SHORT TERM SCIENTIFIC MISSION (STSM) – SCIENTIFIC REPORT

The STSM applicant submits this report for approval to the STSM coordinator

Action number: FA1408

STSM title: The use of molecular markers for viability assessment of foodborne parasites by RT-qPCR

STSM start and end date: 14/10/2018 to 26/10/2018

Host: Laboratory of Parasitology-Mycology, EA 7510 (Reims Champagne-Ardenne University)

Grantee name: Tamirat Temesgen

1. PURPOSE OF THE STSM

Foodborne illnesses are major public health concern throughout the world and pose a negative effect on the socio-economy. The contamination of food with these parasites results in the outbreaks of diseases among the consumers. Nowadays, it has become common to have outbreaks of cyclosporiasis, a foodborne disease caused by *Cyclospora cayetanensis*, every year in the U.S. As of October 1, 2018, 2,299 cases of cyclosporiasis were recorded in 33 different states between May–August (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2018/c-082318/index.html). The control of such outbreaks requires standard laboratory methods that could be used for source attribution by detecting the parasite from the food matrices.

Detection of a parasite from food matrices is not enough but needs to be assessed for its infectivity. Viability of the parasites is used as an indicator for infectivity although it is difficult to say all viable parasites are able to cause infection. Invitro tests such as vital dye inclusion/exclusion, sporulation and excystation tests are used for such purpose among others. The gold standard method for evaluating the infectivity is by using animal bioassay. However, it is expensive, ethically challenging, and time consuming. Therefore, it is highly needed to have an alternative to the animal bioassay.

Reverse transcription quantitative PCR (RT-qPCR) is a potential technique that could be exploited for assessing the viability of foodborne parasites. It is a variant of qPCR that works by forming a complementary DNA (cDNA) from mRNA, by the help of reverse transcriptase enzyme, and amplifies the cDNA. RT-qPCR is the most sensitive technique for quantification of mRNA levels, mostly used in gene expression studies. It has different applications in different fields and here we aim to use it for assessing the viability of selected foodborne parasites. The technique could be very useful for difficult to culture or non-culturable microorganisms, which is especially true for parasites. There has been attempts to develop a method based on RT-qPCR to assess the viability of bacteria (Martínez-Blanch et al., 2011) and parasites (Travaillé et al., 2016), and also yeasts. Given the importance of viability issue, RT-qPCR might develop into a useful method that could be potential alternative to the laborious animal bioassays.

The aim of the STSM was to acquire technical skills in the RT-qPCR and apply it for the development of a standard lab method for the assessment of viability of selected foodborne parasites.

2. DESCRIPTION OF THE WORK CARRIED OUT DURING THE STSM

2.1. Sample preparation

Parasite

Toxoplasma gondii (ME 49 type II strain collected in December 2016) stored in Sulfuric acid (2%) was used in the present STSM. The suspension of *T. gondii* was prepared by washing the sample with water twice. The concentration of the suspension was estimated by diluting it 1:10 in SDS (0.5%) from which 10 µL of the diluted suspension was transferred KOVA™ Glasstic™ Slide 10 with Grids and the oocysts were counted under microscope (400x magnification). Then five different concentrations (10^2, 10^3, 10^4, 10^5, and 10^6) of the oocysts were used for preparation of the calibration curve.

Spiking of the fresh produce samples

In the present study, basil and lamb's lettuce were spiked with about 1 million oocysts of *T. gondii*. About 30 g of the produce were weighed into a box and transferred to a stomacher bag. The suspension containing approximately 1 million oocysts (47.3 µL) was spread on a randomly selected four leafs of the basil and lamb's lettuce. It was then allowed to dry for 2 h at room temperature and put in stomacher bag. The bag is sealed and kept in the fridge overnight.

Washing of the produce

The produce were washed with 150 ml of 1M Glycine buffer (pH 5.5) by stomaching for 1 min. the wash solution was then transferred from the other side of the bag in to a falcon tube (200 mL) and the produce was then further rinsed by using 50 ml of the washing buffer. The tube was then centrifuged at 2500 rcf for 30 min at 15°C. The sediment was then submitted for nucleic acid extraction.

