Combined microscopy-PCR EURL-AP Proficiency Test 2018

Final version

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Table of content

Summary

The European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) organised the present proficiency test for assessing the ability of the NRL network with respect to the detection of processed animal proteins (PAPs) in feed using both light microscopy and PCR according to Commission Regulation EU/51/2013. For the second time, the study also evaluated the correct application of the SOP (Standard Operating Procedures) on operational protocols for the combination of light microscopy and PCR with respect to the type of feed and the composition of the samples.

The total number of participating laboratories was 33 (26 NRLs and 7 labs outside the NRL network). On the exception of one participant, all laboratories delivered results. The study was based on a set of seven samples (to be analysed by light microscopy and/or by PCR) consisting of blank feed matrices or feed fortified with terrestrial processed animal proteins and/or fishmeal.

Regarding the correct implementation of the SOP on operational protocols for the combination of light microscopy and PCR, 89 % of the NRLs were complying. This is an improvement compared to the study conducted in 2017, which revealed that only 64 % of NRLs were complying.

Regarding the detection of PAPs by light microscopy the overall results indicated an excellent level of global performance for 77 % of the NRLs, a satisfying level of global performance for 8 % of the NRLs and 15 % of underperforming NRLs. The present test revealed sensitivity problems for the detection of low adulteration level of porcine PAP and of starfish meal. These problems were also noted for non-EU participants. Focus on continuous training to overcome the lack of experience with new materials would help solving such issues.

Concerning the PCR results, the global performance of the NRL network was in all comparable to the one computed for light microscopy. Four underperforming NRLs were pointed by the present study. The study also highlighted a close dependency of the PCR results on the results obtained by light microscopy which are orientating the decision to perform or not PCR tests. A similar situation was also observed for non-EU participants.

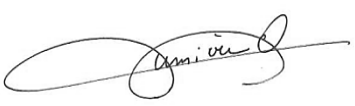
Keywords :

Processed animal proteins – Light microscopy – PCR - Proficiency test – Qualitative analysis

This report identified by an ISBN has been prepared from a draft version sent for revision and comments to the participants on the 28th February 2019. After reception of the comments on the 20th March 2019, it was amended accordingly and approved by the signature of the organisers.

ISO 17043 coordinators signature for approval:

Olivier Fumière *Pascal Veys*



1. Foreword

European Union Reference Laboratories (EURL) were created in order to ensure a high level of quality and a uniformity of the results provided by European control laboratories. On 15th March 2017, the European Parliament and the Council adopted Regulation EU/625/2017 [1], improving the effectiveness of the official food and feed controls while redefining the obligations of the relevant authorities and their obligations in the organization of these controls.

On March 2011, Commission Regulation EC/208/2011 [2] renewed the nomination of the Walloon Agricultural Research Centre as European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP, http://eurl.craw.eu). It has to develop the following priority axes:

(i) To provide National Reference Laboratories (NRLs) with detailed analytical methods, including reference methods for the network of Member State NRLs;

(ii) To coordinate application by NRLs of the methods by organizing interlaboratory studies;

(iii) To develop new analytical methods for the detection of animal proteins in feedingstuffs (light microscopy, near infrared microscopy, PCR, immunology …);

(iv) To conduct training courses for the benefit of NRL staffs from Member States and future Member States;

(v) To provide scientific and technical assistance to the European Commission, especially in cases of disputed results between Member States.

In this framework, the EURL-AP has been organising since 2006 yearly proficiency tests for the assessment of the implementation of the reference methods for the detection of animal proteins in feed as described by Commission Regulation EU/51/2013 [3] amending Annex VI of Commission Regulation EC/152/2009 [4]. The present study report is part of this activity scope.

2. Introduction

According to modified Annex VI of Commission Regulation EC/152/2009 [4] official controls for the detection of animal proteins in feed inside the EU have to be performed by light microscopy and/or PCR since June 2013. Standard Operating Procedures (SOP) are supporting the implementation of the two methods. The SOP on operational protocols for the combination of light microscopy and PCR [5] defines which of the methods shall be used alone or in combination according to the type of feed and its composition.

The objective of the present proficiency test was strictly to evaluate within the network of 26 NRLs the analytical performance to detect processed animal proteins (PAPs) in feed by light microscopy and PCR. Moreover, the compliance with the legal requirements imposed by the SOP on operational protocols for the combination of light microscopy and PCR was evaluated. Participation of the NRLs is mandatory.

In addition and on proposal of the Commission, invitations to participate to this test were also sent to a limited number of official control labs outside the EU. Non-EU participants were asked to apply also light microscopy and PCR although strict following of Annex VI of Commission Regulation EC/152/2009 and related SOPs was not imposed to them.

3. Material and methods

3.1. Study organisation

Twenty six NRLs and seven laboratories outside this EU network participated to the study. A detailed list of the 33 participating labs is included in Annex 1.

Official announcement (Annex 2) of the study was made on the 7th September 2018 to all participants.

