EUROPEAN UNION REFERENCE LABORATORY FOR PARASITES

WORK PROGRAMME 2013

The 2013 working programme of EURL for Parasites (EURLP) consists of the following activities:

1. Multi-years ad hoc activities

1.1 *Trichinella*

1.1.1 To increase and maintain the serum bank of *Trichinella*-infected pigs

1.1.2 To establish a *Trichinella*-positive standard pig serum

1.1.3 To increase and maintain the serum bank of *Trichinella*-infected humans

1.1.4 To standardize the production of *Trichinella* antigens for serology

1.1.5 To produce reference *Trichinella* antigens for serology

1.1.6 Maintenance of reference strains of *Trichinella* in vivo

1.1.7 Screening of commercial kits to detect anti-*Trichinella* IgG in pig sera

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1.2 Anisakidae

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1.3 *Echinococcus*

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1.3.2 To maintain and improve the serum bank of *Echinococcus*-infected humans

1.3.3 Diagnostic activity with the accredited method

1.4 Other Cestodes

1.4.1 To maintain and improve the genetic bank of zoonotic cestodes such as those of the genus *Taenia* and *Diphyllobotrium*

1.5 Trematodes

1.5.1 To maintain and improve the genetic bank of zoonotic trematodes of the Opisthorchidae family

1.5.2 To maintain and improve the serum bank of *Opisthorchis*-infected humans

1.5.3 To produce reference *Opisthorchis* antigens for serology

1.5.4 Diagnostic activity with the accredited methods

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1.6.1 To maintain and improve the genetic bank of protozoa of the genus *Cryptosporidium*

1.6.2 Diagnostic activity with the accredited method

1.7 *Giardia*

1.7.1 To maintain and improve the genetic bank of protozoa of the genus *Giardia*

1.7.2 Diagnostic activity with the accredited method

1.8 *Toxoplasma gondii*

1.8.1 To establish a genetic bank of *Toxoplasma gondii* isolates

2. Research

2.1 Barcoding of zoonotic and non zoonotic helminths and protozoa parasitizing domestic animals and foodstuffs (multi-years)

2.2 The 'omic' project (multi-years)

2.3 Identification of *Toxoplasma gondii* proteins specific for the oocyst stage (multi-years)

2.4 Identification of polymorphic microsatellites in *Trichinella spiralis* and *Trichinella britovi* (multi-years)

2.5 Identification of the *Trichinella*-specific antigens most frequently recognized by sera from *Trichinella* infected humans and pigs by western blotting (two-years)

2.6 Molecular identification and characterization of *Trichinella spiralis* excretory/secretory antigens from muscle larvae (multi-years)

2.7 Population study of *Echinococcus granulosus* sensu stricto and *Echinococcus canadensis* in Europe (multi-years)

2.8 Development of analytical methods for the identification of taenidae eggs in the definitive host (dogs) faeces (multi-years)

2.9 Development of a molecular test to identify *Dientamoeba fragilis* in human and animal faecal samples (two-years)

2.10 Hazard identification by antigen characterization for fish nematodes other than those of the *Anisakis* genus (multi-years)

2.11 Validation of methods to detect Anisakidae larvae in fishery products and evaluation of their performance by Ring Tests (multi-years)

3 Interlaboratory comparison studies

3.1 *Trichinella*

3.1.1 PT on *Trichinella* larva detection in meat samples (multi-years)

1. Multi-years ad hoc activities

1.1 *Trichinella*

1.1.1 To increase and maintain the serum bank of *Trichinella*-infected pigs

Serum and/or meat juice samples will be collected from *Trichinella*-infected pigs, from pigs infected with other parasites, and from pigs known to be *Trichinella*-free. All samples will be tested by the validated ELISA, distributed in aliquots, lyophilised and stored at +4°C. The database of the serum bank will be updated accordingly. Pig serum samples from different world regions and pig races (infected and not infected with *Trichinella*) will be collected, in order to obtain control sera and to refine the most appropriate cut-off value to be used for serological studies on different swine races. If the EURLP will receive a high request of reference serum samples, SPF pigs will be experimentally infected with *Trichinella* spp. larvae. Before the infection, sera will be collected from each pig. After the infection, the kinetics of anti-*Trichinella* antibodies will be followed and, when the serum conversion will be detected (approximately 20-25 days p.i.), pigs will be sacrificed and sera will be collected, tested, distributed in aliquots and lyophilised

Objectives:	*availability of* Trichinella-*positive pig sera for the validation of serological tests*
Expected outputs:	*a statistically significant number of well characterized pig sera*
Performance indicators:	*increase of the available pig sera*

1.1.2 To establish a *Trichinella*-positive standard pig serum

The EURLP aims at establishing a collaboration with the Institute for Reference Material and Measurements (IRMM), Joint Research Centre of the European Commission, to develop an international standard *Trichinella*-positive pig serum. The potential reference material, already available at the EURLP, needs to be further characterized with respect to several parameters of relevance. Indeed, if the reference material is to be used as an arbitrary standard, it must be ensured that its production is reproducible. Thus, the ELISA targets would have to be quantified in the candidate reference material through collaborative studies.

