EuroReference
journal of Reference
anses
French agency for food, environmental and occupational health & safety

Journal No. 4
December 2010

Research for reference

Identification of early *Trichinella spiralis* antigens and their application in developing an ELISA for the diagnosis of trichinellosis in pigs

A. Zocevic, S.A. Lacour, B. Giovani, P. Mace, I. Vallée, P. Boireau, ANSES Maisons-Alfort Laboratory for Animal Health, ENVA, UPEC, JRU BIPAR, Maisons-Alfort, France

A. Zocevic, S.A. Lacour, B. Giovani, P. Mace, I. Vallée, P. Boireau (2010). Identification of early Trichinella spiralis *antigens and their application in developing an ELISA for the diagnosis of trichinellosis in pigs, EuroReference, No. 4, ER04-10R01.*
http://www.anses.fr/euroreference/numero4/PN50I0.htm

Trichinellosis is a parasitic zoonosis caused by eating raw or undercooked meat from animals contaminated with larvae of the nematode's genus *Trichinella*. Although direct detection of the parasite is currently the only recommended method for official inspections of carcasses at slaughterhouses, the introduction of serological methods in the new regulation regarding parasitic zoonoses in Europe is encouraging their development.

Trichinella and trichinellosis

Trichinellosis is a zoonosis caused by consumption of raw or undercooked meat from animals contaminated with larvae of the nematode's genus *Trichinella*. It is worldwide distributed and closely related to culinary and food habits (Blaga *et al.,* 2007). Human trichinellosis is a regularly emerging/re-emerging disease and a study estimates that 11 million people are contaminated on different continents (Dupouy-Camet, 2000). Domestic pigs remain the world's main source of the parasite's transmission to humans but the real reservoir seems to be wild carnivores (Devine, 2003; Pozio, 1998).

Trichinella is a singular nematode since it is the only multicellular parasite that develops in a strictly intracellular habitat in animals. *Trichinella* uses a specific niche, the muscle cell of vertebrates. This nematode belongs to the Trichinellidae family, which includes twelve related species/genotypes in two phylogenetically distinct groups: firstly, encapsulated trichinae (*T. spiralis*, *T. nativa*, T.T6, *T. britovi*, T.T8, *T. murrelli*, T.T9, *T. nelsoni*, T.T12) that infect mammals, and secondly, non-encapsulated trichinae (*T. pseudospiralis*, *T. papuae*, *T. zimbabwensis*) that infect mammals as well as birds and reptiles (Pozio, 2001).

Trichinella undergoes an auto-heteroxenous biological cycle: it takes place entirely in the same host, which is successively the definitive host and the intermediate host. Digestion of contaminated meat, eaten raw or undercooked, releases L1 larvae that penetrate the intestinal epithelium where they develop through L2, L3 and L4 larva stages into sexually adult worms within a few hours (Figure 1). Mated females release NewBorn Larvae (NBL), which enter striated muscles *via* the lymphatic and blood circulation between 4 and 6 days post-infestation (pi). These NBL penetrate muscle cells and induce their dedifferentiation into nurse cells, surrounded or not by a protective collagen capsule. Simultaneously, NBL evolve into Muscle Larvae (ML), which corresponds to the infectious stage of *Trichinella*'s development (Figure 2).

European regulation

Although direct detection of the parasite is currently the only recommended method for official inspections of carcasses at slaughterhouses, serology remains an interesting solution since

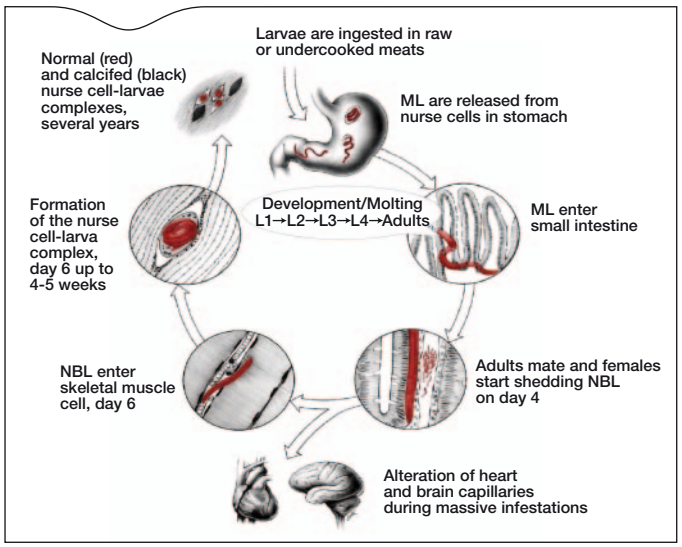

Figure 1. Life cycle of *T. spiralis* (modified from Despommier *et al.,* 2000) (JRU BIPAR, ANSES Maisons-Alfort Laboratory for Animal Health).