On the 18th October 2018, the sample sets were shipped to the participants. On the same day the Excel report forms containing the instructions (Annex 3) were communicated to all participants – downloadable from the EURL-AP intranet for the NRLs or sent by email to the non-EU participants who have no access to this intranet.

The deadline for the delivery of the results was fixed in the announcement and in the instructions at the 16th November 2018.

Within the instructions, some general recommendations were delivered to the participants:

- Laboratories participating to the proficiency test were themselves responsible to reach appropriate homogeneity of the sample sub-portions that had to be taken from the whole sample vial for analysis. Precautions to avoid laboratory cross-contamination were also highlighted.
- Results had to be encoded by way of an Excel report form (Annex 3). Participants were asked to carefully read the instructions on how to fill in the result form and to testify they did it prior to encoding their results. No other support for communicating the results was accepted.
- Participants were asked to sign the summarized results sheet that is automatically generated when filling the form and to return it by email to the EURL-AP. Only when both the Excel file and a copy of the summarized results sheet were received by the EURL-AP were results taken into consideration.
- Participants were notified that results arriving later would not be accepted.

On the exception of one laboratory outside the EU, which did not deliver its results, all results were delivered on time to the organiser.

Twenty eight participants returned results for both microscopic and PCR analyses. The proficiencies of NRLs and other participants were evaluated separately in this report.

3.2. Material

3.2.1. Description of the samples

Six different test materials were prepared for the study. The composition of the sample set was established taking into account the following considerations:

- Use of feed matrices intended to different farmed animals (with the indication on the vial label) for assessing the correct interpretation and implementation of the operational schemes as described in the SOP on operational protocols for the combination of light microscopy and PCR [5].
- Use of aquafeeds as matrices for assessing the detection capabilities of terrestrial PAPs because since the 1st June 2013 non-ruminant PAPs are authorized in aquafeed according to Commission Regulation EU/56/2013 [6].

Each participating lab received seven samples of about 50g each. Each sample was labelled with the type of feed matrix. Each sample was assigned with a unique random number. Details of the sample set are indicated in Table 1.

Table 1: Composition of the sample set.

Sample	Label	Material	Nr of replicates	Expected results * Microscopy: Terrestrial particles	Expected results * Microscopy: Fish particles	Expected results * PCR: Ruminant DNA
1	Aquafeed for trout juveniles	Fishfeed I + 0.05 % porcine PAP	2	+	+	-
2	Aquafeed with haemoglobin powder	Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder	1	NA	NA	+
3	Aquafeed for trout juveniles	Fishfeed I + 0.05 % ruminant PAP	1	+	+	+
4	Feed for fattening pig	Feed for fattening pig	1	-	-	NA
5	Starter feed for broilers	Starter feed for broilers + 1 % starfish	1	-	+	NA
6	Milk replacer	Milk replacer + 0.5 % fishmeal	1	-	+	NA
Total			**7**			

(* Explanations on expected results are described in section 3.4, NA = not applicable)

Expected results were internally determined based on the known composition of the samples (presence or absence of PAP) and the results obtained during the homogeneity study.

The labels were aimed at defining which analyses had to be performed for each sample in agreement with the binding SOP on operational protocol. No other information was delivered to the participants for determining the correct analytical choice.

3.2.2. Materials used in the preparation of the samples

Five commercial matrices were used:

- Fishfeed I was a **compound feed for trout juveniles**. It was composed of fishmeal, fish oil, maize starch, wheat gluten, protein concentrate from pea and feed complements (vitamins, minerals). Its sediment content was of 0.8 %. This feed was used for preparing sample 1 and 3.
- Fishfeed II was a **complete sinking feed for carp**. It consisted of soy feed, wheat germ, toasted soybeans, peas, fishmeal, haemoglobin powder, melasse, fish oil and calcium carbonate. The sediment content of the mixture was about 0.7 %. It was used for preparing sample 2.
- Feed for fattening pig was a **compound feed for pig**. It contained barley, wheat, maize, soybean flour, calcium carbonate, lard, lysine, bicalcium phosphate, salt and premix. Its sediment was of 1.1 %. It was used for preparing sample 4.
- Starter feed for broilers was a **compound feed for broilers**. It consisted of wheat, soybean oilcake, maize, wheat flour, extruded soybean, wheat middlings, sunflower seed oilcake, rapeseed oilcake, barley, calcium carbonate, premix of additives, dicalcium phosphate, sodium bicarbonate and sodium chloride. Its sediment was of 4.1 %. It was used for preparing samples 5.
- Milk replacer was a **complete feeding for calves** made of lactoserum, protein concentrate from lactoserum, wheat gluten, rapeseed oil, copra oil, palm oil, wheat flour, soya oil, inactive beer yeast extract and vitamins, minerals and antioxidants. Its percentage of sediment was of 0.7 %. This feed was used for sample 6.

Adulterant material used:

- A **pure porcine PAP** was used for preparing sample 1. This PAP was used in previous proficiency test 2015, 2016 and 2017 [7, 8, 9]. Its bone content was of about 14 % and its purity was checked by microscopy and PCR.

