Report of the first interlaboratory comparison of a serological test to detect anti-*Trichinella* IgG in swine sera

October-December, 2006

Table of contents

1. Introduction

Nematode worms of the genus *Trichinella* are zoonotic parasites circulating in most of the European countries in both wild and domestic animals (Pozio and Murrell, 2006). Humans acquire the infection by the consumption of raw or undercooked meat from pigs, horses, wild boars and other game animals (Pozio et al., 2003). According to the Commission Regulation (EC) No 2075/2005 (Commission Regulation EC No 2075/2005), all animals which are potential carrier of *Trichinella* infective larvae should be tested at the slaughterhouse according one of the approved test. However, this regulation allows that a pig holding or a category of pig holdings that has been officially recognised by the competent authority as free from *Trichinella* in accordance with the procedure set out in the Chapter II of Annex IV of the regulation, can derogate avoiding to test animals originating from these holdings.

At the same time, the competent authority shall implement a monitoring programme covering domestic swine, horses and other animal species susceptible for *Trichinella* coming from holdings or categories of holdings recognised as free from *Trichinella* or from regions where the risk of *Trichinella* in domestic swine is recognised as negligible, in order to verify that the animals are effectively free from *Trichinella*.

The frequency of testing, the number of animals to be tested and the sampling plan shall be laid down in the monitoring programme. The monitoring programme may include serological methods as an additional tool once a suitable test is validated by the Community reference laboratory.

The Community Reference Laboratory for Parasites (CRLP) has developed an ELISA to detect anti-*Trichinella* IgG in swine sera using excretory/secretory antigens according to the World Animal Health Organization (OIE) manual (Office International des Epizooties, 2000). A laboratory ring trial is an essential element of laboratory quality assurance. The analysis of an external quality control test material allows individual laboratories to compare their analytical results with those from other laboratories while providing them objective standards to perform against. The CRLP has organised a ring trial for the network of appointed NRLs for parasites to determine the sensitivity of the ELISA to detect anti-*Trichinella* IgG in swine sera.

2 Scope

It is one of the core duties of the CRLP to organise ring trials, as is stated in Regulation (EC) No 882/2004 of the European Parliament and of the Council (Commission Regulation No. 882/2004). The scope of this comparison is to test the competence of the appointed NRLs to analyse anti-*Trichinella* IgG antibodies in serum samples of pig origin, and at the same time to validate the test involving not only laboratories of the European Union, but also European laboratories of countries with an high endemicity for this zoonosis and extra-European laboratories which are skilful in this specific field.

3 Time frame

The ring trial was first announced to NRL network at the meeting of the Trichimed workpakage of the European project MED-VET-NET which was held in Glasgow on 11-12 August, 2006. An email to inform all the potential participants to confirm their interest to participate at the ring trial was sent on 5th of September, 2006. On 9 October, the samples were dispatched to participants by an international courier and an electronic copy of the forms was sent by email on the same day. Reporting deadline was 31 October, 2006.

4 Test material

4.1 Microtiter plate

The commercially available ELISA 96-well microtiter plates (Nunc-Immuno Plate Maxisorb, Brand Products, Denmark) code N. 442404, was used.

4.2 Conjugate

The anti-swine IgG goat antibodies peroxidase labeled (Kirkeegard &Perry Laboratories, Gaithersburg, Maryland, USA) code 14-14-06, was used. The conjugate was rehydrated with 1 mL of reagent quality water and the vial was rotate until the lyophilized pellet was totally dissolved. Optimal working concentration was determined experimentally to be 1/20000 dilution. The conjugate was stored frozen in 10 µL aliquots and no further manipulation of the material took place until the dispatch.

4.3 Antigens

Excretory/secretory antigens (ES Ag) produced at the CRLP, was used. Excretory /secretory (E/S) antigens obtained in the CPRL from *T. spiralis* muscle larvae following published protocols (Gamble, HR 1996 Chapter 3.5.3. Trichinellosis. In: Manual of Standards for Diagnostic Tests and Vaccines. List A and B diseases of mammals, birds and bees. Office International Des Epizooties, Paris, 477-480). Before the dispatch, 80 frozen E/S antigen aliquots of 60µL each, were lyophilized during 48 hours and afterwards the aliquots were stored at +4°C without further manipulation until the forwarding.

4.4 Control and test sera

Two vials of 60µg of two negative control sera (codes NC1-NC2), six vials of 60µg of six positive control sera (codes PC1-PC6) and ten vials of 60µg of ten serum samples which should be tested (the Ring Trial Panel identified by sample codes US1-US10) were provide by the CRLP. All control and test sera were freeze-dried. The origin of the serum panel is shown in Table 1.

Table 1. Data on serum panel.

SAMPLES	ORIGIN	SPECIES	L/G (DIAPHRAGM)
NC1	pool from non-infected spf pigs	-	0
NC2	pool from non-infected spf pigs	-	0
PC1	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	723
PC2	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	512
PC3	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	653
PC4	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	814
PC5	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	827
PC6	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	314
US1	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	827
US4	experimentally infected spf pig with 20,000 MSL	*T. spiralis*	653
US2	naturally infected, Croatia	*T. spiralis*	21
US9	naturally infected, Croatia	*T. spiralis*	88
US3	naturally infected, Romania	*T. spiralis*	15
US5	naturally infected, Romania	*T. spiralis*	2
US 7	naturally infected, Italy	*T. britovi*	34
US 8	naturally infected, Italy	*T. britovi*	4
US 6	non infected, from an Italian slaughterhouse	-	0
US 10	non infected, from an Italian slaughterhouse	-	0

All the material will be referred to as CRLP kit.

CRLP kits were sent to each of the participant laboratories with the request to test the sera for IgG antibodies against *Trichinella* sp. according to the attached protocol for the indirect ELISA and, to test the same serum panel with commercially available and in-house tests, whenever possible.

4.5 Preparation

In order to assure the homogeneity of the serum samples, an unique batch for each serum was prepared and dispensed in 100 µL aliquots into coded plastic cryo-vials. Immediately, each serum aliquot was freeze-dried under vacuum conditions and the process was completed within 48 hours. Twelve vials were rejected during the production process, five vials per each sample were held for stability studies. The preparations were subjected to a preliminary in-house testing to verify the immunological reactivity after lyophilization. CRLP kits were sent in polystirene containers under dry ice.

To estimate the suitability of the different conditions under which the CPRL kits would be stored and distributed to the participant laboratories, three CRLP kits were stored at +4°C, -20°C and -80°C, respectively, for 24 hours; in addition, one CPRL kit was stored under dry ice for 72 hours. To check the material stability during the time, three CRLP kits were tested one week before the forwarding, one week after the forwarding and at the end of the ring trial.

4.6 Distribution

The CRLP kits were dispatched to the participant laboratories on 9th October, 2006. The results deadline was set at the end of October, 2006. All participant laboratories, but one, sent the results within 4 weeks.
Each CRLP kit consisted of:

- an ELISA plate x 1 (NUNC code 442404)
- 10 µL of conjugate (KPL Peroxidase-Labeled Affinity Purified Antibody to swine IgG, code 141406)
- 100 µL x 6 of lyophilised sera from 6 pigs positive for *Trichinella spiralis* (positive control sera) with the codes PC1 – PC6
- 100 µL x 2 of lyophilised sera from 2 pigs negative for parasites including *Trichinella* sp. (negative control sera) with the codes NC1 – NC2;
- 100 µL x 10 of lyophilised sera from 10 pigs, which have been tested for *Trichinella* by HCL-pepsin digestion (unknown samples) with the codes S1 – S10;
- 60 µg x 2 of excretory/secretory antigens from *Trichinella spiralis* muscle larvae, batch 1/2004;
- Hard copies of forms which should be filled in and send back to CRLP:
  1) general information on the Ring Trial;
  2) laboratory description;
  3) the code assigned to the laboratory;
  4) a list of instruments, reagents and materials for the indirect ELISA;
  5) the procedure for the indirect ELISA;
  6) the check of the package content and its condition of preservation;
  7) an accompanying letter

5 Instructions to participants

Practical instructions were given to all the participants in two forms that accompanied the samples (a copy was also forwarded by email). A list of instruments as well as a list of chemicals and disposable material required to perform the ELISA procedure, was also included. To make comparable the results obtained by the different laboratories involved in the ring trial, all the participants should mark the protocol step by step or, on the contrary, describe the variation.