Objectives:	*availability of a* Trichinella-*positive reference pig serum for the validation of serological tests*
Expected outputs:	*reference* Trichinella-*positive pig serum*
Performance indicators:	*identification of* Trichinella *antigens recognized by the reference serum*

1.1.3 To increase and maintain the serum bank of *Trichinella*-infected humans

Serum samples and/or blood spots will be collected from infected people during trichinellosis outbreaks occurring in different European countries or outside Europe. Serum samples from people with a confirmed diagnosis of trichinellosis will be tested by ELISA and western blot, distributed in aliquots, lyophilised and stored at +4°C. The database of this serum bank will be updated accordingly.

Objectives:	*availability of* Trichinella-*positive human sera for the validation of serological tests*
Expected outputs:	*a statistically significant number of well characterized human sera*
Performance indicators:	*increase of the available human sera*

1.1.4 To standardize the production of *Trichinella* antigens for serology

A standard protocol for the production of Excretory-Secretory (ES) antigens will be distributed to participating laboratories along with an isolate of *Trichinella* that will be used in parallel with an in-house isolate for ES production. Each participating laboratory will infect ten rats at a dose of 2500 L1 per rat. Thirty-five days post infection, rats will be sacrificed and *Trichinella* larvae will be collected by artificial digestion. Recovered larvae will be enumerated and immediately processed for in vitro culture according to the standard protocol provided. Recovered ES products will be assessed for protein content and aliquoted for freezing (-80 C) and lyophilizing. ES products will be analyzed on two-dimensional gels, stained for protein and glycoprotein components and images digitized and compared. To test for antigenicity, ES components separated in two-dimensions will be blotted onto nitrocellulose and probed with sera from *Trichinella* infected animals. Initially, these sera will be from experimentally-infected pigs, but pending the outcome of initial experiments, low responding and putative cross-reacting sera from naturally-infected pigs will be tested as well. The expectation is that, by following standard methods, participating laboratories will produce ES antigens that are similar in composition. If differences in antigen composition will be found, further studies will be designed to identify the source of these differences.

Objectives:	characterization of Trichinella *standard ES antigens*
Expected outputs:	*International Guidelines for the production of* Trichinella *standard ES antigens*
Performance indicators:	*Drafts of the Guidelines*

1.1.5 To produce reference *Trichinella* antigens for serology

Excretory/secretory (E/S) antigens will be produced from *Trichinella* spp. larvae in order to supply NRLs with the reference antigens for diagnostic purposes.

Objectives:	*Supply NRLs, labs in developing countries and EURLP with* Trichinella *ES antigens*
Expected outputs:	*Production of* Trichinella *ES antigens*
Performance indicators:	*Number of milligrams of produced* Trichinella *ES antigens*

1.1.6 Maintenance of reference strains of *Trichinella in vivo*

Reference strains for each species or genotype of *Trichinella* identified so far will be maintained in laboratory animals. Fresh mouse carcasses infected with *Trichinella* species/genotypes will be provided to laboratories for training and for typing of wild isolates. *Trichinella* spp. larvae from reference strains will be stored in ethyl alcohol and forwarded to laboratories as reference material.

Objectives:	*further development of the Bio-bank of* Trichinella *parasites for European, extraeuropean, and international institutions*
Expected outputs:	*production of reference material*
Performance indicators:	*Number of* Trichinella *species and genotypes maintained in vivo*

1.1.7 Screening of commercial kits to detect anti-*Trichinella* IgG in pig sera

According to the Commission Regulation (EC) 2075/2005, a serological test to monitor the circulation of *Trichinella* infections in pig herds may be used once a suitable test is validated by the EURLP. The ELISA test has been validated by the EURLP and can be used to monitor the circulation of *Trichinella* parasites in pig

herds. A plethora of commercial kits to detect anti-*Trichinella* IgG in swine are now commercially available, but none of them has been validated by the EURLP. Since one of the core duties of the EURLP is to give critical advices, we plan to invite the companies producing ELISA kits for the detection of anti-*Trichinella* IgG in swine sera to provide us with their kits in order to determine their performance and, in particular, their sensitivity, specificity, inter- and intra-assay variation, reproducibility and robustness, using a panel of known pig sera with different levels of IgG.

Objectives:	*Availability on the EU market of reliable commercial kits*
Expected outputs:	*Increased diagnostic quality*
Performance indicators:	*Number of evaluated kits*

1.1.8 Diagnostic activity with accredited methods

Diagnostic samples provided by NRLs or third countries will be tested with the following accredited tests (www.accredia.it/accredia_labsearch.jsp?ID_LINK =293& area=7&&):

i. Identification of anti-*Trichinella* IgG antibodies in swine sera (MI-01 rev. 5, 2009)
ii. Identification of anti-*Trichinella* IgG antibodies in human sera (MI-03 rev. 2, 2009)
iii. Detection of *Trichinella* larvae in meat samples by digestion (EC 2075/2005)
iv. Identification of parasites of the genus *Trichinella* by a multiplex-PCR analysis (MI-02 rev. 4, 2009).

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Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Confirmatory diagnoses*
Performance indicators:	*Number of tested samples*

1.2 Anisakidae worms

1.2.1 To increase and maintain the collection of Anisakidae worms and their genomic DNAs

Reference larvae will be collected directly from naturally infected fish; the DNA will be extracted and stored at -20°C. Alternatively, reference larvae will be requested to European and extra-European laboratories having an expertise in this subject. The database of this collection will be updated accordingly.