- A **pure ruminant PAP** was used for preparing sample 3. This PAP presenting a high bone content of 60.7 % was used in previous proficiency test 2017 [9]. Its purity was controlled by PCR.
- A **skimmed milk powder** was used for sample 2. PCR analyses proved it only to be from bovine origin.
- A **starfish meal** with a sediment of 27.5 % was used for sample 5.
- A **fishmeal** originating from Chili and having a bone content of about 13.8 % was used for sample 6. PCR analyses proved it only to be from fish origin.

3.2.3. Description of the mixing procedures

To avoid presence of interfering material, a cleaning of the rooms where the samples were handled was performed prior to sample preparation, mixing of the materials and filling of the vials.

Blank matrix was conditioned first in order to avoid contamination.

On the exception of the matrices for samples 5 and 6, all other matrices were ground, all or in part, at 2 mm before any other treatment. All samples were prepared by successive dilutions.

3.3. Qualitative analysis

Analyses of qualitative proficiency testing were applied following ISO 13528 [10].

3.3.1. Light microscopy

Qualitative analysis concerned the detection of terrestrial animal and/or fish material.

Results are expressed by the participants in three formulations according to regulation EU/51/2013 [3] amending regulation EC/152/2009 [4]:

- Positive (= presence of microscopically detectable animal material)
- Negative (= absence of any microscopically detectable animal material)
- Below LOD (= low level presence of microscopically detectable animal material with a risk of false positive result)

Considering the risk of false positive results, all results expressed as below LOD have to be assimilated to negative ones as by definition they cannot be certified as positive *sensu stricto*. This allows an on-off, or binary result analysis.

These binary results were analysed by classical statistics: accuracy, sensitivity and specificity. All those statistics were expressed as fractions.

Accuracy is the fraction of correct positive and negative results; it was calculated by the following equation:

$$\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}$$

where *PA* is the number of correct positive results (Positive Agreements), *NA* the number of correct negative results (Negative Agreements), *ND* the number of false negative results (Negative Deviations) and *PD* the number of false positive results (Positive Deviations).

Sensitivity is the ability of classifying positive results as positive, it was calculated as follows:

$$\text{Sensitivity } SE = \frac{PA}{PA + ND}$$

Specificity is the ability of classifying negative results as negative, it was calculated as follows:

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

The *AC, SE* and *SP* were calculated separately for each laboratory and for each requested parameter (detection of terrestrial animal material, detection of fish material) for the estimation of its proficiency. A consolidated *AC* over both parameters was used to rank each participant. Finally a global *AC* was also calculated for each material in order to estimate the performance of the network.

3.3.2. PCR

Qualitative analysis concerned the detection of ruminant DNA.

The participants delivered Ct values (in cycles) to compare to a cut-off value (in cycles) set at 15 copies of the target and validated by a quality criterion (the cut-off Ct value must correspond to a number of copies of the target > 9.00 copies). For each sample, DNA is extracted from 2 test portions. The results obtained from the 2 test portions must be consistent, in the sense that both Ct values should be close to each other and on the same side compared to the cut-off value. A Ct value < cut-off value corresponds to a positive result. Respectively, a Ct value ≥ cut-off value corresponds to a negative result. Results are expressed by the participants in two formulations:

- Present (= presence of ruminant DNA detected)
- Absent (= no ruminant DNA detected)

As for the light microscopy, these binary results were analysed by classical statistics (accuracy, sensitivity and specificity) with the same formulae as presented in 3.3.1.

3.4. Performance criteria

Evaluation of the performance and scoring were applied as recommended by ISO 13528 [10].

The performance was assessed on two different aspects: the correct implementation of the legislation (i.e. choice of method to apply in accordance with the SOP on operational protocol) and the analytical results.

Results from analyses which should not have been performed according to the legislation were not considered for the analytical proficiency assessment but well for the evaluation of the implementation of the legislation. This is notably justified by the fact that if doing so the number of analyses would have been different among participant thus excluding any ranking of them.

The absence of an analytical result while legally imposed was considered as incorrect for both legal and analytical performance assessment. On the other hand, an analytical error leading to a logical stop choice in the operational protocol was not considered as an error in the implementation of the legislation.

3.4.1. Legislation

The first performance evaluation concerned the correct implementation of the operational protocols that have to be followed, depending on the type of feed being analysed, in order to control the application of the prohibitions laid down in Article 7 and Annex IV to Regulation (EC) N°999/2001 [11] (feed ban). The final destination of the compound feed or feed material determines which of the two operational protocols has to be followed: the one for feed or feed material intended for farmed animals others than aquaculture and fur animals, and the second one for feed or feed material intended for aquaculture animals. For the present test the only information conditioning the protocol to follow was the mention on the label referring to the type of feed.

The performance criteria for the legal implementation were decided as:

- **Complying** if no error was recorded concerning the operational scheme applied.
- **Non complying** if one or more errors were recorded concerning the operational scheme applied.

