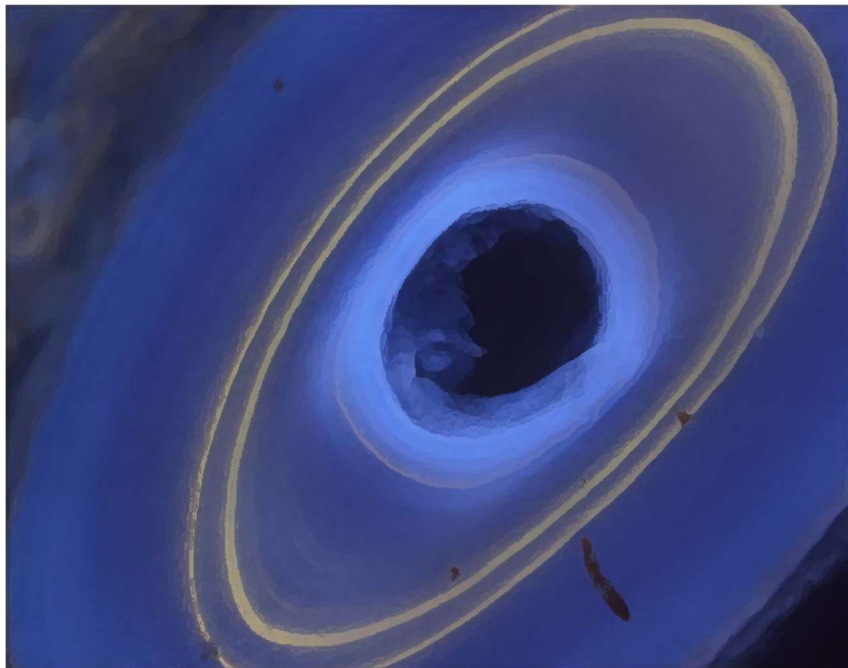


Inter-laboratory comparison:

**TETRACYCLINE BIOMARKER DETECTION
& AGE CLASS DETERMINATION
IN FOX TEETH**



EURL for Rabies

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1 INTRODUCTION

Tetracycline is a group of broad-spectrum antibiotic commonly used as biomarker of oral rabies vaccines bait uptake. After its consumption, the molecules are indeed incorporated into bones and teeth and can be detected using epi-fluorescence microscopy that leads to their use as a bait uptake marker (Brochier, Kieny et al. 1991).

International organisations (European Commission 2002, WHO 2018) recommend the evaluation of bait-uptake in target species sampled in vaccinated areas to assess the efficacy of oral rabies vaccination (ORV) programs. In the European Union (EU), all oral vaccines used (Lysvulpen® (SAD Bern-SAD B19 strain) from Bioveta, Rabigen® (SAG2 strain) from Virbac, Fuchsoral® (SAD B19 strain) and Rabitec® (SPBN GASGAS) from CEVA) include tetracycline in their composition. A first inter-laboratory comparison on tetracycline detection was organised in 2010 by the European Union Reference Laboratory (EURL) for rabies, showing an unexpected high proportion of discordant results among National Reference Laboratories (Robardet and Cliquet, 2011; Robardet et al., 2012). Further trials were thus organised in 2012, 2014 and 2017 and produced better results. This report presents the inter-laboratory comparison results of the fifth session organised in 2022.

2 SCOPE

One of the principal duties of the EURL for Rabies is to organise inter-laboratory comparisons for the benefit of the NRLs, as stated in the Commission Regulation (EU) N°415/2013 of 6 May 2013 laying down additional responsibilities and tasks of the EURL for Rabies and amending Commission Regulation (EC) No N°737/2008 designating the EURL for Rabies. The scope of this inter-laboratory test is to compare the laboratory results of NRLs in detecting the tetracycline in red fox teeth and determining the animal age class of tested animals. Those tests are key techniques in the evaluation of oral vaccination effectiveness.

3 GENERAL INFORMATION

3.1 Identification of coordinator and staff involved in the study

- **Report Validation**: F. Boué
- **EURL director and proficiency test Coordinator**: E. Robardet
- **Technical Staff**: C. Caillot

- **Administrative Staff:** L. Damoiseaux

3.2 Instruction to participants

The inter-laboratory comparison was announced to NRLs by e-mail on 02 September 2022. The panels sending departed from Nancy on 04 October 2022. Reporting result deadline was established on 31 October 2022.

