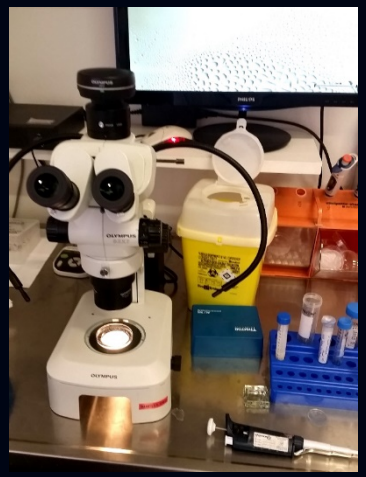
11th PROFICIENCY TESTING on:

“Detection of *Echinococcus* sp. worms in the intestinal mucosa of the definitive host”

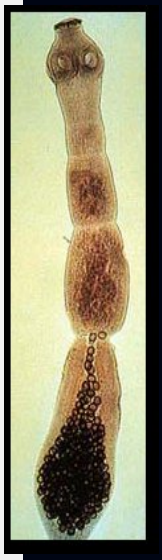
Adriano Casulli

PT on *Echinococcus* in the intestinal mucosa is accredited in a quality system according to ISO 17043 standard;



Participants (N=27)

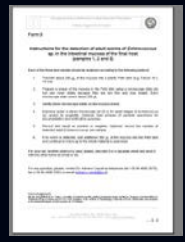
LABORATORIES	COUNTRY
Institute of Public Health, Tirana	**Albania**
Institut für Veterinärmedizin, Innsbruck	**Austria**
Institute of Tropical Medicine, Antwerp	**Belgium**
State Veterinary Laboratory, Nicosia	**Cyprus**
State Veterinary Institute, Jihlava	**Czech Republic**
Statens Serum Institut, Copenhagen	**Denmark**
National 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**
Laboratories for Parasitology, Fish and Bee Diseases, Budapest	**Hungary**
Agrifood and Biosciences Institute, Celbridge	**Ireland**
Istituto Zooprofilattico Sperimentale of Sardinia, Sassari	**Italy**
Institute for Experimental Pathology Keldur	**Iceland**
Laboratory of Food and Environmental Investigations, National Diagnostic Centre, Riga	**Latvia**
National Food And Veterinary Risk Assessment Institute, Vilnius	**Lithuania**
National Veterinary Institute, Oslo	**Norway**
National Veterinary Research Institute, Pulawy	**Poland**
National Veterinary Institute, Lisbon	**Portugal**
Institute for Diagnosis and Animal Health, Bucharest	**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**
National Veterinary Institute, SVA, Uppsala	**Sweden**
Animal and Plant Health Agency, Hutton, York	**UK**
AgriFood and Biosciences Institute (AFBI), Coneywarren, Omagh (Northern Ireland)	**UK**



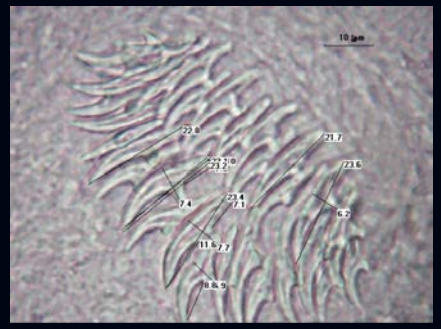
In each package the following forms were added:

Form 1 – Check of the package content and its condition of preservation

Form 3 – Instructions for the detection of *Echinococcus* spp



Form 4 - Results



Preparation of samples

- Foxes shot by hunters were collected and the intestines were stored frozen at -80°C for two weeks.
- The mucosa of the small intestine of foxes (found negative and those from non endemic areas) was collected and cleaned.
- Mucosa samples were homogenised with 70% ethanol (ratio 2:1), aliquoted and spiked with 0, 12 and 25 worms (spiked in double check).

