Report on the eighth NRL Proficiency Testing to detect adult worms of *Echinococcus* sp. in the intestinal mucosa of the definitive host

March-April, 2015

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1 Introduction

Cestode worms of the genus *Echinococcus* are zoonotic parasites circulating in most of European countries in both wild and domestic animals (Eckert et al., 2001). Humans acquire the infection by the ingestion of eggs shed by dogs, which can contaminate raw or undercooked vegetables and fruits, fomities, and the dog coat. Herbivore and omnivore animals (e.g. sheep, goats, cattle, pigs) are the intermediate hosts of parasites belonging to *Echinococcus granulosus* complex; whereas, sylvatic rodents are the intermediate hosts of parasites belonging to *Echinococcus multilocularis*. Humans can accidentally acquire the infection as an intermediate host, even if they represent a dead end of the parasite cycle. Domestic dogs and sylvatic canids (e.g. red foxes and raccoon dogs) act as final hosts of *E. multilocularis*. Domestic and stray dogs and rarely wolves, act as final hosts of *E. granulosus*. The incidence of infection greatly varies from one to another MS. In endemic EU countries, the incidence can reach 6.3 cases for 100,000 inhabitants (Pozio, 2008).

2 Scope

One of the core duties of EURLP is to organise proficiency testings (PTs), as stated in Regulation (EC) No 882/2004 of the European Parliament and of the Council (Commission Regulation No. 882/2004). The scope of this PT is to test the competence of the appointed NRLs to identify adult worms or their parts, e.g. proglottids and rostrellum, of *Echinococcus* sp. in the intestinal mucosa collected from the gut of the final host, and to differentiate this worm from other material of parasitic and non-parasitic origin present in the matrix.

3 Time frame

The proficiency testing (PT) was announced to NRLs by email on 15 January, 2015 and the deadline to send the participation form was 21 February, 2015. From 23 February, 2015, the general information on PT organization was available on EURLP web site at the following address: http://www.iss.it/crlp/index.php?lang=2&id=136&tipo=28. On Monday 16 March, 2015, the PT packages were dispatched to participants by an international courier. The date to submit the PT results was 30 March, 2015. PT reports with evaluations were delivered to NRLs on 10 April, 2015.

4 Test material

From November, 2014 to February, 2015, carcasses of red foxes shot by hunters in Italy, were sent to the Parasitic Diseases of Farm Animals Department of Veterinary Medicine and Animal Production University of Naples Federico II, Italy.

Carcasses were forwarded in individual plastic bags at +4 °C. The intestinal tract was removed and stored at −20 °C. Then for safety reasons (i.e. to kill *Echinococcus* embryos eventually present in the gut), the intestinal tract was frozen at −80 °C for 7 days before examination. After freezing, the gut was thawed

at room temperature and the middle and posterior parts of the intestine were collected and tested by sedimentation and counting technique (SCT) according to a previous published protocol (Mathis et al., 1996). When the sample resulted negative, the anterior third part of the intestine was opened and the mucosa was scraped and autoclaved for the reduction of bacterial activity. The mucosa of the small intestine of 30 foxes found to be negative was spiked with 9 or 19 worms in order to prepare weakly or highly positive samples, respectively (**Annex 1**). No spiked mucosa was used as negative control sample. Adult worms were kindly provided by Dr. T. Sreter of the NRL of Hungary (**Annex 1**).

The test material forwarded to each laboratory consisted of three vials containing:

1. Mucosa spiked with 9 *Echinococcus* adult worms, this sample being considered a weakly infected sample;
2. Mucosa spiked with 19 *Echinococcus* adult worms, this sample being considered a highly infected sample;
3. Negative mucosa, this sample being considered the negative control.

All samples were delivered within 24-36 hours. The following forms were included in the package:

1) information on PT and its purpose (**Annex 2**);
2) package content and its condition of preservation (**Annex 3**)
3) instructions for the detection of *Echinococcus* sp. adult worms (**Annex 4**);
4) results (**Annex 5**);
5) laboratory code.

5 Instructions to participants

Practical instructions were given to all the participants in the Form 3 (**Annex 4**) and in the accompanying letter. To make results obtained by laboratories comparable, all participants had to follow the protocol step by step or describe the modification made, if any. It was requested to qualitatively and quantitatively evaluate the samples by Sedimentation and counting Technique (SCT) (**Annex 6**) (Eckert et al., 2001).