Instructions were given to all participants in an accompanying letter of the test material send explaining that samples had to be stored at -20°C from the reception till the start of analysis and to undertake the tetracycline detection and animal age evaluation by using its own routine procedure. It was also requested to consider for the analysis of this session that all the animals were collected during autumn season.

In parallel to the testing was requested to send acknowledgement and results (result and technical form describing the procedure used) through online forms.

3.3 Participating laboratories

NRLs from EU Member States and reference laboratories from bordering countries previously involved in ORV programs co-financed by the European Commission (EC) were invited to take part in this test. Sixteen laboratories wished to participate and returned their results (Table 1). Two laboratories expressed the wish to take part in the test but were not in measure to realise the testing during the timeframe of the study.

Table 1: Participating laboratories of the fifth inter-laboratory comparison session of tetracycline determination

COUNTRY	NRL	Contact name
ALBANIA	Rabies Laboratory - Institute of Food Safety and Veterinary	Valentin SHTJEFNI
BOSNIA AND HERZEGOVINA	Veterinary Institute of the Republic of Srpska	Sonja NIKOLIC
BULGARIA	Bulgarian Food Safety Agency - National Diagnostic and Research Veterinary Institute	Reneta PETROVA
ESTONIA	Estonian Veterinary and Food laboratory	Katrin PEIK
FINLAND	Finnish Food Authority	Marja ISOMURSU
FRANCE	Nancy Laboratory for Rabies and Wildlife	Emmanuelle ROBARDET

GREECE	Virology Laboratory, Department of Molecular Diagnostics, FMD, Virological, Rickettsial and Exotic diseases	Konstantia	TASIOUDI
HUNGARY	National Food Chain Safety Office, Veterinary Diagnostic Directorate, Virology Laboratory	Peter	MALIK
LATVIA	Institute of Food Safety, Animal Health and Environment BIOR	Zanete	ZOMMERE
LITHUANIA	National Food and Veterinary Risk Assessment Institute	Viktoras	MASKALIOVAS
MONTENEGRO	Diagnostic Veterinary Laboratory	Nikola	PEJOVIC
NORTH MACEDONIA	Faculty of Veterinary Medicine in Skopje	Iskra	CVETKOVIKJ
POLAND	National Veterinary Research Institute	Marcin	SMRECZAK
ROMANIA	Institute for Diagnosis and Animal Health	Vlad	VUTA
SERBIA	Institute of Veterinary Medicine of Serbia	Branislav	KURELJUŠIĆ
SLOVAKIA	State Veterinary and Food Institute - Veterinary Institute Zvolen	Slavomir	JERG

4 INTER-LABORATORY TEST ITEMS

4.1 Panel composition and preparation of the items

Jaws used in this inter-laboratory comparison session were collected on red foxes sampled in the field in Romania in 2021 (Sample 1, 2, 3, 5 and 6) and in Croatia, Italy, and Poland for the 'mix' batch (Sample 4; see below). For each animal, lower jaw was collected and stored at -20°C. Each jaw was divided in two parts. The first half jaw was analysed for tetracycline detection and animal age determination by two independent readers and the second half jaw was stored for the inter-laboratory test evaluation. Twenty panels were constituted according to the number of laboratories willing to participate. The panel of this session was constituted of the following 6 samples:

- **Sample 1:** 1 juvenile (<1 year) negative TTC jaw,
- **Sample 2:** 1 adult (1-2 years) positive TTC jaws,
- **Sample 3:** 1 adult (2-3 and >3 years) positive TTC jaw,
- **Sample 4:** 1 mix of age and positive TTC jaw,
- **Sample 5:** 1 juvenile (<1 year) negative TTC jaw,
- **Sample 6:** 1 juvenile (<1 year) negative TTC jaw.

4.2 Identification of the proficiency test items

For each panel, all items were coded randomly. The code was constituted by the date of the inter-laboratory test campaign, the identification of the laboratory and the unique specific code of the item. Each item was dully labelled.

4.3 Homogeneity and Stability

Microstructures of increments of tooth cement used for age determination and tetracycline marking being permanent, there is no stability issue on teeth samples stored at -20°C.

As no significant difference in the presence of tetracycline in the right and left canines and premolars of marked animals has been demonstrated, there is no homogeneity issue between the participant and the organiser results on the samples (Algeo, Norhenberg et al. 2013).

4.4 Distribution of the samples

Shipment was achieved in dry ice by an international agreed carrier under UN3373 requirements in accordance with both the International Air Transport Association (IATA 2009) and the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR 2009). Laboratories have declared receiving all the samples in appropriate conditions.