In addition, laboratories which already established an own ELISA to detect anti-*Trichinella* sp. IgG in pig sera, were strongly encouraged to test the same panel of serum samples with their own in house ELISA or with the commercial ELISA kit they are using at the laboratory.

6 Evaluation of the results

6.1 General observations

The OIE recommendations [Principles of validation of diagnostic assays for infectious diseases. In: O.I.E. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). 5th edition, 2004, pp. 21-29] were followed to interpret the results. The cut off was determined as the mean ± 3 SD of the optical density values of 860 serum samples from *Trichinella* sp. free pigs from different endemic and non-endemic European regions. The ELISA index (Ie) was calculated according to the following equation:

$$\textbf{Ie} = \frac{\text{OD mean duplicate sample} - \text{OD mean duplicate blanks}}{\text{OD mean duplicate highest positive control} - \text{OD mean duplicate blanks}} \times 100\%$$

The cut off value calculated on the basis of the above equation and expressed as **Ie**, resulted to be 18%. Consequently, US sera with **Ie** values < of 18% were considered to be negative; whereas, US sera with **Ie** values ≥ of 18% were considered to be positive.

A test was considered valid whenever:

- OD of NC1 and NC2 were higher than OD of blanks
- OD of NC1 and NC2 were lower than the cut off value according to CRLP validation
- the highest values of OD correspond to PC1 and/or PC2 (at 1/50 dilution)

The results were evaluated separately if they originated from laboratories which followed the proposed protocol without any modification (referred as type A), or laboratories which followed the proposed protocol with some modifications (referred as type B), or laboratories which used in house or commercial ELISA (referred as type C).

6.2 Statistical Analysis

For a statistical analysis, a normal mixed effect model (Pinheiro and Bates, 2000) was used in which the serum and the laboratory have been considered random effects, while the dilution effect was considered fix. The goal of this model was to explore to what extent the percentage of “true positive” and “true negative” sera might depend on the above mentioned effects. In the random effect models, a major role is played by the variance parameters of the random effects. In this respect, it was compared the variance associated with the lab and serum effects. A smaller value of serum variance would imply the robustness of the proposed ELISA against discrepancies in the protocol.

6.3 Results and scores

Twenty-three laboratories jointed to the ring trial (see page 11). The laboratory answers to the questionnaires (forms 1-6, see Annexe 2) are reported below:

Features	No. and codes of laboratories
Declaration of shipping problems	1 (lab #3)
Package with dry ice still present at the receipt	20 labs (all but lab # 11)
The conjugate still frozen at the receipt	20 labs (all but lab # 11)
CRLP test performed	21 labs (all but lab # 12)
CRLP test performed without any modification	9 labs (labs# 1,4, 6,8,13,14,15,18,21)
CRLP test performed with some modification (washing, conjugate, substrate, etc.)	14 labs (labs# 2,5,7,8,9,10,11, 16,17*, 18,19, 20*,22,23) *invalided: B > NC1 and NC2
Other test/s performed with the same panel of sera	12 labs (labs #2,6,7*,8,10,12,15,17,18,19, 21,23) *invalided: lack of interpretation criteria

6.3.1 Type A laboratories

The **Ie** values obtained from the OD value of each serum dilution in each laboratory, are displayed in the Annex 3

Number of positive and negative sera recognized by each laboratory.

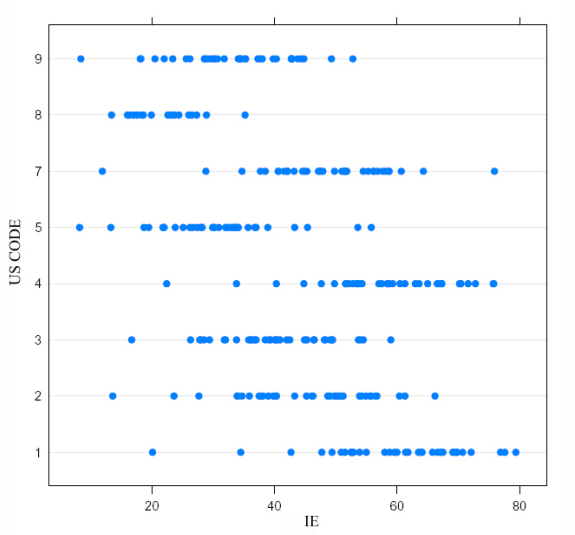
Lab code	Positive sera	Negative sera	No. of false negative	No. of false positive
1	30/30	9/10	0	1
4	30/30	10/10	0	0
6	30/30	10/10	0	0
8	28/30	10/10	2	0
13	30/30	9/10	0	1
14	30/30	10/10	0	0
15	22/30	10/10	8	0
18	29/30	10/10	1	0
21	30/30	9/10	0	1

Percentage of test agreement at different serum dilutions.

Lab code	% test agreement at different dilutions			
	1/50	1/100	1/200	1/400
1	100	100	90	100
4	100	100	100	100
6	100	100	100	100
8	90	90	100	100
13	100	100	90	100
14	100	100	100	100
15	90	90	90	50
18	100	90	100	100
21	100	100	90	100

As a preliminary step, data from true positive and true negative sera were separately analysed. For each group, several models were considered in order to assess the influence of the experimental factors. The explorative analysis of **Ie** values for the US positive sera suggests that there is a serum effect (Figure 1).

Figure 1



For the US positive sera, different **Ie** values were detected for the same serum dilution among labs, suggesting a lab effect which, however, is lower than that of the serum (Figure 2).

Figure 2

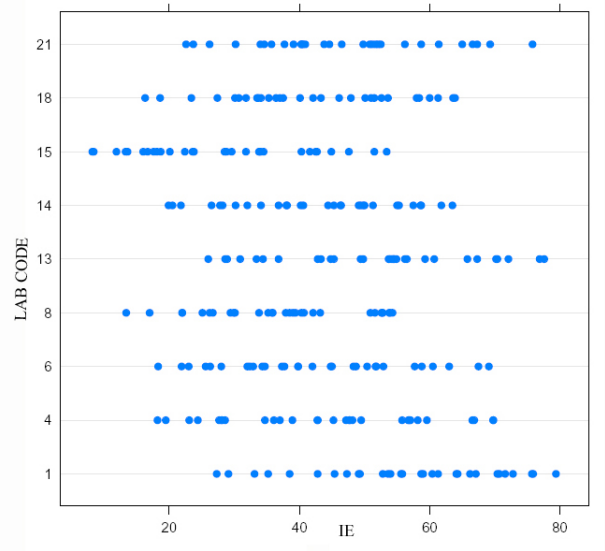
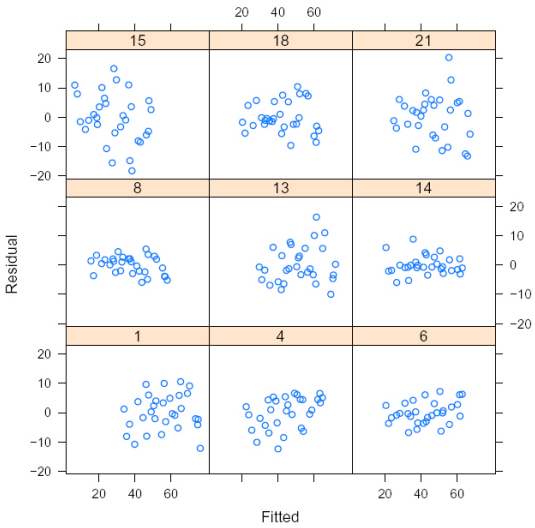


Figure 3



For the positive sera analysed in type A laboratories, the following linear mixed effect model was ended up:

Ie = β0 + Serum + Lab + β3 Dil + β4 Dil^2,

where, 'Serum' and 'Lab' represent random normal effects, 'β0' is the mean effect of all the considered variables and 'σ_S' and 'σ_L' are the standard deviations; while, 'Dil' represents the variable dilution which enters the equation with a quadratic effect.

The greater value of σ_S compared to that of σ_L, confirms the exportability of the test. The variance of the sera is greater than the variance of the labs and there is not interaction between both these effects. Moreover, the graphical residual analysis, indicates that Laboratory #15 behaves substantially different from the other labs (Figure 3).

This model was implemented using the R library **lme4**, provided by Pinheiro and Bates (2000). The random effects Labs and Sera had an estimated standard deviation equal to 7.91 and 14.03, respectively. The fixed effects (intercept, dilution and square of dilution) were all significant. The larger value of 'σ_S' compared to that of 'σ_L', confirms the exportability of the test. There was no evidence of significant interaction between the lab and serum factors.

