STSM Scientific Report

Subject:

T. gondii LAMP for detection of oocyst in shellfish (blue mussels)

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1. Purpose of the STSM

As filtering organisms, bivalve mollusks are exposed and accumulate considerable amount of pollutants. Due to their environmental dissemination and resistance, *T. gondii* oocysts are potential contaminants, and it has been shown that mollusks are able to accumulate the oocysts in their tissues. Consistent with this, few studies reported the presence of oocysts in mussels, oysters and clams using molecular methods based on PCR. Therefore, bivalve mollusks are considered as food vehicles for protozoan infections in humans.

As protozoa do not multiply in the environment, extraction of the pathogens from the mollusk matrices is a key step, and detection methods need to be specific and sensitive enough to detect low loads of organisms. Distinct procedures depending on the mollusk species and/or the tissues have been described, with considerable variability in oocyst recovery. Usually, the first step consists in a tissue homogenization performed on pooled organs or hemolymph, or on whole organisms. This step is sometimes associated to trypsin digestion. Then, *T. gondii* oocysts are usually concentrated by centrifugation, which can be combined to a purification step by sucrose flotation. However, a widely accepted optimized remains to be defined.

The detection of *T. gondii* oocysts in mollusks is performed by molecular biology techniques (i.e., PCR, real-time PCR). These approaches are considered to be specific, rapid, and may provide information on genotypes of detected oocysts. In addition, they support simultaneous detection of different parasites.

Interest for alternative molecular methods such as loop-mediated Isothermal Amplification (LAMP) for detection of pathogens in complex matrices is growing. LAMP is a nucleic acid amplification method that amplifies double stranded DNA genomes under isothermal conditions. LAMP benefits from several advantages : i) the method does not require a costly thermos-cycler, ii) amplification can be completed in less than 1 hr, iii) it does not require labeled probes because amplifications can be monitored visually (turbidimetry), or staining by a fluorescent DNA-binding agent, and iv) it supports multiplexing. LAMP has been successfully compared to culture methods for the detection of food pathogens and the method proved to be robust enough to detect pathogens in complex food matrices. Concerning protozoa detection, LAMP is considered to have a lower detection limit than qPCR in blood, faeces, water or soil samples.

Considering the expertise of Dr Marco Lalle from Istituto Superiore di Sanita in Roma, on LAMP based assays to detect *T. gondii* oocysts in meat and fresh produce, we wishes to benefit from its knowledge to be able to implement LAMP assays in our laboratories. Then, first objective of the STSM is to develop a LAMP assay whatever the targeted microorganism and to implement LAMP. The second objective is to implement LAMP assays on artificially contaminated blue mussels and to evaluate the potential of LAMP as an alternative of real-time PCR to detect *T. gondii* oocysts in mollusks.

2. Description of the work carried out during STSM

First, LAMP assays have been run on DNAs extracted according to Actalia's protocols. Two DNA samples extracted from 10 000 *T. gondii* oocysts suspensions with Instagene and Nuclisens protocols have been analyzed including dilutions corresponding to 100 and 10 oocysts. Six DNAs extracted from three different matrices according to Actalia protocols have been also analyzed with LAMP. Then, considering the protocol proposed by Actalia, two types of samples have been tested: whole tissues and hemolymph from blue mussel freshly fished. Each sample have been prepared and divided in two equal parts. One part was not spiked and was considered as control. The second part was spiked with known quantities of *T. gondii* oocysts: 5 and 50 oocysts for hemolymph, 10 and 100 oocysts for tissues. Oocysts have been recovered from each sample using Actalia protocols. Briefly, hemolymph has been centrifuged and pellet containing oocysts have been washed with NaCl solution. Tissues have been digested in Trypsine 1X and filtered in a stomacher bag. Filtrate recovery has been centrifuged to recover oocysts. Pellets from hemolymph and tissues have been resuspended in buffer of Soil kit. DNA extraction has been realized according to adapted ISS protocol using FastPrep for Soil kit. All DNAs extracted from blue mussel assays have been analyzed with LAMP and qPCR.

3. Description of the main results obtained

The aim of the STSM was to evaluate the sensitivity and efficiency of the Loop-Mediated Isothermal Amplification (LAMP) to detect *T. gondii* genome as an alternative of real-time qPCR and to evaluate different DNA extraction procedures. First, DNA extracted by 10000 *T. gondii* oocysts suspensions using two kits in Actalia's lab, Instagen and Nuclisens, were diluted to reach a DNA content corresponding, approximately, to that of 100 and 10 oocysts and assayed with LAMP and qPCR. All of the DNAs were negatives with LAMP (Figure 1), excepted the dilution 10 from Instagene kit, but they were all positives with qPCR (result not shown), although with a high Cq value. This suggests that kits used for DNA extraction should be not suitable for the LAMP, either due to the presence of inhibitors or due to a low efficiency in DNA extraction, being below the detection limit of LAMP (100-10 fg) vs qPCR (10 fg). Moreover, six DNA samples extracted from three different matrices spiked with a large amount of *T. gondii* oocyst (10 000 and 5 000) according to Actalia protocol (i.e., Instagen and Nuclisens) were also negative with both LAMP and qPCR, excepted one sample (result not shown). These results should confirm the hypothesis mentioned above.