Objectives:	*Development of a genetic-bank of* Anisakidae *parasites for European, extraeuropean, and international institutions*
Expected outputs:	*Supply of reference material*
Performance indicators:	*Number of* Anisakidae *worms characterized and stored*

1.2.2 Diagnostic activity with the accredited method

Anisakidae worms isolated from fish products by NRLs and third countries will be identified using the accredited PCR-RFLP test (www.accredia.it/accredia_labsearch.jsp?ID_LINK =293& area=7&&):

i. Identification at species level of parasites of the family Anisakidae by PCR/RFLP (MI-04 rev. 1, 2010)

Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Confirmatory diagnoses*
Performance indicators:	*Number of tested samples*

1.3 Echinococcus

1.3.1 To maintain and improve the genetic bank of the genus *Echinococcus*

Adult, larval and egg stages will be collected from different species of final and intermediate hosts originating from different geographical regions. The DNA will be extracted and stored at -20°C. The database of this genetic bank will be updated accordingly.

Objectives:	*Further development of a genetic bank of* Echinococcus *parasites for European, and non-European institutions*
Expected outputs:	*Production of reference material*
Performance indicators:	*Number of* Echinococcus *worms characterized and stored*

1.3.2 To establish a serum bank of *Echinococcus*-infected humans

Serum samples from *E. granulosus* and *E. multilocularis* infected humans with a confirmed diagnosis will be collected, aliquoted and stored at -80°C. The database of this serum bank will be updated accordingly.

Objectives:	*availability of* Echinococcus*-positive human sera for the validation of serological tests*
Expected outputs:	*a statistically significant number of well characterized human sera*
Performance indicators:	*increase of the available human sera*

1.3.3 Diagnostic activity with the accredited method

Echinococcus granulosus larvae, adult worms or eggs detected in intermediate and final hosts by NRLs and third countries, will be identified using the accredited method (www.accredia.it/accredia_labse arch.jsp?ID_LINK =293& area=7&&):

i. Identification of *Echinococcus granulosus* complex at genotype/species level by PCR and sequencing (MI-05 rev. 1, 2010)

Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Confirmatory diagnoses*
Performance indicators:	*Number of tested samples*

1.4 Other Cestodes

1.4.1 To maintain and improve the genetic bank of zoonotic cestodes such as those of the genus *Taenia* and *Diphyllobotrium*

Adult, larval and egg stages of zoonotic cestodes besides those of the genus *Echinococcus* will be collected from infected hosts, both humans and animals. Genomic DNA will be extracted and stored. The DNA will be amplified by PCR and the amplicons will be sequenced. The obtained sequences will be compared with

those present in GenBank. The database of this genetic bank will be updated accordingly.

Objectives:	*development of a genetic bank of Cestodes parasites for European, and non-European institutions*
Expected outputs:	*production of reference material*
Performance indicators:	*Number of Cestode worms characterized and stored*

1.5 Trematodes

1.5.1 To maintain and improve the genetic bank of zoonotic trematodes of the Opisthorchidae family

Adult, larval and egg stages of trematodes of the family Opisthorchidae will be collected from final and intermediate hosts, both humans and animals. Genomic DNA will be extracted and stored. The database of this genetic bank will be updated accordingly.

Objectives:	*development of a genetic-bank of Opisthorchidae parasites for European, and non-European institutions*
Expected outputs:	*production of reference material*
Performance indicators:	*Number of Opisthorchidae worms characterized and stored*

1.5.2 To maintain and improve the serum bank of *Opisthorchis*-infected humans

Serum samples from *Opisthorchis* spp. infected humans with a confirmed diagnosis will be collected, aliquoted and stored at -80°C. The database of this serum bank will be updated accordingly.

Objectives:	*availability of* Opisthorchis*-positive human sera for the validation of serological tests*
Expected outputs:	*a statistically significant number of well characterized human sera*
Performance indicators:	*increase of the available human sera*

1.5.3 To produce reference *Opisthorchis* antigens for serology

Excretory/secretory (E/S) antigens will be produced from *Opisthorchis felineus* adult worms for the in-house serodiagnosis and to supply NRLs and third countries with the reference antigens for diagnostic purposes.

Objectives:	*Supply NRLs, labs in developing countries and EURLP with* Opisthorchis *ES antigens*
Expected outputs:	*Production of* Opisthorchis *ES antigens*
Performance indicators:	*Number of milligrams of produced* Opisthorchis *ES antigens*

1.5.4 Diagnostic activity with accredited methods

Diagnostic samples provided by NRLs or third countries will be tested with the following accredited tests (www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7&&):

i. Detection of anti-*Opisthorchis* antibodies in human serum by indirect ELISA (MI-07 rev 0, 2012)

ii. Identification of *Opisthorchis* sp. by PCR (MI-08 rev 0, 2012)

Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Confirmatory diagnoses*
Performance indicators:	*Number of tested samples*

1.6 *Cryptosporidium*

1.6.1 To maintain and improve the genetic bank of protozoa of the genus *Cryptosporidium*

Cryptosporidium spp. oocysts will be collected from domestic and wild animals, humans and environmental samples. Nucleic acids will be extracted and stored at -20°C until their identification by molecular tools. The database of this genetic bank will be updated accordingly.