We are very grateful to:

- **TAMAS SRETER** (Hungary)
- **JACEK KARAMON** (Poland)
- **ANTTI OKSANEN** (Finland)

for providing worms of *Em* and intestinal mucosa

Criteria for the **qualitative** evaluation

The PT result evaluation is expressed as "**CORRECT**" (detection of one or more *Echinococcus* sp. 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 samples;

The **FINAL EVALUATION** is only based on qualitative evaluation and is expressed as "positive" if the results of all samples are correct or "negative" if at least one result is incorrect.

Criteria for the **quantitative** evaluation

- The z-score was established using the SD of the sample from the average PT values.
- This statistical approach shows the laboratory performance in comparison to the average of the PT.

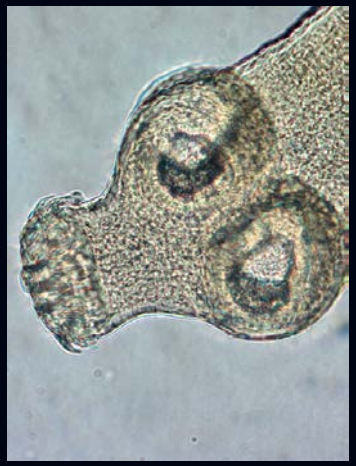
$$z = \frac{X_{lab} - X_{\mathrm{Ref}}}{\hat{\sigma}}$$

Evaluation criteria:

z-score ≤|3|; the laboratory result is **POSITIVE**

|2|<z-score≤|3|, the lab result is **POSITIVE** but with **ALERT**;

z-score > |3|; the laboratory result is **NEGATIVE**.



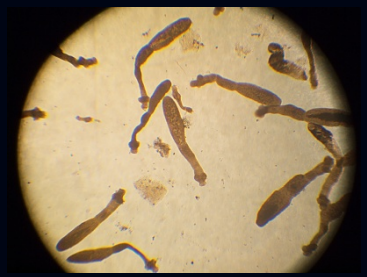
RESULTS (Qualitative evaluation)

The average RECOVERY RATE:

- 10 (range 0-19) [low worm number, n=12]
- 20 (range 2-47) [high worm number, n=25]

The QUALITATIVE evaluation obtained by the NRLs:

- Sample 1 (negative sample): 27 labs (100%) obtained a positive evaluation.
- Sample 2 (25 worms): 27 labs (100%) obtained a positive evaluation.
- Sample 3 (12 worms): 26 labs (96%) obtained a positive evaluation.

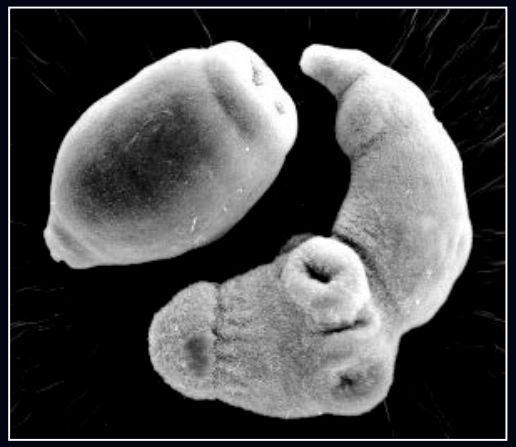


RESULTS (Qualitative evaluation)

LAB CODE	SAMPLE 1 (N=0)	SAMPLE 2 (N=25)	SAMPLE 3 (N=12)	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
E27	Correct	Correct	Incorrect	Negative
E28	Correct	Correct	Correct	Positive

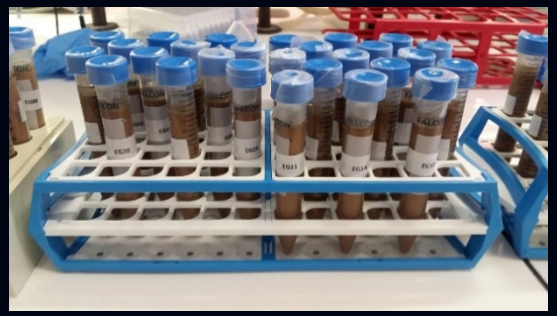
SUMMARY of RESULTS (Qualitative evaluation)

