becomes fluorescent under UV light only if bound to DNA; or ii) by agarose gel electrophoresis with positive reaction showing a typical ladder of bands.

Table A – LAMP Primer Mix

Oligonucleotide sequence	Name
5'-TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG -3'	BIP
5'-TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC -3'	FIP
5'-CCACAGAAGGGACAGAAGTC -3'	F3
5'-TCCGGTGTCTCTTTTTCCAC -3'	B3
5'-TCCAAGACGGCTGGAGGAG -3'	LF
5'-CGGAGAGGGAGAAGATGTTTCC -3'	LB

3. References

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ISO 22174: 2005. Microbiology of food and animal feeding stuffs. Polymerase chain reaction (PCR) for the detection of food-borne pathogens. General requirements and definitions.

ISO/FDI 20837:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for sample preparation for qualitative detection.

ISO/FDI 20838:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for amplification and detection for qualitative methods.

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4. Definitions

529 bp, 529 bp non-coding nucleotide sequence present up to 200-300 folds in the single genome of *Toxoplasma gondii*.

Oligonucleotide (Primer), short sequence (15/30 nucleotide bases) used to amplify a DNA specific fragment.

LAMP Mix, mix of 6 oligonucleotides necessary to specifically amplify the 529 bp sequence.

Reference DNA, purified DNA from *Toxoplasma gondii* tachizoytes.

Positive control for the DNA extraction, pork meat balls (5 g±1g) spiked with *Toxoplasma gondii* tachizoytes (10.000 tachizoytes /5 g meat) analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session.

Sample DNA, DNA extracted from a single sample.

Positive control for the amplification, eference DNA; this control is used in the amplification session to verify the efficacy of the LAMP.

Negative control for the amplification, reagent grade water; this control is used in the amplification session to verify the absence of contamination in the LAMP reaction.

The definitions and terminology used in the ISO 22174 standard are applied in the present method.

5. Devices/instruments

5.1 Vortex.

5.2 Incubation oven with rotisserie, temperature range 45÷100°C.

5.3 Bench top refrigerated centrifuge for 1.5 mL tubes, minimum 10,000 xg.

5.4 Freezer ≤-15°C.

5.5 Thermoblock with vibration, temperature range 25÷100°C.

5.6 PCR thermocycler.

5.7 Refrigerator, temperature range 1÷8°C.

5.8 Horizontal electrophoretic apparatus.

5.9 Analytical balance, readability 0.1 g.

5.10 UV transilluminator.

5.11 Digital imaging system.

5.12 Adjustable volume pipettes, volume range: 1-10 µL, 2-20 µL, 20-100 µL, 50-200 µL, 200-1000 µL.

5.13 Reagent grade water system production.

5.14 Orbital shaker.

5.15 Ethanol 95-100%

6. Reagents and chemicals

6.1 **Proteinase K** Commercial solution (i.e. DNeasy Blood & Tissue Kit, Qiagen, 20 mg/ml). Store according to the manufacturer's recommendations.

6.2 **Incubation buffer.** Commercial solution (i.e. AL buffer, DNeasy Blood & Tissue Kit, Qiagen). Store according to the manufacturer's recommendations.

6.3 **Lysis buffer.** Commercial solution (i.e. DNeasy Blood & Tissue Kit, Qiagen). Store according to the manufacturer's recommendations.

6.4 **Washing buffers.** Commercial solution (i.e. DNeasy Blood & Tissue Kit, Qiagen). Once prepared according to the manufacturer, label as "AW1" and "AW2". Store according to the manufacturer's recommendations.

6.5 **Recovery column.** Commercially available material (i.e. DNeasy Blood & Tissue Kit, Qiagen), labelled as "DNeasy Mini spin columns").

6.6 **Collection tubes.** Commercially available material (i.e. DNeasy Blood & Tissue Kit, Qiagen, 2 ml tubes labelled as Collection tube).

