



Deliverable D-JRP- TOXOSOURCES-WP4.1

**SOP for a novel source-
attributing serological
method**

**Work package 4 of
JRP22-FBZ4.1-
TOXOSOURCES**

Responsible Partners:
UCM, ISS, RKI, SSI



GENERAL INFORMATION

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Authors	Gema Álvarez García (UCM), Luis M. Ortega Mora (UCM), Nadia Mª López Ureña (UCM), Furio Spano (ISS), Frank Seeber (RKI), Břetislav Koudela (VETUNI Brno), Rebecca Davidson (NVI), Radu Blaga (ANSES) and Pikka Jokelainen (SSI).
Other contributors	TOXOSOURCES consortium
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D-JRP-TOXOSOURCES-WP4.1

SOP FOR A NOVEL SOURCE-ATTRIBUTING SEROLOGICAL METHOD

BACKGROUND

This is a public deliverable of One Health EJP Joint Research Project:

JRP22-FBZ4.1-TOXOSOURCES – *Toxoplasma gondii* sources quantified

(<https://onehealthjep.eu/jrp-toxosources/>);

Work Package:

JRP-TOXOSOURCES-WP4 Serology method based on novel antigens to discriminate *T. gondii* infections acquired from oocysts

Task:

JRP-TOXOSOURCES-WP4-T2 Development of a novel stage-specific antigen-based ELISA to diagnose oocyst- and bradyzoite-driven *T. gondii* infections

Project Leader: Pikka Jokelainen, SSI; Deputy Project Leader: Joke van der Giessen, RIVM.

WP Leader: Furio Spano, ISS; Deputy WP Leader: Frank Seeber, RKI.

Task Leader: Gema Álvarez García, UCM; Deputy Task Leader: Luis Miguel Ortega Mora, UCM.

Contacts: Pikka Jokelainen, PIJO@ssi.dk; Gema Álvarez García, gemaga@ucm.es; Luis Miguel Ortega Mora, luis.ortega@ucm.es; Furio Spano, furio.spano@iss.it; Frank Seeber, seeber@staff.uni-marburg.de

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 WP4 explores serology for source-attribution of *T. gondii* infections.

Objectives of TOXOSOURCES WP4:

- ✓ To identify *T. gondii* antigens with source-attributing potential (proteins of interest, POIs)
- ✓ To explore serological methods that discriminate oocyst- from tissue cyst-driven infections
- ✓ To estimate the prevalence of oocyst-driven infections in humans and animals

As part of the work done in TOXOSOURCES WP4.T2, an exhaustive selection of reference sera panel from livestock (pig and sheep) has been carried out in the consortium. These panels of sera came from experimental infections carried out in pigs and sheep by several participant groups in the consortium (UCM, ANSES and VETUNI Brno) prior to the beginning of the project. The experimental groups comprised animals infected with different doses and strains of *T. gondii*, and the infective stages used were oocysts or tissue cysts. The sera were characterized by a wide range of in-house and commercially available serological tests (ELISAs, indirect immunofluorescence test, Western blot) that contributed to harmonization of the serological tools employed for samples from pigs and sheep. Reference sera from these panels were selected for the screening of proteins of interest (POIs) from WP4.T1, provided by ISS and RKI. These POIs were either oocyst wall- or sporozoite-specific proteins. Finally, a screening pipeline composed of sequential steps was followed and two out of 15 screened POIs became selected as preliminary promising candidates to develop an ELISA test. Both selected POIs proved to be putative oocyst markers when tested with sera from experimental infections.

This Deliverable reports on the work done and highlights the key achievements of the process.



PURPOSE

The purpose of this work is to develop a source-attributing serological method that can be applied to estimate the proportion of oocyst-driven infections in humans and animals used for human consumption.

Outcome is a SOP of the method that can be applied in pilot studies.

EXPERIMENTAL WORK AND EVALUATION OF THE KEY STEPS OF THE METHOD

A workflow (Fig. 1) was formulated and published (Alvarez-García et al., 2021), and followed in this work.