6 Participating laboratories

Twenty-seven NRLs agreed to participate (**Annex 7**).

7 Evaluation criteria

7.1 Qualitative evaluation

The PT result evaluation is expressed as "**correct**" (detection of one or more *Echinococcus* sp. adult worms in spiked samples or no worm in not spiked samples) or "**incorrect**" (false positive or false negative results), irrespective of the

number of worms in the sample/s. The **final evaluation** is only based on qualitative evaluation and is expressed as "**positive**" if the results of all samples were correct or "**negative**" if at least one result was incorrect.

7.2 Quantitative evaluation

No consistent data exist in the scientific literature for the quantitative evaluation of the sedimentation and counting technique; therefore, a z-score was established using the standard deviation of the sample result from the average PT values. This statistical approach allows to evaluate laboratory performance in comparison to the overall average performance of this PT.

The Z score was calculated by the formula: $$z = \frac{X_{lab} - X_{\mathrm{Ref}}}{\hat{\sigma}}$$

where:
X_{lab} is the number of worms found in the sample by the laboratory;
X_{ref} is the number of worms spiked in the sample;
σ is the standard deviation (i.e., 1.65 for the sample # 1; and 6.99 for the sample # 3) calculated from the quantitative results obtained in this PT.

Evaluation criteria:
If the z-score is "≤ |3|", the laboratory result is "**positive"**; however, if the z-score is "|2| < z-score ≤ |3|", the result is still positive but the laboratory should be alerted to start preventive actions to avoid a future negative performance; if the z-score is "> |3|", the laboratory result is "**negative"**.

8 Results

The average recovery rate of adult worms for the weakly spiked (n=9) sample was 7 (range 1-10), whereas the average recovery rate was 15 (range 1-20) for the highly spiked sample (n=19).

8.1 The qualitative evaluation obtained by the NRLs was (**Annex 8**):

- Sample 1 (spiked with 9 worms): 27 laboratories (100%) obtained a positive evaluation.

- Sample 2 (spiked with 19 worms): 27 laboratories (100%) obtained a positive evaluation.

- Sample 3 (negative sample): 27 laboratories (100%) obtained a positive evaluation.

8.2 Quantitative evaluation obtained by the NRLs was (**Annexes 9 and 10**):

- Sample 1 (spiked with 9 worms): 26 laboratories (out of 27) obtained a positive evaluation (3 with alert) and 1 laboratory a negative evaluation.

- Sample 2 (spiked with 19 worms): 26 laboratories (out of 27) obtained a positive evaluation (3 with alert) and 1 laboratory a negative evaluation.

9 Conclusions

The results show that the personnel of NRLs participating in the PT, are skill to detect adult worms of *Echinococcus* spp. both in qualitative and quantitative test, even with a low worm burden. Quantitative evaluation was introduced using the z-score as statistical approach for the evaluation of the performance. Since no consistent data exist in the scientific literature on the detection limits of these parasites in the intestinal content by SCT, data originating from this and further PTs will be very important to establish the detection limit of this technique. The comparison of the results during the last 4 years, demonstrates that detection capabilities of NRL personnel are improving over time (**Annex 11**).

10 References

Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (2001). WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. World Organisation for Animal Health, Paris, France, pp. 1- 265.