Regarding the sample set labelling, the expected operational protocols were:

Table 2: Expected operational protocols and analyses to perform.

Sample	Label	Operational protocol for the analysis of feed or feed material intended for
1	Aquafeed for trout juveniles	Aquaculture animals (microscopy and PCR)
2	Aquafeed with haemoglobin powder	Aquaculture animals (PCR only)
3	Aquafeed for trout juveniles	Aquaculture animals (microscopy and PCR)
4	Feed for fattening pig	Farmed animals other than aquaculture and fur animals (microscopy)
5	Starter feed for broilers	Farmed animals other than aquaculture and fur animals (microscopy)
6	Milk replacer	Farmed animals other than aquaculture and fur animals (microscopy)

3.4.2. Light microscopy

Considering the sample set composition, the expected results are indicated in Table 1.

Samples 1 and 3 are to be declared positive for both terrestrial and fish material presence.

Samples 5 and 6 are to be declared negative for terrestrial material presence and positive for fish material presence.

Sample 4 is to be declared negative for both parameters.

Sample 2 shall not be analysed by light microscopy because the sample label mentions that this sample contains haemoglobin powder.

Based on these considerations, the following performance criteria were decided for the light microscopy:

- **Excellent** level of global performance = consolidated AC superior to 0.90, i.e. having no ND for terrestrial material.
- **Satisfying** level of global performance = consolidated AC below 0.90 and having no more than 2 wrong results including a maximum of 1 ND for terrestrial material.
- **Underperforming** level of global performance = consolidated AC below 0.90 and having either more than 2 wrong results or 2 ND for terrestrial material.

3.4.3. PCR

As for light microscopy, the expected results are indicated in Table 1. Sample 1 is considered to be declared negative for the presence of ruminant DNA.

Samples 2 and 3 are considered to be declared positive for the presence of ruminant DNA. Sample 2 contains skimmed milk powder which is the source of ruminant DNA. The ruminant PAP content of sample 3 (Fishfeed I + 0.05 % ruminant PAP) is below 0.1 %. The method is usually sensitive enough to detect the presence of ruminant DNA in that sample but it could be considered as a more challenging sample for the participants.

Samples 4, 5 and 6 should not be analysed by PCR because the sample label mentions that it is a feed other than fishfeed.

Concerning the PCR, the performance criteria were decided as:

- **Excellent** level of global performance = no wrong result for the detection of ruminant DNA.
- **Satisfying** level of global performance = no more than 1 wrong result for the detection of ruminant DNA.
- **Underperforming** level of global performance = 2 wrong results or more for the detection of ruminant DNA.

3.5. Homogeneity study

Homogeneity study has been carried out for all materials used. Table 3 summarizes the results.

Table 3: Homogeneity study – Results.

Sample	Material	Light microscopy			PCR			
		Nr of replicates	Terrestrial	Fish	Nr of replicates	Ruminant	Porcine	Fish
1	Fishfeed I + 0.05 % porcine PAP	10	+	+	10	-	+	+
2	Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder	10	-	+	10	+	+	+
3	Fishfeed I + 0.05 % ruminant PAP	10	+	+	10	+	ND	+
4	Feed for fattening pig	10	-	-	3	-	ND	ND
5	Starter feed for broilers + 1 % starfish	10	-	+*	3	-	ND	-
6	Milk replacer + 0.5 % fishmeal	10	-	+	3	+	ND	+

(Legend: ND = not tested, + = systematically detected, - = systematically not detected, * = starfish particles)

The homogeneity was studied by light microscopy on 10 g of sample material for each replicate. Analyses of replicates were performed following strictly EC/152/2009. For PCR analysis of each replicate a double extraction was performed on 100 mg of sample material.

Sample 1 (Fishfeed I + 0.05 % porcine PAP) showed always fish particles as well as terrestrial bones. No feathers or blood particles were detected. This sample was systematically negative for the presence of ruminant DNA and positive for the presence of porcine and fish DNA.

Sample 2 (Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder) was systematically detected as positive for fish material and blood particles. Neither terrestrial bones nor feathers were recorded. PCR revealed it as positive for ruminant, porcine and fish DNA.

Sample 3 (Fishfeed I + 0.05 % ruminant PAP) showed always fish particles as well as terrestrial bones. No feathers or blood particles were detected. PCR analyses revealed the sample as positive for both ruminant and fish DNA.

Sample 4 (Feed for fattening pig) was free from any traces of animal origin. PCR revealed the sample as negative for ruminant DNA.

Sample 5 (Starter feed for broilers + 1 % starfish) was systematically showing starfish particles and no other type of animal traces. PCR analyses revealed the sample as negative for both ruminant and fish DNA.

Sample 6 (Milk replacer + 0.5 % fishmeal) was negative for terrestrial PAP particles but always positive for fish fragments. PCR analyses showed the sample as positive for ruminant and fish DNA.

Results from the homogeneity study allowed declaring the samples as fit for their purpose.