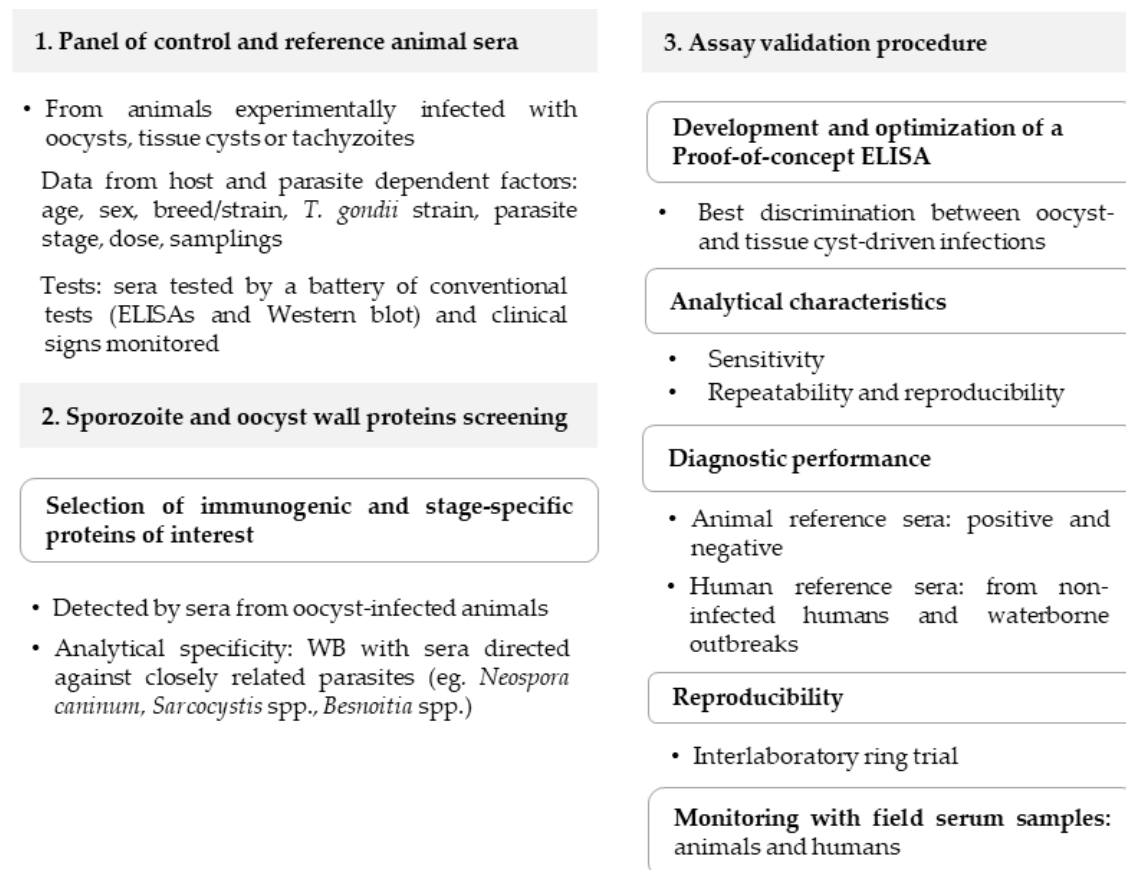


Figure 1: Workflow to develop an assay to differentiate oocyst-driven infections vs. tissue cyst-driven infections from a One Health perspective and according to OIE guidelines (Álvarez-García et al., 2021).



Panel of control sera

Sera from experimentally infected pigs and sheep were analyzed for selection as control sera. The following data were recorded: age, sex, breed/strain, *T. gondii* strain, parasite stage, dose, and sampling details.

Pig sera

First, sera from three different experimental infection studies carried out in pigs were characterized by a range of serological tests: three commercial ELISAs (IDScreen®, PrioCHECK™ and Pigtype®), an in house ELISA (TgSALUVET ELISA 2.0), a Western Blot (TgSALUVET WB) and an IFAT. The different experimental groups included pigs infected with tissue cysts, pigs infected with oocysts and non-infected animals. The experimental infections differed in the parasite dose and *T. gondii* strains employed. The comparative study evidenced differences in the diagnostic performance among tests and the need of harmonizing the serological techniques to obtain comparable and reliable results.

Next, reference sera from two experimental infection studies were selected, based on an intense antibody response developed by all infected pigs, and which was maintained during the monitoring period (6-8 weeks post-inoculation). The selected positive control sera were from pigs that seroconverted, had detectable antibody levels by all serological tests and showed a characteristic pattern of *T. gondii* antigen recognition by Western blot. The selected negative control sera tested negative by all serological techniques.