Number of participant laboratories	**26**
Number of participants that passed the PT	25
Number of participants that failed the PT	1

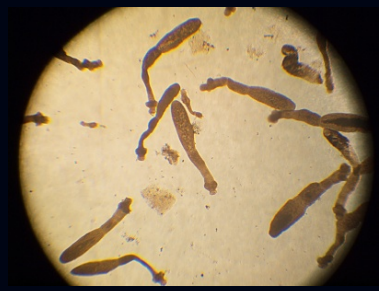


RESULTS low burden (Quantitative evaluation)

Lab Code	Sample (N=12)	Z-SCORE	EVALUATION
E1	2 *	-2,71	**Positive with alert**
E2	10	-0,54	Positive
E3	7	-1,35	Positive
E4	12	0,00	Positive
E5	12	0,00	Positive
E6	10	-0,54	Positive
E7	4	-2,17	**Positive with alert**
E8	12	0,00	Positive
E9	11	-0,27	Positive
E10	19	1,90	Positive
E11	7	-1,35	Positive
E12	13	0,27	Positive
E13	9	-0,81	Positive
E14	11	-0,27	Positive
E15	12	0,00	Positive
E16	11	-0,27	Positive
E17	6	-1,62	Positive
E18	10	-0,54	Positive
E19	12	0,00	Positive
E20	10	-0,54	Positive
E21	11	-0,27	Positive
E22	9	-0,81	Positive
E23	10	-0,54	Positive
E24	9	-0,81	Positive
E25	11	-0,27	Positive
E27	0	-3,25	**Negative**
E28	7	-1,35	Positive

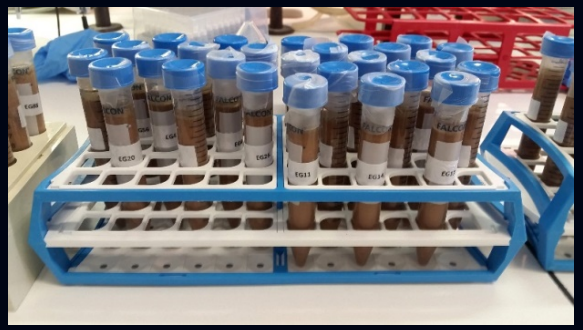


* Delayed delivery



RESULTS high burden (Quantitative evaluation)

Lab Code	Sample (N=25)	Z-SCORE	EVALUATION
E1	2 *	-2,84	Positive with alert
E2	25	0,00	Positive
E3	47	2,71	Positive with alert
E4	23	-0,25	Positive
E5	19	-0,74	Positive
E6	20	-0,62	Positive
E7	7	-2,22	Positive with alert
E8	25	0,00	Positive
E9	25	0,00	Positive
E10	12	-1,60	Positive
E11	17	-0,99	Positive
E12	24	-0,12	Positive
E13	23	-0,25	Positive
E14	16	-1,11	Positive
E15	24	-0,12	Positive
E16	14	-1,36	Positive
E17	18	-0,86	Positive
E18	22	-0,37	Positive
E19	19	-0,74	Positive
E20	22	-0,37	Positive
E21	19	-0,74	Positive
E22	15	-1,23	Positive
E23	24	-0,12	Positive
E24	21	-0,49	Positive
E25	26	0,12	Positive
E27	20	-0,62	Positive
E28	8	-2,10	Positive with alert



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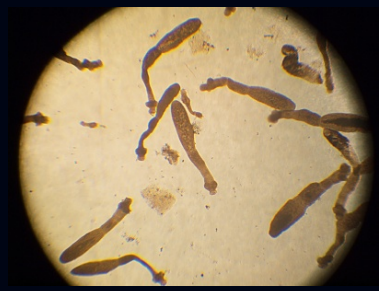
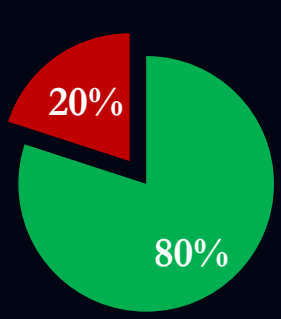
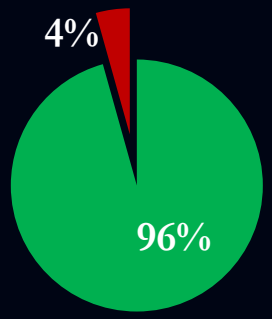