5 RESULTS

5.1 Laboratory results on tetracycline detection

The Table 2 records results of tetracycline examinations obtained by each participating laboratory. The Table 3 summarizes the results by sample category.

Table 2: Results on tetracycline (TTC) detection. Red: discordant result. Green: concordant result.

	JUVENILE and TTC NEGATIVE			ADULT and TTC POSITIVE			ADULT and TTC POSITIVE			MIX and TTC POSITIVE			JUVENILE and TTC NEGATIVE			JUVENILE and TTC NEGATIVE		
code_lab	code_1	status	observed	code_2	status	observed	code_3	status	observed	code_4	status	observed	code_5	status	observed	code_6	status	observed
L01	22090814	NEGATIVE	NEGATIVE	22090488	POSITIVE	POSITIVE	22090882	POSITIVE	POSITIVE	22090191	POSITIVE	POSITIVE	22090029	NEGATIVE	NEGATIVE	22090962	NEGATIVE	NEGATIVE
L02	22090809	NEGATIVE	NEGATIVE	22090643	POSITIVE	POSITIVE	22090249	POSITIVE	POSITIVE	22090192	POSITIVE	POSITIVE	22090636	NEGATIVE	NEGATIVE	22090753	NEGATIVE	NEGATIVE
L03	22090539	NEGATIVE	NEGATIVE	22090948	POSITIVE	POSITIVE	22090961	POSITIVE	POSITIVE	22090102	POSITIVE	POSITIVE	22090744	NEGATIVE	NEGATIVE	22090756	NEGATIVE	NEGATIVE
L04	22090947	NEGATIVE	NEGATIVE	22090851	POSITIVE	POSITIVE	22090173	POSITIVE	POSITIVE	22090151	POSITIVE	POSITIVE	22090447	NEGATIVE	NEGATIVE	22090696	NEGATIVE	NEGATIVE
L05	22090520	NEGATIVE	NEGATIVE	22090937	POSITIVE	POSITIVE	22090475	POSITIVE	POSITIVE	22090379	POSITIVE	POSITIVE	22090434	NEGATIVE	NEGATIVE	22090031	NEGATIVE	NEGATIVE
L06	22090830	NEGATIVE	NEGATIVE	22090004	POSITIVE	POSITIVE	22090791	POSITIVE	POSITIVE	22090691	POSITIVE	POSITIVE	22090739	NEGATIVE	NEGATIVE	22090290	NEGATIVE	NEGATIVE
L07	22090654	NEGATIVE	NEGATIVE	22090527	POSITIVE	POSITIVE	22090258	POSITIVE	POSITIVE	22090111	POSITIVE	POSITIVE	22090168	NEGATIVE	NEGATIVE	22090578	NEGATIVE	NEGATIVE
L08	22090038	NEGATIVE	POSITIVE	22090085	POSITIVE	POSITIVE	22090215	POSITIVE	POSITIVE	22090611	POSITIVE	POSITIVE	22090936	NEGATIVE	NEGATIVE	22090465	NEGATIVE	NEGATIVE
L09	22090124	NEGATIVE	NEGATIVE	22090427	POSITIVE	POSITIVE	22090236	POSITIVE	POSITIVE	22090063	POSITIVE	POSITIVE	22090485	NEGATIVE	NEGATIVE	22090112	NEGATIVE	NEGATIVE
L11	22090223	NEGATIVE	NEGATIVE	22090990	POSITIVE	POSITIVE	22090324	POSITIVE	POSITIVE	22090316	POSITIVE	POSITIVE	22090449	NEGATIVE	NEGATIVE	22090451	NEGATIVE	NEGATIVE
L12	22090525	NEGATIVE	NEGATIVE	22090532	POSITIVE	POSITIVE	22090406	POSITIVE	POSITIVE	22090041	POSITIVE	POSITIVE	22090695	NEGATIVE	NEGATIVE	22090863	NEGATIVE	NEGATIVE
L13	22090879	NEGATIVE	POSITIVE	22090203	POSITIVE	POSITIVE	22090326	POSITIVE	POSITIVE	22090057	POSITIVE	POSITIVE	22090400	NEGATIVE	NEGATIVE	22090305	NEGATIVE	POSITIVE
L14	22090517	NEGATIVE	NEGATIVE	22090931	POSITIVE	POSITIVE	22090154	POSITIVE	POSITIVE	22090610	POSITIVE	POSITIVE	22090946	NEGATIVE	NEGATIVE	22090142	NEGATIVE	NEGATIVE
L15	22090918	NEGATIVE	NEGATIVE	22090758	POSITIVE	POSITIVE	22090055	POSITIVE	POSITIVE	22090701	POSITIVE	POSITIVE	22090056	NEGATIVE	NEGATIVE	22090404	NEGATIVE	NEGATIVE
L17	22090216	NEGATIVE	POSITIVE	22090769	POSITIVE	POSITIVE	22090926	POSITIVE	POSITIVE	22090663	POSITIVE	POSITIVE	22090876	NEGATIVE	NEGATIVE	22090762	NEGATIVE	POSITIVE
L18	22090059	NEGATIVE	NEGATIVE	22090944	POSITIVE	POSITIVE	22090058	POSITIVE	POSITIVE	22090189	POSITIVE	POSITIVE	22090624	NEGATIVE	NEGATIVE	22090296	NEGATIVE	NEGATIVE