Sheep sera

Sera from an experimental infection study carried out in pregnant sheep with *T. gondii* oocysts were characterized by a range of serological tests: four commercial ELISAs (IDScreen®, PrioCHECK™, Pigtype®, IDEXX®), an in house ELISA (TgSALUVET ELISA 2.0), and a Western Blot (TgSALUVET WB). The results showed that Western Blot is a reference-specific test and Tg SALUVET ELISA 2.0 is an accurate technique for this host species. All ELISAs needed readjustment to improve their performance and make results comparable. It is noteworthy that anti-*N. caninum* IgG cross-reacted with *T. gondii* antigens used in commercial ELISAs when using the cut-offs suggested by the manufactures. Accordingly, cross-reactivity with *N. caninum* or other closely related parasites (e.g., *Besnoitia caprae* and *Sarcocystis* spp.) should be taken into consideration for the development and standardization of serological techniques.

The selected positive control sera were from sheep that seroconverted, had detectable antibody levels by all serological tests, and showed a characteristic pattern of antigen recognition by Western blot. The selected negative control sera tested negative by all serological techniques.



Screening of proteins

A total of 18 recombinant sporozoite- or oocyst wall-specific proteins were screened. After protein quantification by the Bradford method, proteins were electrophoresed, electrotransferred to nitrocellulose membranes and visualized by Ponceau red staining. Next, Western blot under reducing and non-reducing conditions were carried out with the selected control sera. Those proteins were selected that i) were able to differentiate infections by oocysts vs tissue cysts; ii) were recognized after the infection, and iii) showed similar results regardless of the panel of pig sera analysed. Two candidate proteins were then put forward to the assay validation procedure.

Assay validation procedure

The development of a proof of concept serological tool is based on Western blot results. The variables tested include protein concentration, combination of proteins, blocking solution, primary antibody dilution, secondary antibody type, dilution of substrate and time of incubation.

Further steps

Further steps to be carried out include assessment of analytical specificity of selected POIs, and exploring the adaptation of the assay to sera from sheep and humans. The finalized SOP will be shared with partners involved in WP4-T3 for exploring the prevalence of oocyst-driven infections in animals and in humans.

DISSEMINATION AND IMPACT

The work is done in international collaboration, including partner institutes and researchers across sectors and disciplines, and both experienced and early-career scientists have participated in the work.

The progress of the work has been presented to TOXOSOURCES consortium at regular Consortium meetings. Selected parts of this work and its results were presented as oral contributions at the 6th PhDay at the Faculty of Veterinary in the Complutense University of Madrid (VETINDOC) (October 14th, 2020), the ApicoWplexa virtual meeting "Diagnosis of apicomplexans parasites" (February 18th, 2021) and at the 13th European Multicollloquium of Parasitology (October 12th-16th, 2021), by Nadia María López-Ureña, as oral contribution at the 16th Workshop of the National Reference Laboratories for Parasites (November 24th, 2021) by Furio Spano, and as a poster at the Zoonoses 2021 - International Symposium on Zoonoses Research, Berlin (October 16th, 2021) by Frank Seeber on behalf of the co-authors.

The results will be part of scientific article(s) that will be published Open Access, and some will be part of Nadia María López-Ureña's doctoral thesis at the Faculty of Veterinary at the University Complutense of Madrid. A review on the present knowledge and existing gaps related to stage-specific serology for source-attribution of



T. gondii infections was authored and published Open Access (Álvarez-García et al., 2021). The final SOP will be made available Open Access.

The objective of TOXOSOURCES-WP4 is ambitious and identified as high-risk-high-gain. However, the rigorous pipeline designed and followed for the serological tool development, together with an exhaustive selection of POIs and control sera, is producing rigorous results that can confirm or discard the feasibility of using serology for source-attribution of *T. gondii* infections.

REFERENCES

Álvarez-García, G; Davidson, R; Jokelainen, P; Klevar, S; Spano, F; Seeber, F. Identification of oocyst-driven *Toxoplasma gondii* infections in humans and animals through stage-specific serology-current status and future perspectives. *Microorganisms* 2021, 9, 2346. doi: 10.3390/microorganisms9112346.