3.6. Stability of the samples

Internal stability studies performed on similar samples from past studies have demonstrated that such samples were stable over time (years) for both light microscopic and PCR analyses. There are no reasonable elements which would indicate that present samples should be unstable.

4. Results

Gross results for microscopy and PCR from all participants are to be found in Annex 4 and 5 respectively.

4.1. Legal compliance

Several cases of non-compliance were found, however in a lesser extend compared to preceding year.

Labs 4, 17 and 18 did not apply correctly the SOP on operational protocols. These participants performed microscopic analysis that is not authorised on sample 2. Indeed the label on this sample is mentioning an aquafeed containing haemoglobin powder. In such case only PCR has to be performed.

Lab 8 was also non-compliant, not regarding the implementation of the SOP on the operational protocols but for considering that fish material detection had not to be performed by microscopy for sample 6, labelled as milk replacer. This is an infringement to point 2.1.5. of regulation EU/51/2013 [3] which imposes to report on the presence of constituents from both terrestrial animal and fish origin. The laboratory commented to have detected fish particles in the samples and mentioned that fishmeal is authorised in milk replacers. The authorised or unauthorised criterion of an ingredient does not prevent from reporting it when it is detectable by the method.

About the correct implementation of PCR analysis, lab 14 did not follow the SOP for one of the replicates of sample 1. This is likely explained by the fact that on this replicate only a limited (<LOD) number of terrestrial bones were detected. However the SOP [5] imposes to perform PCR analysis as soon as terrestrial particles are detected without referring to any threshold.

4.2. Microscopy results

4.2.1. Qualitative analyses from the NRLs

4.2.1.1. Results and performance of the network

Table 4 summarizes the results submitted by the 26 NRLs for the sample types submitted to microscopic analysis.

Table 4: Global results expressed as accuracy (AC) – light microscopy

Sample	Material	n	AC	
			Terrestrial	Fish
1	Fishfeed I + 0.05 % porcine PAP	52	0.808 (10)	1.000
2	Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder	NA	NA	NA
3	Fishfeed I + 0.05 % ruminant PAP	26	1.000	1.000
4	Feed for fattening pig	26	1.000	1.000
5	Starter feed for broilers + 1 % starfish	26	1.000	0.731 (7)
6	Milk replacer + 0.5 % fishmeal	26	0.962 (1)	1.000

Accuracy means sensitivity in case of ND and specificity in case of PD. In brackets the number of ND or PD. (Legend: n = number of results; NA = not applicable).

The overall results, expressed in terms of global accuracy (AC) reveal the excellence of the NRL network for the detection of PAPs. The percentage of total error only accounted for 12 % of the total responses. This percentage is a bit higher than the one observed last year [9] but finds its origin from only two situations.

The first main source of error is related to sample 1, the fishfeed fortified at 0.05 % with porcine PAP. A problem of sensitivity is evidenced for the detection of the porcine PAP. From a total of 10 false negative results, 6 are situations were effectively nothing is has been detected while 4 out of them are cases of few findings of terrestrial bones classified as <LOD.

The second main source of error is also a sensitivity issue but for fish material. It concerns the difficulty to identify starfish meal. Seven cases of false negative results were noted whereof 5 with no detection at all and 2 cases of <LOD. This is not surprising since starfish meal is not a quite common feed ingredient.

4.2.1.2. Detailed review of results per sample

Sample 1: Fishfeed I + 0.05 % porcine PAP

ND for terrestrial particles:

- Labs 4, 9 and 18 failed at detecting the porcine PAP on both replicates
- Lab 3 and 14 reported few bones (<LOD) in one replicate while in the other replicate findings of bones allowed a positive results
- Lab 17 did not detect enough bones on both replicates (<LOD)

Some comments were also made by these participants:

Lab 17 described two "feather like" fragments. Similarly two feather fragments were also reported by lab 14. Lab 21 mentioned also the presence of blood as well as mouse hair, these findings were only limited to one replicate and not in the other.

Lab 22 found mites in both replicates.

Sample 2: Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder

No microscopic analysis had to be realised.

Sample 3: Fishfeed I + 0.05 % ruminant PAP

No error was noted.

Lab 22 found the presence of mites.

Sample 4: Feed for fattening pig

No error was noted.

However two cases of <LOD were reported for fish particles:

- Lab 14: 3 fishbones and 2 muscles, based on two determinations
- Lab 17: bone and gill fragments, based on three determinations

Insect particles were found in this sample by lab 20 but not classified as terrestrial.

Sample 5: Starter feed for broilers + 1 % starfish

ND for fish particles:

- Labs 4, 9, 20, 21 and 24 failed at detecting any fish particle.
- Labs 3 and 8 observed some fish particles but <LOD, based each on two determinations.

Insect particles were found in this sample by labs 16 and 20 but not classified.

Some participants also reported the presence of shell fragments (labs 1 and 7). However the feed matrix was not containing shell grits, the origin of such fragments – or fragments identified as such – is probably to be found in the starfish meal.