Moreover, a graphical residual analysis (Figure 2), indicates that Lab 15 behaves substantially different from the other labs.

To establish a score for each type A laboratory, an analysis of the casual effects was evaluated according to Pinheiro and Bates (2000). The coefficient of each laboratory is displayed in Table 1.

6.3.2 Type B laboratories

The **Ie** values obtained from the OD value of each serum dilution in each laboratory, are displayed in the Annex 4

Number of positive and negative sera recognized by each laboratory.

Lab code	Positive sera	Negative sera	False negatives	False positives	Lab code	Positive sera	Negative sera	False negatives	False positives
2	30/30	10/10	0	0	16*	30/30	9/10	0	1
5	30/30	9/10	0	1	16*	30/30	9/10	0	1
7	29/30	10/10	1	0	16*	30/30	9/10	0	1
8	30/30	10/10	0	0	18*	16/30	10/10	14	0
9	30/30	10/10	0	0	18*	27/30	10/10	3	0
10	28/30	10/10	2	0	19	29/30	10/10	1	0
11	28/30	8/10	2	2	22	30/30	10/10	0	0
					23	30/30	9/10	0	1

*Different test condition used in the same laboratory

Percentage of test agreement at different serum dilutions.

Lab code	Dilution sera				Lab code	Dilution sera			
	1/50	1/100	1/200	1/400		1/50	1/100	1/200	1/400
2	100	100	100	100	16a	100	100	90	100
5	100	100	90	100	16b	100	100	90	100
7	100	90	100	100	16c	100	100	90	100
8	100	100	100	100	18	80	80	60	40
9	100	100	100	100	18a	100	90	100	80
					19	100	90	100	100
10	100	100	100	80	22	100	100	100	100
11	100	90	90	80	23	100	100	90	100

To evaluate the 'protocol' effect, data from both type A and type B laboratories were compared using the same model as above. This has been done by enlarging the above model via the addition of a "protocol" fixed effect, that is:

Ie = β0 + Serum + Lab + β3Prot + β4Dil + β5Dil^2,

The Chi Square test for comparing the two nested models (with and without the 'Protocol' effect) provides significant differences between the models (p = 0.0038). This should be interpreted as an indication to perform the ELISA for *Trichinella* IgG antibodies as much as possible according to the CRLP protocol. Moreover, the addition of laboratories which introduced modifications to the CRLP protocol, produces a greater variance for the lab effect.

σ_L= 10.37 (where σ_L is the laboratory variance)

It should be noted that the modifications of this protocol were highly variable in both their number and importance; consequently, the present study does not give information on the statistical influence of each variation.

Considering the negative sera only, where a lower variability among the type A and type B labs can be expected, the variance of the lab effect is smaller than the variance of the serum effect. The Chi Square test for comparing the two nested models (with and without the 'Protocol' effect) provides a slight significant difference between the models (p = 0.07) (Figure 4)

Finally to evaluate the ELISA carried out in each laboratory, a score value has been calculated for each laboratory. The score was obtained according to the following equation:

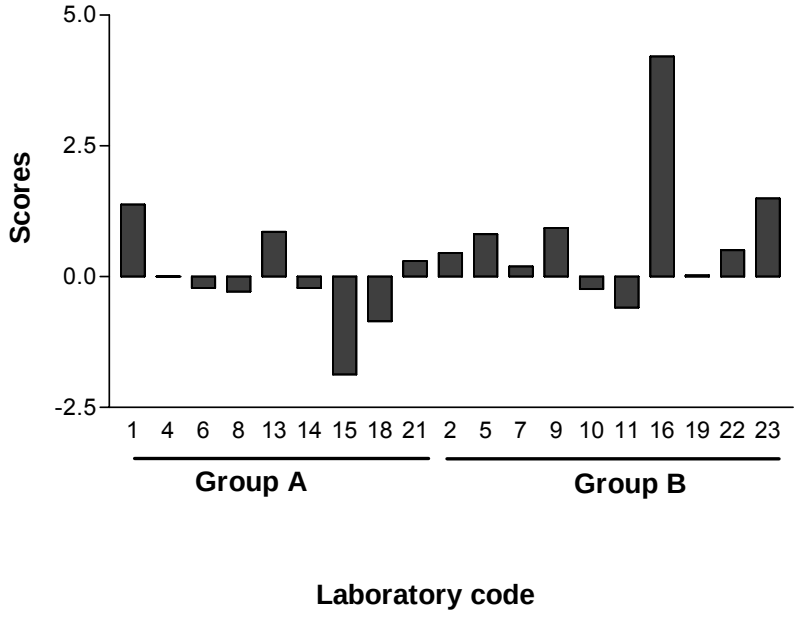
$$S = \frac{\text{Laboratory coefficient} - \text{CRLP coefficient}}{\text{Residual SD}}$$

Where the laboratory coefficient, related to the variance of each lab, is that showed in Table 1, and the 'Residual SD' is a measure of the casual uncertainty (see Table 1).

Table 1. Laboratory coefficients

Laboratory code	Laboratory coefficient	Laboratory code	Laboratory coefficient
1	41.94577	5	37.18419
4	30.38966	7	32.00318
6	28.55934	9	38.14013
8	28.00027	10	28.40780
13	37.59347	11	25.42253
14	28.58870	16	65.60207
15	14.71288	19	30.57090
18	23.25969	22	34.64263
21	32.89533	23	42.92962
2	34.17934	Residual SD	

Figure 4. Performance evaluation of the laboratories. Score values close to '0.0' show a higher laboratory performance.



6.3.3 Type C laboratories

Comparison of the CRLP test and other in house tests performed in 7 laboratories.

Lab code	Serum dilution	No. of positive samples in agreement with CRLP/total positive samples tested	% test agreement of total samples	No. of total samples in agreement with CRLP/total tested
4	1/50	30/30	100	40/40
6	1/50	12/30	55	22/40
8	1/50	26/30	90	36/40
8	1/100	8/8	100	10/10
17	1/50	9/30	48	19/40
19	unknown	30/30	100	40/40
21	1/100	8/8	100	10/10

Comparison of the CRLP test with commercial tests used in other laboratories

Lab code	Test (serum dilution)	No. of positive samples in agreement with CRLP/total positive samples tested	No. of total samples in agreement with CRLP/total tested (%)
2	C (1/20)	8/8	10/10 (100)
6	A (1/50)	17/30	27/40 (67.5)
10	C (1/50)	26/30	36/40 (90)
10	C (1/20)	8/8	10/10 (100)
12	C (1/20)	8/8	10/10 (100)
12	C (1/40)	8/8	10/10 (100)
15	B (1/50)	15/30	25/40 (62)
23	B (1/50)	23/30	33/40 (82.5)

*A = test not yet on the market; B and C = commercial tests/sera dilution

The results were not statistically evaluated, because the protocols used for each in house or commercial kit were too different.

7 Conclusions

The results show that the ELISA developed at the CRLP is a reproducible test allowing its use on the field to monitor pigs for the infection of *Trichinella* sp. at the farm level. The results obtained in each participating laboratory show the capability of most laboratories to reach a good standard even if the test was performed in different conditions. The reproducibility and robustness of this test suggest that it can be used in both endemic and non-endemic areas for *Trichinella* sp. infection and on different pig races.

8 Acknowledgements

Authors would like to thank Prof. Brunero Liseo for the statistical analysis of the data. We also thanks Dr. A. Ludovisi and Mr. M. Amati for their assistance to prepare the samples. The laboratories participating in this ring trial, listed below, are kindly acknowledged.