6.7 **Microcentrifuge tubes.** Commercially available molecular biology grade material, 0,2 mL, 1,5 mL, 15 mL, 50 mL.

6.8 **Elution buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store according to the manufacturer's recommendations.

6.9 **LAMP reaction mix 2x**. Buffer obtained by mixing commercial solutions, 10X ThermoPol DF (detergent-free) and 100 mM $MgSO_4$; 5M Betaine; 100% Tween 20 (to prepare 1 ml of 2X reaction buffer mix: 320 µl of betaine 5M, 200 µl of 10X ThermoPol DF; 120 µl of $MgSO_4$ 100mM, 2 µl of Tween 20 100% and 358 µl reagent grade H_2O). Do not add Tween 20 detergent if it is in the 10x ThermoPol. Aliquote the mix and store at -20 °C (5.4) up to 24 months.

6.10 **Deoxynucleotide (dNTP) Solution Mix**. Commercial solution (100 mM) of deoxynucleotide (dNTP) mix. Each dNTP is used 25 µmol for each amplification reaction. Store at -20 °C (5.4).

6.11 **Bst DNA polymerase (Large Fragment).** Bst DNA polymerase is a commercially available enzyme suitable for LAMP reactions. Store according the manufacturer's instructions.

6.12 **Oligonucleotides.** Commercial preparation (*Table B*); the lyophilized products is reconstituted with reagent grade H_2O (6.17), according to the manufacturer's recommendations, at a concentration of 100 pmol/µL; the lyophilized product can be stored frozen for up to 20 years; the reconstituted product can be stored frozen up to 10 years.

6.13 **LAMP Primers Mix.** Oligonucleotides mixture used for LAMP reactions (6.7). The mixture is obtained combining a defined volume of the oligonucleotides reported in *Table A* in reagent grade H_2O (6.17); the final concentration corresponds to 40 pmol/µL for BIP and FIP, 5 pmol/µL for F3 and B3, 20 pmol/µL of LF and LB. 100µL aliquots are prepared and stored frozen up to 10 years.

6.14 **Loading buffer**. Commercial product allowing DNA molecules electrophoresis to be visualized. Store according to the manufacturer's recommendations.

6.15 **Agarose**. Commercial product suitable for performing DNA molecules electrophoresis. Store at room temperature according to the manufacturer's recommendations.

6.16 **TAE solution 50x**. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store according to the manufacturer's recommendations.

6.17 **TAE solution 1x.** 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. Store at room temperature for up to 1 month.

6.18 **DNA intercalating agent**. Commercial product able to fit in the DNA double helix, used to view the amplification product on agarose gel. Store according to the manufacturer's recommendations.

6.19 **L1000.** Commercial product containing markers for DNA molecular size multiple of 50 bp. All commercial products containing molecules in the range 250-1.000 bp and 250-10.000 bp may be used. Store refrigerated according to manufacturer's recommendations.

6.20 **Reagent grade water or Milli-Q.**

6.21 **SYBR Green I.** Commercial product. Dilute 1:100 to prepare the working solution. Store in the dark at -20°C for up to 24 months.

6.22 **Reference sample.** Pork meat balls (5 g±1 g) spiked with *Toxoplasma gondii* tachizoytes (10.000 tachizoytes /5 g meat). Store at -20°C (5.4) up to 2 years.

6.23 **Reference DNA,** purified DNA from *Toxoplasma gondii* tachizoytes. (10ng/µL). Store at -20°C (5.4) up to 10 years.

6.24 **Glass beads,** commercial product (diameter 0.5-1 mm).

7. Procedure

7.1 Sample preparation

The sample (5 g±1 g in disposable 50 mL tube), is inspected to check the tube integrity and no trace of content spread out.

Samples are then stored at -20°C (5.4) for at least 4 days before being tested. In such conditions, the samples are stable for up to 5 years.