Another alternative tried in this study was by using 200 ml of 0.01% Tween-80 in PBS. Four samples were analysed by this solution, two of which was processed by stomaching and the other two were shaken at 200 rpm at 37 degree for 10 min. the eluates were then centrifuged at 3500 rcf for 30 min at 10 degrees. Following the concentration of sediments, two samples (one from each group) was submitted for RNA extraction by FastRNA Pro™ Soil-Direct Kit and the other two by Dynabeads™ mRNA DIRECT™.

Nucleic acid extraction

The gene targets in this STSM included SporoSAG and HSP 70 for the RT-qPCR as well as the repeat region of T. gondii for the qPCR. The expression of HSP 70 was induced by incubating the suspension of oocyst at 45 for 1 hour while the control sample with same number of oocysts was incubated at room temperature.

mRNA extraction

Commercially available kit (Dynabeads™ mRNA DIRECT™) was employed for the extraction of mRNA. After resuspending the pellet in 200 uL of lysis binding buffer, the samples underwent 6 cycles of freezing (-80) for 10 min and thaw (65) for 2.5 min to enhance oocyst wall disruption. The kit's instruction was followed for completing the protocol and the elution volume was 50 uL. The mRNA extract was then treated with DNase and stored at -80 until ready for analysis.

Total RNA extraction

Total RNA was extracted by using FastRNA Pro™ Soil-Direct Kit. The manufacturers instruction was followed appropriately. For this procedure, the disruption of the oocyst wall was facilitated by bead beating of the sample in a lysing matrix tube provided with the kit. The final elution volume was 100 uL. The eluate was DNase treated and stored at -80 until ready for analysis.

DNA extraction

DNA was also extracted for calculating the percentage viability of the oocysts in the suspension. For this purpose, the kit used was InstaGene ™ Matrix. The elution volume for the DNA was 90 uL and it was stored at -80 until ready for analysis.

Quality assessment of the RNA extracts

The quality of RNA extract was assessed by spectrophotometry (Pharmacia GenQuant). Gel electrophoresis was also used to evaluate the quality of the RNA extracts.

RT-qPCR and qPCR

The reaction conditions of the assay included a total reaction volume of 25 µL with 2 µL template. The primers and a probe used in this study and the set-up is summarized in the table 1. It was a one-step assay combining both reverse transcription and qPCR.

Table 1. Experimental conditions of the RT-qPCR and qPCR

Assay type	RT-qPCR		qPCR
Gene target	SporoSAG	HSP70	Repeat region
Fwd primer (5'→3')	CGGACAAATGTGGCGTACAC	CTTACCTCGGCAAGGAAGTG	AGAGACACCGGAATGCGATCT
Rev primer (5'→3')	GTGATCTTGCGCCGAACAC	TACCAGCATCCTTGGTAGCC	CCCTCTTCTCCACTCTTCAATTCT
Probe (5'→3')	TTCTCGTCAAAGCGGCACCACAGG	CCGTCATTACCGTTCCTGCGTACTTCA	ACGCTTTCCTCGTGGTGATGGCG
Primer conc.	0.4 µM	0.4 µM	0.4 µM
Probe conc.	0.2 µM	0.2 µM	0.2 µM
Thermal profile	50°C for 30 min 1× 95°C for 15 min 1× 95°C for 15 s and 60°C for 60 s 40×		95°C for 3 min 1× 95°C for 15 s and 60°C for 60 s 40×

The TaqMan probes were labelled with a fluorescent dye FAM at the 5' end and nonfluorescent dye BHQ1 at the 3' end.

