Deliverable D-JRP-TOXOSOURCES-WP3.3

Report on the ring trial of WP3

Workpackage 3 of JRP22-FBZ4.1-TOXOSOURCES

Responsible Partners:

ISS, VRI, SSI

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REPORT ON THE RING TRIAL OF WP3

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.

As part of the work done in TOXOSOURCES WP3, Standard Operating Procedure (SOP) "Molecular detection of *T. gondii* oocysts contamination in ready-to-eat salads by multiplex qPCR" has been prepared.

The steps of the work are described in Deliverables:

Deliverable D-JRP-TOXOSOURCES-WP3.1 Report on available analytical procedures for detection of *Toxoplasma gondii* in fresh produce and list of promising analytical procedures
https://zenodo.org/record/3778719#.YHVhCegzaUk
and
Deliverable D-JRP-TOXOSOURCES-WP3.2 SOP on detection of *T. gondii* in selected fresh produce matrix
https://zenodo.org/record/4405243#.YHVhiOgzaUk

and supporting review was published:

Slana et al. Molecular Methods for the Detection of *Toxoplasma gondii* Oocysts in Fresh Produce: An Extensive Review. Microorganisms 2021, 9(1), 167; https://doi.org/10.3390/microorganisms9010167; https://zenodo.org/record/4647840#.YHVh7ugzaUk

Moreover, the work was selected for an oral presentation at the 3rd Annual Scientific Meeting of the One Health EJP, which will be held in June 2021:

Presenter: Marco Lalle
Title of the presentation: MOLECULAR METHOD FOR DETECTION OF *TOXOPLASMA GONDII* OOCYSTS IN LEAFY-GREEN VEGETABLES: METHOD SELECTION, VALIDATION AND SOP DEVELOPMENT.

The SOP describes a selected method to detect the presence of *T. gondii* oocysts contamination in leafy green vegetables (mixed salad) by detecting *T. gondii* genomic DNA using a hydrolysis probe-based qualitative multiplex real-time PCR targeting both the B1 gene and the 529 repetitive element. At the time of submitting this Deliverable, the SOP is in the course of implementation in TOXOSOURCES consortium partner laboratories (UCM; PIWET; UoS; SSI; NVI; VRI; ANSES; BfR; INIAV). The goals of the task JRP-TOXOSOURCES-WP3-T1 have been reached, and the number of laboratories across Europe that are implementing the harmonised method is higher than originally planned.

This deliverable reports on the detailed workplan for the organization of a Ring Trial (RT) to complete the validation process of the SOP and to assess the performance of the consortium partners to apply the implemented SOP.

PURPOSE

The aim of the planned ring trial (RT) is to evaluate the performance of laboratories in detecting the presence of *T. gondii* oocysts in leafy green vegetables (mixed salad) and to provide a solid base for the delivery of homogeneous and reliable data in the course of the multicenter pilot survey (task JRP-TOXOSOURCES-WP3-T3). Data from the RT will also provide further validation for the SOP, specifically for the different steps of the procedure: oocyst recovery from the matrix, DNA extraction, and qPCR.

RT ITEMS

RT items will consist of three panels of samples:

- Panel 1:
  5 vials containing suspension (sterile PBS supplemented with antibiotics/antimycotics) with or without enumerated *T. gondii* oocysts, to be used for spiking of leafy salads at each laboratory.
- Panel 2:
  5 vials containing vegetable sediment spiked with suspension with or without *T. gondii* oocysts in different concentrations;
- Panel 3:
  3 vials containing gDNA extracted from vegetable sediment previously spiked with suspension with or without *T. gondii* oocysts.

All vials will be individually labeled with distinct alphanumeric codes.

Additionally, positive and negative control DNAs will be provided.

RT items will be prepared as follows:

- *Toxoplasma gondii* oocyst suspension (in 1.5 ml vial) consists of oocysts (*T. gondii* genotype II) isolated from cat faeces using a published protocol (Wainwright et al., 2007), and further purified by sucrose density gradient. The oocysts are counted (5 replicates) on a hemocytometer (Kova Glasstic slides), diluted at the desired concentration, and re-counted (10 replicates). Aliquots of 100 uL of the suspension are prepared and stored short-term at 4°C until shipping.
- Vegetable sediment is prepared as reported in the SOP and aliquots (250-300 µl) of pooled pellets are prepared so that they correspond to 30 gr of leafy salad. Pellets are stored frozen till use. Immediately prior to shipping, pellets are thawn, individually spiked with suspension with or without *T. gondii* oocysts, and sealed.
- Pooled DNA is purified from vegetable sediment (spiked with suspension with or without *T. gondii* oocysts) as reported in the SOP. DNA aliquots (40 µl) are transferred into clean tubes and sealed.

The homogeneity of RT items will be ensured by providing all participants aliquots of the same *T. gondii* oocyst suspensions, vegetable sediments and DNA preparations.