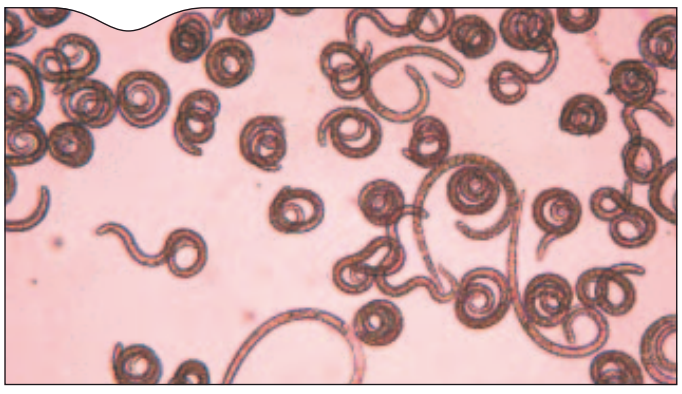

Figure 2. Microscopic observation (magnification 80X) of *T. spiralis* ML (JRU BIPAR, ANSES Maisons-Alfort Laboratory for Animal Health).

Research for reference

it is easier to implement, costs less and can be automated. However, the European Union regulation (EC) no. 2075/2005 introduces serological methods exclusively for epidemiological survey and monitoring purposes of *Trichinella*-free pig holdings in the EU once, a suitable test has been validated by the European Union Reference Laboratory concerning parasites (Istituto Superiore di Sanita, Rome, Italy).

Issues

The only serological test currently available for trichinellosis purposes is an indirect ELISA based on Excretory/Secretory (E/S) antigens from the ML stage of *Trichinella* (Nockler *et al.,* 2009). The E/S ELISA provides a high degree of sensitivity for the detection of *Trichinella* pig infection. However, the existence of an early "blind window of time" in the detection involves the occurrence of false negative results for recently infected animals (less than one month) (Nockler *et al.,* 1995). Furthermore, animals with light infections are not detected consistently (Nockler *et al,* 1995). Therefore, the development of an alternative test to the E/S ELISA would be useful. This test should allow an earlier detection of the infection, notably for the diagnosis of domestic animals. It should also be highly specific, notably for the diagnosis of wild animals.

Objectives and strategy

The objectives of the work initiated were:

- to identify and characterise very early antigens from *Trichinella* invasive stages present in the intestinal mucosa;
- to develop a more sensitive and specific ELISA enabling an earlier detection of trichinellosis in pigs than the E/S ELISA.

The strategy set up required two steps. The first involved the construction of complementary DNA libraries from intestinal invasive stages of *T. spiralis* worms, which is the most commonly *Trichinella* species identified in European pig farms and over the world (Pozio & Murell, 2006). To our knowledge, no genes specifically expressed before the 3-day-old adult stage have been described so far (Boireau *et al.,* 1997; Liu *et al.,* 2007; Wu *et al.,* 2009). Therefore, based on the data available in the literature, three specific targets were chosen to be analysed towards their antigenicity: the pre-adult 14 hours pi (stage L2) and 20 hours pi (stage L3) larval stages (Khan, 1966) as well as the "young adult" 48 hours pi stage. The second step involved immunoscreening in a prokaryotic system in order to select the corresponding antigens. To do this, the parallel use of a polyclonal serum and antibodies produced in the supernatant of intestinal tract of pigs infested with *T. spiralis* larvae was chosen (Figure 3).

Results

Over a hundred clones were collected and sequenced. The results obtained highlighted the potential role of proteins such as serine proteases and heat shock proteins during the early stages of infection. From the analysis of the identified sequences, two new, specific targets were selected and tested towards their antigenicity. The recombinant proteins expressed in E. coli were purified and their antigenicity was demonstrated by Western blot. For that reason, a prototype ELISA based on these antigens was developed (Figure 4). For pigs with a high infesting dose (20,000 ML) tested here, this ELISA has the same sensitivity as the E/S ELISA currently used as a reference. Furthermore, detection is possible one to two

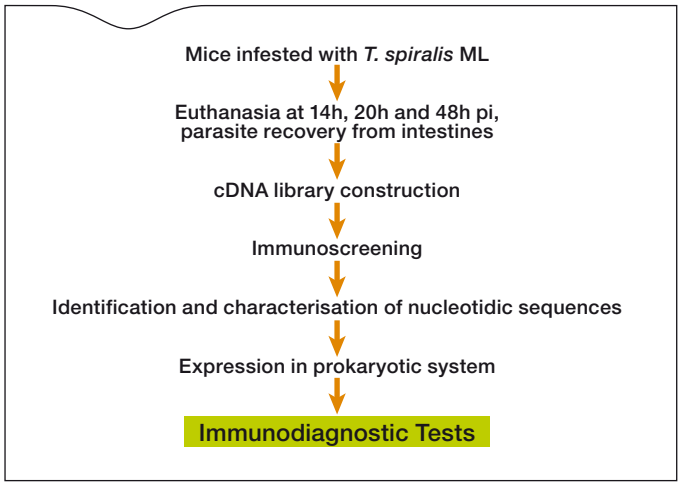

Figure 3. Description of the strategy implemented to identify *T. spiralis* immunodominant antigens (JRU BIPAR, ANSES Maisons-Alfort Laboratory for Animal Health).