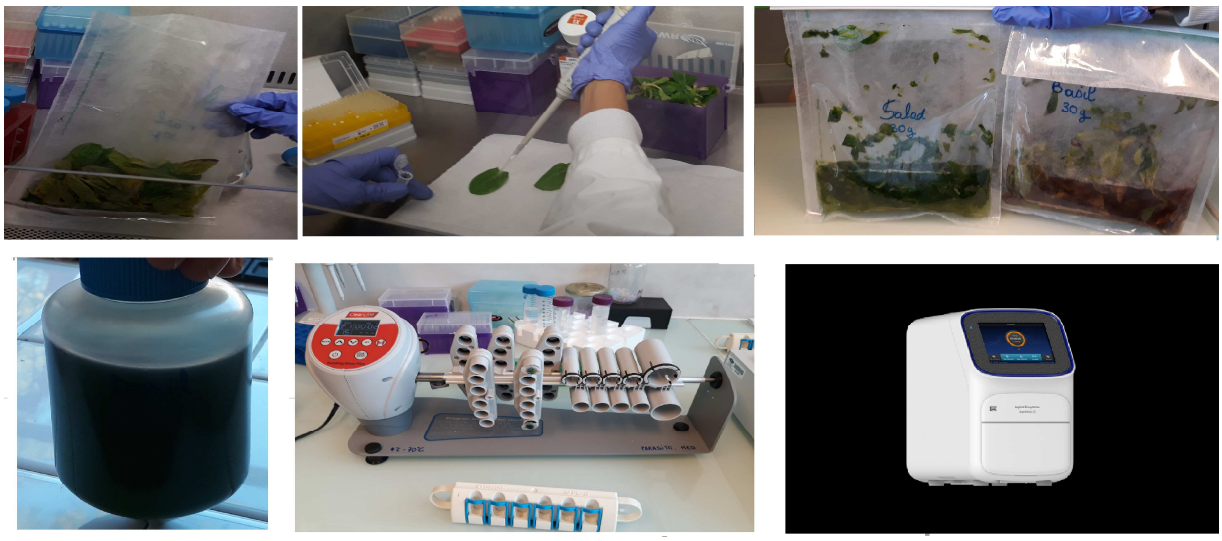


Figure. Pictorial representation of the RT-qPCR Protocol

3. DESCRIPTION OF THE MAIN RESULTS OBTAINED

In this section the main results of the RT-qPCR is presented. The quality of RNA extracted by using the FastRNA kit was evaluated by Spectrophotometry and gel-electrophoresis and the result showed that the purity of the extract was not good enough as shown in table 2 and figure 1. It seems that the purity of the RNA extract was better for the sample processed by stomaching as compared to shaking. But the total concentration of RNA was found to be higher in the sample processed by shaking.

Table 2. Summary of the Quality assessment of the RNA extract by using FastRNA Pro Soil Direct kit

Parameter	**Sample processing of the lamb's lettuce**	
	Stomaching	Shaking
A260 (AU)	0.489	0.693
A280 (AU)	0.319	0.488
A260/280	1.5	1.4
Purity (%)	85	78
RNA conc. (µg/mL)	39.2	55.4

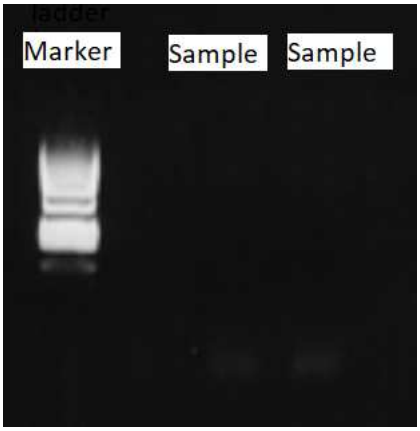


Figure 1. Gel electrophoresis of the RNA extracts

The calibration curve prepared from the suspensions showed that there was a good linearity, explained by the coefficient of determination (r^2 0.994). However, the efficiency was out of an acceptable range (Eff. 141% calculated from the slope) (Figure 2).

It is agreed that the efficiency of an ideal assay should be in the range of 90-110%. The efficiency obtained in the present assay might indicate there was inhibition. It could also be attributed to variations during RNA extraction because the calibration curve was not done by diluting the stock RNA rather by diluting the oocysts just from the very beginning.