The proper identification of starfish fragments was only reported by a few participants:

- Lab 17 identified them correctly even mentioning a very low presence of fishbones.

- Lab 7 identified fragments of starfish endoskeleton and also mentioned a low presence of fishbones. About its findings of the few fishbones, the participants also described them as presenting few typical lacunae.
- Lab 18 strictly referred to starfish, without even mentioning other type of fish particles.

Sample 6: Milk replacer + 0.5 % fishmeal

PD for terrestrial particles:

- Lab 17: blood and whey powder

Three participants (labs 1, 5 and 6) declared the sample as positive for terrestrial material based on the identification of milk powder. These results were assimilated to correct answers.

Insect particles were found in this sample by lab 16 but not classified as terrestrial.

4.2.1.3. Individual performances of NRLs in qualitative analysis

Individual performance parameters were assessed for each participant by calculating the accuracy, sensitivity and specificity over the blind sample set. This was calculated separately for both the detection of terrestrial material and of fish material. Results are to be found in Tables 5 and 6. A ranking of the labs was prepared based on the consolidated accuracy.

Tables 5 (left) and 6 (right): NRL proficiencies regarding the detection of terrestrial and fish material respectively. Ranking follows AC values for primary key and SE for second key.

Terrestrial

lab code	AC	SE	SP
1	1.000	1.000	1.000
2	1.000	1.000	1.000
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
8	1.000	1.000	1.000
10	1.000	1.000	1.000
11	1.000	1.000	1.000
12	1.000	1.000	1.000
13	1.000	1.000	1.000
15	1.000	1.000	1.000
16	1.000	1.000	1.000
19	1.000	1.000	1.000
22	1.000	1.000	1.000
23	1.000	1.000	1.000
25	1.000	1.000	1.000
27	1.000	1.000	1.000
20	1.000	1.000	1.000
21	1.000	1.000	1.000
24	1.000	1.000	1.000
3	0.833	0.667	1.000
14	0.833	0.667	1.000
4	0.667	0.333	1.000
9	0.667	0.333	1.000
18	0.667	0.333	1.000
17	0.500	0.333	0.667

Fish

lab code	AC	SE	SP
1	1.000	1.000	1.000
2	1.000	1.000	1.000
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
10	1.000	1.000	1.000
11	1.000	1.000	1.000
12	1.000	1.000	1.000
13	1.000	1.000	1.000
14	1.000	1.000	1.000
15	1.000	1.000	1.000
16	1.000	1.000	1.000
17	1.000	1.000	1.000
18	1.000	1.000	1.000
19	1.000	1.000	1.000
22	1.000	1.000	1.000
23	1.000	1.000	1.000
25	1.000	1.000	1.000
27	1.000	1.000	1.000
3	0.833	0.800	1.000
4	0.833	0.800	1.000
8	0.833	0.800	1.000
9	0.833	0.800	1.000
20	0.833	0.800	1.000
21	0.833	0.800	1.000
24	0.833	0.800	1.000

A general ranking of the NRLs was also performed on a consolidated evaluation including their proficiency in detecting both terrestrial and fish materials through the set of blind samples (Table 7).

Table 7: General NRL proficiency regarding the detection of terrestrial and fish material. Ranking follows AC values as primary key and SE as second key. Lines in blue refer to satisfying NRLs.

Consolidated			
lab code	**AC**	**SE**	**SP**
1	1.000	1.000	1.000
2	1.000	1.000	1.000
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
10	1.000	1.000	1.000
11	1.000	1.000	1.000
12	1.000	1.000	1.000
13	1.000	1.000	1.000
15	1.000	1.000	1.000
16	1.000	1.000	1.000
19	1.000	1.000	1.000
22	1.000	1.000	1.000
23	1.000	1.000	1.000
25	1.000	1.000	1.000
27	1.000	1.000	1.000
8	0.917	0.875	1.000
20	0.917	0.875	1.000
21	0.917	0.875	1.000
24	0.917	0.875	1.000
14	0.917	0.875	1.000
3	0.833	0.750	1.000
18	0.833	0.750	1.000
17	0.750	0.750	0.750
4	0.750	0.625	1.000
9	0.750	0.625	1.000

Twenty labs out of 26 NRLs (77 %) performed very well.

Two NRLs performed satisfyingly (8 %). Six NRLs (15 %) were underperforming for microscopic analyses. In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), the underperforming participants are asked to report on the origin of their errors as well as on the actions they will undertake in order to solve the problems. The two participants that performed satisfyingly are also asked to report on the origin of their errors because of the false negative result for terrestrial material.

4.1.2. Qualitative analyses and individual performances the non-EU participants

Individual performances from the 6 participants outside the EU were assessed exactly as in previous section (4.2.1.3). A ranking of those labs was prepared as well based on the consolidated accuracy. Results are to be found in Tables 8 and 9 (next page).