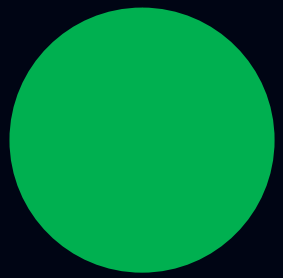
TREND: QUALITATIVE EVALUATION (2019)



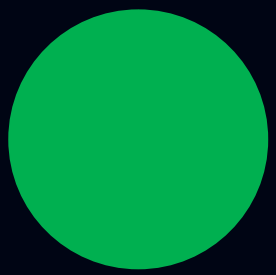
2012



2013

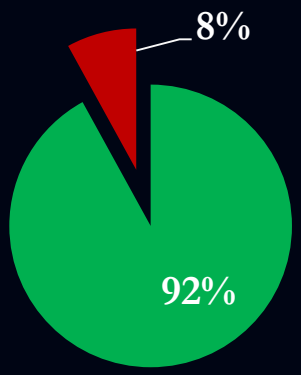


2014

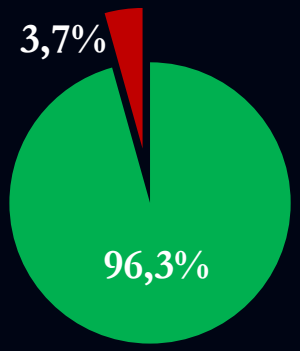


2015

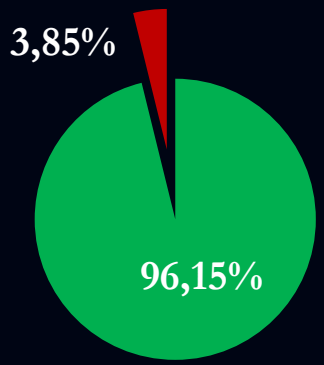
Positive Negative



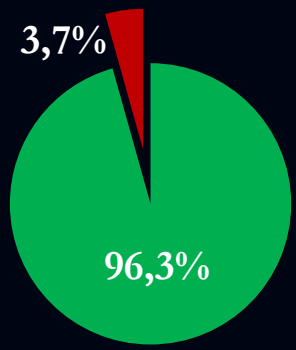
2016



2017



2018



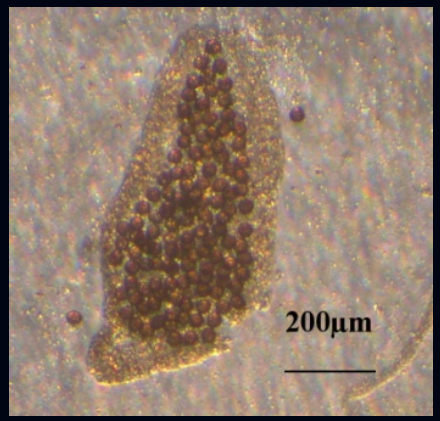
2019

outcomes….

- The experience derived from the PT carried out **in 2019**, showed that the personnel of NRLs are skill to detect this parasite in a qualitative test.