weeks earlier compared to the E/S ELISA. In addition, detection is observed beyond 20 weeks with the developed ELISA (Figure 5). Regarding moderate (1,000 ML) and low (200 ML) infestations, the sensitivity of the prototype ELISA is below that of the E/S ELISA for pigs tested. However, the use of these new antigens in association with NBL1 and 411 proteins, previously identified in the laboratory (Boireau *et al.,* 2006), could increase the sensitivity of the test.

The results obtained have been published in a patent: Zocevic, A., Lacour, S.A., Giovani, B., Mace, P., Vallee, I., Boireau, P. Antigènes polypeptidiques de *Trichinella* et leurs applications. Patent 1000660. 17th February 2010.

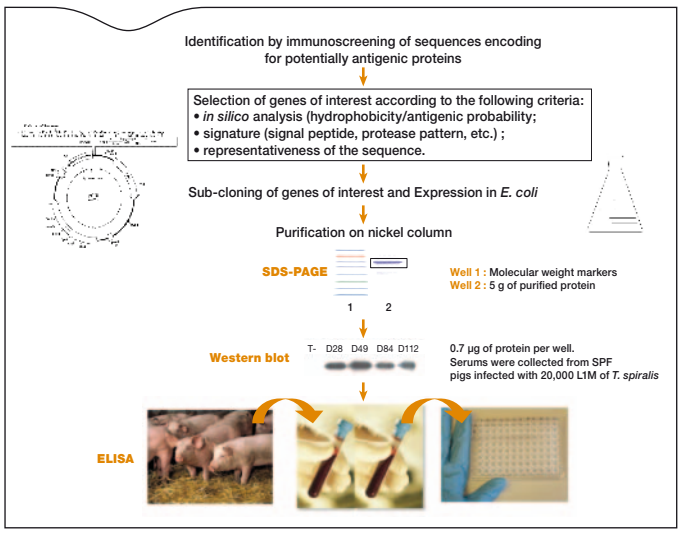

Figure 4. Diagram showing the key steps up to the development of the prototype ELISA (JRU BIPAR, ANSES Maisons-Alfort Laboratory for Animal Health).