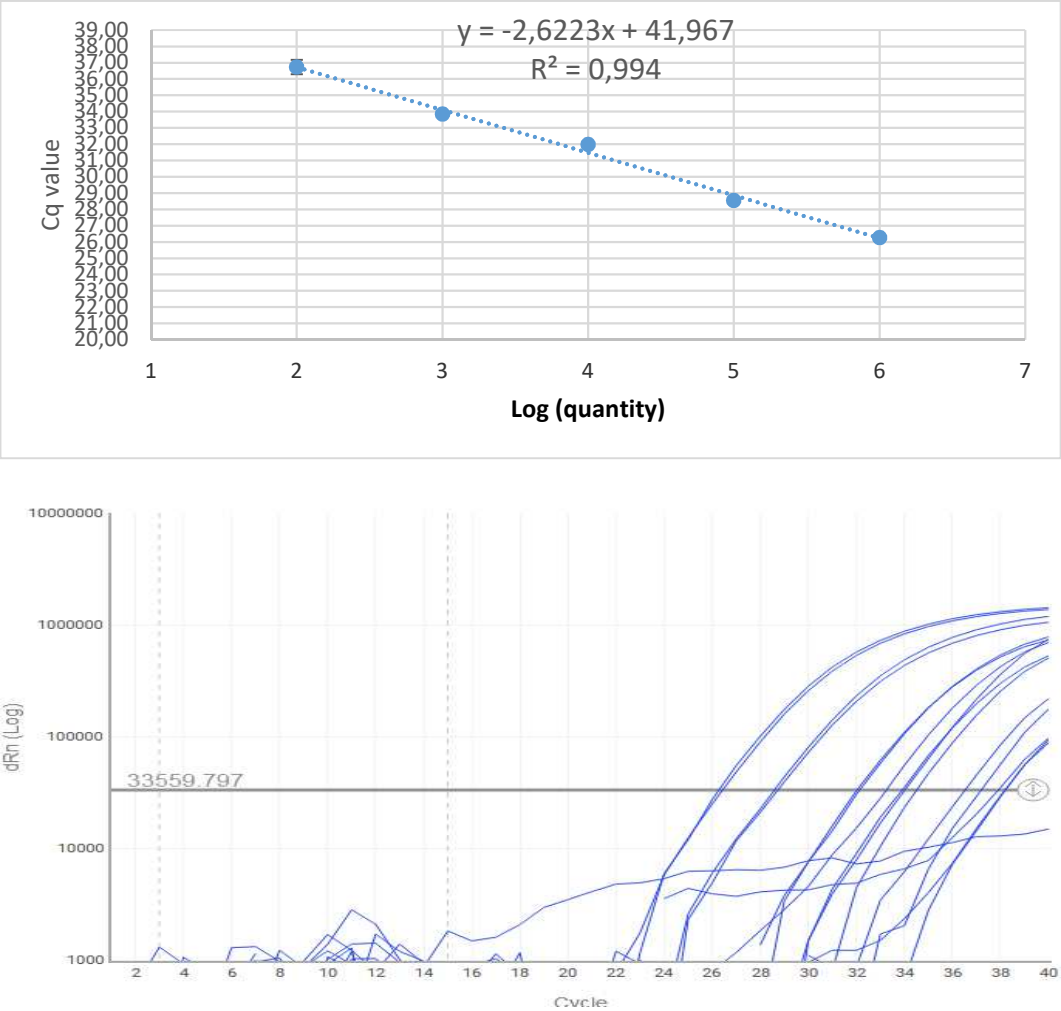


Figure 2. Calibration curve (top) and amplification plot (bottom) prepared from the suspension by the RT-qPCR assay

The spikes of basil and lamb's lettuce were also analysed with the RT-qPCR assay and the result showed that there was amplification from the 10 fold diluted template for both basil and lettuce. However, the standard deviation of Cq for the Lamb's lettuce replicate was too

In general, it was noted that the RT-qPCR results were difficult to reproduce, i.e. repeated measurements of the same samples resulted in different results (sometimes positive and the other time no amplification).

Table 3. RT-qPCR results of the spiked basil and lamb's lettuce

Sample	Adjusted Cq	Mean Cq	Deviation
Lamb's lettuce 1/10	34,76	32,94	2,58
Lamb's lettuce 1/10	31,11		
basil 1/10	34,74	34,57	0,24
basil 1/10	34,40		

The qPCR for the DNA isolates from the oocyst suspension also showed an inefficient reaction that seems there was inhibition. It followed that the highest concentration (6 log) template showed higher Cq than the template of 5 Log concentration. As a result, the 6-log point was excluded from the calibration curve, yet the efficiency of the assay was not within the acceptable limit (140%) (Figure 3). Again, the inefficiency could be likely due to the way the calibration points are prepared, i.e. dilution of oocysts rather than the DNA template. This means that it is not necessarly the inefficiency of the qPCR assay rather could also be due to DNA extraction inefficiency.