Objectives:	*further development of a genetic bank of* Cryptosporidium *parasites for European, and non-European institutions*
Expected outputs:	*production of reference material*
Performance indicators:	*Number of* Cryptosporidium *isolates characterized and stored*

1.6.2 Diagnostic activity with accredited method

Diagnostic samples provided by NRLs or third countries will be tested with the following accredited test (www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7&&):

i. Identification at the species level of oocysts of C*ryptosporidium* spp. by PCR/RFLP (MI-06 rev 1, 2011)

Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Confirmatory diagnoses*
Performance indicators:	*Number of tested samples*

1.7 *Giardia*

1.7.1 To maintain and improve the genetic bank of protozoa of the genus *Giardia*

Giardia spp. cysts will be collected from domestic and wild animals, humans and environmental samples. Nucleic acids will be extracted and stored at -20°C. The database of this genetic bank will be updated accordingly.

Objectives:	*development of a genetic bank of* Giardia *parasites for European, and non-European institutions*
Expected outputs:	*production of reference material*
Performance indicators:	*Number of* Giardia *isolates characterized and stored*

1.7.2 Diagnostic activity with accredited method

Diagnostic samples provided by NRLs or third countries will be tested with the following accredited test (www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7&&):

i. Identification at the assemblage level of cysts of *Giardia duodenalis* by PCR/RFLP (MI-09 rev 0, 2012)

Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Confirmatory diagnoses*
Performance indicators:	*Number of tested samples*

1.8 *Toxoplasma gondii*

1.8.1 To establish a genetic bank of *Toxoplasma gondii* isolates

A panel of *Toxoplasma gondii* reference strains will be produced and implemented over time. The strain collection will include tachyzoites belonging to either of the three major genotypes, denominated I, II and III, which account for approximately 95% of *T. gondii* strains circulating in Europe and North America. In addition, parasite isolates of the so-called "atypical" genotypes will be collected. On request, viable tachyzoites, genomic DNA or tachyzoite protein lysates of any given strain will be supplied to European laboratories.

Objectives:	*development of a genetic bank of* T. gondii *parasites for European, and non-European institutions*
Expected outputs:	*production of reference material*
Performance indicators:	*Number of* T. gondii *isolates characterized and stored*

2 Research

2.1 Barcoding of zoonotic and non zoonotic helminths and protozoa parasitizing domestic animals and foodstuffs

The use of short DNA sequences as a barcode to differentiate taxa and to discover new species, is becoming a popular technique in the scientific community. There are many possible applications of DNA barcoding, from biodiversity studies to food tracking. Our task will be the identification of specific DNA regions that could be used for the identification at the species, genus or family level and the evaluation of their potential for a large scale application. In the field of food-borne parasites, we will continue to focus our attention on: 1) the liver flukes circulating in freshwater fish in Europe; 2) nematode larvae resembling *Trichinella* that are often collected during the digestion of muscle samples will be also identified at the species, genus or family level; and 3) cestode cysts detected in livestock tissues to distinguish between those belonging to zoonotic parasites from those which do not infect humans.

Objectives:	*Diagnostic support to NRLs and developing countries*
Expected outputs:	*Identification of 'unusual' foodborne parasites*
Performance indicators:	*Number of tested samples*

2.2 The 'omic' project

The advent of the so-called high-throughput sequencing (also termed next-generation sequencing or NGS) techniques, that can produce millions of sequences at once, has opened totally new chances for the improvement of the diagnostic tools and epidemiological information. The declinations of this technology are numerous and range from genomics (the elucidation of the complete nucleotide sequence of an organism) to transcriptomics (the inventory of the genes expressed by an organism) to proteomics (the identification of proteins expressed that are), only to mention a few, and are collectively referred to as "omics". The increasing capacity in data generation make "omics" affordable to laboratories. In parallel to NGS technologies, the necessity to analyze huge amount of data have boosted the development of softwares in the expanding area of bioinformatics. In the field of parasitology, NGS have been applied to many protozoa and helminthes of both human and veterinary

importance, and have provided a new wealth of information. It is our believe that the EURLP should consider the application of NGS in its activities. With an emphasis on foodborne parasites of particular relevance in the European scenario, the aims will be 1) to generate new data, starting with the elucidation of the genomic sequences, 2) to learn and use the most relevant approaches in bioinformatics, 3) to develop new molecular-based tools with improved specificity and sensitivity, and 4) to distribute to NRLs the acquired knowledge and the newly developed tools. Although the genome of *Trichinella spiralis* has been characterized, there is no information for any of the 11 other species/genotypes, and in particular for *T. britovi* the most important etiological agent of trichinellosis in Europe. Using advanced molecular and supercomputing technologies, the principal aim of the proposed research is to investigate the molecular epidemiology of *T. britovi* on a global scale and explore the mechanisms or processes involved in transmission and parasite-host interactions. In order to obtain new markers for molecular epidemiology of zoonotic Anisakidae worms, the complete nuclear genome will also be determined. The availability of genomic data will have strong implications for the molecular diagnosis, prevention and control of anisakidosis.

Objectives:	*Acquisition of data for the development of diagnostic tools and for epidemiological investigations*
Expected outputs:	*Important basic information on foodborne parasites circulating in the EU*
Performance indicators:	*Produced genomic data*

2.3 Identification of *Toxoplasma gondii* proteins specific for the oocyst stage

A significant fraction of *Toxoplasma gondii* infections in humans is driven by environmentally resistant oocysts shed by cats and ingested along with contaminated food or water. Despite the essential epidemiological role played by this parasite stage, currently available serologic assays cannot discriminate between infections caused by *Toxoplasma* oocysts or by bradyzoite-containing tissue cysts present in undercooked or raw meat. To try and fill this gap in the serology of toxoplasmosis, we will assess the immunogenic potential in natural infections of a panel of 10 distinct oocyst/sporozoite proteins currently under study at the EURLP, to possibly pave the way towards a serologic test able to identify oocyst-driven infections. The serodiagnostic value of the novel proteins will be assessed through western blot and ELISA assays, using recombinant protein fragments and a panel of *Toxoplasma*-positive sera derived from i) human subjects exposed to either oocysts or bradyzoites and ii) experimentally-infected animals exposed to known parasite infective stages. The screening will include *T. gondii* proteins specifically expressed in the oocyst wall or in sporozoites, as well as a set of proteins whose expression is dramatically upregulated in sporozoites compared to the other parasite invasive stages (bradyzoites and tachyzoites).