9 Participating laboratories

	Country
NRL for parasites, Institut für Veterinärmedizin	Austria
NRL for parasites, Institute of Tropical Medicine Antwerp	Belgium

Canadian Food Inspection Agency	Canada
University of Zagreb	Croatia
NRL for parasites, State Veterinary Laboratory	Cyprus
NRL for parasites, University of Veterinary and Pharm Sciences Brno	Czech Rep
NRL for parasites, Danish Food and Veterinary Institute	Denmark
NRL for parasites, Finnish Food Safety, Evira	Finland
NRL for parasites, Laboratoire d'études et de recherches en pathologie animale et zoonoses, AFSSA	France
NRL for parasites, Federal Institute for Risk Assessment, BfR	Germany
NRL for parasites, Center of Athens Veterinary Institutions	Greece
NRL for parasites, Central Veterinary Institute	Hungary
NRL for parasites, Istituto Superiore di Sanità	Italy
NRL for parasites, National Veterinary Laboratory	Lithuania
NRL for parasites, National Institute of Public Health and the Environment, RIVM	Netherlands
University of Utrecht	Netherlands
NRL for parasites, National Institute of Health	Portugal
Institute for the Application of Nuclear Energy, INEP	Serbia
NRL for parasites, National Veterinary Institute	Slovenia
Instituto de Salud Carlos III	Spain
NRL for parasites, Statens Veterinärmedicinska Anstalt	Sweden
Vetsuisse Faculty and Faculty of Medicine	Switzerland
USDA, ARS, BARC, APDL	USA

Laboratories not appearing on the above list did not register for this ring trial, either because they were not appointed at the time of the test, or because of technical problems.

10 References

1. Pozio, E., Murrell, K.D. (2006). Systematics and epidemiology of *Trichinella*. Advances in Parasitology 63:367-439.
2. Pozio E., Gomez Morales M.A., Dupouy Camet J. (2003). Clinical aspects, diagnosis and treatment of trichinellosis. Expert Review of Anti-infective Therapy 1:471-482.
3. Commission Regulation (EC) No 2075/2005 of 5 December 2005 laying down specific rules on official controls for Trichinella in meat. L338/60-82.
4. Office International des Epizooties. Manual of standards for diagnostic tests and vaccines, Paris, France, 322-327, 2000.
5. Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.
6. Pinheiro, José C. and Bates, D., (2000) Mixed-Effects Models in S and S-plus. Springer, New York.

Annex 1

Invitation letter to laboratories

ISTITUTO SUPERIORE DI SANITA'
COMMUNITY REFERENCE LABORATORY FOR PARASITES
DIRECTOR: DR. EDOARDO POZIO

9th October, 2006

Object: Ring Trial to detect anti-*Trichinella* IgG in swine sera by an ELISA

Dear All,

It is a great pleasure that all of you accepted to participate in this first ring trial for the detection of anti-*Trichinella* IgG in swine sera by an ELISA.

In this envelop, you will find the following forms:

1) few general information on the Ring Trial (1 page);
2) laboratory description (1 page)
3) the code assigned to your laboratory; this code should be written on the top right corner of the sheets of the items 1, 2, 4, 5 and 6;
4) a list of instruments, reagents and materials for the indirect ELISA (3 pages);
5) the procedure for the indirect ELISA (1 page);
6) the check of the package content and its condition of preservation (1 page).

Forms at the items 1, 2, 4, 5 and 6 should be filled in and sent me back by email, whereas a signed hard copy should be sent by priority mail or a courier, together with the photocopy of the original report/s containing OD values of all tests performed on serum samples provided by us. We greatly appreciate if you would provide us also with an electronic form of the OD report(s).

If you will use the same sera I sent you with other in-house or commercial kits, please, provide me all the information of the procedures, a photocopy of the OD report/s and the instructions of commercial kits even if they are not on the market yet.

For any question, please, contact Dr. M.A. Gomez Morales (tel +39 06 4990 3379) by telephone or email (gomez@iss.it) or myself.

Best Regards

Edoardo Pozio

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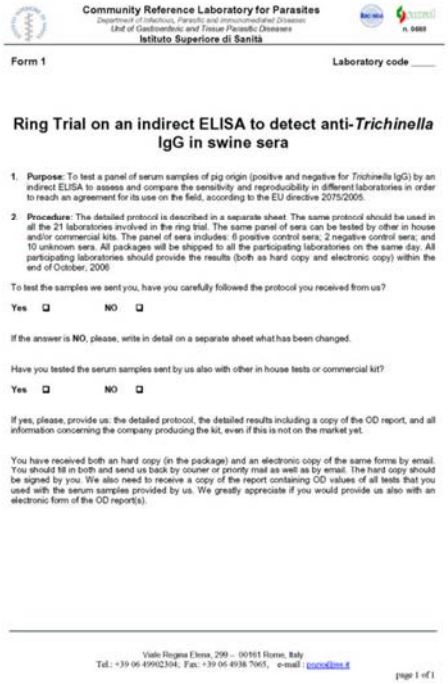
Tel.: +39 06 49902304 ; Fax: +39 06 4938 7065
e-mail: pozio@iss.it

Forms sent to participating laboratories



Community Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastrointestinal and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 1 Laboratory code _____

Ring Trial on an indirect ELISA to detect anti-*Trichinella* IgG in swine sera

1. **Purpose:** To test a panel of serum samples of pig origin (positive and negative for *Trichinella* IgG) by an indirect ELISA to assess and compare the sensitivity and reproducibility in different laboratories in order to reach an agreement for its use on the field, according to the EU directive 2075/2005.
2. **Procedure:** The detailed protocol is described in a separate sheet. The same protocol should be used in all the 21 laboratories involved in the ring trial. The same panel of sera can be tested by other in house and/or commercial kits. The panel of sera includes: 8 positive control sera; 2 negative control sera; and 10 unknown sera. All packages will be shipped to all the participating laboratories on the same day. All participating laboratories should provide the results (both as hard copy and electronic copy) within the end of October, 2006

To test the samples we sent you, have you carefully followed the protocol you received from us?

Yes ☐ NO ☐

If the answer is **NO**, please, write in detail on a separate sheet what has been changed.

Have you tested the serum samples sent by us also with other in house tests or commercial kit?

Yes ☐ NO ☐

If yes, please, provide us: the detailed protocol, the detailed results including a copy of the OD report, and all information concerning the company producing the kit, even if this is not on the market yet.

You have received both an hard copy (in the package) and an electronic copy of the same forms by email. You should fill in both and send us back by courier or priority mail as well as by email. The hard copy should be signed by you. We also need to receive a copy of the report containing OD values of all tests that you used with the serum samples provided by us. We greatly appreciate if you would provide us also with an electronic form of the OD report(s).

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page 1 of 1

Community Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastrointestinal and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 2 Laboratory code _____

Laboratory description

Is your lab accredited according to UNI CEI EN ISO/IEC 17025:2005? Yes ☐ No ☐

If yes, in which year has been the laboratory accredited? _____

How many samples have you tested by serology last year?

- for viral diseases _____
- for bacterial diseases _____
- for parasitic diseases _____
- for other diseases _____

What kind of test do you use for serology:

commercial kit ☐ in-house test ☐ both ☐

If you are routinely using in-house tests, was the antigen prepared in the lab? Yes ☐ No ☐

What kind of serological tests are you using in the lab (e.g., ELISA, IF, Wb, IHA)?

What kind of serological test/s are you using in the lab to detect anti-*Trichinella* antibodies in swine sera?

Commercial kit ☐ In-house test ☐ Other (specify) _____ No test ☐

Please provide, in a separate sheet, the detailed protocol, including reagents and equipments, that you are routinely using for the in house test and that you will use in parallel with serum samples we sent you.

How many scientists and technicians are working in the lab? Scientists _____ Technicians _____

How many scientists and technicians are working on serology? Scientists _____ Technicians _____

How long is the experience of this/these person/s in this specific diagnostic field? ___ (months/years)

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page 1 of 1

Community Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastrointestinal and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 3

The code of your laboratory is .....

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page 1 of 1

Community Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastrointestinal and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 4 Laboratory code _____

Instruments, reagents and materials required for the detection of anti-*Trichinella* antibodies in swine serum by an indirect ELISA

1 Instruments

The following instruments are needed to prepare the reagents to perform the ELISA procedure. To make comparable the results obtained by laboratories involved in the ring trial, we have to verify which instruments have been used. Thus you are requested to mark the column YES in case of use of the relevant instrument or, on the contrary, describe the variation in the following column.

DESCRIPTION	YES	VARIATION
Adjustable pipettes (volumes: 1 ÷ 1000 µL)		
Balance (0.01-100gr)		
Automatic plate washer (if is strongly recommended, if available, if not, please, notify it)		
ELISA plate microtiter reader		
Freezer -20-30°C		
Ice maker		
Incubator 37°C		
Magnetic stirrer		
Adjustable volume dispenser (e.g. Multipette Eppendorf®)		
pH meter		
Pipette aid		
Refrigerator +4°C ± 2°C		
Vortex		

2 Reagents and materials

The procedure to prepare the reagents is described step by step in the following table. To make comparable the results obtained by laboratories involved in the ring trial, we must verify that the preparation of reagents was carefully followed. Thus you are requested to mark the column YES if you strictly followed the indications (ex: pH, volume, reagents) or, alternatively, describe in the column VARIATION any variation you brought to the step.