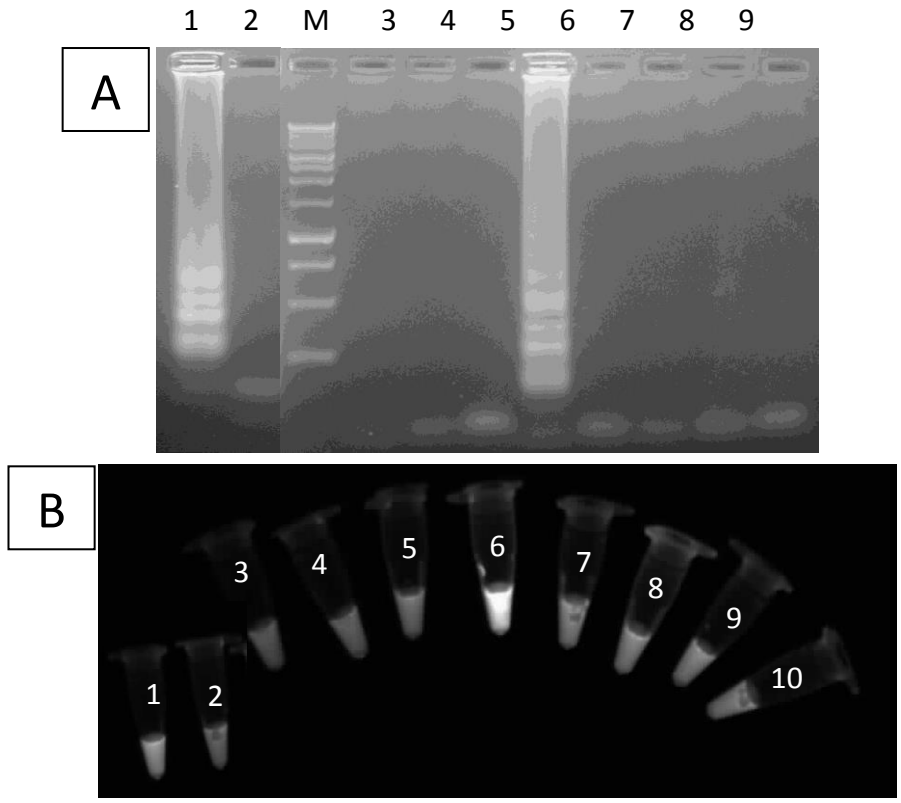

Figure 1: Evaluation of LAMP method on DNAs extracted with Instagene and Nuclisens protocol

(A) Electrophoretic analysis of the LAMP. (B) Visualization of amplification under UV-light with SYBR green 1/100 staining. Lane 1: Positive control (10 ng); lane 2 : negative control (water); lane M, 1 kb ladder used as size marker; lanes 3-4: DNA sample 1 extracted with Instagene corresponding to 100 and 10 oocysts respectively; lanes 5-6: DNA sample 2 extracted with Instagene corresponding to 100 and 10 oocysts respectively; lanes 7-8: DNA sample 1 extracted with Nuclisens corresponding to 100 and 10 oocysts respectively; lanes 9-10: DNA sample 2 extracted with Nuclisens corresponding to 100 and 10 oocysts respectively.

The second goal of the STSM was to implement LAMP assays on artificially contaminated blue mussels, from local market. Hemolymphs and tissues of mollusks were collected and spiked with known quantities of *T. gondii* oocysts: 5 (±1) and 50 with hemolymph, 10 (±1) and 100 with tissues. Unspiked samples were used as control to confirm the specificity of the assays. The recovery of oocysts from blue mussels was carried out according to Actalia protocols, with some modification. Then, DNA extraction was performed according to ISS protocol on pellets from tissues and hemolymphs samples using FastPrep for Soil kit. DNAs were analyzed with both LAMP and qPCR. All DNAs from contaminated hemolymph were positive with LAMP and qPCR, including those contaminated with the lowest amount of oocysts (Figure 2). Controls were negative. Same results were obtained with LAMP on DNA samples extracted from tissues. Hence, DNAs extracted from low spiked mussels with FastPrep and Soil kit were totally positive with LAMP but not always with qPCR (1 out of 2) whereas DNA samples extracted from high spiked mussels with Instagen and Nuclisens protocols were always negative with LAMP. This suggests that FastPrep is more suitable for LAMP assays including the low level of contamination than Instagen or Nuclisens protocols. LAMP seems

to be more sensitive (robust) than qPCR. This results should be confirmed a larger panel of samples. In summary, appropriate DNA extraction protocols allows to obtain positive results from high and low contaminated mussels with LAMP, in particular the FastPrep protocol with Soil kit. In addition, this molecular tool appears to be more sensitive than qPCR and these results should be confirmed with further experiments.