Positive samples: 16/16 (100%) laboratories provided satisfactory results.

Negative samples: 13/16 laboratories (81%) provided satisfactory results.

Table 3: Summary of the inter-laboratory results on tetracycline detection.

Tested samples	N participating laboratories	% laboratories with satisfactory results	Binomial proportion confidence interval	N samples analysed	% discordant results	Binomial proportion confidence interval
TTC Positive Adult	16	100 (n=16)	[79.4 – 100]	32	0 (n=0)	[0.0 – 10.9]
TTC Positive Mix	16	100 (n=16)	[79.4 – 100]	16	0 (n=0)	[0.0 – 20.6]
Total TTC Positive	16	100 (n=16)	[79.4 – 100]	48	0 (n=0)	[0.0 – 7.4]
TTC Negative Juvenile	16	81 (n=13)	[54.4 – 95.6]	48	10 (n=5)	[3.5 – 22.7]
Total TTC Negative	16	81 (n=13)	[54.4 – 95.6]	48	10 (n=5)	[3.5 – 22.7]
Total	16	81 (n=13)	[54.4 – 95.6]	96	5 (n=5)	[1.7 – 11.7]

Five false positive results were detected in negative juvenile samples (10% of the negative juvenile samples). No false negative results was detected.

For the whole inter-laboratory test, 13 laboratories (81%) showed entire satisfactory results. A total of 5 discordant result (5%) was detected on the 96 total tested samples.

In conclusion, the overall success rate of the tetracycline detection test of this session is satisfactory (81% of laboratories succeeded in all tests). The success rate of laboratories on positive and negative samples was 100% and 81% respectively.

5.2 Laboratory results on age determination

Various age classes were provided in the panel test (Juvenile samples: [0-1[year; Adult samples: [1-2[years; [2-3[years; ≥ 3 years). It was asked to participating laboratories to determine the juvenile or adult status of each sample. The dates of death of animals were the autumn for all analysed samples. The Table 4 summarizes the results by sample category while the Table 5 records results of age determination obtained by each participating laboratory.

Adult coded samples: 16/16 laboratories (100%) provided satisfactory results.

Juvenile coded samples: 12/16 laboratories (75%) provided satisfactory results.

Table 4: Summary of the inter-laboratory results on age determination: Juvenile/Adult

Tested samples	N participating laboratories	% laboratories with satisfactory results	Binomial proportion confidence interval	N samples analysed	% discordant results	Binomial proportion confidence interval
Total adult samples	16	100 (n=16)	[79.4 – 100]	32	0 (n=0)	[0.0 – 10.9]
Juvenile samples	16	75 (n=12)	[47.6 – 92.7]	48	10 (n=5)	[3.5 – 22.7]
Total	16	75 (n=12)	[47.6 – 92.7]	80	6 (n=5)	[2.1 – 14.0]

Six discordant results on age determination were detected in juvenile samples (6% of the total samples) while no discordant results were detected in adult samples.

Seventy five percent of laboratories estimated the correct age class on all samples. Five discordant results (6%) were detected on the total of 80 samples analysed for age estimation. Most of the discordant results were detected in juvenile samples (<1 year) identified as adult samples.

Table 5: Results on animal age determination. Red: discordant result; Green: concordant result.