Step	DESCRIPTION	YES	VARIATION
2.1	**Analytical grade water**		
2.2	**Phosphate buffered saline (PBS), pH 7.3 ± 0.2** KH_2PO_4 0.34 g Na_2HPO_4 1.21 g NaCl 8.0 g Analytical grade water up to 1000 mL Dissolve compounds in 750 mL of analytical grade water under magnetic stirring. Check the pH (7.3 ± 0.2) and then bring the solution to the final volume; store refrigerated.		

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page 1 of 3

2.3	**Carbonate buffered saline, pH 9.6 ± 0.2** Na_2CO_3 1.12g $NaHCO_3$ 2.92g Analytical grade water up to 1000 mL Dissolve compounds in 750 mL of analytical grade water under magnetic stirring. Check the pH (9.6 ± 0.2) and then bring the solution to the final volume; store at room temperature. If it needs, clear the solution by filtration.		
2.4	**Washing solution** Tween 20 1 mL Analytical grade water up to 2000 mL The solution should be prepared immediately before use, as follows: add 1 mL of Tween 20 into a 2 L flask, bring it to the volume with analytical grade water, and mix by magnetic stirring until the solution is clear. If stored refrigerated, the solution should be utilised within 24 hs.		
2.5	**Blocking solution** BSA 0.25 g Tween 20 0.05 mL PBS up to 50.00 mL The solution should be prepared immediately before use, as follows: weight 0.25 g BSA (bovine serum albumin) directly in a 50 mL tube, add 40 ml of PBS buffer and mix by vortexing until the BSA is completely dissolved. Add 0.05 mL Tween 20, mix by vortexing and bring to volume. If stored refrigerated, the solution can be utilised within 24 hs.		
2.6	**Sera and conjugate diluter** BSA 1.00g Tween 20 0.05mL PBS up to 100 mL The solution should be prepared immediately before use, as follows: weight 0.50 g BSA directly in a 50 mL tube, add 40 ml of PBS buffer and mix by vortexing until BSA is completely dissolved. Add 0.025 mL Tween 20, stir by vortexing and bring it to volume. If stored refrigerated, the solution can be utilised within 24 hs.		
2.7	**Stop solution** HCl 1N in analytical grade water Prepare the solution under a chemical hood; store at room temperature		
2.8	**TMB (3, 3', 5, 5' tetra-metil-benzidine) peroxidase substrate, Kirkegaard & Perry Laboratories, cod. 50-76-00, Gaithersburg, Maryland 20879-4174, USA.** This substrate is recommended, if not available, any other peroxidase substrate can be used (please, notify it)		
2.9	**96-wells flat bottomed microtiter plate** (provided by the CRLP)		
2.10	**Excretory/secretory antigens (ES Ag)** (provided by the CRLP) The antigens should be reconstituted using 60µL of analytical grade water. Then, the reconstituted antigens should be brought to a final volume of 12mL with carbonate buffer saline pH 9.6 (see 2.3). The reconstitution and the subsequent dilution steps should be performed on ice immediately before use.		

2.11	**Anti–swine IgG goat antibodies peroxidase labeled (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA)** (provided by the CRLP) The conjugate should be used at 1/20,000 dilution using the conjugate diluter (see 2.6). The dilution should be prepared on ice immediately before the use.		
2.12	**Anti-*Trichinella* sp. seropositive control sera** (provided by the CRLP) 100 µL x 6 of lyophilised sera from 6 pigs positive for *Trichinella spiralis* identified with codes PC1 – PC6 Each serum should be reconstituted using 100 µL of analytical grade water. Each positive control serum should be used at 1/50 dilution in duplicate using the appropriate diluter (see 2.6). The reconstitution and the subsequent dilution should be performed on ice immediately before use.		
2.13	**Anti-*Trichinella* sp. seronegative control sera** (provided by the CRLP) 100 µL x 2 of lyophilised sera from 2 pigs negative for *Trichinella spiralis* identified with codes NC1 – NC2. Each serum should be reconstituted using 100 µL of analytical grade water. Each negative control serum should be used at 1/50 dilution in duplicate using the appropriate diluter (see 2.6). The reconstitution and the subsequent dilution should be performed on ice immediately before use.		
2.14	**Unknown swine sera** (provided by the CRLP) 100 µL x 10 of lyophilised sera from 10 pigs, which have been tested for *Trichinella* by HCL-pepsin digestion (unknown samples) identified with codes US1 – US10. Each serum should be reconstituted using 100 µL of analytical grade water. Each serum should be tested at 1/50, 1/100, 1/200 and 1/400 dilutions in duplicate using the appropriate diluter (see 2.6). The reconstitution and the subsequent dilution should be performed immediately on ice before use.		

Form 5 **Laboratory code _____**

Procedure for the detection of anti-*Trichinella* antibodies in swine serum by an indirect ELISA

The procedure is described step by step in the following table. To make comparable the results obtained by laboratories involved in the ring trial, the operative protocol must be carefully followed. Thus you are requested to mark the column YES, if you strictly followed the indications (ex. pH, volume, incubation temperature, time) or, alternatively, describe in the column VARIATION any variation you brought to the step.

Step	DESCRIPTION	YES	VARIATION
1.0	Fill the microtiter plate with 100µL per well of ES Ag in carbonate buffered saline (see 2.3), incubate for 1 hour at 37°C		
2.0	Wash 3 times in the automatic plate washer with the washing solution (see 2.4)		
3.0	Add 200 µL per well of blocking solution (see 2.5), incubate for 1 h at 37° C		
4.0	Wash 3 times in the automatic plate washer with the washing solution (see 2.4)		
5.0	Add 100µL per well of each diluted sample as reported in the following scheme, and incubate for 30 min at 37°C		
5.1	Each serum dilution should be in duplicate		
5.2	Positive (PC) and negative (NC) control sera should be diluted 1/50		
5.3	Unknown sera (US) should be diluted 1/50, 1/100, 1/200, and 1/400		
5.4	For the duplicate of US10, only the wells for the dilution 1/50 and 1/100 are available		

ELISA plate scheme

	1	2	3	4	5	6	7	8	9	10	11	12
A	$PC1_{50}$	$PC1_{100}$	$US1_{50}$	$US1_{100}$	$US2_{50}$	$US2_{100}$	$US3_{50}$	$US3_{100}$	$US4_{50}$	$US4_{100}$	$US5_{50}$	$US5_{100}$
B	$PC2_{50}$	$PC2_{100}$	$US1_{200}$	$US1_{400}$	$US2_{200}$	$US2_{400}$	$US3_{200}$	$US3_{400}$	$US4_{200}$	$US4_{400}$	$US5_{200}$	$US5_{400}$
C	$PC3_{50}$	$PC3_{100}$	$US1_{50}$	$US1_{100}$	$US2_{50}$	$US2_{100}$	$US3_{50}$	$US3_{100}$	$US4_{50}$	$US4_{100}$	$US5_{50}$	$US5_{100}$
D	$PC4_{50}$	$PC4_{100}$	$US1_{200}$	$US1_{400}$	$US2_{200}$	$US2_{400}$	$US3_{200}$	$US3_{400}$	$US4_{200}$	$US4_{400}$	$US5_{200}$	$US5_{400}$
E	$PC5_{50}$	$PC5_{100}$	$US6_{50}$	$US6_{100}$	$US7_{50}$	$US7_{100}$	$US8_{50}$	$US8_{100}$	$US9_{50}$	$US9_{100}$	$US10_{50}$	$US10_{100}$
F	$PC6_{50}$	$PC6_{100}$	$US6_{200}$	$US6_{400}$	$US7_{200}$	$US7_{400}$	$US8_{200}$	$US8_{400}$	$US9_{200}$	$US9_{400}$	$US10_{200}$	$US10_{400}$
G	$NC1_{50}$	$NC1_{100}$	$US6_{50}$	$US6_{100}$	$US7_{50}$	$US7_{100}$	$US8_{50}$	$US8_{100}$	$US9_{50}$	$US9_{100}$	$US10_{50}$	B
H	$NC2_{50}$	$NC2_{100}$	$US6_{200}$	$US6_{400}$	$US7_{200}$	$US7_{400}$	$US8_{200}$	$US8_{400}$	$US9_{200}$	$US9_{400}$	$US10_{400}$	B