Tables 8 (left) and 9 (right): non-EU lab proficiencies regarding the detection of terrestrial and fish material respectively. Ranking follows AC values for primary key and SE for second key. (Legend: NA: not applicable)

Terrestrial			
lab code	AC	SE	SP
29	1.000	1.000	1.000
30	1.000	1.000	1.000
33	0.833	0.667	1.000
35	0.833	0.667	1.000
31	0.667	0.333	1.000
32	0.500	0.000	1.000

Fish			
lab code	AC	SE	SP
30	1.000	1.000	1.000
32	1.000	1.000	1.000
29	0.833	0.800	1.000
31	0.833	0.800	1.000
33	0.833	0.800	1.000
35	NA	NA	NA

The error details are described per sample:

Sample 1: Fishfeed I + 0.05 % porcine PAP

ND for terrestrial particles:

- Lab 32 failed at detecting the porcine PAP on both replicates
- Lab 31 reported a result classified as <LOD in one replicate while in the other no terrestrial particle was detected
- Lab 33 did not detect the porcine PAP in one replicate
- Lab 35 reported a result classified as <LOD in one replicate.

Sample 2: Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder

No microscopic analysis had to be realised.

Sample 3: Fishfeed I + 0.05 % ruminant PAP

ND for terrestrial particles:

- Lab 32 failed at detecting terrestrial material.

Sample 4: Feed for fattening pig

No error was noted.

Sample 5: Starter feed for broilers + 1 % starfish

ND for fish particles:

- Lab 31 recorded a <LOD case.
- Labs 29 and 33 failed at detecting any fish particle.

Sample 6: Milk replacer + 0.5 % fishmeal

No error was noted.

A general ranking as for the NRL network was established (Table 10).

Two participants performed excellently and two performed satisfyingly (line in blue in Table 10). The two other participants were classified as underperforming (lines in red in Table 10) according to the applied criteria.

Table 10: General non-EU lab proficiency regarding the detection of terrestrial and fish material. Ranking follows AC values as primary key and SE as second key. Lines in blue refer to satisfying results. Lines in red refer to underperforming results.

Consolidated			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
29	0.917	0.875	1.000
33	0.833	0.750	1.000
35	0.833	0.667	1.000
31	0.750	0.625	1.000
32	0.750	0.625	1.000

4.3. PCR results

4.3.1. Qualitative analyses from the NRLs

4.3.1.1 On the respect of the instructions

The NRLs seem to stick generally to the SOPs. No deviation is to notice this year.

4.3.1.2 Overview of results and global performance of the network

Table 11 summarizes the results provided by 26 NRLs for the three sample types submitted to qualitative PCR analysis.

Table 11: Global results expressed as accuracy (AC) –PCR

Sample	Material	n	AC
1	Fishfeed I + 0.05 % porcine PAP	52	0.827 (9)
2	Fishfeed II with haemoglobin meal + 0.5 % skimmed milk powder	26	1.000
3	Fishfeed I + 0.05 % ruminant PAP	26	0.962 (1)

Accuracy means sensitivity in case of ND and specificity in case of PD.
The absence of a PCR result when expected is considered as a deviation (ND or PD).
In brackets the number of false results. (Legend: n = number of results)

Sample 1 was present in duplicate in the sample set. It was an aquafeed containing 0.05 % w/w of pig PAP. The PCR result expected for the presence of ruminant DNA was negative. Only two false positive results were recorded by the same lab (lab 8). The other deviations (7 out of 9) were due to an absence of result consecutive to the lack of detection of terrestrial PAP by microscopy.

Sample 2 was a fishfeed containing haemoglobin meal. This sample type was present in duplicate in the sample set. According to the SOP about the operational protocols for combination of the methods (light microscopy and PCR) and due to the presence of blood product in the composition of the sample, the detection of ruminant DNA by PCR was requested. The presence of ruminant DNA was correctly concluded by the 26 NRLs.

Sample 3 was a fishfeed containing 0.05 % in mass fraction of ruminant PAP. Twenty-five out of the 26 NRLs (96 %) detected correctly the presence of ruminant DNA.

4.3.1.3 Individual performances of NRLs in qualitative analysis

Individual performances were assessed for each participant by calculating the accuracy, sensitivity and specificity over the samples. A ranking of the labs was prepared based on the accuracy. Results are to be found in Table 12 that summarizes the results obtained by the participants for the analyses of the three sample types (sample type 1, 2 and 3) representing a total of 4 samples.

Table 12: NRL proficiencies regarding the detection of ruminant material. Ranking follows AC values. Cell in blue refers to a satisfying NRL. Cells in red refer to underperforming NRLs.