Research for reference

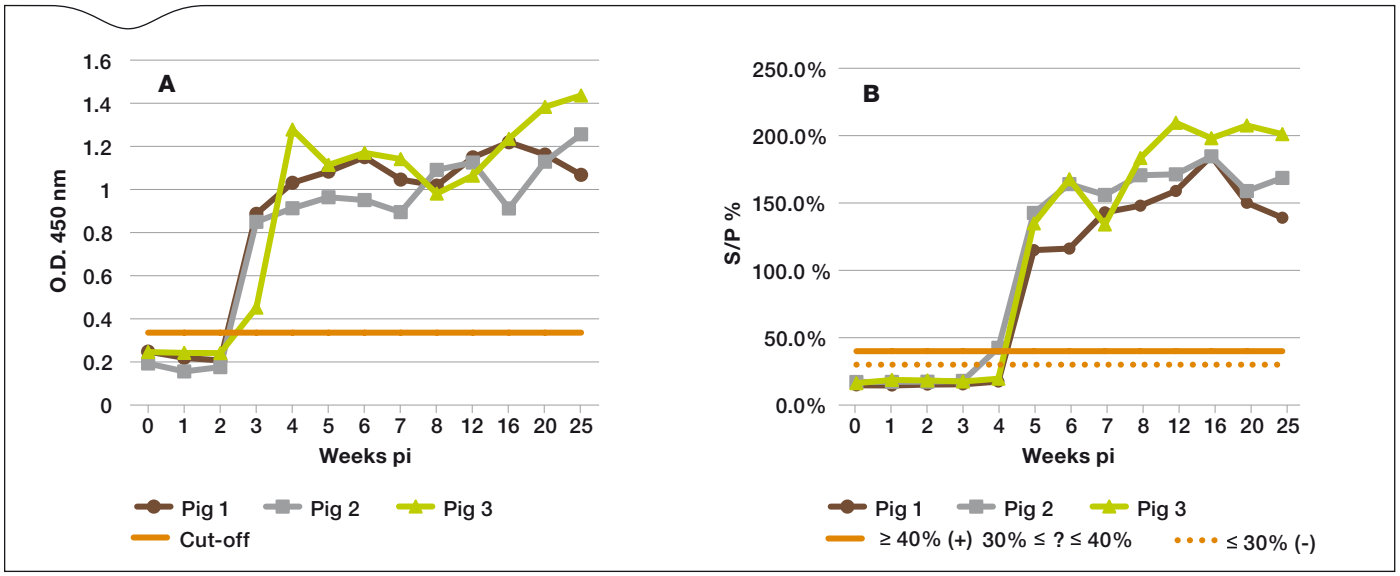

Figure 5. (A) Kinetics of optical density (O.D.) measured by the developed ELISA. (B) Kinetics of the humoral response measured by the E/S ELISA (Pourquier® ELISA Trichinella Serum Screening, Institut Pourquier, Montpellier, France) and expressed in S/P %, which is defined as follows: (O.D. 450 value of the sample) / (O.D. 450 value of the positive control) x 100. *Serums were collected from specific pathogen free pigs experimentally infested with 20,000 ML of* T. Britovi**.**

Conclusion

Using an original approach, several genes, which probably play a key role in the invasion of the intestine and the survival of the parasite, were identified. Furthermore, two antigens are possible candidates for the diagnosis by ELISA of pig trichinellosis. Consequently, additional analyses will be carried out. Pig vaccination tests with these antigens are planned in the light of results obtained with other recombinant antigens also available in the laboratory.

References

Blaga, R., Durand, B., Antoniu, S., Gherman, C., Cretu, C.M., Cozma, V., Boireau, P., 2007, A dramatic increase in the incidence of human trichinellosis in Romania over the past 25 years: impact of political changes and regional food habits. Am J Trop Med Hyg 76, 983-986.

Boireau, P., Vayssier, M., Fabien, J.F., Perret, C., Calamel, M., Soule, C., 1997, Characterization of eleven antigenic groups in *Trichinella* genus and identification of stage and species markers. Parasitology 115, 641-651.

Boireau, P., Mingyuan, L., Baoquan, F. *et al.* Utilisation de deux antigènes identifiés chez *Trichinella*, NBL1 et 411, pour le diagnostic précoce de la trichinellose. Patent 0601058. 07 February 2006.

Despommier, D.D., Gwadz, R.W., Hotez, P.J., Knirsch, C.A., 2000, *Trichinella* spiralis. In: "Parasitic Diseases" Despommier, D.D., Gwadz, R.W., Hotez, P.J., Knirsch, C.A. (ed) Apple Trees Productions, LLC. New York, 125-132.

Devine, R., 2003, La consommation des produits carnés. INRA Productions Animales 16, 325-327.

Dupouy-Camet J., 2000, Trichinellosis: a worldwide zoonosis. Vet Parasitol 93, 191-200.

Gamble, H.R., Pozio, E., Bruschi, F., Nockler, K., Kapel, C.M., Gajadhar, A.A., 2004, International Commission on Trichinellosis: recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man. Parasite 11, 3-13.

Khan, Ali Z., 1966, The postembryonic development of *Trichinella spiralis* with special reference to ecdysis. J Parasitol 52, 248-259.

Liu, M.Y., Wang, X.L., Fu, B.Q., Li, C.Y., Wu, X.P., Le Rhun, D., Chen, Q.J., Boireau, P., 2007, Identification of stage-specifically expressed genes of *Trichinella spiralis* by suppression subtractive hybridization. Parasitology 135, 1-13.

Nockler, K., Reckinger, S., Broglia, A., Mayer-Scholl, A., Bahn P., 2009, Evaluation of a Western Blot and ELISA for the detection of anti-*Trichinella*-IgG in pig sera. Vet Parasitol 163, 341-347.

Nockler, K., Voigt, W.P., Protz, D., Miko, A., Ziedler, K., 1995, Diagnosis of trichinellosis in living pigs using indirect ELISA. Berl Munch Tierarztl Wochenschr 108, 167-174.

Pozio, E., 1998, Trichinellosis in the European Union: Epidemiology, Ecology and Economic Impact. Parasitol Today 14, 35-38.

Pozio, E., 2001, New patterns of *Trichinella* infection. Vet Parasitol 98, 133-148.

Pozio, E., Murrell, K.D., 2006, Systematics and epidemiology of *Trichinella*. Adv Parasitol 63, 367-439.

Wu, X.P., Fu, B.Q., Wang, X.L., Yu, L., Yu, S.Y., Deng, H.K., Liu, X.Y., Boireau, P., Wang, F., Liu, M.Y., 2009, Identification of antigenic genes in *Trichinella spiralis* by immunoscreening of cDNA libraries. Vet Parasitol 159, 272-275.