	JUVENILE and TTC NEGATIVE			ADULT and TTC POSITIVE			ADULT and TTC POSITIVE			Mix and TTC POSITIVE			JUVENILE and TTC NEGATIVE			JUVENILE and TTC NEGATIVE		
code_lab	code_1	status	observed	code_2	status	observed	code_3	status	observed	code_4	status	observed	code_5	status	observed	code_6	status	observed
L01	22090814	Juvenile	Juvenile	22090488	Adult	Adult	22090882	Adult	Adult	22090191	Mix	Juvenile	22090029	Juvenile	Juvenile	22090962	Juvenile	Juvenile
L02	22090809	Juvenile	Juvenile	22090643	Adult	Adult	22090249	Adult	Adult	22090192	Mix	Juvenile	22090636	Juvenile	Juvenile	22090753	Juvenile	Juvenile
L03	22090539	Juvenile	Juvenile	22090948	Adult	Adult	22090961	Adult	Adult	22090102	Mix	Adult	22090744	Juvenile	Juvenile	22090756	Juvenile	Juvenile
L04	22090947	Juvenile	Juvenile	22090851	Adult	Adult	22090173	Adult	Adult	22090151	Mix	Juvenile	22090447	Juvenile	Juvenile	22090696	Juvenile	Juvenile
L05	22090520	Juvenile	Juvenile	22090937	Adult	Adult	22090475	Adult	Adult	22090379	Mix	Juvenile	22090434	Juvenile	Adult	22090031	Juvenile	Juvenile
L06	22090830	Juvenile	Juvenile	22090004	Adult	Adult	22090791	Adult	Adult	22090691	Mix	Adult	22090739	Juvenile	Juvenile	22090290	Juvenile	Adult
L07	22090654	Juvenile	Juvenile	22090527	Adult	Adult	22090258	Adult	Adult	22090111	Mix	Adult	22090168	Juvenile	Juvenile	22090578	Juvenile	Juvenile
L08	22090038	Juvenile	Juvenile	22090085	Adult	Adult	22090215	Adult	Adult	22090611	Mix	Juvenile	22090936	Juvenile	Juvenile	22090465	Juvenile	Juvenile
L09	22090124	Juvenile	Juvenile	22090427	Adult	Adult	22090236	Adult	Adult	22090063	Mix	Adult	22090485	Juvenile	Juvenile	22090112	Juvenile	Juvenile
L11	22090223	Juvenile	Juvenile	22090990	Adult	Adult	22090324	Adult	Adult	22090316	Mix	Adult	22090449	Juvenile	Juvenile	22090451	Juvenile	Juvenile
L12	22090525	Juvenile	Juvenile	22090532	Adult	Adult	22090406	Adult	Adult	22090041	Mix	Adult	22090695	Juvenile	Juvenile	22090863	Juvenile	Juvenile
L13	22090879	Juvenile	Juvenile	22090203	Adult	Adult	22090326	Adult	Adult	22090057	Mix	Adult	22090400	Juvenile	Juvenile	22090305	Juvenile	Adult
L14	22090517	Juvenile	Juvenile	22090931	Adult	Adult	22090154	Adult	Adult	22090610	Mix	Juvenile	22090946	Juvenile	Juvenile	22090142	Juvenile	Juvenile
L15	22090918	Juvenile	Juvenile	22090758	Adult	Adult	22090055	Adult	Adult	22090701	Mix	Adult	22090056	Juvenile	Juvenile	22090404	Juvenile	Juvenile
L17	22090216	Juvenile	Adult	22090769	Adult	Adult	22090926	Adult	Adult	22090663	Mix	Adult	22090876	Juvenile	Juvenile	22090762	Juvenile	Adult
L18	22090059	Juvenile	Juvenile	22090944	Adult	Adult	22090058	Adult	Adult	22090189	Mix	Adult	22090624	Juvenile	Juvenile	22090296	Juvenile	Juvenile

5.3 Inter-annual comparison

The success rate in tetracycline detection appears comparable from 2012 to 2022 (Figure 1). Result comparisons indeed show a higher success rate of laboratories for tetracycline detection in 2012, 2014, 2017 and 2022 compared to the first session performed in 2010 (81% in 2022, 92% in 2017, 82% in 2014, 86% in 2012 and 25% in 2010). Considering the total number of tested samples, the proportion of discordant results in 2022 (5%) is lower than the proportion of discordant results observed in 2010 (26%) but does not differ from that of 2012, 2014 and 2017 (2%, 4% and 1% respectively).

Regarding age determination, the success in age class determination between 2017 and 2022 appears comparable. Result comparisons show a higher success rate of laboratories for tetracycline detection in 2017 and 2022 compared to the 2012 session (75% in 2022, 69% in 2017, 25% in 2014, 7% in 2012 and 44% in 2010) (Figure 2). Considering the total number of tested samples, this session recorded an identical amount of discordant result on age determination as in 2017. The number of discordant results of 2022 (6%) and of 2017 (6%) are lower to one's observed in 2014 and 2012 (30% for both year).