For sample shipping, the 1.5 ml tubes are plugged and sealed with plastic paraffin film, individually identified with a code, and vacuum sealed in a plastic bag. Each RT panel set is put inside a polystyrene carton with an adequate number of ice packs to guarantee the maintenance of an internal temperature between +4°C and +15°C during the transport. Insulating material is added to avoid undesired shaking of package content during transport.

No other material, reagent or consumable will be provided by RT organizer.

As a matrix for spiking, participants will use locally commercially available ready-to-eat mixed (3 ingrediens) leafy salad (including e.g., green and red baby lettuce, arugula, baby spinach, romaine baby lettuce) that is NOT labelled as organic. The participating laboratories buy the salad themselves, from a local store. The mix should not contain carrots or other vegetables or herbs, only leaves of leafy salads. The participants report the composition of the salad used. The participants buy at least 200 gr of the same brand and batch (lot) with an appropriate best-before date: the analysis is done before the best-before date. If salad package size is less than 200 gr, several bags of the same batch (lot) to obtain a homogeneous matrix is acceptable.

RT PROCEDURE

The RT participants are informed about the date of delivery of the RT items in advance by email. Delivery is done on Monday to ensure the packages are delivered to the participants within the week.

On the day of shipping, each participant receives an individual laboratory code and a link to an online form were the following information must be reported:

1. Participant information and RT item checklist
2. Materials and methods used
3. Results

The on-line form will remain active up to RT deadline, which is indicated in the email to the participants.

In addition, a protected Excel file will be provided to report in detail relevant specific RT results (e.g., Ct values, qPCR reaction dynamic parameters).

Upon arrival in the laboratory, the packaging and its contents are checked for correctness and completeness. The samples are advised to be immediately stored as follows:

- Panel 1 at +4°C until the spiking will be done (within 24-48h);
- Panel 2 and Panel 3 at -20°C until the DNA extraction and qPCR will be performed.

The analysis is to be done in compliance with the SOP. Use of reagents other than those exemplified in the SOP (e.g. DNA extraction kit; qPCR Mastermix) is acceptable providing that their characteristics are consistent with the expected performance of the test.

The following remarks are asked to be taken into account:

i) for DNA extraction, it is recommended to use kits based on a bead-beating step. If a different DNA extraction method (e.g. kits based on silica-membrane) is used, including several freeze and thaw cycles before DNA extraction is indicated in the SOP;

ii) for the qPCR, all DNA samples shall be run in triplicate, including positive and negative controls and the standard curve.

SPIKING OF MIXED SALAD

Performing the spiking and the oocyst recovery step are done as soon as possible, within 24-48h, after sample arrival. The obtained sediment pellet can be stored at -20°C before proceeding with DNA extraction.

Before proceeding with spiking, the matrix is tested to ensure it is *Toxoplasma gondii* -free by applying the SOP on, at least, 30 gr of salad. Alternatively, salad control (unspiked) can be treated and tested in the same session as spiked samples.

Spiking procedure:

1) Prepare 5 filter bags (or 6 if an unspiked control is ran in the same session) on a stand or in a beaker.
2) In each filter-bag, weigh out 30 g ± 1g of the leafy mixed salad. Keep the bags propped upright to minimize the risk of leakage or splashing during spiking.
3) Spin-down each tube of the Panel 1 (few seconds at max speed) to ensure collecting all drops and oocysts from the tube walls.
4) Gently pipet up and down several times to mix the suspension. Do not vortex, to avoid the suspension spreading on the tube walls.
5) Dispense the entire suspension (approximately 100 µl) in 10 droplets (≈10 µl each) distributed across different areas of the sample (aiming for the center of leaves to ensure that the suspension does not run off).
6) Leave to dry at room temperature for 2-3 h with the filter bag open.
7) Close the bag by folding the edge, secure with adhesive tape and place at 4°C overnight.
8) The day after, start processing the samples.

CRITERIA FOR EVALUATION OF THE RESULTS

Evaluation of the results is qualitative: the participant have to correctly identify RT items as positive or negative.

REPORTS

Within 10 working days after the due date to submit results, an individual report will be provided to each participant including the following information: i) expected result (positive/negative); ii) reported result (positive/negative); iii) final evaluation, and iv) comments based on the laboratory performance.

A Final Report including results obtained by all participants is compiled. The Final Report will be discussed with all participants during an E-meeting and the results are disseminated in collaboration with TOXOSOURCES WP1.

REFERENCES

- Deliverable D-JRP-TOXOSOURCES-WP3.1 Report on available analytical procedures for detection of *Toxoplasma gondii* in fresh produce and list of promising analytical procedures https://zenodo.org/record/3778719#.YHVhCegzaUk
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- Wainwright et al. Chemical inactivation of *Toxoplasma gondii* oocysts in water. J Parasitol. 2007 93(4), 925-31. https://doi.org/10.1645/GE-1063R.1