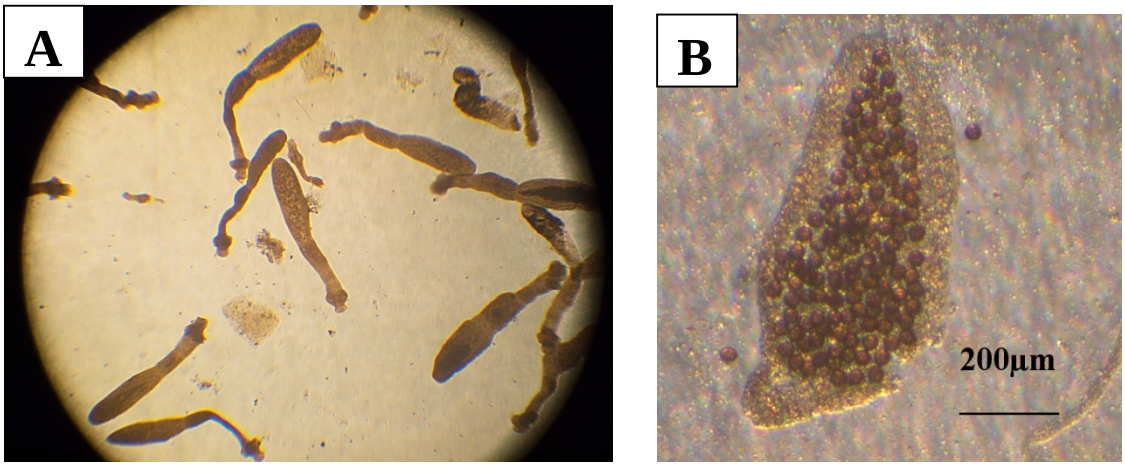
Mathis A, Deplazes P, Eckert J. (1996). An improved test system for PCR-based specific detection of Echinococcus multilocularis eggs. J Helminthol. 70:219-22.

Pozio, E. (2008). Epidemiology and control prospects of foodborne parasitic zoonoses in the European Union. Parassitologia 50:17-24.

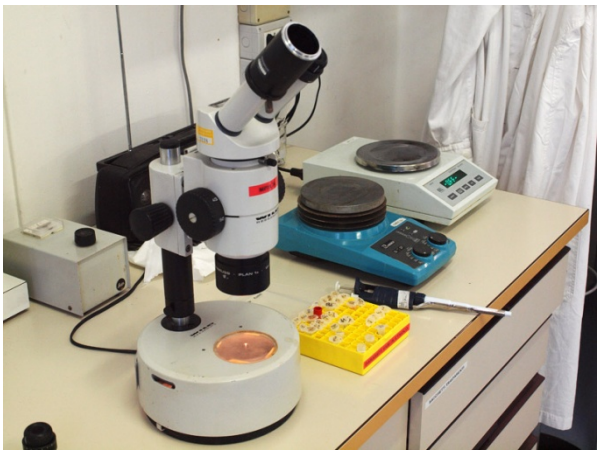
Annex 1

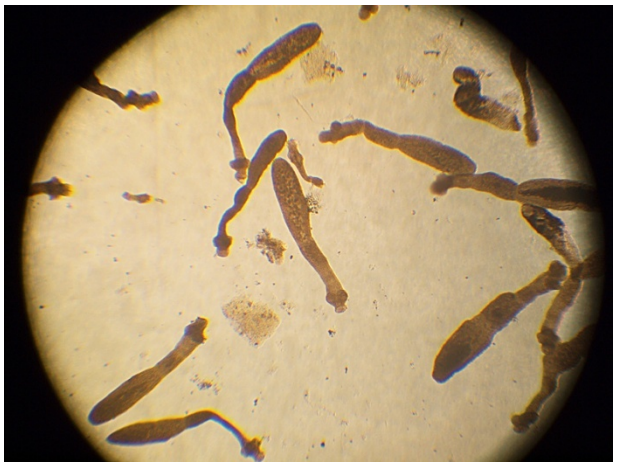
Preparation of the test material

Necropsy of a fox carcass: collection of the gut



Adult worms of *Echinococcus* (a) and a proglottid (b) isolated from the intestinal mucosa of a fox





Adult worms of *Echinococcus* spiked in the intestinal mucosa

The three PT samples forwarded to the 27 participating labs

Annex 2

Email sent to NRL to inform about the 2015-PTs and their purposes

Dear All,

Following your request during the 2014 Workshop, the EURLP will organize the following five PTs in 2015:

- Proficiency Testing on the identification of Trichinella larvae at the species level by a molecular method
- Proficiency Testing for the detection of Echinococcus sp. adult worms in the intestinal mucosa of the definitive host
- Proficiency Testing for the detection of Anisakidae L3 larvae in fish fillets
- Proficiency Testing to detect Trichinella spiralis larvae in meat samples according to EU Regulation 2075/2005
- Proficiency Testing to detect anti-Toxoplasma IgG in ovine serum samples

The PT samples will be forwarded on **Monday 16 March, 2015**.