Objectives:	*Development of a diagnostic test to detect* Toxoplasma *oocysts in cat faeces and in environmental samples and to distinguish human infection caused by cysts or oocysts ingestion*
Expected outputs:	*Production of MAb specific for* Toxoplasma *oocyst wall proteins*
Performance indicators:	*number of MAbs produced*

2.4 Identification of polymorphic microsatellites in *Trichinella spiralis* and *Trichinella britovi*

The genetic structure of *T. spiralis* isolates circulating within restricted geographical areas (e.g. Extremadura region, Spain) will be investigated by the analysis of the microsatellite variability. In the last years, approximately 50 isolates were collected from wild boars of Extremadura. The muscle larvae will be genotyped using the 18 genetic markers described to date. The search for novel microsatellites will continue using the collection of DNA supercontigs from the whole genomic sequencing (WGS) project now available on public databases. The use of genetic markers from the genome of *T. spiralis* has proven to be ineffective in the study of the genetic variability of *T. britovi*. For this reason, we plan to develop a specific *T. britovi* WGS project. The project will include: 1. Circularization-Ready Fragments. Genomic DNA will be sheared into 20 Kb, 8 Kb or 3 Kb fragments and adaptors will be added to the end of each fragment; 2. Circularized DNA. DNA will be circularized; 3. Paired Ends Library Construct. The circularized DNA will be fragmented and fragments containing the added adaptors will be isolated and amplified for sequencing; 4. Paired Ends Library. The resulting library will consist of true paired end reads with two end tags averaging over 150 bp and separated by 20 Kb, 8 Kb or 3 Kb; and 5. Assembly. Assembly of sequences in supercontigs.

Objectives:	*Development of a specific* Trichinella britovi and T. spiralis *WGS to study of the genetic variability of this species*
Expected outputs:	*Production of genetic markers specific for* Trichinella britovi *and* T. spiralis
Performance indicators:	*number of genetic markers produced*

2.5 Identification of the *Trichinella*-specific antigens most frequently recognized by sera from *Trichinella* infected humans and pigs by western blotting

ELISA based on excretory/secretory antigens (ESA) have been considered the most suitable test to detect *Trichinella* infection, but it is not exempt of problems due to cross-reactivity, which yields false positive (FP) results. In light of this problem, a confirmatory test, such as western blotting (Wb), could help to reach the definitive diagnosis. Our purpose is to define the distinctive Wb pattern of *Trichinella* sp. infections based on the specific ESA most frequently recognized by sera of *Trichinella* sp. infected humans and pigs previously identified as positive by ELISA, and to distinguish it from the Wb patterns generated by ELISA FP sera.

Objectives:	*Development of diagnostic confirmatory tests to detect anti-*Trichinella *IgG in human and pig sera*
Expected outputs:	*Identification of ES antigens recognized only by* Trichinella*-infected humans and pigs*
Performance indicators:	*number of Wb patterns*

2.6 Molecular identification and characterization of *Trichinella spiralis* excretory/secretory antigens from muscle larvae

A major problem in the serological diagnosis of nematode infections in animals and humans is the cross-reactivity with other parasitic and non-parasitic antigens. Crude and excretory/secretory antigens (ESA) from *Trichinella spiralis* muscle larvae are widely used for diagnosis but, since these antigens are complex mixtures of molecules, cross-reactivity to other antigenically related parasites may occur. Using immunoblot, a limited set of bands in the ESA have been now proved to be

recognised by 100% of the sera from naturally or experimentally *Trichinella*-infected pigs. As part of the collaboration program between EURLP and the Institute for Reference Material and Measurements (IRMM), which aims to develop an international standard *Trichinella*-positive pig serum, a proteomic approach will be used for the identification and characterization at the molecular level of ESA from muscle larvae. Taking advantage from the recently sequenced *Trichinella spiralis* genome, a mass spectrometry technique will be used. ESA molecules will be immunoprecipitated by Ig present in the temporary reference positive pig sera (or, as control, from temporary reference negative pig sera). Immunoprecipitated materials will be separated on SDS-PAGE and subjected to analysis with nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS). The genes coding for the identified proteins will be then cloned and expressed in bacterial or yeast background and tested by ELISA for their specific reactivity against a large panel of positive and negative pig and human sera. The antigenic portions of the selected protein will be further characterized by PEP-SPOT analysis to allow the synthesis of standardized *Trichinella*-specific antigens for multiple diagnostic tests.