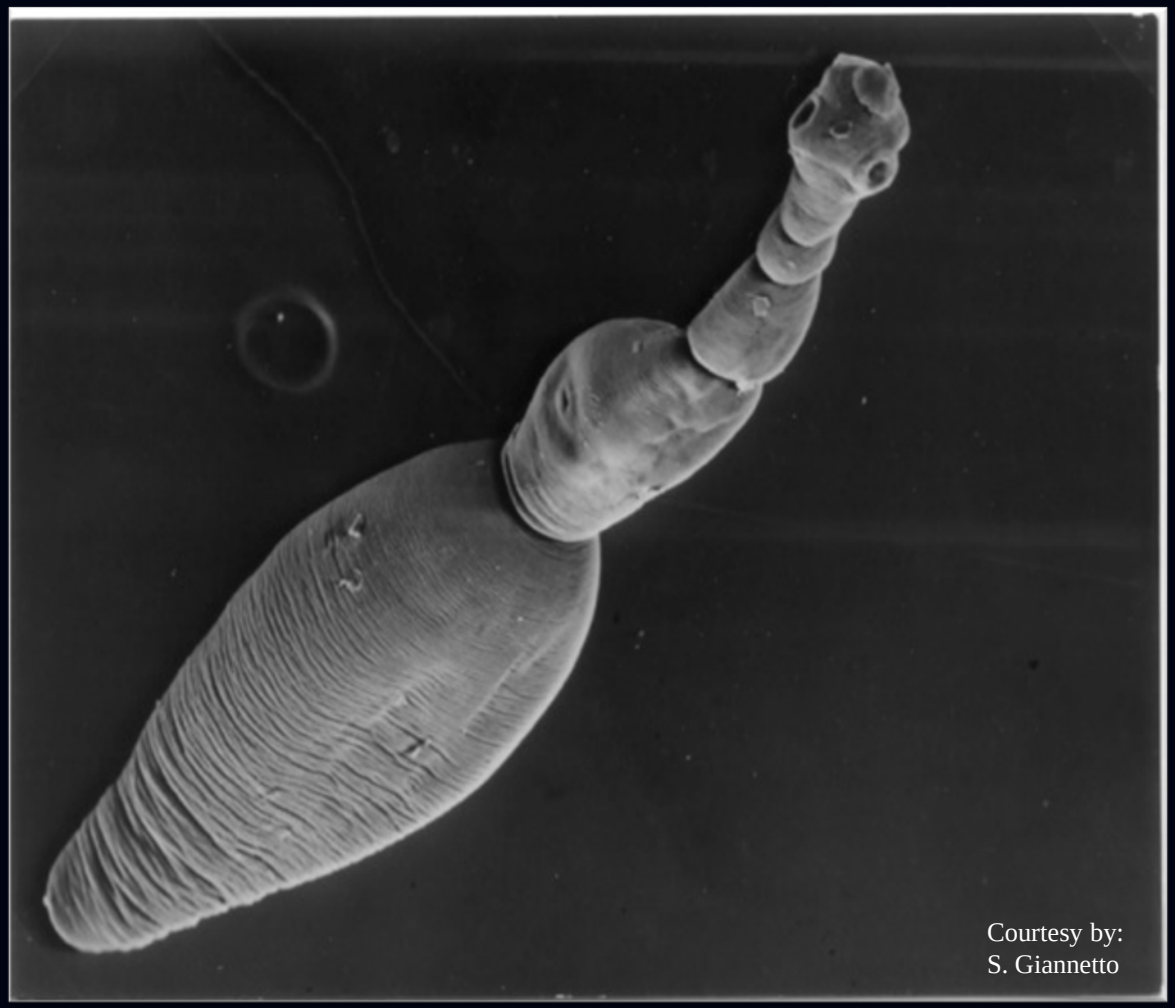
- As for the past years, the **lower capability detection** of the samples could be due to (1) eye focus softening and (2) incorrect application of the SCT, while an overestimation could be due (3) over-counting (separate proglottids detached from adult worms).

- The comparison of the results during the **last 8 years**, demonstrates that the detection capabilities of the personnel is increasing over time reaching a plateau of high standard of quality.