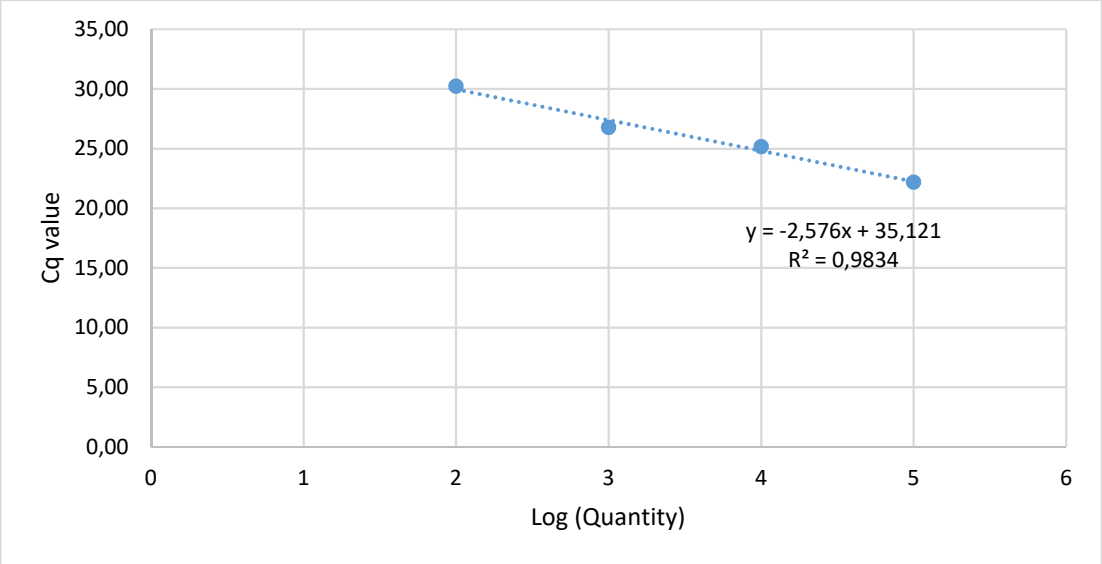


Figure 3. Calibration curve prepared from the suspension by qPCR targeting the repeat region.

The effort to calculate the percentage viability of the oocysts based on the ratio of the estimates of oocyst number in the RT-qPCR to the qPCR assay was not successful due to the poor estimates of the regression parameters. As it is shown in table 4 the percentage viability calculate was over 100% for each test-which is not sensible.

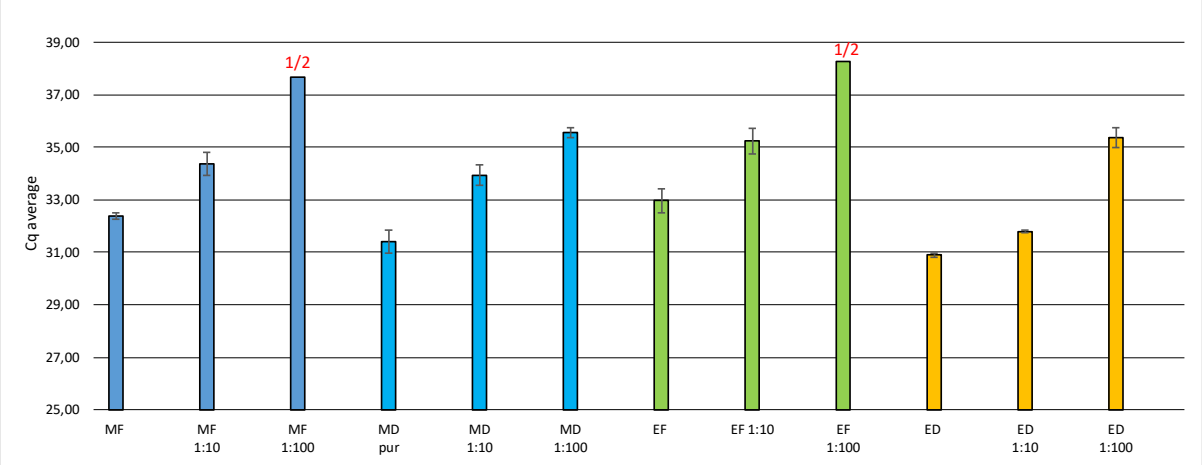
Table 4. Summary of the percentage viability estimate of the Oocysts suspension

No. of oocysts in each tube	Percentage viability
100 000	175 %
10 000	121 %
1 000	102 %
100	177 %

Based on the findings in this study, I suggest that calibration curves should be prepared by dilution of the nucleic acid templates. This is because the parameters from regression estimates (slope and intercept) are very crucial in their use for estimating the percent viability of the oocysts. This means that the accuracy of our results are dependent on these parameters.