Objectives:	*Production of* Trichinella-*specific synthetic antigens*
Expected outputs:	*Identification and characterization of* Trichinella *diagnostic antigens*
Performance indicators:	*Number of identified and characterized proteins*

2.7 Population study of *Echinococcus granulosus* sensu stricto and *Echinococcus canadensis* in Europe

Echinococcus granulosus is known to have a broad geographical distribution and a wide host range. The three known genotypes of *E. granulosus* s. s. are called the sheep (G1), the Tasmanian sheep (G2) and the buffalo (G3) strains. To understand the spread, infectivity and pathogenicity of these genotypes in humans and livestock of EU, a wide panel of samples from different European areas will be identified. To date, studies based on both mitochondrial and nuclear DNA genes revealed that *E. granulosus* sensu lato consists of at least 4 valid species: *E. granulosus* sensu stricto (genotype G1–G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5) and *Echinococcus canadensis* (G6–G10). Genes coding for the RNA polymerase II second largest subunit (rpb2), the phosphoenolpyruvate carboxykinase (pepck) and the DNA polymerase delta (pold) will be selected as targets for nuclear markers in *E. canadensis*, because of their single-copy nature in many eukaryotic organisms.

Objectives:	*Knowledge on spread, infectivity and pathogenicity of* Echinococcus *species and genotypes inducing cystic echinococcosis in EU*
Expected outputs:	*Acquisition of epidemiological indicators*
Performance indicators:	*Number of characterized parasites*

2.8 Development of analytical methods for the identification of taenidae eggs in the definitive host (dog) faeces

Taenia is a relatively large genus containing approximately 42 species and 3 subspecies. In contrast to many other helminth infections, the intra vitam diagnosis of taeniid tapeworm infections cannot reliably be achieved by the microscopical detection of the worm eggs in faecal samples by a routine coprological method (e.g., flotation technique) because eggs of all species of the family Taeniidae (genera *Echinococcus* and *Taenia*) are morphologically indistinguishable from one another.

Developing a molecular method for detecting and distinguishing between taeniid eggs present in faeces is considered to be essential. Flotation technique will be used to concentrate the parasite eggs. DNA will be extracted from 0.5 to 1.0g of pellet derived from the flotation of the faecal sample. Reference sequences will be identified using GenBank with the BLAST system; forward and reverse sequences will be aligned and compared using Accelsys gene 2.5 program. In a first step, the protocol will be applied to faecal samples spiked with *E. granulosus*, *E. multilocularis* and *Taenia* spp. Subsequently, the flotation-amplification method will be validated on naturally infected dogs.

Objectives:	*Development of analytical methods for the identification of taenidae eggs in the definitive host (dogs) faeces*
Expected outputs:	*Identification of a panel of primers for the molecular identification of eggs*
Performance indicators:	*Number of amplified sequences*

2.9 Development of a molecular test to identify *Dientamoeba fragilis* in human and animal faecal samples

Dientamoeba fragilis is a protozoan parasite found in the gastrointestinal tract of humans, and is currently classified as a flagellate, although it lacks a flagellum. The organism has a worldwide distribution and the prevalence of *D. fragilis* in humans varies widely from 0.3% to 52%. In contrast to other pathogenic protozoa which have high prevalence in developing regions of the world, *D. fragilis* is also prevalent in industrialized countries. Apart from its evident association with humans, few reports have suggested the presence of *D. fragilis* in animal hosts. Indeed, the host range of this parasite remains to be determined. Moreover, very little is known on the transmission route(s) of this parasite. Diagnosis of *D. fragilis* has traditionally relied upon microscopy of fixed fecal smears. Because of the "fragile" nature of the organism, prompt fixation of clinical specimens is essential as the trophozoites degenerate rapidly once passed in stool samples. Diagnosis is improved by the use of PCR-based techniques, and in particular, real-time PCR protocols show excellent specificity and sensitivity when applied to human fecal samples, and can be considered as the gold standard in the diagnosis of dientamoebiasis. Recently, we discovered this parasite in farmed swine and in persons working at the farm, suggesting a possible transmission of this parasite from pigs to humans, i.e. a possible new zoonosis linked with swine. We intend to compare two real-time assays that target the 18S and the 5.8S genes in the ribosomal cluster, in order to evaluate their performance on both human and animal isolates available in the genetic bank of our laboratory. This could also provide information on the circulation of the parasite in non-human hosts.

Objectives:	*Development of analytical methods for the identification of* Dientamoeba fragilis *in human and pig faeces*
Expected outputs:	*Identification of species-specific primers*
Performance indicators:	*number of isolates identified*

2.10 Hazard identification by antigen characterization for fish nematodes other than those of the *Anisakis* genus

Crude extract and excretory/secretory (ES) antigens from *Pseudoterranova* spp. and *Contracaecum* spp. will be prepared from worms harvested from infected fish and fish products by several cycles of homogeneization-sonication followed by extraction

in phosphate buffered saline. Hyper-immune sera to the parasites antigens will be obtained in rabbits using standard procedures. The antigenic profiles of the parasite extracts will be analyzed by western blotting (WB). The allergenic capacity of the different selected parasite antigens will be determined in animal models. Animals will be immunized with either crude or ES antigens three times together with aluminum hydroxide as adjuvant. Sera will be collected at appropriate intervals and the presence of specific antibodies as well as the class of antibodies will be evaluated by ELISA and WB. Lymphocyte proliferation and cytokine production will be evaluated according to published protocols. In parallel, laboratory animal species will be orally immunized with the selected untreated or heat-treated antigens in presence of cholera toxin or other adjuvant. The evaluation of the immune responses will be carried out as above. To determine the contact between Anisakidae parasites and human beings, by the detection of specific IgE, a panel of sera collected from fish-eating people with history of allergic reactions after fish consumption, will be screened by WB.