2nd PROFICIENCY TESTING on:

"Molecular identification of *Echinococcus* at the species level"



Adriano Casulli

Participants (N=16):

LABORATORIES	COUNTRY
Institute of Tropical Medicine, Antwerp	**Belgium**
Statens Serum Institut, 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**
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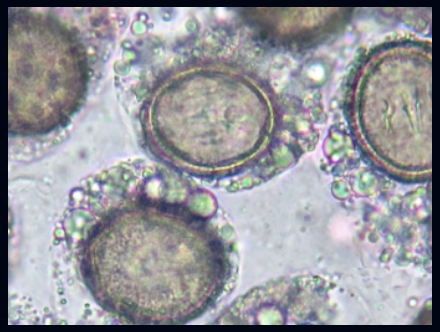
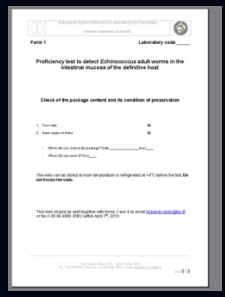
Agrifood and Biosciences Institute, Celbridge	**Ireland**
Istituto Zooprofilattico Sperimentale of Sardinia, Sassari	**Italy**
Laboratory of Food and Environmental Investigations, National Diagnostic Centre, Riga	**Latvia**
National Institute of Public Health and the Environment, Bilthoven	**Netherlands**
National Veterinary Institute, Oslo	**Norway**
National Veterinary Research Institute, Pulawy	**Poland**
Institute for Diagnosis and Animal Health, Bucharest	**Romania**
Veterinary and Food Institute, Bratislava	**Slovak Republic**
Animal and Plant Health Agency, Hutton, York	**UK**

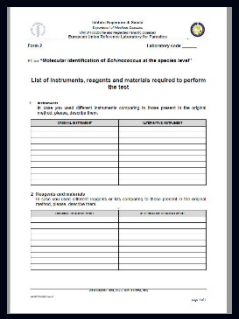


In each package the following forms were added:

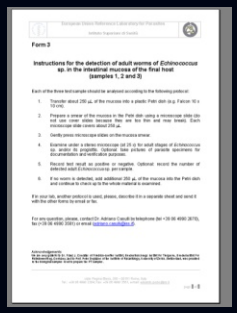
Form 1 – Check of the package content and its preservation



Form 2 – List of instruments, reagents and materials required to perform the test for the detection



Form 3 – Instructions for the detection of *Echinococcus* spp.



Form 4 - Results

Preparation of samples

- *E. granulosus sensu stricto* (metacestode larvae) from animal host and *E. multilocularis* (worms from the intestines of foxes) were stored in 70% ethanol and used for the extraction of high quality DNA using a commercial kit.
- *Echinococcus* parasites were individually identified at species level through molecular analysis (Trachsel et al., 2007; Bowles et al., 1992).
- Homogeneity was ensured by providing participants with aliquots of the same DNA preparations.
- 3 tubes: 2 contained 10 µl DNA of *E. granulosus ss* and *E. multilocularis,* and 1 negative control.
- The 2 ml tubes were plugged and sealed using plastic paraffin film, individually coded and sealed under vacuum in a plastic bag.

RESULTS

1. Positive control
2. Eg 0.5 ul (1:10)
3. Eg 1 ul (1:10)
4. Eg 2 ul (1:10)
5. Eg 0.5 ul
6. Eg 1 ul
7. Eg 2 ul
8. Positive control
9. Negative control
10. Ladder
11. Positive control
12. Em 0.5 ul (1:10)
13. Em 1 ul (1:10)
14. Em 2 ul (1:10)
15. Em 0.5 ul
16. Em 1 ul
17. Em 2 ul
18. Positive control
19. Negative control

Trachsel et al., 2007:

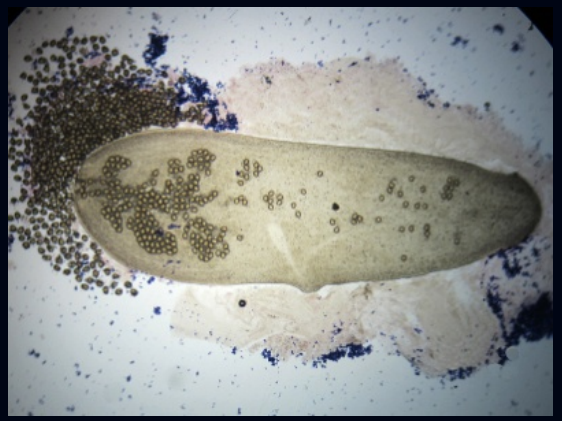
Multiplex-PCR in 30µL total reaction with 0.2 µM of primers (Cest1, Cest2, Cest3, Cest4, Cest5).

