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FIRST EUROPEAN INTERLABORATORY COMPARISON OF TETRACYCLINE AND AGE DETERMINATION WITH RED FOX TEETH FOLLOWING ORAL RABIES VACCINATION PROGRAMS

Emmanuelle Robardet,[1,2] Jean-Michel Demerson,[1] Sabrina Andrieu,[1] and Florence Cliquet[1]

[1] French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Nancy Laboratory for Rabies and Wildlife, World Health Organization Collaborating Centre for Research and Management in Zoonoses Control, World Organization for Animal Health (OIE) Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies, European Union Reference Institute for Rabies Serology, Technopôle Agricole et Vétérinaire, BP 40009, 54220 Malzéville cedex, France

[2] Corresponding author (email: emmanuelle.robardet@anses.fr)

ABSTRACT: The first European interlaboratory comparison of tetracycline and age determination with red fox (*Vulpes vulpes*) tooth samples was organized by the European Union Reference Laboratory for rabies. Performance and procedures implemented by member states were compared. These techniques are widely used to monitor bait uptake in European oral rabies vaccination campaigns. A panel of five red fox half-mandibles comprising one weak positive juvenile sample, two positive adult samples, one negative juvenile sample, and one negative adult sample were sent, along with a technical questionnaire, to 12 laboratories participating on a voluntary basis. The results of only three laboratories (25%) were 100% correct. False-negative results were more frequently seen in weak positive juvenile samples (58%) but were infrequent in positive adult samples (4%), probably due to differences in the ease of reading the two groups of teeth. Four laboratories (44%) had correct results for age determination on all samples. Ages were incorrectly identified in both adult and juvenile samples, with 11 and 17% of discordant results, respectively. Analysis of the technical questionnaires in parallel with test results suggested that all laboratories cutting mandible sections between the canine and first premolar obtained false results. All the laboratories using longitudinal rather than transverse sections and those not using a mounting medium also produced false results. Section thickness appeared to affect the results; no mistakes were found in laboratories using sections <150 μm thick. Factors having a potential impact on the success of laboratories were discussed, and recommendations proposed. Such interlaboratory trials underline the importance of using standardized procedures for biomarker detection in oral rabies vaccination campaigns. Several changes can be made to improve analysis quality and increase the comparability of bait uptake frequencies among member states.

Key words: Age determination, fox teeth, oral rabies vaccination follow-up, tetracycline.

INTRODUCTION

Biomarkers are indicators commonly used in wildlife and disease management programs requiring the monitoring of oral treatment. Within the framework of oral rabies vaccination programs, various biomarkers have been assessed in the field. Tetracycline (Hanlon et al., 1989; Cliquet and Aubert, 2004; Slate et al., 2009; Zienius et al., 2011), sulphadimethoxine (a broad-spectrum antimicrobial, short-term antemortem seromarker; Hanlon et al., 1993), iophenoxic acid (relatively long-lived seromarker lasting 6–12 wk; Baer et al., 1985; Follmann et al., 1987; Trewhella et al., 1991), and rhodamine B (antemortem external marker; Farry et al., 1998; Fry et al., 2009) are the most frequently cited.

Despite this wide range of biomarkers, tetracycline remains the most commonly used in rabies field studies. Due to poor reliability, feasibility, or their high cost, other markers have not been used on a large scale for assessing bait uptake rates in vaccinated populations. Tetracycline—initially developed for its antibiotic properties—is typically incorporated in the bait casing as a biomarker for monitoring vaccine and contraceptive bait consumption in carnivores and rodents (Linhart and Kennelly, 1967; Johnston and Voigt, 1982;

Johnston et al., 1988; Creekmore et al., 1998; Schmit et al., 2010). After consumption, the molecule is incorporated into bones and teeth, and this interaction creates a line in the bone that can be observed using epifluorescence microscopy (Milch et al., 1958). International organizations recommend evaluating bait uptake in field target species sampled in rabies-vaccinated areas to assess the efficacy of the vaccination program by detecting tetracycline in the teeth or bones of collected animals (European Commission, 2002; World Organization for Animal Health [OIE], 2005, 2008; World Health Organization [WHO], 2005).