Comparison of two washing and RNA extraction protocols

The snap comparative test between two washing methods and two RNA extraction methods showed an interesting result. In this STSM the simple shaking of the lamb's lettuce soaked with 200 ml of 0.01% Tween-80 in PBS on the shaker at 200 rpm for 10 minutes combined with the Dynabeads RNA extraction kit showed a better result as compared to the rest of combination trials (Figure 4).



Key: MF- Stomaching + FastPrep; MD- Stomaching + Dynabeads; ED- Shaking +Dynabeads; EF-Shaking + FastPrep

Figure 4. Comparison four combinations of the washing protocols and RNA extraction protocols.

In this comparison, it could be seen that the Dynabeads extraction protocol is better than the FastPrep protocol in the combinations tested. It seems also that Kits showed different performance when combined with different washing protocols. The dynabeads seems to work better with shaking not stomaching while the FastPrep works better with stomaching not shaking. However, we cannot make definitive conclusion from such snap tests. Therefore, I recommend the lab to plan a well-designed experiment to test this interesting point. Such studies would benefit from applying multifactorial experimental design.

HSP70 as a target for the RT-qPCR

In the present STSM, an alternative target for the RT-qPCR was also tested by using the HSP70. The findings of the study showed that there was no significant difference in Cq between the induced and control samples (Figure 5). It is not clear if the heat shock was not sufficient, this might need further tests with different options of temperature and duration.

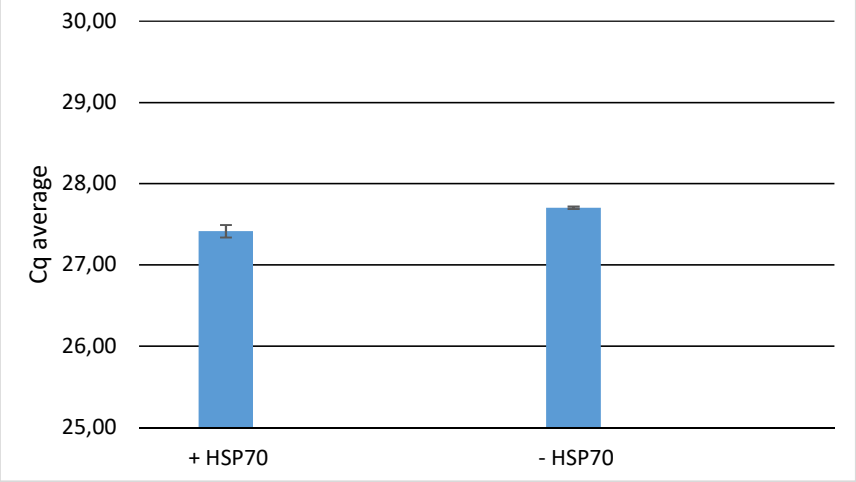


Figure 5. Induced HSP 70 (heat shock 45 for 1h) and control sample.

A rough comparison of the sensitivity of the two targets tested (SporoSAG and HSP70) in this STSM indicated that there was no huge difference but SporoSAG had lower Cq (26 vs 27.4).

FUTURE COLLABORATIONS

The present STSM would be a baseline for future collaboration because it would be very easy to communicate and share ideas. Such collaborations would avoid duplication of efforts and contribute to the efficient use of resources.

Acknowledgements

I would like to thank the host institute, Laboratory of Parasitology-Mycology, EA 7510 (Reims Champagne-Ardenne University) for accepting my proposal. I would like to say thank you to all who contributed to this achievement, Prof. Isabelle, Dr. Stephanie, Dr. Sandie, and Angelique.