We will inform you by email when the PT procedures and related forms for each PT (Participation Request Form) will be available on the website of the EURLP (http://www.iss.it/crlp/)

Kind Regards,

Edoardo Pozio

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Annex 3

Package content and its condition of preservation

European Union Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 1 **Laboratory code** _____

PT on "Detection of Echinococcus spp. adult worms in the intestinal mucosa of red foxes (*Vulpes vulpes*)"

Check of the package content and its condition of preservation

Insert sample codes:

1. _____ ❑
2. _____ ❑
3. _____ ❑

The content of the package has been forwarded refrigerated/frozen

- When did you receive the package? Date _____hour____
- When did you open it? hour____
- Which temperature did you measure inside the package when you opened it? _____

Additional instructions:

Annex 4

Instructions for the detection of *Echinococcus* sp. adult worms in the intestinal mucosa

European Union Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 3 **Laboratory code _____**

PT on "Detection of Echinococcus spp. adult worms in the intestinal mucosa of red foxes (*Vulpes vulpes*)"

Method: "Sedimentation and Counting technique (SCT)"

Procedure

The procedure is described step by step in the following table. To make comparable the results obtained by laboratories involved in the PT, 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.	Transfer the whole sample into a glass bottle containing 1L of saline solution (0.9% NaCl);		
2.	After a sedimentation of 15 min, discharge the supernatant and refill the bottle with saline solution;		
3.	Repeat step 2, until the solution is sufficiently clear (usually 2-3 times);		
4.	Examine the sediment in 5-10 ml portions in a Petri dish with a counting grid under a stereomicroscope (25x).		

Annex 5

Results

European Union Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form n. 4 **Laboratory Code _____**

PT on "Detection of Echinococcus spp. adult worms in the intestinal mucosa of red foxes (*Vulpes vulpes*)"

Results

SAMPLE CODE	ADULT WORM NUMBERS	NOTES

The PT results are to be submitted within 14 days after the shipment date

Date _____

Analyst

Name _____

Surname_____

signature

Annex 6

Sedimentation and counting Technique (SCT) (Eckert et al., 2001)

After –80 °C freezing the whole gut for no less than 5 days, counting can be done with the deep-frozen material after washing the intestinal mucosa using a dilution counting technique:

1. incise the intestine longitudinally and examine macroscopically for large helminths, and then cut the gut into 20 cm long segments;
2. transfer the segments of the intestine to a glass bottle containing 1 L of saline solution. After vigorous shaking for a few seconds, strip the mucosa between two pressed fingers, and remove the segments of the intestine from the flask.
3. allow the intestinal material to sediment for 15 min several times until the sediment is sufficiently cleared from coloured particles.
4. examine the sediment in small portions of 5-10 mL in rectangular plastic dishes with a counting grid (for example, 9 cm × 9 cm Falcon®, No. 1012) under a stereomicroscope at a 120x magnification.

Annex 7

National Reference Laboratories (NRL) participating at the PT for *Echinococcus* sp.

National Reference Laboratory	Country
Institut für Veterinärmedizin, Innsbruck	Austria
Institute of Tropical Medicine, Antwerp	Belgium
National Diagnostic and Research Veterinary Institute, Sofia	Bulgaria
State Veterinary Laboratory, Nicosia	Cyprus
Danish Food and Veterinary Institute, Copenhagen	Denmark
Estonian Veterinary and Food Laboratory, Tartu	Estonia
Finnish Food Safety, Evira, Oulu	Finland
Technopole Agricole et Vétérinaire, Malzeville	France
Friedrich-Loeffler-Institut, Institut für Epidemiologie, Wusterhausen	Germany
Centre of Athens Veterinary Institutions, Athens	Greece
Laboratories for Parasitology, Fish and Bee Diseases, Budapest	Hungary
Agrifood and Biosciences Institute, Celbridge	Ireland
Istituto Zooprofilattico Sperimentale of Sardinia, Sassari	Italy
Laboratory of Food and Environmental Investigations, National Diagnostic Centre, Riga	Latvia
National Food And Veterinary Risk Assessment Institute, Vilnius	Lithuania
National Veterinary Laboratory, Albertown, Marsa	Malta
National Institute of Public Health and the Environment, Bilthoven	Netherlands
National Veterinary Institute, Oslo	Norway
National Veterinary Research Institute, Pulawy	Poland
National Veterinary Institute, Lisbon	Portugal
Faculty of Veterinary Medicine, Skopje	The former Yugoslav Republic of Macedonia
Institute for Diagnosis and Animal Health, Bucarest	Romania
University of Ljubljana, Veterinary Faculty, Ljubljana	Slovenia
Veterinary and Food Institute, Bratislava	Slovak Republic
Laboratorio Central de Sanidad Animal de Santa Fe, Granada	Spain
Veterinary Laboratory Department of Agriculture & Food Laboratories, Omagh	UK
Veterinary Laboratories Agency, Suffolk	UK