LAB Codes	LITERATURE REFERENCES	MOLECULAR METHODS	MOLECULAR MARKERS	SEQUENCING	METHOD MODIFICATIONS
E1	• Bowles et al 1992	PCR	cox1	YES	45 cycles
E2	**1)** Abbasi et al 2003 (Eg); **2)** Dinkel et al 1998 (Em); **3)** Trachsel et al 2007	**1)** PCR **2)** Nested-PCR **3)** Multiplex-PCR	**1)** tandem-repeated DNA seq (EgG1HaeIII) **2)** Mt-encoded 12S rRNA **3)** rrnS, nad1	NO NO NO	1) Mix vol 25 ul; primers only 1 pair (1121a, 1122a); 2) Mix vol 25 ul, 30 cycles; 3) Mixture vol 25 ul; Primer Cest5 -8uM
E3	• Trachsel et al 2007	Multiplex-PCR	(small sub-unit of ribosomal RNA) rrnS, nad1	NO	Primers C1-C4 0.5 uL (from 100 uM primer stock) in a 50 uL reaction. Primer C5 0.5 uL (from 160 uM primer stock) in a 50 ul reaction. PCR conditions: 30 sec 72°C and final extension for 10 min 72°C
E4	• Trachsel et al 2007	Multiplex-PCR	rrnS, nad1	NO	25 uL reaction mix. 5 ul DNA. 61°C annealing temperature.
E6	• in House PCR (primers by Trachsel '07)	Single-PCRs	rrnS	YES	Primers E. m. -H15 F; E. m. -H17 R; E. g. s.l.-Cest4 F; E. g. s.l.-Cest5 R; Taenia spp.-Cest3 F; Taenia spp.-Cest5 R. 10x PCR Rxn Buffer; dNTPs, 50 mM MgCl2; Taq Polym
E7	• Bowles et al 1992 (EURLP method)	PCR	cox1	YES	NO
E8	• Trachsel et al 2007	Single-PCR	rrnS, nad1	NO	1x GoTaq Flexi Buffer, 2mM MgCl2, 0,2 mM dNTP, 0,4uM primer pairs, 1,25 U Go Taq Hot Polymerase, 2 ul template DNA. Total vol 25ul
E9	• Bowles et al 1992 (as in Umhang et al 2013)	PCR	cox1	YES	PCR mix: 22 ul/reaction with 0.8 uM of each primer. DNA 3ul. PCR: 94°C 2 min; 35X (94°C 30sec, 52°C 30 sec, 72°C 30 sec); 72°C 7 min.
E10	• In house PCR/RFLP (Geysen et al 2007)	SemiNested-PCR, RFLP	Internal Trascribed Spacer (ITS2)	NO	Primers developed in house
E12	• Trachsel et al 2007	Multiplex-PCR	rrnS, nad1	NO	NO
E13	• Laurimaa et al 2015	Multiplex-PCR	tRNA-Ile/Lys, 12S rRNA	NO	primers 0,4 µM; dNTP-s 0,6 mM of each
E16	• Trachsel et al 2007	Multiplex-PCR	rrnS, nad1	NO	
E17	**1)** Bowles/McManus 1993; **2)** Em: Stieger et al 2002 (Dinkel'98) + Øines et al '14	**1)** PCR **2)** PCR, qPCR	**1)** nad1 **2)** Mt-encoded 12S rRNA	YES YES	PCR cycling: annealing @ 46°C
E22	• Trachsel et al 2007	Multiplex-PCR	rrnS, nad1	NO	NO
E27	• Bowles et al 1992	PCR	cox1	YES	NO
E28	**1)** Bowles et al 1992 **2)** Bowles et al 1992 (EURLP method)	PCR	cox1	YES	NO