Objectives:	*Identification of allergens in fish nematodes of the genera* Pseudoterranova *and* Contracaecum
Expected outputs:	*Characterization of antigens*
Performance indicators:	*Number of identified and characterized allergens*

2.11 Validation of methods to detect Anisakidae larvae in fishery products and evaluation of their performance by Ring Tests

Methods based on UV and RT-PCR technologies will be subjected to an inter-laboratory trial to demonstrate their suitability for the industrial use. For this purpose reference materials will be used. The digestion method will be considered the confirmatory method for the UV-microtech development. Specificity, sensitivity and repeatability of the methods will be determined. The efficiency of the methods in larval detection will be evaluated. The most sensitive, specific and repeatable method(s) will be selected to organize a Ring Trial involving at least five experienced laboratories to evaluate reproducibility of the test/s and reliability of data produced by each laboratory.

Objectives:	*Identification of suitable methods to detect Anisakidae larvae in fish products*
Expected outputs:	*evaluation of the specificity, sensitivity and repeatability of the methods*
Performance indicators:	*number of samples per PT*

3 Interlaboratory comparison studies

According to the requests of NRLs expressed in the course of the seventh NRL workshop, held in Rome from 28 to 29 May, 2012, four proficiency tests (PTs) will be organised by the EURLP in the course of 2013.

Objectives:	*See the Excel file for the Performance Indicators*
Expected outputs:	*See the Excel file for the Performance Indicators*
Performance indicators:	*See the Excel file for the Performance Indicators*

3.1 *Trichinella*

3.1.1 PT on detection of *Trichinella* larva in meat samples

The seventh PT on the detection of *Trichinella* larvae in meat samples, will be organised among NRLs to evaluate the competence of NRLs. Test samples (100 or 35 g meatballs made with diaphragm tissue from pigs and/or horses) will be spiked with a known number of *T. spiralis* larvae obtained from experimentally infected mice. Each NRL will receive samples containing two different numbers of *Trichinella* larvae, plus a negative control sample. Samples will be packed and sent as bio-hazardous material in cool freeze containers to ensure a stable temperature. Every participating partner in the proficiency test will be notified in advance about the timetable and when to receive the test panels along with the protocol. The test results from each laboratory will be evaluated, compared to those of the previous years, and possible critical points will be identified and corrected.

3.1.2 PT on *Trichinella* larva identification

The PT will be organised among NRLs to evaluate their skill to properly identify *Trichinella* larvae at the species level. *Trichinella* larvae from reference strains representing the species circulating in Europe and those which have been occasionally imported from non-EU countries into Europe, will be collected from infected mice. Vials will be coded and forwarded to participating labs for molecular identification by a PCR derived method according to the PCR method used in each laboratory. Participant laboratories will be invited to identify single larvae instead of a pool of larvae.

3.2 *Echinococcus*

3.2.1 PT on the detection of *Echinococcus* adult worms in intestinal contents

For the fourth time, this PT will be organised among NRLs to detect adult worms or their portions of *Echinococcus* sp., spiked in the natural matrix (intestinal content). Each NRL will receive three samples. Samples will be packed and sent as bio-hazardous material in cool freeze containers to ensure a stable temperature. Every participating partner in the PT will be coded (lab code) and notified in advance about the timetable and when to receive the test panels along with the protocol. The test results from each laboratory will be evaluated, compared to those of the previous years, and possible critical points will be identified and corrected.

3.3 Anisakidae

3.3.1 PT on the detection of Anisakidae larvae in fish fillets

A PT to detect Anisakidae larvae in fish fillets by digestion will be organised for the second time. Anisakidae larvae will be collected from naturally infected fish on the market. A known number of larvae will be spiked in fish fillets. Samples will be packed and sent as bio-hazardous material in cool freeze containers to ensure a stable temperature. Every participating partner in the PT will be coded (lab code) and notified in advance about the timetable and when to receive the test panels along with the protocol. Participating NRLs will digest the fish fillets and count the larvae. The test results from each laboratory will be evaluated, compared to those of the previous years, and possible critical points will be identified and corrected.

4 Workshop

In the first half of 2013, a two day-workshop will be held at the Istituto Superiore di Sanità of Rome, or in another place, to present and discuss the results of the PTs and other issues including epidemiological problems related to foodborne parasitic zoonoses occurring in the MS. Some experts in the field of foodborne parasitic zoonoses will be invited to present the most recent acquisitions on the epidemiology, diagnosis and control of these pathogens.

Objectives:	*See the Excel file for the Performance Indicators*
Expected outputs:	*See the Excel file for the Performance Indicators*
Performance indicators:	*See the Excel file for the Performance Indicators*

5 Visit to NRLs

Qualified personnel of the EURLP will visit NRLs to assist them as required by circumstances. The selection of the NRLs will be done with an agreement among NRL, EURLP and the Commission. The outcome of the visits will be reported to the Commission.

Objectives:	*See the Excel file for the Performance Indicators*
Expected outputs:	*See the Excel file for the Performance Indicators*
Performance indicators:	*See the Excel file for the Performance Indicators*

6 Training for the personnel of NRLs and developing countries

On request by NRLs within EU or by governmental institutions of developing countries, personnel will be hosted at the EURLP to be trained on different detection methods of foodborne parasites and quality control systems.