Annex 8

Qualitative evaluation of the PT results

LAB CODE	SAMPLE 1 (N=9)	SAMPLE 2 (N=0)	SAMPLE 3 (N=19)	FINAL EVALUATION
E1	Correct	Correct	Correct	Positive
E2	Correct	Correct	Correct	Positive
E3	Correct	Correct	Correct	Positive
E4	Correct	Correct	Correct	Positive
E5	Correct	Correct	Correct	Positive
E6	Correct	Correct	Correct	Positive
E7	Correct	Correct	Correct	Positive
E8	Correct	Correct	Correct	Positive
E9	Correct	Correct	Correct	Positive
E10	Correct	Correct	Correct	Positive
E11	Correct	Correct	Correct	Positive
E12	Correct	Correct	Correct	Positive
E13	Correct	Correct	Correct	Positive
E14	Correct	Correct	Correct	Positive
E15	Correct	Correct	Correct	Positive
E16	Correct	Correct	Correct	Positive
E17	Correct	Correct	Correct	Positive
E18	Correct	Correct	Correct	Positive
E19	Correct	Correct	Correct	Positive
E20	Correct	Correct	Correct	Positive
E21	Correct	Correct	Correct	Positive
E22	Correct	Correct	Correct	Positive
E23	Correct	Correct	Correct	Positive
E24	Correct	Correct	Correct	Positive
E25	Correct	Correct	Correct	Positive
E26	Correct	Correct	Correct	Positive
E27	Correct	Correct	Correct	Positive

Annex 9

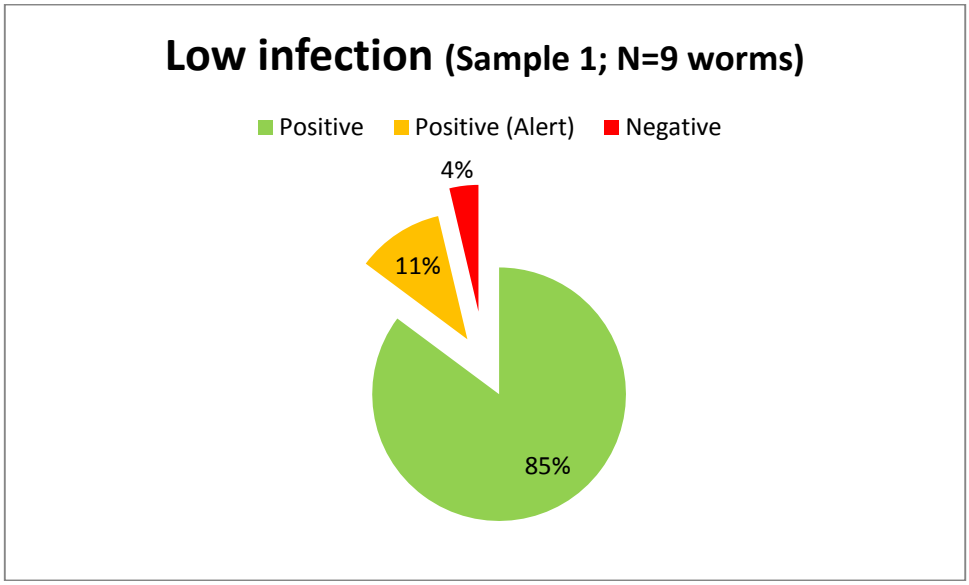
Quantitative evaluation of the PT results