When considering individual results of participating laboratories (Table 6 and Table 7), it appears that 2/3 (for TTC detection) and 2/4 (for age estimation) laboratories harbouring a discordant results are laboratories having never participating in the inter-laboratory comparison before. One laboratory harbouring at least a discordant result in age determination presented discordant results for two consecutive sessions.

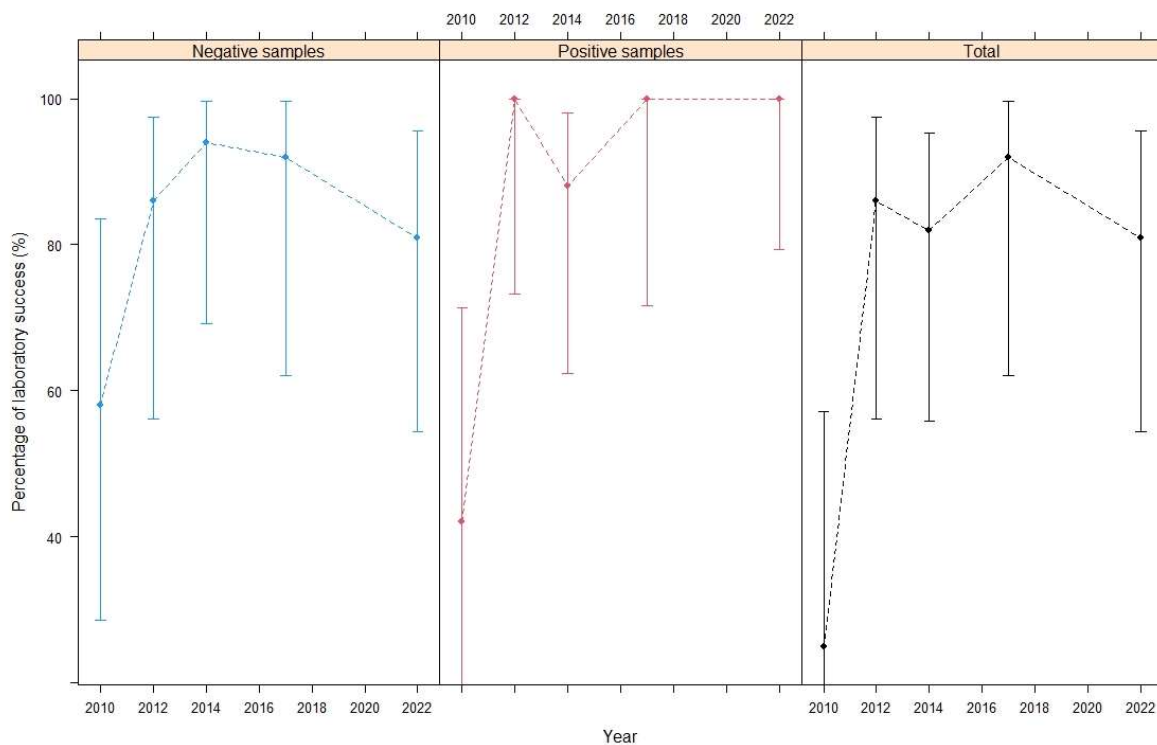


Figure 1: Evolution of inter-laboratory results on tetracycline detection: proportion of laboratories succeeding in the test

