COST STSM SCIENTIFIC REPORT

1. Aim and objectives of the STSM

Toxoplasma gondii is an intracellular parasite capable of infecting animals, humans, and being of the most prevalent parasites worldwide. Infected pig meat is a source of *T. gondii* infection for humans and animals in many countries. Can be transmitted to humans by the ingestion of contaminated fresh or processed foods. Moreover, can be transmitted through the consumption of raw meat or meat which have suffered a minimal thermal process or by consumption of meat products.

T. gondii is able to cause infections with severe symptoms, especially in immunocompromised patients (Tenter et al., 2000). While studies indicate a relatively high rate of infection in farm animals, the infection is subclinical, with the exception of sheep which is associated with neonatal abortion and sickness (Iovu et al., 2008). This parasite also causes mortality in pigs, especially neonatal pigs. Most pigs acquire *T. gondii* infection postnatal by ingestion of oocysts from contaminated environment or ingestion of infected tissues of animals (Dubey et al., 2009). The aim of this study was to evaluate the presence of *T. gondii*, to identify associated risk factors and to isolate *T. gondii* from slaughtered pigs in Spain. The main objective was to harmonize the methodology for the detection and characterization of *T. gondii.*

2. Description of the work undertaken

The work had been undertaken in the Universidad de Burgos (Burgos, Spain), where my work has been supervised by Prof. Dr. David Rodríguez-Lázaro.

Sera sample collection. A total of 976 samples from slaughterhouses where collected from 21st July 2015 to 29th October 2015 from all over Spain (241 sera, 244 muscle -diaphragm pillars-, 245 feaces and 246 liver). There have been pigs slaughtered in 20 different abattoirs and reared in 209 farms from 29 different provinces of the total 52 in Spain. All pigs were raised for meat production.

The animals were sampled at the slaughter line, during thoracic stick exsanguinations, by members of the research team who collected the samples. The samples were kept in a freezer at -20°C until use.

ELISA test. Sera samples collected at the slaughter line were examined for *T. gondii* antibodies. *T. gondii*-specific IgG antibodies were detected by commercial ELISA kit (PrioCHECK® Toxoplasma Ab SR). The kit includes ELISA plates coated with cell culture derived *T. gondii* tachyzoite-antigen, a peroxidase-labelled anti-small ruminant secondary antibody, tetramethyl benzidine (TMB) as a chromogenic substrate, control sera and buffer solutions. Sera were diluted starting at 1:25. Positive and negative controls were included in each run. Sera reactive at ≥ 1:25 were considered positive.

DNA extraction. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen). The protocol was performed using 250 mg of tissue disrupted by zirconia beads in a fast Prep instrument. PCR was performed with extract called SV-PCR (small volume). DNA was eluted with 100 µL of elution solution. After DNA extraction, samples were kept at +4°C for up to 48 h or frozen at −35°C for use within the week.

Detection by real-time PCR. SYBR® Green was used to detect fluorescence in real-time PCR using the Real-Time PCR System (Applied Biosystems). The primer used (Invitrogen) amplified a 529 bp region that is repeated 200-300 times in the genome of the parasite. For the assay, each reaction mix had a final volume of 20 µL and contained 10 µL of SYBR Green PCR Master Mix (Applied Biosystems), 1.5 µL of each primer at a concentration of 10 pmol/µL, 5 µL of DNA and 2 µL of sterile distilled water. The reaction was carried out as follows: 5´ hot start at

95°C, 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1.5´, followed by a final extension for 7´ at 72°C. Every sample was tested in duplicate. Absence of inhibitors was checked for each well by using an internal positive control.

Primers (Edvinsson et al., 2006)	5'CACAGAAGGGACAGAAGT3'
	5'TCGCCTTCATCTACAGTC3'

Microsatellite analysis. Genotyping was carried out using standard protocols as described by Ajzenberg et al. (2002). For each microsatellite, primers were designed to amplify sequences with a size of less than 200 nucleotides and containing the microsatellite. The forward primers were 5´end labeled with fluorescein (6-FAM or HEX) to allow sizing of PCR products with an automatic sequencer.