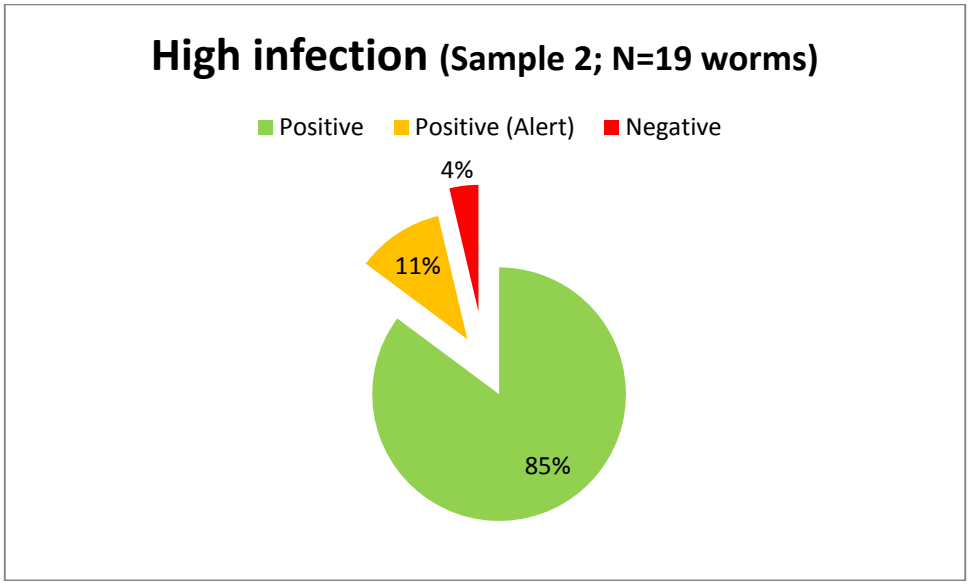
Lab Code	Sample (N=9)	z-score (N=9)	Evaluation	Sample (N=19)	z-score (N=19)	Evaluation
E1	6	-1,30	Positive	18	-0,18	Positive
E2	8	-0,43	Positive	19	0,00	Positive
E3	3	-2,61	Positive (Alert)	14	-0,91	Positive
E4	10	0,43	Positive	16	-0,55	Positive
E5	7	-0,87	Positive	14	-0,91	Positive
E6	1	-3,47	Negative	5	-2,56	Positive (Alert)
E7	8	-0,43	Positive	19	0,00	Positive
E8	6	-1,30	Positive	16	-0,55	Positive
E9	8	-0,43	Positive	20	0,18	Positive
E10	8	-0,43	Positive	19	0,00	Positive
E11	7	-0,87	Positive	18	-0,18	Positive
E12	6	-1,30	Positive	11	-1,46	Positive
E13	3	-2,61	Positive (Alert)	1	-3,29	Negative
E14	8	-0,43	Positive	18	-0,18	Positive
E15	9	0,00	Positive	22	0,55	Positive
E16	6	-1,30	Positive	19	0,00	Positive
E17	10	0,43	Positive	14	-0,91	Positive
E18	9	0,00	Positive	14	-0,91	Positive
E19	6	-1,30	Positive	6	-2,37	Positive (Alert)
E20	9	0,00	Positive	18	-0,18	Positive
E21	7	-0,87	Positive	9	-1,83	Positive
E22	3	-2,61	Positive (Alert)	5	-2,56	Positive (Alert)
E23	10	0,43	Positive	18	-0,18	Positive
E24	7	-0,87	Positive	21	0,37	Positive
E25	7	-0,87	Positive	20	0,18	Positive
E26	6	-1,30	Positive	16	-0,55	Positive
E27	9	0,00	Positive	15	-0,73	Positive

Z-score evaluation: if the z-score is “≤ |3|”, the laboratory result is “positive”; however, if the z-score is “|2| < z-score ≤ |3|”, the result is still positive but the laboratory should be alerted to start preventive actions to avoid a future negative performance; if the z-score is “> |3|”, the laboratory result is ”negative”.

Annex 10

Graphic representation of the PT quantitative results for 2015





Annex 11

Trend of the PTs using quantitative results with low and high infected samples from 2012 to 2015.