Age estimation is a second criterion regularly analyzed along with tetracycline detection to assess vaccination effectiveness across age categories. Age estimation, based on dental eruption and morphology, is considered a suitable method (Harris, 1978). The technique consists in counting the number of cementum lines and observing dentine width, which increases with age. Furthermore, dentine is deposited inward into the pulp cavity, gradually filling it (centripetal growth). A large pulp cavity is consequently observed in young animals only. Cementum is deposited over the root dentine (centrifugal growth; Morris, 1972; Goddard and Reynolds, 1993).The first dark-stained line appears in tooth cementum during January–March of the year following birth (Goddard and Reynolds, 1993). The lines consequently appear as characteristic annual rings of paler opaque areas in summer and darker transparent areas in winter (Grue and Jensen, 1973; Van Lancker et al., 2005; Roulichova and Andera, 2007). As one dark line is produced per year, it is feasible to determine age with an interval of 1 yr per age category. Determining age can help establish whether the tetracycline mark and thus bait consumption occurred during the current or previous campaigns. When associated with antibody levels, this can also indicate the timing and efficacy of yearly booster immunizations.

Both age and tetracycline determination techniques have consequently been widely used within Europe since the first oral rabies vaccination programs in Switzerland in 1978 (Steck et al., 1982). No comparative assessment of laboratory analyses had been organized before this study. As it is important to evaluate the reliability and comparability of biomarker frequency detection, the European Union (EU) Reference Laboratory (EURL) for rabies initiated an interlaboratory proficiency test (ILPT) to compare tetracycline and age determination using red fox (*Vulpes vulpes*) teeth under its European mandate (Vassilou, 2008). To our knowledge, this is the first such study comparing these techniques and their performance at the EU level.

MATERIALS AND METHODS

Laboratories

National reference laboratories (NRLs) from the EU and several bordering countries involved in oral vaccination programs were invited by the EURL to take part in this ILPT. Participation was voluntary. The study, held in 2010, included 12 participating NRLs from member states, including the EURL for rabies and one laboratory from a country outside of the EU. Staff in all the participating laboratories, including the EURL, performed the test blind coded.

Panel composition

The lower jaws (mandible) used for the ILPT were collected from red foxes sampled in the field. They were part of a collection from the last oral vaccination campaign in eastern France, near the border of Germany, in 2005 (Cliquet and Aubert, 2004). The lower jaw of each animal was collected, stored at −20 C, and divided into two. The EURL determined tetracycline and age using the first half-mandible. An IsoMet® (Reutlingen, Germany) saw was used to obtain a transverse section of an undecalcified tooth to allow both age and tetracycline determination according to Johnston and Watt (1980) and Johnston et al. (1987). As there is no significant difference in the presence of tetracycline in the right and left mandible halves (Algeo et al., in press), the second half-mandible was used for the ILPT on the basis

of both the age and tetracycline results obtained by the EURL on the first half. Four intact mandible groups were created: "mandibles from adult/juvenile foxes positive/negative for tetracycline (TC)." Fifteen panels were constituted, each consisting of two positive adult half-mandibles, one positive juvenile half-mandible, one negative adult half-mandible, and one negative juvenile half-mandible. The positive adult specimens produced a strong tetracycline signal, while the positive juvenile samples had only a weak signal. The selection of juvenile samples with a weak signal depended on the sample collection, and there were not enough juvenile mandibles with a strong tetracycline signal collected to be included in the study. One positive control (cross section of a positive tooth) and one negative control (cross section of a negative tooth) were also included in the panel. The two controls were transverse sections of an undecalcified canine root prepared by the EURL. These sections, approximately 150 μm thick and without any treatment or prior mounting, were prepared using an IsoMet diamond saw. Participating laboratories were in charge of mounting the sections. All samples were blind coded, and different individual laboratory codes were attributed to each participant.

Distribution of samples

The half-mandibles were shipped in dry ice to ensure sample stability by an internationally approved carrier under UN3377 requirements in accordance with the International Air Transport Association (IATA, 2009) and the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR, 2009).