Legend

$PC1_{50}$ = positive control serum No. 1, diluted 1/50; $NC1_{50}$ = negative control serum No. 1, diluted 1/50
$US1_{50}$ - $US1_{400}$ = unknown serum No. 1, diluted 1/50 – 1/400; B = Blank

6.0	Wash 3 times in the automatic plate washer with the washing solution (see 2.4)		
7.0	Add 100µL per well of the diluted anti-swine IgG peroxidase labelled antibodies to each well and incubate for 1h at 37° C		
8.0	Wash 3 times in the automatic plate washer with the washing solution (see 2.4)		
9.0	Add 100µL per well of TMB substrate (see 2.8), incubate for 10 min at room temperature		
10.0	Stop the reaction by adding 50µL per well of the stop solution (see 2.7) to each well and read the reaction in the ELISA plate microtiter reader at 450nm		

Form 6 **Laboratory code _____**

Check of the package content and its condition of preservation

1. ELISA plate x 1 (NUNC code 442404) ☐
2. 10 µL of conjugate (KPL Peroxidase-Labeled Affinity Purified Antibody to swine IgG, code 141406) ☐
3. 100 µL x 6 of lyophilised sera from 6 pigs positive for *Trichinella spiralis* (positive control sera) with the codes PC1 – PC6; ☐
4. 100 µL x 2 of lyophilised sera from 2 pigs negative for parasites including *Trichinella* sp. (negative control sera) with the codes NC1 – NC2; ☐
5. 100 µL x 10 of lyophilised sera from 10 pigs, which have been tested for *Trichinella* by HCL-pepsin digestion (unknown samples) with the codes S1 – S10; ☐
6. 60 µg x 2 of excretory/secretory antigens from *Trichinella spiralis* muscle larvae, batch 1/2004 ☐
7. Hard copies of forms which should be filled in and send back to CRLP ☐
8. Hard copy of the detailed instruction to perform the ELISA which should be filled in and send back to CRLP ☐

The content of the package has been forwarded with dry ice

- When did you receive the package? Date _____hour____
- When did you open it? hour____
- Was dry ice still present in the package? **Yes** ☐ **No** ☐
- Was the conjugate still frozen? **Yes** ☐ **No** ☐

The conjugate should be stored frozen at -20°C before use.

The ELISA plate, the E/S lyophilised antigens and the lyophilised sera should be stored at +4°C before use.

Annex 2

Material stability

The material stability was tested as follows:

- at + 4°C or - 20°C or - 80°C for 24 hrs before the assay;
- the plate was stored under dry ice for 72 hs before the assay;
- during the ring trial:
    - one week before the forwarding
    - one week after the forwarding
    - at the end of the ring trial

The stability of the OD of the control sera at +4°C, -20°C, -80°C was evaluated as the mean of raw OD values in duplicates of 3 plates (one per each temperature condition).

Stability of OD values of control sera stored at different temperatures.

Serum code	Mean of OD values± SD[a]	CV (%)[a]
PC1	2.520 ± 0.045	1.7
PC2	2.210 ± 0.008	0.3
PC3	1.889 ± 0.032	1.6
PC4	1.724 ± 0.022	1.2
PC5	1.672 ± 0.035	2.1
PC6	1.823 ± 0.089	4.8
NC1	0.289 ± 0.021	7.4
NC2	0.254 ± 0.009	3.8

[a]Data are referred to 1/50 dilution; CV allowed: positives 15%; negatives 25%.

The stability of the of the O.D. of "unknown" sera at +4°C, -20°C, -80°C was evaluated as the mean of raw O.D. values in duplicates of 3 plates (one per each temperature condition)

Stability of OD values of "unknown" sera stored at different temperatures.

Code	Mean ± SD[a]	CV[a] (%)	Code	Mean ± SD[a]	CV[a] (%)
US1	1.864 ± 0.034	1.8	US6	0.242 ± 0.021	8.7
US2	1.530 ± 0.045	2.9	US7	1.516 ± 0.019	1.3
US3	1.335 ± 0.043	3.2	US8	0.707± 0.015	2.1
US4	1.778 ± 0.011	0.6	US9	1.129± 0.028	2.5
US5	1.127 ± 0.036	3.2	US10	0.248 ± 0.067	2.7

[a]Data are referred to 1/50 dilution; CV allowed: positives 15%; negatives 25%.

The stability of the O.D. of control sera at three different times was evaluated as the mean of raw O.D. values in duplicates of 3 plates (one per each day)

Stability of OD values of control sera stored at different times.

Code	Mean ± SD[a]	CV[a](%)
PC1	2.562 ± 0.064	2.4
PC2	2.274 ± 0.053	2.3
PC3	1.846 ± 0.044	2.3
PC4	1.690 ± 0.061	3.6
PC5	1.788 ± 0.150	8.3
PC6	1.830 ± 0.094	5.1
NC1	0.280 ± 0.056	20
NC2	0.260 ± 0.055	21

[a]Data are referred to 1/50 dilution; CV allowed: positives 15%; negatives 25%.

The stability of OD values of 'unknown' serum at three different times was evaluated as the mean of raw O.D. values in duplicates of 3 plates (one per each day)

Stability of OD values of "unknown" sera stored at different temperatures.

Code	Mean ± SD[a]	CV[a](%)	Code	Mean ± SD[a]	CV[a](%)
US1	1.822 ± 0.067	3.7	US6	0.232 ± 0.055	24
US2	1.511 ± 0.142	9.4	US7	1.521 ± 0.094	6.2
US3	1.335 ± 0.031	2.3	US8	0.719± 0.073	10
US4	1.777 ± 0.073	4.1	US9	1.207± 0.069	5.7
US5	1.100 ± 0.130	12	US10	0.275 ± 0.013	5.1

[a]Data are referred to 1/50 dilution; CV allowed: positives 15%; negatives 25%.

The stability of OD values of control sera after the contact with the CO_2 developed from the dry ice was evaluated as the mean of raw O.D. values in duplicates of 2 plates. The greatest difference allowed for positive samples ≤ 0.400 and for negative samples ≤ 0.150.

Stability of OD values of control sera stored in contact with the CO_2 from the dry ice.

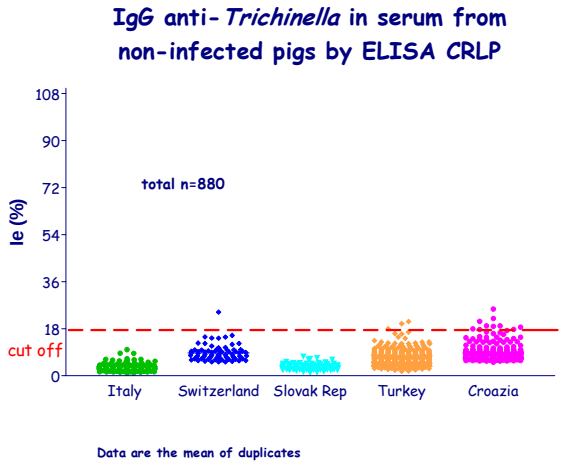
Code	OD (Mean duplicates) in standard conditions	OD (Mean duplicates) after 72hs under dry ice	Differences
PC1	2.488	1.415	1.073
PC2	2.213	1.015	1.198
PC3	1.879	0.694	1.185
PC4	1.745	0.605	1.14
PC5	1.712	0.561	1.151
PC6	1.925	0.764	1.161
NC1	0.265	0.268	0.003
NC2	0.260	0.239	0.021

The stability of OD values of 'unknown' sample after contact with dry ice was evaluated as the variation of raw O.D. of duplicates of 2 plates. The greatest difference allowed for positive samples ≤ 0.400 and for negative samples ≤ 0,150.

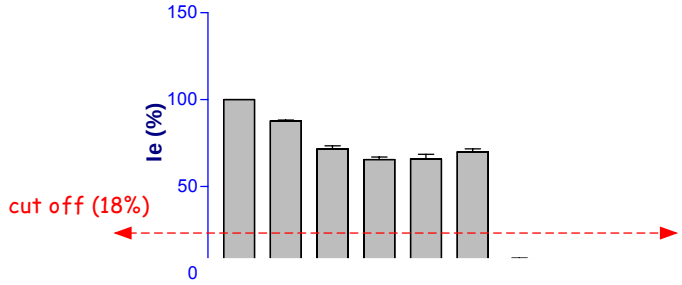
Stability of OD values of 'unknown' sera stored in contact with the CO_2 from the dry ice.