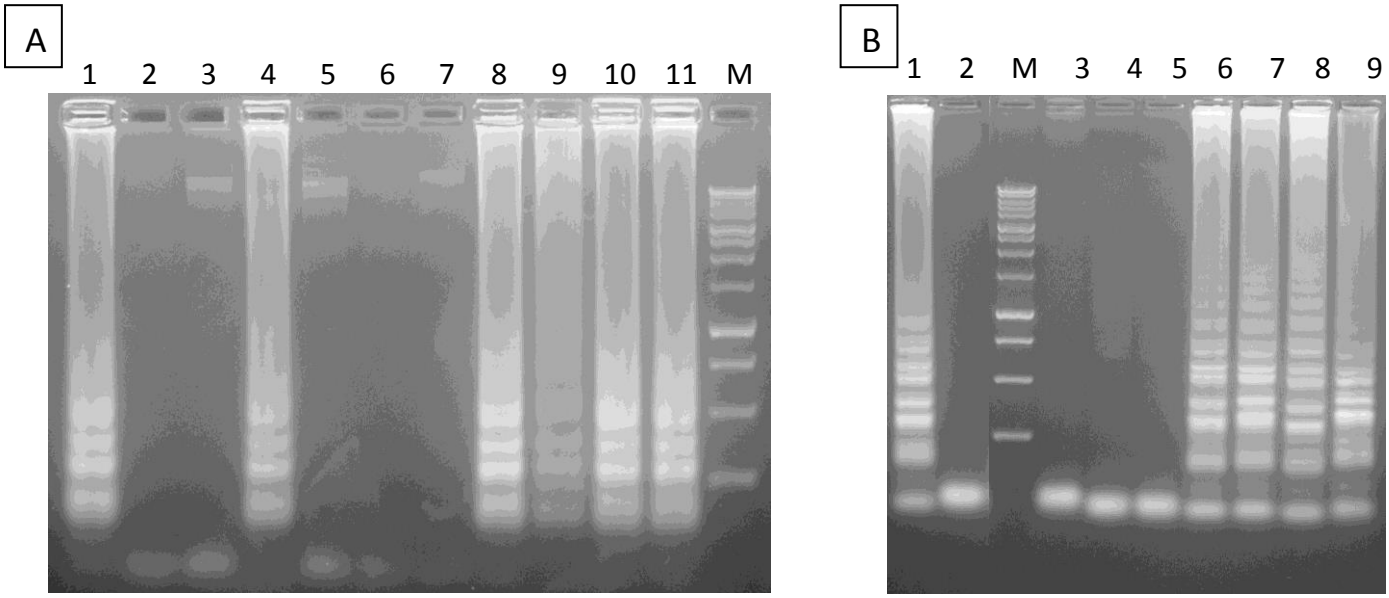

Figure 2: LAMP assays with DNAs extracted from hemolymph and tissue of mollusk

(**A**) Electrophoretic analysis of the LAMP on DNAs extracted from hemolymph. Lane 1 and 4: Positive control (10 ng); lane 2 and 3: negative control (water); lane 5-7, DNAs extracted from hemolymphs 3, 8, 15 without spikes, respectively; lanes 8-9, DNAs extracted from hemolymphs 3 and 8 spiked with 5 oocysts, respectively; lane 10-11, DNAs extracted from hemolymphs 15 and 8 spiked with 50 oocysts, respectively; lane M, 1 kb ladder used as size marker. (**B**) Electrophoretic analysis of the LAMP on DNAs extracted from tissue. Lane 1 : Positive control (10 ng); lane 2 : negative control (water); lane M, 1 kb ladder used as size marker; lane 3-5, DNAs extracted from tissues 5, 19, 20 without spikes, respectively; lane 6-7, DNAs extracted from tissues 19 and 5 spiked with 10 oocysts, respectively; lane 8-9, DNAs extracted from tissues 5 and 20 spiked with 100 oocysts, respectively.

4. Future collaboration possibilities with the host institution

Depending of the results of this mission, we want to collaborate and communicate with host institution to apply appropriated DNA extraction protocol from blue mussel.

5. Foreseen publications/articles to result from the STSM

The results obtained during this mission should be presented in the annual meeting within COST Action based on Roma in May 2017.