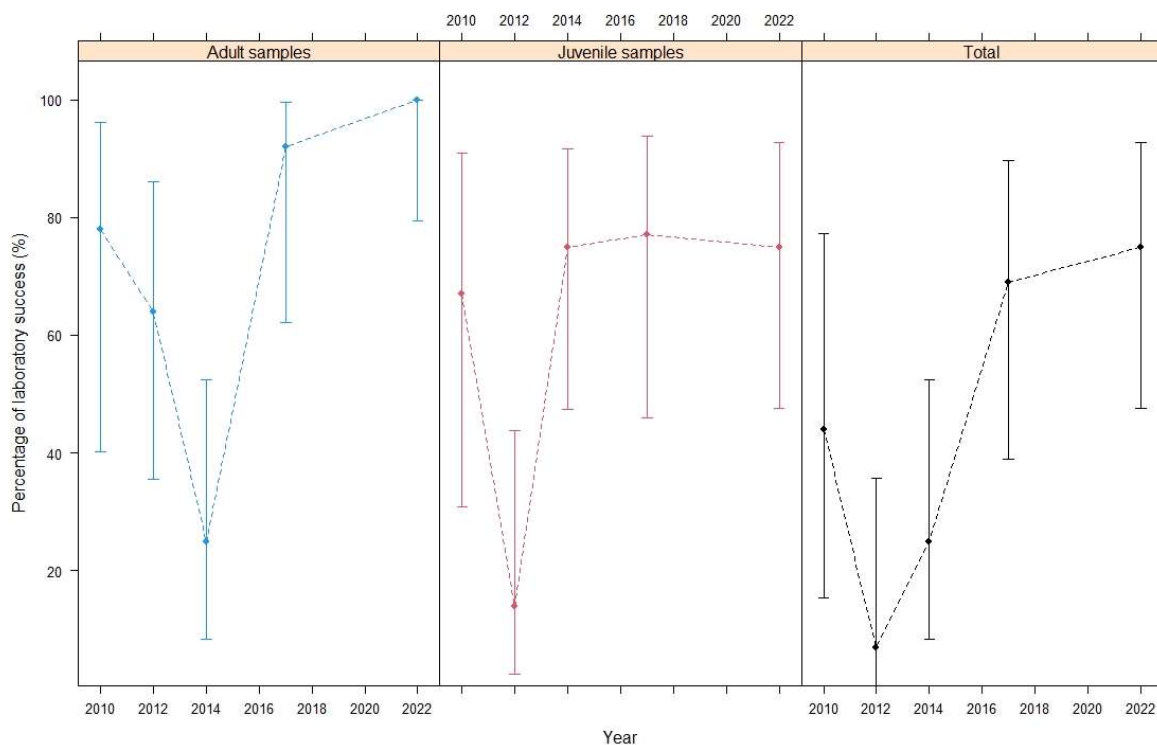


Figure 2: Evolution of inter-laboratory results on age estimation: proportion of laboratories succeeding in the test

Table 6: Evolution of tetracycline results comparisons in participating laboratories. Red: evaluation with at least one discordant result; Green: concordant results.

Laboratory code	2010	2012	2014	2017	2022
L01					
L02					
L03					
L04					
L05					
L06					
L07					
L08					
L09					
L11					
L12					
L13					
L14					
L15					
L17					
L18					

Table 7: Evolution of age estimation comparisons in participating laboratories. Red: evaluation with at least one discordant result; Green: concordant results.

Laboratory code	2010	2012	2014	2017	2022
L01					
L02					
L03					
L04					
L05					
L06					
L07					
L08					
L09					
L11					
L12					
L13					
L14					
L15					
L17					
L18					

6 DISCUSSION

6.1 Tetracycline detection: reminder of important points to consider for proper detection

Some critical factors are known to affect the results if modified. To ensure and maintain a high accuracy level of laboratories in the detection of tetracycline in teeth it should be reminded that:

- **Tissue analysed: Both bone and canine must be visible on the section.**

Because tetracycline is more likely deposited in bones and then canine teeth, transverse sections of the tooth must be cut through the root area of the canine to include tooth and bone tissues. Under this condition, and if both the bone and canine tooth are examined, the sensitivity of tetracycline detection is around near 100% (Hanlon, Niezgoda et al. 1999).

- **Jaw section:** When the method includes a teeth extraction step (i.e.: adult sample analysis using Buehler Isomet saw), **jaw section must be performed between C1 and C2 or between C2 and C3.** A cut between B and C1 could damage the canine root, making no more possible the section at the end of the root, where annuli cementum lines are the most visible and distinguishable.

If the method does not include a prior extraction step of the teeth (i.e.: sample analysis using Biopro Osteotom, small juvenile sample analysis using Buehler isomet saw, etc.), the canine root sections are performed directly into the jaw.

- **Teeth section:** Teeth section must preferably be performed transversally. Transverse sections allow a higher number of cuts to identify the position of tetracycline lines with adjacent annuli. **The cut should be made 2 or 3 mm from the end of the root and perpendicular to the root axis** in order to maximize the visibility of successive cementum lines. If the cut is made too far from the end of the root, the cementum annuli may be too closely spaced to identify individual annuli.

- **Thickness of the section: $\approx 150 \mu\text{m}$ is estimated as the optimal thickness for the analyses of both tetracycline and age determination.** A study of Johnston (1987) recommended cutting sections ranging from $60 \mu\text{m}$ to $150 \mu\text{m}$ thick based on the experience of personnel at the Ontario Ministry of Natural Resources. In the experience of the EURL for

rabies, the optimal thickness section is around 150 µm for both tetracycline detection and age determination.