Test procedure and technical questionnaire

Laboratories were asked to use their own routine procedure for determining the presence of tetracycline and evaluating the age of animals sampled. A technical questionnaire on the techniques used was sent to the participating laboratories at the same time as the panels. These questionnaires were designed to analyze the variability of methods used by the participants for each step of the procedure. Information requested included the laboratory's number of years of experience in tetracycline detection, the number of samples routinely tested per year, and explanations of sample preparation, including extraction technique, location of the mandible section, longitudinal or transverse section, number of sections per sample, material used, section thickness, use of a control slide, the microscope's wavelength excitation filter, use of a mounting medium, and number of independent readers.

RESULTS

Laboratory results of tetracycline analysis

All participating laboratories ($n=12$) carried out the tetracycline detection test. All samples were tested except two negative juvenile samples that were considered untestable (Table 1a). Only three laboratories (25%) produced 100% correct results for tetracycline detection. Of all 56 half-mandibles tested, 26% gave incorrect results. No difference was observed in the level of false-negative (22%) and false-positive (32%) results. Eleven laboratories (92%) provided 100% correct results for positive adult samples, while five laboratories (42%) provided correct results for the mandibles from juvenile foxes with a weak tetracycline signal. Seven of 12 laboratories (58%) provided 100% correct results for adult samples negative for tetracycline, and most (80%) also provided 100% correct results for negative juvenile-coded samples. There appear to be more false-positive results in adult samples (42%) than in juvenile samples (20%).

Laboratory results for age determination

Nine of 12 participating laboratories estimated the animal age class of the samples. Four of nine laboratories (44%) estimated the correct age class for all samples analyzed using undecalcified samples without staining. Seven laboratories (78%) provided correct results for adult-coded samples, while six (67%) provided correct results for juvenile-coded samples without any obvious difference between groups (Table 1b). The proportion of incorrect results ranged from 11% (positive/adult; positive/juvenile; negative/adult) to 22% (negative/juvenile). Only four laboratories (44%) estimated the correct age class for all samples, and six incorrect results (13%) were reported for

TABLE 1. Interlaboratory results on (a) tetracycline and (b) age determination. Discordant results appear in bold.[a]

Laboratory code	Positive/ adult		Positive (weak)/ juvenile	Negative/ adult	Negative/ juvenile	Positive	Negative	General
a) Tetracycline detection								
L01	pos	pos	pos	**pos**	**pos**	S	**NS**	**NS**
L02	**neg**	pos	**neg**	neg	neg	**NS**	S	**NS**
L03	pos	pos	pos	neg	neg	S	S	S
L04	pos	pos	pos	neg	neg	S	S	S
L05	pos	pos	**neg**	**pos**	neg	**NS**	**NS**	**NS**
L06	pos	pos	**neg**	**pos**	not done	**NS**	**NS**	**NS**
L07	pos	pos	**neg**	neg	not done	**NS**	S	**NS**
L08	pos	pos	pos	**pos**	neg	S	**NS**	**NS**
L09	pos	pos	pos	neg	neg	S	S	S
L10	pos	pos	**neg**	**pos**	**pos**	**NS**	**NS**	**NS**
L12	pos	pos	**neg**	neg	neg	**NS**	S	**NS**
L13	pos	pos	**neg**	neg	neg	**NS**	S	**NS**
% (*n*/*n*) laboratories with satisfactory results	91.7 (11/12)		41.7 (5/12)	58.3 (7/12)	80.0 (8/12)	41.7 (5/12)	58.3 (7/12)	25.0 (3/12)
% (*n*/*n*) samples with discordant results	4.2 (1/24)		58.3 (7/12)	41.7 (5/12)	20.0 (2/10)	22.2 (8/36)	31.8 (7/22)	25.8 (15/58)