Table 1. Microsatellite markers, PCR primers and allelic polymorphism

Marker	Definition	Repeat sequence	PCR primers 5´-3´	Number of alleles	Size range of alleles (bp)
TUB 2	Beta-tubulin gene of TgRH tachyzoite	$(TG)_8$	1 **HEX**-CCAAGTTCTTCCGTCATTTC 2 CCTCATTGTAGAACACATTGAT	3	122–126
TgM-A	Myosin A gene of TgRH tachyzoite	$(TG)_9$	1 **6-FAM**-CATGTCCCTGTCGGTTTCTC 2 CGTAAATGCGGATGGAAACT	4	115–121
W35487[b]	TgESTzy77d12.r1 TgRH tachyzoite cDNA	$(CT)_{10}$	1 **6-FAM**-TGCTGCGGTCTTTTCTCTTC 2 AACATGCCGTTCCCTTCC	3	5–101
N60608[b]	TgESTzy20b09.r1 TgRH tachyzoite cDNA	$(TA)_{13}$	1 **6-FAM**-GAATCGTCGAGGTGCTATCC 2 AACGGTTGACCTGTGGCGAGT	7	131–145
N82375[b]	TgESTzy52d03.r1 TgRH tachyzoite cDNA	$(TA)_{13}$	1 **6-FAM**-TGCGTGCTTGTCAGAGTTC 2 GCGTCCTTGACATGCACAT	10	107–131
N83021[b]	TgESTzy58c09.r1 TgRH tachyzoite cDNA	$(TA)_{11}$	1 **6-FAM**-ACAACGACACCGCTATCTC 2 CTCTCTATACACAGACCGATTGG	10	125–161
N61191[b]	TgESTzy27f04.r1 TgRH tachyzoite cDNA	$(TA)_{11}$	1 **6-FAM**-CCGTATCACCAGATCATGTT 2 CTCTCACCTGATGTTGATGTAA	11	120–160
AA519150[b]	TgESTzz34e04.r1 TgME49 bradyzoite cDNA	$(TA)_{13}$	1 **HEX**-GTTGTCTATGCTGTCGTGCG 2 CACCCATAAACGGTTACTGGTC	16	134–170

[a]Number of dinucleotide repeats as published in GenBank for the corresponding strain.
[b]GenBank accession number.

The amplification reaction mixture consisted of 10 mM Tris–HCl , 50 mM KCl, 1.5 mM $MgCl_2$ solution (Applied), 0.2 mM dATP, dCTP and dGTP (Roche), 0.4 mM dUTP (Roche), 4 pmol of each primer, 5% (vol./vol.) dimethyl sulfoxide, 0.6 U Fast Start *Taq* DNA polymerase (Roche), 0.2 U uracyl DNA glycosylase (Roche) and 2.4 ml DNA in a 20 mL reaction volume. The PCR conditions were as followed: 2´ min at 50°C for uracyl DNA glycosylase action, 3´ at 94°C for initial denaturation, 35 cycles of denaturation at 94°C, annealing at 52°C for the *TgM-A* gene, 54°C for the *TUB2* gene, and for *AA519150*, *W35487*, *N61191*, *N82375* and *N83021*, and at 55°C for EST *N60608*, and extension at 72°C for 30 s each. The final cycle was followed by an additional 10´ at 72°C.

After this, PCR products were first separated by electrophoresis in 1.5% agarose gel. One microlitre of each PCR product was mixed with 0.5 mL of the red dye labelled GeneScan size standard ROX350 (Applied Biosystems) and 24.5 mL of deionised formamide. This mixture was then denatured and run on a polyacrylamide gel POP4 (Applied Biosystems) in a 47 cm/50 mm capillary for genetic analysis. Signals were read with an automatic sequencer (Applied Biosystems).