- **Number of sections performed per sample:** minimum 2 sections must be performed by sample.
- **Tetracycline wavelength excitation:** It must be ensured that the filter added to the microscope used for the observation allows a wavelength excitation at 390nm.
- **Mounting medium:** Using a mounting medium could increase the accuracy of the reading step. Mounting media provide better contrast and allow observation of the entire section in a single plan (Robardet, Demerson et al. 2012).
- **Control slides:** Control slides must be examined before the test slides to ensure that the equipment is operating satisfactorily. Tetracycline lines will appear on positive control and test slides as more or less intense yellow lines on the bluish background.
- **Number of readers:** Two independent readers should examine the slides and confront their results in order to minimize the impact of individual interpretation error.

6.2 Age determination: reminder of important points to consider for proper estimation

Age estimation based on dental eruption and morphology is considered to be a suitable method for age determination. The technique consists to count the number of cementum lines and to observe dentine width. Dentine width increases with age and is deposited in the dental cavity which is gradually filled up (centrifugal growth). Large pulp cavity is consequently observed in young animals only. Cementum is deposited over the dental root annuli (centripetal growth) (Morris 1972).

The first dark-staining line appears in tooth cementum during January to March of the year following the birth (Goddard and Reynolds 1993). The lines consequently appear annuli as characteristic annual rings consisting of paler (summer) opaque and darker (winter) transparent areas (Grue and Jensen 1973, Harris 1978, Goddard and Reynolds 1993, Van Lancker, Van Den Berge et al. 2005, Roulichova and Andera 2007). As one dark line is produced per year, age determination by age class with interval of one year is feasible (Figure 3).

Moreover, it is important to note that estimating the age of individuals collected between January and March is hazardous, since this is the period of growth of the dark-staining line, it is almost impossible to distinguish juveniles from adults. However, given the dates of vaccination performed in Europe (September-October and April-May), and the recommended vaccination control period carried out one month later, such sampling (collection of foxes between January and March) is fortunately unlikely to occur.

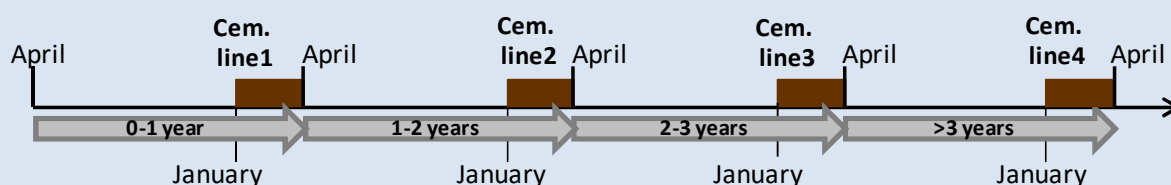


Figure 3: Schema showing the period of the apparition of the successive dark line in cementum over the time and corresponding fox age estimation.

Thus, for animals sampled **in autumn**, the **last cementum line appearing is pale**.

No dark cementum line means the animal is <1 year old.

One cementum dark line -----is 1-2 years old.

Two cementum dark lines -----is 2-3 years old.

Three cementum dark lines -----is >3 years old.

For animals sampled **in spring**, the **last cementum line appearing is black**.

No cementum line (neither pale nor dark) means the animal is <1 year old.

One cementum dark line-----is 1-2 years old.

Two dark cementum dark lines-----is 2-3 years old.

Three cementum dark lines -----is >3 years old.

7 CONCLUSION

The fifth inter-laboratory session has revealed that 13/16 participating laboratories (81%) presented 100% concordant results in the tetracycline detection test. This is a stable proportion compared to previous sessions (2017, 2014, and 2012) but with a higher result compared to the first session of 2010.

Considering the age determination of the samples, 12/16 laboratories (75%) estimated a correct age class on the whole panel. This is a comparable result with the 2017 session, with a higher performance compared to 2012 session.

These results demonstrate a constant satisfactory level of performance of the laboratories in both detection of tetracycline and age determination since the 2017 session. They are encouraging and demonstrate the laboratories capacity and the satisfactory results comparability for bait uptake estimations performed at EU level in the frame of oral vaccination campaigns.

Frequently, misinterpretations of age estimation are observed between juveniles samples (0-1 year) and adult samples of 1-2 years. Considering the birth period of cubs in Europe in March-April, for animals sampled in October-November, after the summer season, harbouring one pale line should have been aged >1-2 years and not 1-2 years old. This observation highlights the importance of taking into account the age of death of the animal for proper age estimation.

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