b) Age determination

	Positive/ adult		Positive (weak)/ juvenile	Negative/ adult	Negative/ juvenile	Adult	Juvenile	General
L01	A	A	J	A	J	S	S	S
L02	A	A	J	A	**A**	S	**NS**	**NS**
L03	A	**J**	J	**J**	J	**NS**	S	**NS**
L04	A	A	J	A	J	S	S	S
L08	A	A	J	A	J	S	S	S
L09	A	A	J	A	J	S	S	S
L10	A	A	**A**	A	J	S	**NS**	**NS**
L12	A	**J**	J	A	J	**NS**	S	**NS**
L13	A	A	J	A	**A**	S	**NS**	**NS**
% (*n*/*n*) laboratories with satisfactory results	77.8 (7/9)		88.9 (8/9)	88.9 (8/9)	77.8 (7/9)	77.8 (7/9)	66.7 (6/9)	44.4 (4/9)
% (*n*/*n*) samples with discordant results	11.1 (2/18)		11.1 (1/9)	11.1 (1/9)	22.2 (2/9)	11.1 (3/27)	16.7 (3/18)	13.3 (6/45)

[a] pos = positive result; neg = negative result; S = satisfactory results; NS = unsatisfactory results; A = adult; J = juvenile.

the total of 45 samples analyzed for age estimation. With the exception of one laboratory, laboratories with incorrect age determination results also obtained incorrect tetracycline detection results.

Technical questionnaire

All but one laboratory ($n=11$) returned the technical questionnaire. As several parts of questionnaires were not completed, the number of responses varies by question.

Experience level of participating laboratories: More than half the laboratories (six of 11) had 3 to 6 yr of experience in tetracycline determination. The other five participants had 0, 2, 15, 20, or 22 yr of experience, respectively. False tetracycline determination results did not depend on years of experience.

Current techniques in oral vaccination program follow-up: Eight participating laboratories were involved in oral vaccination programs in 2010, while three laboratories did not

use these techniques that year. Two no longer performed these analyses as their countries had obtained rabies-free status, and oral vaccination programs had been stopped. The number of unsuccessful laboratories in the ILPT was proportional to the number of participating laboratories, whether they were in countries where oral vaccination was in progress (six of eight) or not (two of three).

Number of samples tested per year: The number of samples tested per year was highly heterogeneous. The maximum, >2,000 samples per year, was reached by four of 11 laboratories, while the minimum was observed for two laboratories with no tetracycline or age determination analyses in 2010. Incorrect results were not correlated with the quantity of samples analyzed during the year except for the laboratories with zero analyses ($n=2$), which both produced incorrect results.

Sample preparation: All the laboratories were accustomed to performing analyses on the lower mandible, but the mandible section location varied according to the laboratory. Four of 10 laboratories cut the transverse section from between C and P1 (Fig. 1; line A): one between P1 and P2 (Fig. 1; line B); three between P2 and P3 (Fig. 1; line C); and two after P3 (Fig. 1; line D). The number of unsuccessful laboratories was higher in the group cutting the mandible between C and P1 (four of four) than in the group cutting between P1 and P3 (one of four). Every laboratory answering the questionnaire used an IsoMet diamond saw for tooth sectioning (11/11).The section was longitudinal for two of nine laboratories and transverse for seven of nine laboratories. All longitudinal sections of the lower mandible tended to be peripheral rather than axial. This could be explained by the fact that when the lower mandible half is sectioned, it is almost impossible to cut an axial section of the tooth due to the curvature of the lower mandible. Unlike

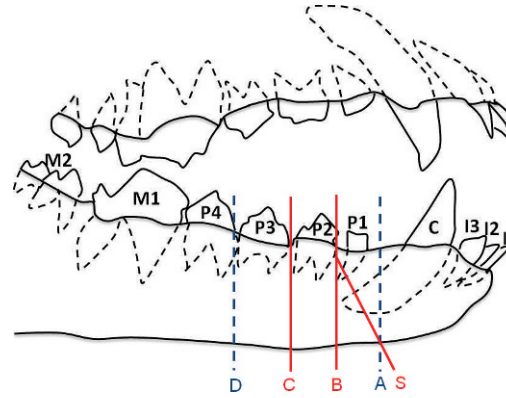

FIGURE 1. Schematic view of a fox (*Vulpes vulpes*) half-mandible. A, B. C, and D: mandible sections performed by participating European Rabies laboratories. B and C: correct location of the mandible section. S: correct location of the tooth section.