Code	OD (mean duplicates) standard conditions	OD (mean duplicates) after 24 hours in dry ice	Differences
US1	1.830	0.695	1.135
US2	1.583	0.493	1.09
US3	1.362	0.606	0.756
US4	1.791	0.659	1.132
US5	1.157	0.318	0.839
US6	0.210	0.210	0
US7	1.537	0.547	0.99
US8	0.736	0.292	0.444
US9	1.183	0.386	0.797
US10	0.256	0.254	0.002

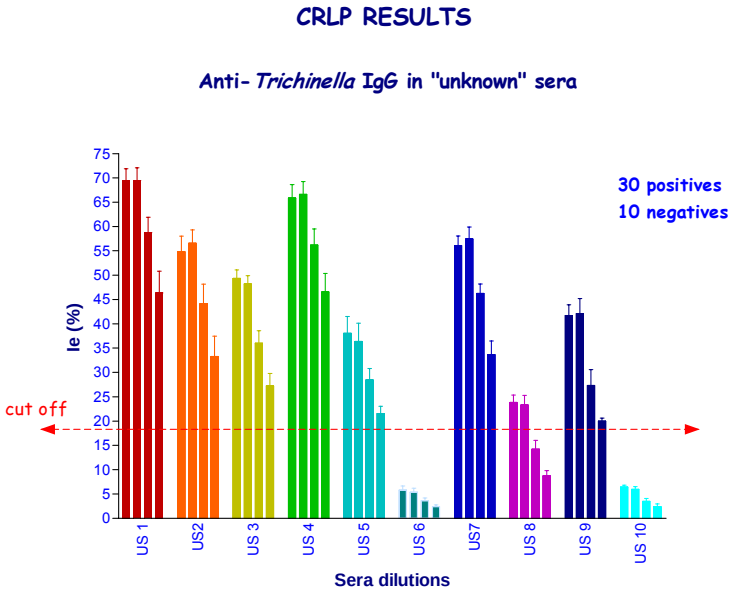
Ie values of control sera from non-infected pigs tested at the CRLP



Ie values of OD of positive (PC) and negative (NC) control sera obtained at the CRLP.



Ie values detected at the CRLP of 'unknown' sera (US) used for the ring trial.



Annex 3

Ie values for 'US' sera at the four dilutions from group A laboratories

Serum code	Laboratory code								
and dilution	**1**	**4**	**6**	**8**	**13**	**14**	**15**	**18**	**21**
US1 1/50	**70.7%**	**73.2%**	**67.5%**	**52.6%**	**65.8%**	**63.5%**	**42.7%**	**52.6%**	**52.5%**
US1 1/100	**64.1%**	**70.8%**	**69.1%**	**52.7%**	**72.1%**	**61.8%**	**51.5%**	**58.0%**	**61.4%**
US1 1/200	**79.4%**	**56.7%**	**58.8%**	**53.9%**	**76.9%**	**55.0%**	**34.5%**	**63.9%**	**66.6%**
US1 1/400	**67.1%**	**34.6%**	**52.9%**	**50.9%**	**77.6%**	**49.4%**	**20.1%**	**60.0%**	**69.3%**
US2 1/50	**60.4%**	**57.5%**	**50.4%**	**40.3%**	**53.9%**	**50.0%**	**34.0%**	**37.5%**	**40.3%**
US2 1/100	**61.3%**	**59.7%**	**48.7%**	**37.9%**	**56.5%**	**49.1%**	**33.9%**	**46.1%**	**49.8%**
US2 1/200	**66.2%**	**48.1%**	**39.8%**	**39.0%**	**54.9%**	**46.3%**	**23.6%**	**50.1%**	**51.2%**
US2 1/400	**55.6%**	**40.8%**	**34.7%**	**35.9%**	**54.2%**	**38.1%**	**13.6%**	**43.3%**	**50.8%**
US3 1/50	**59.0%**	**51.7%**	**48.3%**	**39.4%**	**53.7%**	**46.4%**	**44.9%**	**36.4%**	**40.5%**
US3 1/100	**54.0%**	**49.7%**	**42.0%**	**35.8%**	**49.4%**	**40.2%**	**42.5%**	**40.1%**	**40.9%**
US3 1/200	**49.1%**	**39.3%**	**32.0%**	**33.8%**	**54.5%**	**36.8%**	**28.5%**	**37.0%**	**46.5%**
US3 1/400	**38.5%**	**33.9%**	**26.3%**	**29.4%**	**45.3%**	**27.8%**	**16.7%**	**31.8%**	**39.1%**
US4 1/50	**71.6%**	**68.2%**	**63.0%**	**54.3%**	**59.3%**	**58.7%**	**40.3%**	**53.6%**	**52.1%**
US4 1/100	**72.8%**	**70.1%**	**60.5%**	**52.8%**	**67.3%**	**58.6%**	**53.4%**	**58.4%**	**67.3%**
US4 1/200	**75.7%**	**578%**	**51.7%**	**53.8%**	**70.4%**	**57.5%**	**33.8%**	**63.6%**	**65.0%**
US4 1/400	**70.4%**	**51.0%**	**44.8%**	**51.6%**	**70.2%**	**49.8%**	**22.4%**	**61.3%**	**75.8%**
US5 1/50	**55.8%**	**42.8%**	**32.4%**	**29.9%**	**33.4%**	**32.0%**	**18.7%**	**30.1%**	**26.2%**
US5 1/100	**53.6%**	**40.9%**	**32.9%**	**26.7%**	**36.8%**	**34.1%**	**23.8%**	**33.5%**	**35.7%**
US5 1/200	**45.4%**	**31.4%**	**28.0%**	**25.1%**	**43.3%**	**28.2%**	**13.3%**	**33.7%**	**30.2%**
US5 1/400	**33.1%**	**23.5%**	**21.9%**	**22.0%**	**30.9%**	**21.8%**	**8.2%**	**27.4%**	**34.0%**
US6 1/50	**7.5%**	**7.5%**	**4.4%**	**4.2%**	**8.5%**	**6.9%**	**8.4%**	**3.1%**	**4.5%**
US6 1/100	**5.8%**	**6.9%**	**4.4%**	**3.3%**	**7.4%**	**5.3%**	**5.9%**	**3.3%**	**4.6%**
US6 1/200	**4.3%**	**4.8%**	**3.3%**	**3.1%**	**6.0%**	**3.9%**	**4.8%**	**3.1%**	**3.3%**
US6 1/400	**3.0%**	**2.9%**	**2.5%**	**3.0%**	**5.4%**	**2.7%**	**3.3%**	**2.2%**	**2.6%**
US7 1/50	**58.7%**	**57.1%**	**57.7%**	**43.2%**	**56.2%**	**55.3%**	**47.6%**	**47.9%**	**44.6%**
US7 1/100	**75.9%**	**59.7%**	**51.8%**	**42.1%**	**54.5%**	**51.3%**	**41.6%**	**51.5%**	**58.7%**
US7 1/200	**64.3%**	**47.3%**	**45.0%**	**40.7%**	**60.7%**	**45.3%**	**28.8%**	**51.0%**	**56.2%**
US7 1/400	**47.3%**	**37.3%**	**37.7%**	**38.5%**	**49.8%**	**40.6%**	**11.9%**	**42.1%**	**51.7%**
US8 1/50	**35.2%**	**25.7%**	**23.0%**	**17.0%**	**28.9%**	**26.5%**	**17.6%**	**18.6%**	**23.7%**
US8 1/100	**27.3%**	**25.3%**	**18.3%**	**13.4%**	**26.0%**	**19.9%**	**16.0%**	**16.3%**	**22.6%**
US8 1/200	**19.3%**	**16.9%**	**15.2%**	**11.4%**	**19.4%**	**13.7%**	**11.6%**	**12.8%**	**19.2%**
US8 1/400	**13.0%**	**9.7%**	**9.6%**	**9.9%**	**14.7%**	**10.4%**	**5.7%**	**9.5%**	**14.6%**
US9 1/50	**49.3%**	**43.0%**	**39.8%**	**35.2%**	**42.8%**	**44.4%**	**31.8%**	**34.1%**	**40.3%**
US9 1/100	**52.8%**	**45.7%**	**37.3%**	**30.1%**	**44.8%**	**38.0%**	**29.6%**	**35.3%**	**43.8%**
US9 1/200	**42.8%**	**33.4%**	**34.3%**	**26.2%**	**34.4%**	**30.2%**	**18.1%**	**30.7%**	**37.7%**
US9 1/400	**29.1%**	**18.8%**	**25.6%**	**22.0%**	**28.6%**	**20.5%**	**8.4%**	**23.4%**	**34.6%**
US10 1/50	**7.9%**	**7.1%**	**6.1%**	**5.5%**	**9.3%**	**8.7%**	**12.9%**	**4.0%**	**5.2%**
US10 1/100	**5.4%**	**6.8%**	**5.5%**	**4.8%**	**7.1%**	**6.7%**	**14.6%**	**4.3%**	**5.2%**
US10 1/200	**4.6%**	**4.2%**	**4.5%**	**4.2%**	**6.9%**	**4.7%**	**8.7%**	**3.5%**	**6.1%**
US10 1/400	**2.7%**	**3.0%**	**3.0%**	**3.9%**	**5.9%**	**3.3%**	**3.8%**	**2.8%**	**3.9%**

