SHORT TERM SCIENTIFIC MISSION (STSM) – SCIENTIFIC REPORT

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

Action number: FA1408
STSM title: Comparison of serological methods to detect antibodies specific to *Toxoplasma gondii* in bovine sera
STSM start and end date: 05/04/2018 to 16/04/2018
Grantee name: Helga Waap

PURPOSE OF THE STSM

Toxoplasmosis is caused by *Toxoplasma gondii*, a highly successful apicomplexan protozoan capable of infecting all warm-blooded animals. *T. gondii* is one of the most common zoonotic parasites worldwide and nearly 1/3 of the human population has been exposed. Cats and other felids are the only known definitive hosts. Toxoplasmosis is an important abortive pathogen in sheep and goats, causing significant economic losses to the livestock industry. In humans, infection may cause abortion or serious congenital disease, fatal disease in immunocompromised individuals, as well as ocular and neurocognitive disorders in immunocompetent hosts. Human infection occurs mainly by the oral route: either by ingestion of oocysts excreted into the environment by cats and other felids, or by ingestion of tissue cysts contained in meat products which are consumed raw or undercooked. A multicenter study carried out in Europe estimated that 30-60% of seroconversions in pregnant women were linked to the consumption of raw or undercooked meat while 6-7% were attributable to soil contact. The sources of infection are therefore multiple and the risk factors still poorly identified. Risk control and management require precise identification of the sources of the parasite. Though meat-producing animals like pigs, sheep and goats are most often association to transmission, several authors demonstrated the presence of *T. gondii* tissue cysts in cattle meat. Serological data for cattle are available from many parts of the world, especially from Europe, including, France, Spain, Switzerland, the Czech Republic and the Netherlands, with infection rates ranging between (7.8 and 83.3%). Also, there is evidence for transplacental transmission in cattle and the potential of *T. gondii* as a bovine abortive pathogen. Depending on cultural and individual food habits, bovine meat is frequently served raw or undercooked in many traditional dishes such as steak tartar, carpaccio or rare steak. Though the potential risk associated to the consumption of these meat products is generally acquainted, the role of bovines in the transmission of *T. gondii* to humans remains uncertain. The absence of validated diagnostic tools to assess *T. gondii* infection in cattle is a major gap in the comprehension of disease transmission. Ideally, all tests should be validated for the species for which they are intended.

Though several serological tests, including the Modified Agglutination Test (MAT), Immunofluorescent Antibody Test (IFAT), Enzyme Linked Immunosorbent assay (ELISA), and Western blot, were developed to assess *T. gondii* infection in different animal species, so far, few attempts were made to analyze and compare their performance in cattle. Therefore, evaluation of performance characteristics of available tests would strongly contribute to a more critical interpretation of previous studies and to the implementation of adequate surveys to determine the true serological prevalence of infection or exposure in cattle. This will ultimately facilitate epidemiological risk factor analysis and contribute to the definition of disease control measures. The aim of this study was to evaluate the diagnostic performance of the in-house p30 enzyme-linked immunosorbent assay (p30-ELISA) and an in-house p30 Western blot and a whole antigen Western blot using a panel of well-characterized sera from naturally exposed cattle in Portugal.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

Serological tests were performed using 616 cattle sera randomly selected from a bovine serum bank available at the National Institute for Veterinary and Agrarian Research (INIAV), Portugal. Blood samples were obtained between 2012 and 2013 from several farms in different geographical areas in Portugal. Samples were collected in the frame of the national plan for the eradication of brucellosis and kindly made available by the national veterinary authority DGAV.

All sera were tested with the in house *T. gondii* p30-ELISA utilized at the Friedrich Loeffler Institut. Briefly, the wells of 96-well Polysorb ELISA plates (Nunc) were coated with affinity-purified native *T. gondii* p30 antigen and incubated for 1 hour at 37ºC. Plates were washed and blocked with 1% casein in PBS-0.05% Tween (PBS-T) during 30 minutes at 37ºC. Sera and positive and negative controls were diluted 1:100 in the blocking buffer and distributed in duplicates on plates. Positive and negative controls were included in each plate in each testing round. After 30 minutes incubation, the plates were washed with PBS-T and developed with Peroxidase-conjugated AffiniPure Rabbit Anti-Bovine IgG [H+L] during 30 minutes at 37ºC. The reaction was revealed for 15 minutes at 37ºC with TMB substrate solution. The colorimetric reaction was stopped with 2M H_2SO_4. Optical densities were read at 450 nm on a microplate reader. Index values for each sample were determined using the following formula: average sample OD (S) minus average negative control OD (N) divided by the average positive control OD (P) minus the average negative control OD (S – N)/(P – N).