the laboratories using transverse sections, every laboratory cutting longitudinal sections produced incorrect results. The laboratories prepared one ($n=4$), two ($n=3$), or three ($n=2$) sections per tooth. The number of unsuccessful laboratories in the ILPT remained proportional to the number of participating laboratories in each of the three groups, showing no evidence that the number of sections prepared affected the quality of results. The thickness of the tooth section varied between 50 μm and 500 μm. Laboratories producing false-positive or false-negative results used a section 150 μm or 200–500 μm thick, while the results of laboratories using a section ranging from 50 μm to 80 μm thick were correct.

Detection of tetracycline

Tetracycline has an excitation wavelength of 390 nm (Fig. 2a). Three laboratories indicated a wavelength range outside the tetracycline excitation wavelength but identified the positive samples nonetheless. This supports the hypothesis of confusion by participants in the transcription of the wavelength reported in the questionnaire. Half the laboratories (five of 10) used a mounting medium before reading. Laboratories using mounting media produced

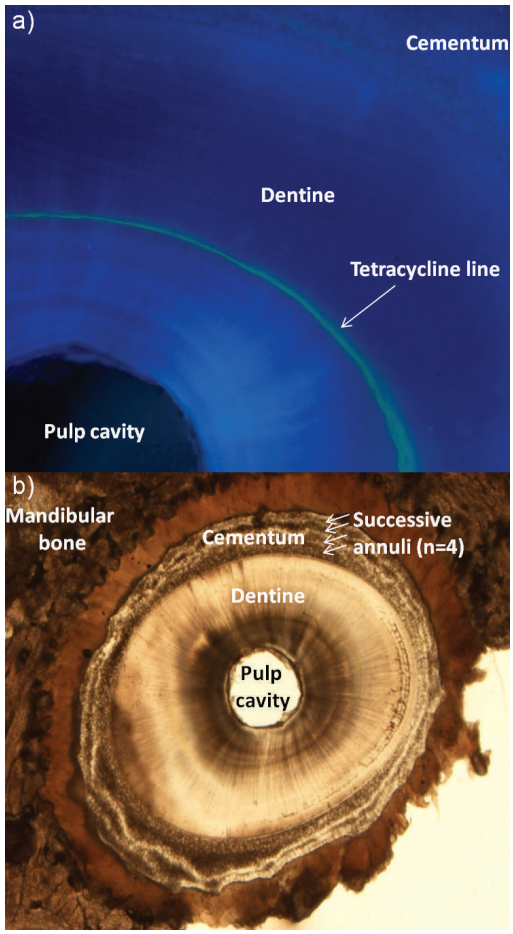

FIGURE 2. Transverse section of a fox's (*Vulpes vulpes*) canine tooth showing (a) yellow fluorescent line of tetracycline at 390-nm wavelength excitation and (b) four successive annuli in cementum, indicating an age estimation of 4 yr.

more correct results (one of four laboratories with incorrect results) than those that did not use mounting media (five of five laboratories with incorrect results).

Seven of 11 laboratories used neither positive nor negative control slides before reading. The number of unsuccessful laboratories in the ILPT was proportional to the number of participating laboratories not using controls. One reader was used per reading session in four laboratories and two in six laboratories. One laboratory used two or more readers. The number of unsuccessful laboratories was proportional to the number of participating laboratories in both groups. The location of the tetracycline, the number of tetracycline lines, and the date of animal death all help to determine the period of bait uptake. It seems that most laboratories did not analyze such factors and consequently did not estimate when and how many times vaccine baits were consumed.

Age determination

Seven laboratories routinely estimated animal age in the context of oral rabies vaccination follow-up studies. Nine laboratories evaluated animal age for the ILPT. Of eight laboratories (one laboratory did not answer the questionnaire), three used age classes with intervals of 1 yr, while the others simply distinguished juveniles from adults. Laboratories estimating age classes (0–1; 1–2; 2–3; >3 yr) analyzed the date of animal death, the diameter of the pulp cavity, the presence of cementum, and the number of dark lines in the cementum (the critical element that reveals yearly growth and therefore the animal's age; Fig. 2b).