LAB CODE	SAMPLE 1 *E. granulosus*	SAMPLE 2 Negative	SAMPLE 3 *E. multilocularis*	FINAL EVALUATION
E1	Correct	Correct	Correct	Positive
E2	Correct	Correct	Correct	Positive
E3	Correct	Correct	Correct	Positive
E4	Correct	Correct	Incorrect	Negative
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
E12	Correct	Correct	Correct	Positive
E13	Correct	Correct	Correct	Positive
E16	Correct	Incorrect	Incorrect	Negative
E17	Correct	Correct	Correct	Positive
E22	Correct	Correct	Correct	Positive
E27	Correct	Correct	Correct	Positive
E28	Correct	Correct	Correct	Positive

SUMMARY of RESULTS (Qualitative evaluation)

Number of participant laboratories	**16**
Number of participants that passed the PT	14
Number of participants that failed the PT	2



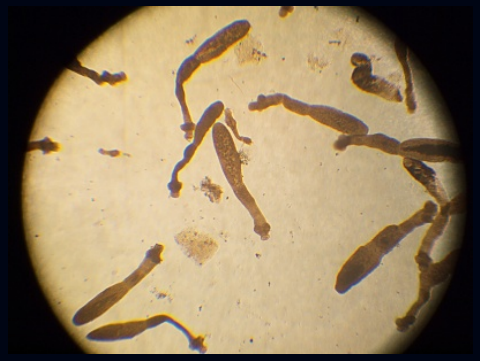
RESULTS (Qualitative evaluation)

The QUALITATIVE evaluation obtained by the NRLs:

Sample 1 (*E. granulosus*, G1): 16 labs (100%) obtained a positive evaluation.

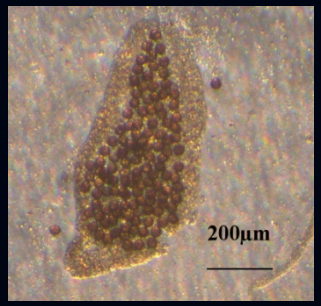
Sample 2 (negative): 15 labs (94%) obtained a positive evaluation.

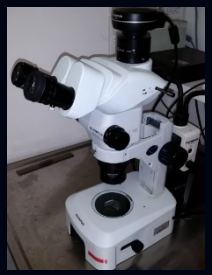
Sample 3 (*E. multilocularis*): 14 labs (87.5%) obtained a positive evaluation.



outcomes….

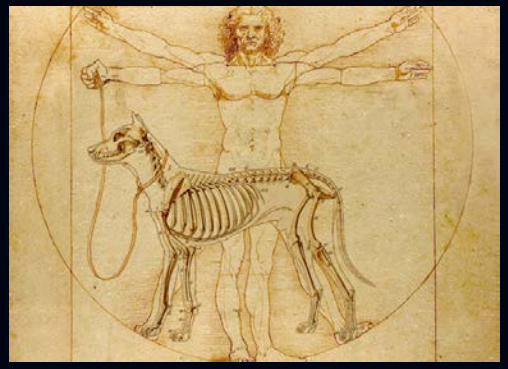
- The experience derived from 2nd PT on molecular detection of *Echinococcus* carried out in 2019, showed that the personnel of NRLs are skill to detect this parasite by molecular approach.





ACKNOWLEDGMENTS:

- Simona Cherchi (“worming” the samples / packing PTs)
- Maria Interisano (mixing the mucosa / packing PTs)
- Alessia Possenti (supervising: molecular PT & quality system)
- Azzurra Santoro (preparation molecular & SCT PTs/ data managing / packing PTs)
- Federica Santolamazza (preparation molecular & SCT PTs / data managing / packing PTs)
- Francesca Tamarozzi (preparation molecular PT /data managing)



Thanks for the attention!