Preliminary cut-off points for positive and equivocal sera were determined corresponding to the 95^{th}, 90^{th} and 85^{th} percentiles of results. A subpanel of sera comprising all samples with positive and equivocal results, as well as a randomly selected number of negative sera was assayed by *T. gondii* whole antigen and p30 protein Western blot for comparison of results. In addition, in order to assess potential cross-reactivity with *Neospora caninum*, the sub-panel of sera was tested by the in house p38 *N. caninum* ELISA.

The Western blot was carried out as follows: briefly, 100µl of the antigen suspension containing 2 x 10^8 tachyzoites or p30 protein were loaded on 12.5% polyacrylamide gels. Proteins were separated by vertical gel electrophoresis and electrotransferred to PVDF membranes. Whole antigen or p30 protein membrane

strips were placed into Western blot trays, soaked in blocking buffer and added with sera and controls to obtain a dilution of 1:100. After 1 hour, membrane strips were washed with PBS-T and incubated with Peroxidase conjugated AffiniPure Rabbit Anti-Bovine IgG [H+L]. After washing, membranes were developed in chloro-1-naphthol substrate solution. The reaction was stopped when immunodominant antigens were clearly visible.

N. caninum ELISA plates were coated with p38 protein and blocked with horse serum. The reaction was developed with Monoclonal anti-bovine IgG Clone BG-18 Biotin Conjugate diluted 1:2000. The colorimetric reaction was revealed with Extravidin® Peroxidase Conjugate and stopped with 2M H_2SO_4. Washing procedures and incubation steps were similar to the *T. gondii* ELISA.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

Geographical origin, as well as animal and farm data were retrieved for each sample. Sera were from a total of 37 herds located in 32 civil parishes belonging to 24 municipalities in the NUTS2 regions Norte (n=261), Centro (n=149), Lisboa (n=20) and Alentejo (n=186). The number of animals assayed per farm ranged between 1 and 24 (mean=16.6). The mean herd size was 157.1 and ranged between 9 and 540 animals. The mean age of animals was 5.7 years and ranged between 9 months and 20 years. Six hundred and eleven sera were from female and 5 from male cattle. All animals included in the present analysis had been previously tested by the modified agglutination test for the closely related apicomplexan parasite *B. besnoiti* and were serologically negative.

A preliminary cut-off for seropositivity was set at the 95th percentile, whereas the 90th and 85th percentile were selected to define two threshold levels for equivocal sera. Sera with index values <0.108, 0.108 - 0.207, >0.207 - 0.466 and >0.466 were classified as negative, equivocal 2, equivocal 1 and positive, respectively.

Of the 616 animal tested by the *T. gondii* p30 ELISA, 31 (5%) were positive, 31 (5%) were classified as equivocal 1 and 31 (5%) as equivocal 2. At least one positive animal was found in 14 of the 37 farms tested (37.8%). Further 5 farms (13.5%) had animals classified as equivocal 1 and 2, and 2 farms (5.4%) had animals classified as equivocal 2 only. Among positive farms, 12 (85.7%) also had animals classified either as equivocal 1 or 2 or both.

In the absence of a reference standard, agreement between the *T. gondii* p30 ELISA and *T. gondii* p30 Western blot for the subpanel of sera (n=121) was assessed using the following measures: overall agreement, positive percent agreement, negative percent agreement and Cohen's Kappa. Considering the different cut-off levels, the following results were obtained:

- 95th percentile cut-off: overall percent agreement = 52.9%; PPA = 27.6%; NPA = 76.2%, k = 0.04
- 90th percentile cut-off: overall percent agreement = 70.2%; PPA = 72.4%; NPA = 68.2%, k = 0.41
- 85th percentile cut-off: overall percent agreement = 71.1%; PPA = 96.6%; NPA = 47.6%, k = 0.43

Comparison of the *T. gondii* p30 ELISA with whole antigen Western blot was inconclusive, due to a high variability in the pattern of immunodominant antigens; therefore, further details of this analysis were not evaluated.

Testing of the subpanel of sera by the *N. caninum* p38-ELISA, gave 13 positive (10.7%) and 3 (2.5%) ambiguous results. Eleven animals with positive and 2 animals with ambiguous result in the p38-ELISA were also positive or equivocal for *T. gondii*. The presence of specific antibodies to *T. gondii* was confirmed by the *T. gondii* p30 Western blot for 11 of the sera.

FUTURE COLLABORATIONS (if applicable)

The results of the study will be reassessed and further exploited. The panel of sera will be analyzed by the immunofluorescent antibody test (IFAT) at INIAV and results compared with the outcomes of the present work. The study should allow a joint publication and/or presentation on a scientific meeting.

The study will allow further collaboration related to the study of *T. gondii* infection in cattle and other animal species (e.g. in risk factor studies or studies on the prevalence of viable *T. gondii*).