Objectives:	*See the Excel file for the Performance Indicators*
Expected outputs:	*See the Excel file for the Performance Indicators*
Performance indicators:	*See the Excel file for the Performance Indicators*

7 Update of the website of the EURL for parasites

The web site will be improved by developing a database for the creation of a register on human cases of cystic echinococcosis. This is a pilot project, involving, at the beginning, only Italian patients enrolled by specialized centers of the Italian National Health System. The aim is to collect clinical data and to highlight risk factors and the true prevalence of human echinococcosis in Italy, and hopefully, to extend the register at EU level in endemic countries. As a matter of fact, echinococcosis is a neglected diseases, widespread in some regions but underestimated by official reports, since its notification to health authorities is not compulsory. The website will be also updated by publishing the new accredited methods and the technical SOPs developed by the EURLP.

Objectives:	*Continuous improvement of the EURLP web site*
Expected outputs:	*Increase of the available information and its friendly use*
Performance indicators:	*Number of the EURLP web site visitors*

8 Standardization of methods for the detection of parasites in food

Following the launching of the DIS voting/CEN *Enquiry stage* (lasting 5 months) for the standard “Digestion method for the detection of *Trichinella* larvae in meat”

developed by the working group ISO/TC34/SC9/WG6 Sub-Group "*Trichinella*", the standard will undergo the *Approval stage* and will be published, hopefully, at the end of 2013. Concerning *Trichinella* serology, an ISO TS for the "Detection of anti-*Trichinella* antibodies in swine serum by ELISA" will be drafted, and, in the meanwhile, inter-laboratory studies for the standardization of antigens and sera will be carried out. A second meeting of the ISO/TC34/SC9/WG6 Sub-Group "*Trichinella*" will be organized, as well as a workshop on *Trichinella* serology with the participation of epidemiologists.

Objectives:	*to standardize methods for the detection of parasites in food*
Expected outputs:	*Standardization of the Digestion method for the detection of* Trichinella *larvae in meat*
Performance indicators:	*Publication of the 'Digestion method for the detection of* Trichinella *larvae in meat' as an ISO standard*

9 Development of reference materials for diagnostic methods to detect *Trichinella* infections

A collaboration between EURLP and Institute for Reference Material and Measurements (IRMM) Joint Research Centre of the European Commission (Belgium) has started with a double aim: 1.- to carry out stability studies of *Trichinella* positive swine sera already stored at the EURLP based on the isochronous sample storage schemes applied by IRMM for the characterization of reference materials. Stability will be studied at short and a long term, the former is already concluded, the latter is in progress; 2.- To develop a certified reference material based on a reproducible preparation of excretory/secretory antigens which will be the basis for standardisation. To this end two options will be investigated in parallel: a) protocol defining the antigen production (collection and preparation of E/S antigens (at EURLP) based on 1) systematic investigation on the reproducibility of E/S antigen preparation and refinement preparation protocols (at EURLP); 2) systematic studies on the reproducibility of binding the antigens to microtitre plates (at IRMM and at EURLP); b) definition and selection of significant measurands: 1) attempts to identify the various antigens in the E/S extracts (at EURLP) by SDS PAGE and western blotting of *Trichinella* positive swine sera (at EURLP); 2) two dimensional electrophoresis (at IRMM); 3) mass spectrometry and peptide analysis after enzymatic digest (TOF, LC-MS, LC-MS-MS) (at IRMM).

Objectives:	*Evaluation of the stability of lyophilized* Trichinella*-positive pig sera stored at +4°C*
Expected outputs:	*Identification of the best protocol for the storage of reference sera*
Performance indicators:	*results of the long term stability study*

10 Validation of apparatuses and kits

According to the Guidelines for the validation of apparatuses for the detection of *Trichinella* larvae in meat samples by digestion, the EURLP will organize the validation process involving four National Reference Laboratories for Parasites. When the "Guidelines on the requirements of serological kits for the detection of anti-*Trichinella* IgG in pig sera to be used in monitoring programs", will be approved by the DG SANCO, commercial kits will be validated according to the company requests.

Objectives:	*Validation of apparatuses and kits for the diagnoses of foodborne parasites*
Expected outputs:	*Validation of new commercial apparatuses and/or kits*
Performance indicators:	*publication of the technical report of the validation process*

11 Quality assurance system

The continuous improvement of the EURLP Quality Assurance System, is a key factor to assure to the NRLs the highest level of reliability of EURLP services. Therefore, in order to improve the services provided by EURLP to NRLs, new methods for the diagnosis/identification of parasites in food will be developed, validated and submitted to accreditation during the annual surveillance audit carried out by the Italian accreditation body, ACCREDIA.

Objectives:	*See the Excel file for the Performance Indicators*
Expected outputs:	*See the Excel file for the Performance Indicators*
Performance indicators:	*See the Excel file for the Performance Indicators*

12 Support to International Institutions

Qualified personnel of the EURLP will support the ECDC, EFSA, FAO, OIE, WHO, and other international institutions in the field of foodborne parasitic zoonoses.

Objectives:	*See the Excel file for the Performance Indicators*
Expected outputs:	*See the Excel file for the Performance Indicators*
Performance indicators:	*See the Excel file for the Performance Indicators*

13 Meeting at the DG SANCO

The Director of EURL for Parasites or a person appointed by the director, will attend the yearly meeting at the DG SANCO.

Rome, 28th August, 2012

The Director of EURL for Parasites
Dr. Edoardo Pozio