7.2 Method

7.2.1 *DNA extraction from sample.*

- If not otherwise specified, the procedure is carried out at room temperature.
- Each working session requires the DNA extraction of a "reference sample" (6.22) identified as "positive control for the extraction".
- Before starting the procedure, prepare a sufficient volume of Incubation buffer (6.2).

a) Thaw the tubes containing the sample and the "reference sample" (6.22) at room temperature.
b) Add 2.5 g (±0.3 g) of glass beads (6.24).
c) Add 10 mL of Incubation buffer (6.2).
d) Homogenize the sample by vortex (5.1) at maximum speed for 90 sec.
e) Add 200 µL of Proteinase K (6.1).
f) Incubate at 55°C (±3°C) for 16-18h in the incubation oven (5.2) under rotation.
g) Transfer 200 µL of the homogenate in 1.5 ml vial. Transfer the surplus of the homogenate in 15 mL tube (6.7) and store at -20°C (5.4) up to 6 months.
h) Add 200 µL of lysis buffer (6.3) and briefly mix by vortex (5.1).
i) Incubate for 10 min at 70 °C in the thermoblock (5.5). During incubation, shake at 1,400 vibrations/min.
j) Centrifuge (5.3) vials at 13,000 rpm for 1 min.
k) Collect supernatant and transfer in new 1.5 ml vials (6.7).
l) Add 200 µL of ethanol (6.5) and vortex (5.1) briefly.
m) For each sample, put one binding column (6.5) in a collection tube (6.6).
n) Transfer the lysate (i) in a binding column (6.5) and centrifuge (5.3) at 13,000 rpm for 1 min.
o) Discard the collection tube (6.6) and transfer the binding column (6.5) in a new collection tube (6.6).
p) Add 500 µL of wash buffer AW1 (6.4) to the binding column (6.5) and centrifuge (5.3) at 13,000 rpm for 1 min.
q) Discard the collection tube (6.6) and transfer the binding column (6.5) in a new collection tube (6.6).
r) Add 500 µL of wash buffer AW2 (6.4) to the binding column (6.5) and centrifuge (5.3) at 13,000 rpm for 3 min.
s) Transfer the binding column (6.5) in a new 1.5 mL vial (6.7).
t) Add 30 µL of elution buffer (6.8) to the binding column (6.5) and incubate for 1-2 min at room temperature.

u) Centrifuge (5.3) at 13,000 rpm for 1 min., discard the binding column (6.5) and store the 1.5mL vial with the eluted DNA.

v) The obtained DNA will be defined 'DNA/sample' and store frozen (5.4) for up to 10 years.

7.2.2 LAMP amplification

- Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves.
- At each working session, use a positive and a negative amplification control. Use reference DNA (6.23) as positive control and water (6.20) as negative control.

a) Thaw DNA/sample, 2x LAMP reaction mix (6.9), dNTP Mix (6.10), LAMP primer mix (6.13), Bst DNA polymerase (6.11) and positive amplification controls (Reference DNA 6.23).

b) Mark with a progressive number an adequate number of 0.2 mL tubes (6.7).

c) Prepare an adequate cumulative volume of the amplification mix. Calculate the volume on the basis of a single sample amplification mix (*Table B*) and of the total number of samples added of three reactions (one for the positive amplification control, one for the negative one and one for an extra reaction).

Table B – single sample amplification mix: components and volumes

LAMP reaction mix 2X (6.9)	12.5 µL
LAMP Primers Mix (6.13)	6 µL
Bst DNA polymerase (6.11)	1 µL
dNTP mix (6.10)	1.4 µL
H_2O (6.20)	2.1 µL
Total	23 µL

d) Vortex (5.1) the amplification mix and, if necessary, centrifuge (5.3) at maximum speed for a few sec.

e) Transfer 23 µL of the cumulative amplification mix to each tube (point "b").

f) Add 2 µL of the DNA/sample to be tested to each tube.

g) Close the tubes, mix and centrifuge (5.3) at maximum speed for a few sec.

h) Start the amplifying cycle (*Table C*) on the thermocycler (5.7); wait for the temperature to reach 63°C and insert the tubes in the thermoblock by pausing the instrument.