3. Main results

At this moment, we have only detected by anti-*T. gondii* antibodies in the pig sera one positive sample and the result was consistent with the detection by PCR (Figure 1). The commercial ELISA kit evaluated in this study could represent a useful tool for surveillance and reporting system for *T. gondii* in pig populations at the farm level or for diagnosis at the slaughterhouse, contributing to the control of this widespread zoonosis. However, not all the samples were finished and preliminary results are being evaluated. Cultures of oocysts are being carried out but the experiment did not worked properly.

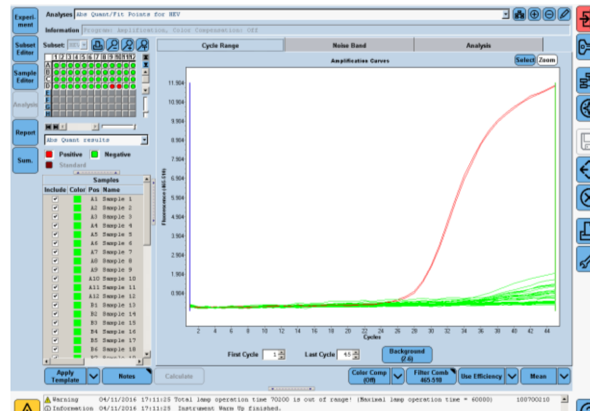

Figure 1. Amplification of DNA samples by real-time PCR. Analyses were performed by real-time PCR targeting 529 bp region

Regarding the typing of this positive sample (Figure 2), the PCR product consisted of a single peak of fluorescence after analysis by GeneScan software. Some additional peaks, due to strand slippage of *Taq* polymerase on microsatellite sequence, were sometimes present, but their fluorescence intensity was always much less important than the main peak. This main peak corresponding to the length of the PCR product was assigned to an allele.

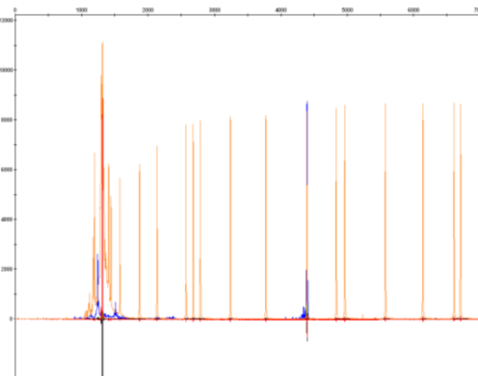

Figure 2. Data analyzed by GeneScan software after electrophoresis on an automated sequencer of PCR amplified DNA

This study wants to bring in evidence, primarily seroprevalence of *T. gondii* infection in pig slaughtered for human consumption and the existence of risk for human contamination through the consumption of raw meat. Human infection with *T. gondii* following meat consumption is very frequently, and already have been demonstrated that

the consumption of sheep meat prepared improperly, is an increased risk of infection transmission to people with tissue cysts (Iovu et al., 2008).

4. Future collaboration possibilities with the host institution

This STSM has provided me new opportunities to study in collaboration with the host institution, Universidad de Burgos. Closer contact between research groups working at Dunarea de Jos University of Galati and Universidad de Burgos have been established, thanks to this STSM. During my stay, I have developed the skills necessary for detection and genotyping of *T. gondii*. The researcher group from Universidad de Burgos performed a serological screening on a large number of pig samples, and with the same ELISA kit, retested the doubtful sera by indirect immunoflorescence. Due to the lack of time to repeat the doubtful results, the analysis is still ongoing.

5. Future plans, including potential future publications

The data collection is ongoing. After completion of this STSM and data gathering, all the knowledge acquired in the host institution will strongly help me in my home institution with our future researches. Moreover, research findings are planned to be published in an international journal or presented in an international conference.

Acknowledgements

I would like to express my sincerely appreciation to my professors Prof. Dr. David Rodríguez-Lázaro for this opportunity and encouragement attending this STSM, as well as support during the STSM. And very special thanks to Dr. Marta Hernández who was also taking care of me, showing me her kind hospitality and helpfulness.

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