Deliverable D-JRP-TOXOSOURCES-WP3.2

SOP on detection of *T. gondii* in selected fresh produce matrix

Workpackage 3 of JRP22-FBZ4.1-TOXOSOURCES

Responsible Partners:
ISS, VRI, SSI

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SOP ON DETECTION OF *T. GONDII* IN SELECTED FRESH PRODUCE MATRIX

BACKGROUND

This is a public deliverable of One Health EJP Joint Research Project:
JRP22-FBZ4.1-TOXOSOURCES – *Toxoplasma gondii* sources quantified (https://onehealthejp.eu/jrp-toxosources/);

Work Package:
JRP-TOXOSOURCES-WP3 Multicentre survey to fill the key existing gap: role of fresh produce (i.e. Ready-to-Eat salads);

Task:
JRP-TOXOSOURCES-WP3-T1 Selection, evaluation and implementation of detection procedure for *T. gondii* oocysts in fresh produce.

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TOXOSOURCES addresses the research question – **What are the relative contributions of the different sources of *T. gondii* infection?** – by using several multidisciplinary approaches and novel and improved methods to yield robust estimates that can inform risk management and policy makers.

TOXOSOURCES WP3 aims to fill the knowledge gap concerning the relevance of fresh produce contamination by *T. gondii* oocysts as an infection source for humans. Objectives of TOXOSOURCES WP3:

- ✓ To identify and assess the most appropriate procedure to detect *T. gondii* oocysts in fresh produce.

- ✓ To provide an overview of *T. gondii* oocysts in fresh produce and the environment.
- ✓ To conduct a risk-based pilot study based on available prevalence data (literature review), data on food production chains, EU trade patterns of selected fresh produce and available consumption data (WP2).
- ✓ To evaluate *T. gondii* oocyst contamination in selected fresh produce commodities by a multicentre pilot survey in representative EU regions.

This deliverable reports on the work to establish a standard operating procedure (SOP) to be implemented among consortium partners and to be applied in a multicenter pilot survey.

EXPERIMENTAL WORK

During the first year of the project, 2020, following the annual work plan and building on the results of the literature review work (D-JRP-TOXOSOURCES-WP3.1; Slana et al., submitted manuscript), comparative experimental work was conducted at two partner institutes, ISS and VRI, for all the key analytical steps necessary for the SOP development. The three key steps are oocyst recovery from fresh produce, DNA extraction, and detection by PCR. Overall, the method aims to detect the presence of *T. gondii* oocyst contamination in leafy green vegetable matrix (mixed salad) by detecting *T. gondii* genomic DNA using a hydrolysis probe-based qualitative multiplex real-time PCR (qPCR) targeting both the B1 gene and the 529 repetitive element. By the experimental work, key characteristics of the method, such as sensitivity, reproducibility, repeatability, ease of handling, availability and costs of reagents and equipment, hands on and turn-around times, were evaluated. Protocol and video tutorials were compiled.

Experiments were performed using the following test material:

i) commercial RTE mixed salads (containing green and red baby lettuce, arugula, baby spinach, roman baby lettuce) from local stores (in Italy and Czech Republic) as fresh produce matrix (amount tested 30 gr/test)

ii) *T. gondii* (genotype II) oocysts previously isolated from cat faeces using the protocol of Wainwright et al. (2007), and further purified by sucrose or cesium chloride density gradient;

iii) genomic DNA (gDNA) extracted from control post-washing vegetable pellet using the FastPrep kit for soil;

iv) serial dilution of gDNA extracted from *T. gondii* oocysts (genotype II)

v) serial dilution of purified plasmid DNA (pDNA) carrying a fragment of the B1 gene, or the 529 repetitive element, or the IAC (Slany et al., 2019).

Mixed salad or the post-washing pellets were artificially spiked with a known number of *T. gondii* oocysts (100; 50; 10). Spiking of the mixed salad was conducted as indicated in the IMPACT spiking guideline (Chalmers et al., in preparation); briefly, mixed salad was placed into a homogenization filter bag and spiked as drops on the leafs; after drying at room temperature, the bags were kept over night at 4°C before proceeding with the test.

The method was tested using the following ranges:

i) plasmid pDNA in the range of 10000-10 copies/reaction;

ii) *T. gondii* gDNA in the range of 1000-0.001 oocyst-equivalents/reaction;

iii) DNA from mixed salad spiked with *T. gondii* gDNA in the range 100-1 oocyst-equivalents/reaction;

iv) DNA extracted from mixed salad previously spiked with *T. gondii* oocysts (in the range of 100-10 oocysts/sample) using 1:50 or 1:10 extracted DNA/reaction.

EVALUATION OF THE KEY STEPS OF THE METHOD

Evaluation of the oocyst recovery step

Oocyst recovery from mixed salad leaves was performed according to Lalle et al. (2018) with minor modifications, in particular using antifoam agent in the washing solution. The procedure implies washing of fresh produce sample in a filter bag by stomaching. Two different stomachers were used. Efficiency was evaluated by comparing Cq values. A step by step protocol was prepared.

Evaluation of the DNA extraction step

DNA extraction from pellets was evaluated using two commercial kits applied after mechanical oocyst disruption by bead-beating (Lalle et al., 2018; Slany et al., 2019) and one kit after chemico-physical disruption by freeze-thawing and proteinase k. Efficiency of DNA extraction was evaluated by comparing the Cq values. A step by step protocol for the different DNA extractions and video tutorials were prepared.

Evaluation of the multiplex qPCR step

The multiplex qPCR described in Slany et al. (2019) was selected for the molecular detection step. Its performance was independently evaluated at ISS and VRI using, initially, serial dilutions of plasmid carrying fragments of the 529RE or the B1 target (from 10000 to 10 copies/reaction). Suitable IAC concentration was previously established at ≈120 copies/reaction (Slany et al., 2019). The following variables were considered: i) four different hot start master mixes were evaluated in terms of performance (sensitivity and specificity); ii) different probe quenchers from different manufacturers (MGBEQ and EQ); and iii) two Real-time Platforms. Parameters recommended in Broeders et al. (2014) were recorded. Specificity was previously tested in silico and with other pathogens (Lalle et al., 2018; Slany et al., 2019) and now with different leafy green matrices. Sensitivity expressed as LOD in multiplex format was reported as haploid genome equivalents (HGE) and calculated using serial dilution of pDNA, serial dilution of genomic DNA and gDNA extracted from vegetable sample artificially spiked with different amount of *T. gondii* oocysts. PCR efficiency (ε) and linearity (R2) were also calculated. Practicability and robustness will be further confirmed with blind samples to be tested in a Ring Trial (RT) among TOXOSOURCES consortium partners. A detailed protocol to perform and evaluate the results was prepared. Cq cut-off values will be consolidated following the SOP implementation in the TOXOSOURCES consortium laboratories and the RT.

FINAL STEPS

The experimental work done was presented to the TOXOSOURCES consortium members who participate in WP3 at an online meeting on November 27th 2020, after which a preliminary version of the SOP and video tutorials were distributed to the TOXOSOURCES partner laboratories. All specific material needed, *T. gondii* oocysts and gDNA and aliquotes of the IAC, were provided by VRI. There was discussion e.g. on the different stomachers used in the laboratories across Europe. The implementation of the method in several laboratories is planned to be completed in early 2021, after which the final SOP as well as all supporting data (following FAIR principle) will be made available.

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