Table C – amplification cycles

Amplification	90 min/63°C
Inactivation	2 min/80°C

i) At the end of the amplification phase, centrifuge (5.3) the tubes at maximum speed for a few sec.

l) Keep tubes on ice or refrigerated (5.8).

7.2.3 Result display and interpretation

7.2.3.1 Fluorescence of the amplification products under UV:

a) add 10 µl of SYBR Green I working solution (6.21) to each vial;

b) control the eventual colour change from orange to green of each vial by visual inspection and then fluorescence on UV transilluminator (5.11).

Amplification test is considered valid if:

i) the positive control of amplification shows colour change from orange to green by visual inspection and fluorescence under UV;

ii) the negative control of amplification shows neither colour change from orange to green by visual inspection no fluorescence under UV.

The test is evaluated anyway by electrophoresis on agarose gel.

7.2.3.2 Agarose gel electrophoresis

a) Add, to each vial, the loading buffer (6.14) according to the manufacturer's instructions.

b) Keep tubes on ice or refrigerated (5.8).

c) Assemble the electrophoresis apparatus according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of samples.

d) Weight (5.10) 1.5 gr of agarose (6.15) and add in 100 mL TAE 1x (6.17) in a glass beaker.
e) Gently resuspend the powder by rotation.
f) Weight (5.10) the solution.
g) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, boil for another 30 sec.
h) Restore with water the volume lost by boiling.
i) Allow the agarose solution to cool.
j) Before it solidifies, add DNA intercalating agent (6.13) according to the manufacturer's instructions
k) Shake gently to dissolve uniformly the DNA intercalating agent and pour the agarose in the gel tray previously prepared (point "c").
l) Wait for the gel to solidify, which requires at least 30 min.
m) Place the tray with the gel in the electrophoresis apparatus.
n) Cover the gel with TAE 1x buffer (6.17) and gently pull out the comb.
o) The first or last well are loaded with 15 µL of the L50 solution (6.14).
p) Load in each well 16 µL of the amplification product (point 7.2.2 "l"), respecting the progressive numbering of the tubes (point 7.2.2 "b").
q) Connect the electrophoresis apparatus with the power supply and set 10 V/cm of gel.
r) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.14), reaches a distance of 1 cm from the gel border.
s) After 30 min, switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if it is possible to easily distinguish all bands of molecular weight marker ranging from 50 to 2000 bp. If the separation is incomplete, continue the run.
t) At the end of the run, transfer the gel to the imaging system and print the result.

The amplification profile after gel electrophoresis is evaluated directly by comparison with the molecular size marker L1000 (6.19) and the positive control of extraction and amplification. The visual evaluation is considered sufficient and appropriate.

The amplification test is considered valid if:
i. the positive control of amplification shows a typical ladder of bands;
ii. the negative control shows no ladder of bands or eventually shows only bands related to oligonucleotides excess or primer dimers;
iii. the positive control of extraction shows a typical ladder of bands.

8. Results

The results is expressed in the test report as follow:

If the amplification product shows a typical ladder of bands, the sample if identified as POSITIVE for the presence of *T. gondii* DNA.

If the test is considered valid, the tested sample will be considered as NEGATIVE both in case it does not show any amplification or it shows an amplification profile not comparable with the positive controls, even if after the amplification the vial results fluorescent under UV examination.

In case of NEGATIVE result, the presence of DNA of *T. gondii* in the matrix is not excluded since the amount of parasite DNA could be below the limit of sensitivity of the method.

9. Characteristics of the method

This method has been characterised in terms of repeatability and specificity. The results of the validation protocol are used to confirm that the method is suitable for the expected aim and are reported in the validation report, which can be sent upon request.

10. Safety measures

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For general safety measures, refer to the CDC guidelines.