DISCUSSION

Aerial oral vaccination campaigns against rabies initiated in 1978 in Switzerland were rapidly expanded to neighboring countries (Aubert et al., 2004). Since this period, oral vaccines have been widely used throughout Europe for controlling rabies epidemics in wildlife. From 1978 to 1999, for example, 151,465,503 baits were dropped throughout Europe (Pastoret et al., 2004). Baits consist of a blister pack attractive to targeted species containing a capsule or a sachet of antirabies vaccine. A quantity of 150 mg of tetracycline per bait is generally used to ensure lifelong marking of bones and teeth of bait consumers (Wachendörfer et al., 1986; Laine et al., 2008; Niin et al., 2008; Sattler et al., 2009).

For oral rabies vaccination campaigns, international institutions such as WHO, the OIE, or the European Commission recommend monitoring vaccination effectiveness

(European Commission, 2002; WHO, 2005; Dodet, 2006; OIE, 2008). Vaccination effectiveness is assessed by monitoring tetracycline in the teeth and bones of the target population to evaluate bait uptake, by the examination of sera to evaluate the level of protection in the target population, and by analyzing the incidence of rabies in animals before, during, and after the oral vaccination campaign. These techniques are consequently widely used across Europe. The results of this first interlaboratory comparison showed an unexpected failure rate of laboratories in tetracycline and age determination, with only 25 and 44% of laboratories, respectively, producing 100% correct results.

False-negative results in tetracycline detection were more frequently detected in weak juvenile samples (58%), whereas they were infrequent in adult samples (4%). Test panels mimicked real test conditions, with strong and weak positive as well as negative samples. The fluorescence of the different samples under test was consequently not fully calibrated. Positive juvenile samples selected for the ILPT had weaker fluorescence than positive adult samples. Because strong fluorescence is easier to detect than weak fluorescence, the proportion of false negatives in juvenile samples could be explained by reading difficulties. The difference in success among laboratories must be interpreted with caution, as the fluorescence within a group of teeth (weak positive/juvenile and positive/adult) was not identical among teeth. It is, therefore, possible that some laboratories received teeth with greater or lesser fluorescence than those of other laboratories.

Moreover, the status of the sent sample was determined in comparison with the EURL's analysis of the first half-mandible. We assumed that, even if no significant difference occurs between the two half-mandibles, there could be an occasional difference in the tetracycline deposition pattern between the two half-mandibles, especially in weak positive samples. This limitation could be partially resolved by including a coded section of tooth of a known status in the panel. This would ensure the status of the sample but would not allow the sectioning quality of laboratories to be assessed, by being based on sectioning starting from half-mandibles and not from a ready-to-use section.

There were 42% false-positive results for adult samples and 17% for juvenile samples. False-positive samples can occur when artifacts left from poor-quality tooth sections are confused with tetracycline fluorescence. From the available information, the reasons for the difference in false positives between juvenile and adult samples are not clear.

Such findings raise questions about the comparability of bait uptake rates previously determined when monitoring oral vaccination campaigns. However, during oral vaccination campaigns—especially when they are held over successive years—most samples are strongly marked. The large proportion of animals with weak signals in our test (20% of weak positive juvenile samples in the panel) is largely overestimated compared to the proportion of weak samples generally observed during oral vaccination campaigns. This has probably led to an overestimation of the proportion of discordant results compare to the one that could be observed during routine analysis. Nevertheless, our overall findings demonstrate a need to improve tetracycline and age determination methods within member states. The failures were not correlated with laboratory experience or quantity of samples analyzed per year. By analyzing the technical questionnaires sent by participating laboratories, four factors were identified as potentially affecting the results.