Marked areas show false positive or false negative values

Annex 4

Ie values for 'US' sera at the four dilutions from group B laboratories

Serum code and dilution	Laboratory code														
	2	**5**	**7**	**8**	**9**	**10**	**11**	**16***	**16***	**16***	**18****	**18****	**19**	**22**	**23**
US 1 1/50	57.1	46.9	53.3	67.5	77.6	49.6	48.0	85.3	85.2	85.2	45.8	70.1	56.5	66.4	57.0
US 1 1/100	60.9	55.9	60.6	60.1	66.9	59.8	46.6	90.4	89.9	89.9	35.7	58.1	55.7	68.9	73.3
US 1 1/200	56.1	65.9	65.6	55.7	69.3	57.7	42.9	77.9	78.3	79.4	23.2	40.3	54.9	66.6	76.6
US 1 1/400	50.5	56.8	56.0	48.1	57.7	48.4	36.7	98.5	98.3	98.1	14.6	30.6	52.9	57.0	66.8
US 2 1/50	47.0	29.4	39.4	53.4	54.3	39.5	37.5	87.0	86.7	86.4	34.7	52.5	44.1	54.1	51.4
US 2 1/100	47.7	38.0	45.0	48.0	53.2	49.7	36.8	97.4	97.7	97.9	23.9	44.7	41.4	55.9	54.0
US 2 1/200	45.0	46.0	47.0	42.9	58.2	43.9	32.9	94.0	94.2	94.4	15.1	29.2	44.8	51.4	59.4
US 2 1/400	42.5	39.2	38.6	34.5	52.3	35.4	26.6	88.6	88.9	88.9	10.4	25.3	38.7	42.0	51.0
US 3 1/50	52.7	46.4	42.9	49.8	51.5	40.3	45.0	85.1	84.9	84.7	60.3	66.0	38.1	49.0	52.1
US 3 1/100	47.2	50.2	39.8	44.0	46.2	45.8	38.6	74.4	74.6	73.9	40.6	54.0	37.7	50.1	54.9
US 3 1/200	42.1	50.0	37.0	35.2	43.7	41.0	29.9	78.0	77.9	77.5	26.5	36.3	33.1	44.0	50.1
US 3 1/400	32.0	39.8	26.3	26.4	33.8	33.4	20.3	61.2	60.5	59.6	16.5	27.9	29.5	35.3	38.5
US 4 1/50	57.0	54.2	63.5	64.2	64.7	45.1	51.8	94.7	94.9	94.7	41.0	68.1	62.2	52.4	66.0
US 4 1/100	58.4	57.5	62.4	61.0	65.4	56.2	50.9	98.9	99.2	99.5	33.7	55.6	63.0	55.6	77.4
US4 1/200	56.1	67.7	64.3	57.2	62.2	58.0	46.5	99.1	99.2	99.5	23.9	42.6	59.9	57.2	79.7
US 4 1/400	52.3	62.7	56.2	50.8	55.6	55.5	38.7	103.9	104.0	104.3	18.1	32.9	53.7	53.7	70.3
US 5 1/50	39.2	39.3	34.4	37.8	40.2	26.7	31.1	59.4	58.7	57.5	3.9	45.0	32.4	30.7	36.9
US 5 1/100	35.9	46.1	33.9	34.7	39.3	30.8	29.8	46.2	45.3	44.0	3.7	31.5	37.5	33.1	43.5
US 5 1/200	29.9	46.6	29.9	27.6	31.3	26.0	23.6	44.8	43.9	42.7	2.1	21.6	29.2	30.8	36.2
US 5 1/400	23.5	42.1	20.4	20.1	25.5	16.9	15.0	38.8	37.9	36.6	1.8	14.1	24.7	21.4	26.7
US 6 1/50	11.9	13.2	6.0	4.6	5.8	7.8	2.2	15.9	15.2	14.1	0.2	0.6	4.9	8.4	5.8
US 6 1/100	7.7	10.8	6.3	3.6	6.4	7.4	3.6	6.8	6.3	5.7	0.1	0.7	4.9	6.0	5.6
US 6 1/200	4.5	10.6	6.0	2.8	3.9	6.7	1.8	5.0	4.7	4.2	0.1	0.5	4.5	4.4	4.6
US 6 1/400	2.4	7.9	6.2	2.3	3.3	6.7	1.8	4.6	4.3	3.9	0.5	0.5	4.0	3.2	3.9
US 7 1/50	55.6	42.3	45.6	51.5	59.1	36.9	47.5	99.6	99.6	99.8	48.6	61.5	43.9	59.5	63.2
US 7 1/100	57.1	62.4	47.4	49.1	59.2	43.8	53.8	104.0	104.0	104.5	32.2	49.8	45.0	62.7	70.9
US 7 1/200	48.5	65.9	47.3	44.5	53.3	40.9	41.9	96.8	97.0	97.0	17.3	33.9	41.9	47.9	66.1
US 7 1/400	37.7	61.5	40.5	36.3	44.1	39.5	33.1	76.8	76.1	75.1	9.7	19.4	35.4	42.1	56.1
US 8 1/50	33.9	24.3	19.0	22.4	25.0	19.7	19.5	58.4	57.7	56.7	14.7	26.8	18.1	28.1	24.5
US 8 1/100	23.3	23.4	16.4	18.3	22.7	18.0	15.2	24.9	23.9	22.6	8.2	15.3	17.6	22.7	24.8
US 8 1/200	16.2	19.8	14.7	14.8	15.0	13.4	28.4	21.0	20.0	18.8	3.2	7.4	12.4	14.2	18.1
US 8 1/400	10.7	14.9	12.5	10.0	10.2	11.7	19.9	14.9	14.1	13.0	1.2	3.2	9.6	9.1	14.3
US 9 1/50	41.5	38.6	35.4	43.6	44.4	27.1	38.4	75.7	75.4	74.8	34.0	50.5	38.5	27.6	46.8
US 9 1/100	42.2	46.7	35.4	37.1	42.2	26.8	34.4	57.0	56.2	55.2	19.4	35.8	35.7	32.0	51.3
US 9 1/200	33.6	42.7	33.0	31.5	31.2	23.3	28.4	54.1	53.3	52.0	10.0	21.5	28.8	26.6	40.8
US 9 1/400	23.2	33.3	26.5	23.4	21.0	17.1	19.9	37.8	36.7	35.4	5.6	11.6	24.2	19.1	31.6
US 10 1/50	14.8	16.3	8.3	6.8	5.0	2.1	2.4	17.2	16.4	15.2	0.4	1.7	4.8	5.0	7.4
US 10 1/100	17.0	13.5	8.3	5.3	5.7	1.5	3.3	7.3	6.9	6.4	0.5	1.2	4.0	3.6	6.8
US 10 1/200	9.1	13.8	7.7	4.1	4.0	0.5	1.8	6.3	5.8	5.2	0.2	0.7	3.6	1.9	5.6
US 10 1/400	4.1	9.1	6.5	3.1	2.9	-0.1	1.6	5.3	4.9	4.4	0.1	0.3	3.9	1.8	3.9

Marked areas show false positive or false negative values

*Laboratory which read the ELISA at three different λ values

**Laboratory which tested the sera with two different protocols