Location of the mandible section: All the laboratories that cut the mandible section between teeth C and P1 (Fig. 1; line A) produced incorrect results. It appeared that a section between P1 and P2 (Fig. 1; line B) and between P2 and P3 (Fig. 1;

line C) provided more reliable results. A cut between C and P1 can damage the canine root, thus making a section from the end of the root, where annual cementum lines are the most visible and distinguishable and unusable. Regarding the EURL experience, the mandible of an adult specimen is sectioned (Fig. 1; line B), and only one of the canine teeth (with its root) will be extracted from the mandible with forceps and then sectioned (Fig. 1, line S). For juvenile specimens, the canine root and bone of the mandible will be directly sectioned in the piece of mandible without prior extraction (Fig. 1, line S).

Direction of the tooth section: Transverse or longitudinal sections can be performed. Transverse sections allow a higher number of cuts to identify the position of tetracycline lines with adjacent annuli (Fig. 2). Longitudinal sections allow more certain identification of the first annulus, which is sometimes not visible in transverse sections because of its highly variable separation from the dentine-cementum junction (Matson and Kerr, 1998).

In this study, transverse sections were commonly used (seven of nine laboratories), and the two laboratories using longitudinal sections obtained incorrect results. The transverse section seems to be easier and more practical to perform by technical staff. The cutting of several successive sections compensated for any lack of visibility. The cut should be made 2 or 3 mm from the end of the root 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. Because tetracycline is more likely to be deposited in bones and canine teeth (Hanlon et al., 1989), transverse sections of the tooth must be cut through the root of the canine to include tooth and bone tissues that would normally be undergoing mineralization and likely to reveal a tetracycline biomarker.

Thickness of the section: No incorrect results were obtained by laboratories using a section 50 to 80 μm thick, unlike laboratories using a section 150 μm or >200 μm thick. A study by Johnston et al. (1987) recommended cutting sections 60 to 150 μm thick based on the experience of personnel at the Ontario Ministry of Natural Resources. In the experience of the EURL for rabies, the optimal section thickness is around 150 μm for both tetracycline detection and age determination. We emphasize that the quality of the cut (thickness, sharpness, and absence of damage to the section) is critical. Poor-quality sections can introduce artifacts during microscopic examination, leading to false-positive results.

Use of a mounting medium: All laboratories (five of five) that did not use a mounting medium obtained incorrect results, while only one laboratory (one of four) that did use a mounting medium obtained <100% correct results. It appears therefore that 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 plane.

Although not identified as affecting results, some other points need to be emphasized. Some laboratories indicated an improbable wavelength range outside the tetracycline excitation wavelength despite identifying positive samples. This suggests some confusion among participants. The wavelengths of the filter combinations recommended by manufacturers for tetracycline fluorescence analysis sometimes differ. This can lead to confusion in the differentiation of tetracycline fluorescence from nonspecific tissue fluorescence in laboratories using different brands of microscope. Such results highlight the need to standardize excitation wavelengths and filter combinations.

Some laboratories have explained their interpretation of tetracycline detection without any information on the date of the animal's death. However, the date of

death, pulp cavity size, and the presence or absence of cementum are determining factors when the objective is to distinguish juveniles from adults. The presence or absence of tetracycline should be interpreted by considering, at the very least, the date of death and date of oral vaccination. If the animal is killed within a few days of bait ingestion, the tetracycline deposit in a tooth may be on the growing edge of the tooth dentine and cementum. Therefore, the tetracycline line may be confused with edge refraction and distortion from the saw cut on the edge of the tooth.

In conclusion, analysis of technical questionnaires has underlined the need to standardize techniques. Our findings emphasize that laboratory performance comparisons and standard operating procedure analyses remain priority tools for detecting a method's critical parameters. This type of study also facilitates standardization of techniques, helps to raise performance, and provides a better comparison of epidemiologic data among countries. This study has led to recommendations on the problematic issues revealed during the ILPT (location of the mandible section, section thickness, etc.); a second ILPT is planned in order to continue harmonizing issues, which could need such revision. A one-day training course is also planned for NRLs from EU member states. Such approaches should facilitate standardization and optimize the technical performance of NRLs throughout the EU.

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