Lab code	AC	SE	SP
1	1.000	1.000	1.000
3	1.000	1.000	1.000
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
10	1.000	1.000	1.000
11	1.000	1.000	1.000
12	1.000	1.000	1.000
13	1.000	1.000	1.000
15	1.000	1.000	1.000
16	1.000	1.000	1.000
17	1.000	1.000	1.000
19	1.000	1.000	1.000
20	1.000	1.000	1.000
21	1.000	1.000	1.000
22	1.000	1.000	1.000
23	1.000	1.000	1.000
24	1.000	1.000	1.000
25	1.000	1.000	1.000
27	1.000	1.000	1.000
14*	0.750	1.000	0.500
2	0.750	0.500	1.000
4*	0.500	1.000	0.000
8	0.500	1.000	0.000
9*	0.500	1.000	0.000
18*	0.500	1.000	0.000

* The absence of PCR result(s) is assimilated to deviation(s)

Table 12 illustrates the excellent level of global performance for 20 labs out of 26 NRLs (77 % of the NRLs) having no false result. Two labs were satisfying: lab 2 returned 1 ND and lab 14 did not give the expected PCR result for one of the samples. Four labs were underperforming: labs 4, 9 and 18 did not deliver 2 PCR results whereas lab 8 obtained 2 PD.

4.3.1.4 Cut-off quality control

A quality control for the number of copies of the ruminant target reached with the Ct value of the cut-off, was developed to minimize the risk of false positive result. A minimum of 9.00 copies at the cut-off was required. Indeed, depending on the variability of the lab (PCR platform + operator), the cut-off value can correspond to a too low number of copies.

This year one participant (lab 6) did not reach the minimum criterion of 9.00 copies (8.18 copies). The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test was 65.4 % (64.0 % in 2017 [9]; 59.3 % in 2016 [8]; 65.4 % in 2015 [7] ; 70.4 % in 2014 [12] ; 55.6 % in 2013 [13]). In the case of lab 6, the too low copy number at the cut-off did not have any influence on the results (no deviation). The 2 positive deviations recorded by lab 8 are not due to a cut-off problem.

4.3.2. Qualitative analyses from the non-EU participants

4.3.2.1. Individual performances

Individual performances were assessed for only two non-EU participants who reported PCR results by calculating the accuracy, sensitivity and specificity over the samples. Their results are to be found in Table 14.

Table 14 : Non-EU participant proficiencies regarding the detection of ruminant material. Ranking follows AC values.

Lab code	AC	SE	SP
29	1.000	1.000	1.000
31*	0.500	1.000	0.000

* The absence of PCR result(s) is assimilated to deviation(s)

Labs 29 obtained excellent results (no deviation).

Concerning Lab 31, two deviations are recorded with the sample 1 (Fishfeed I + 0.05 % porcine PAP). The sample were not analysed by PCR. No terrestrial particle was identified by light microscopy. Under the assumption that this participant is following the SOP related to the operational protocols, it is justifying stopping the analyses.

4.3.2.2. Assessment of the cut-off values

Labs 29 and 31 have cut-off values that comply with the minimum criterion of 9 copies set by the EURL-AP.

5. Discussion and conclusions

This proficiency test is the second one proposed to the NRL network without any indication on the method to be used since the selection of the analytical method, light microscopy and/or PCR, is under the responsibility of the operator. The only leading indication was the label on the vial referring to the type of feed.

Whereas in 2017 about 36 % of the NRLs were non-compliant with the choice of the method according to the SOP on operational protocols for the combination of light microscopy and PCR, this year the non-compliances accounted for only 11 % of the NRLs. Whether or not this is an improvement in the interpretation of the SOP is debatable. Indeed it is probably more related to the change in the instructions regarding point 7.3 of the SOP of which the last paragraph was not to be considered. One participant misperceived the legal obligation to report on the presence of both terrestrial and fish constituents and the authorised or unauthorised characteristic of an ingredient. Laboratories need to report what they detect and not only reporting what is not authorised.

Results obtained from light microscopic analyses were good on the exception of the disclosure of the porcine PAP and the detection of starfish meal. Regarding the lack of sensitivity for the porcine PAP several factors can be evocated as an explanation: the adulteration level of 0.05 %, the masking effect of the matrix containing fishmeal, the low bone content of the porcine PAP and finally recovery issues from the sedimentation. Laboratories that missed the detection of this terrestrial PAP are asked to deliver explanations on their failure. In particular for laboratories that had <LOD results, recovery issues from the sedimentation is suspected to be the cause of the error. About the detection of starfish meal, the sensitivity issue is not surprising. This type of feed material is not common and 27 % of the NRLs failed at detecting it. Effectively a lack of experience most probably explains the sensitivity issue. Nevertheless 3 NRLs succeeded to report the presence of starfish endoskeletal fragments. The need for continuous training and gain of experience is still challenged.

Concerning non-EU participants, only two out of six performed excellently for microscopy. Two performed satisfyingly and finally two revealed to be underperforming when applying the same performance criteria as those for the NRL network. When analysing their results, their encountered problems were similar to the ones of the NRL network, namely a lack of sensitivity for porcine PAP and starfish meal.

For the PCR results, a majority of laboratories (77 %) performed excellently. Two participants returned satisfying results and 4 labs were considered as underperforming. It is to underline that a majority of the deviations are due to the lack of sensitivity from the microscopic observations leading to a stop of the investigations and the absence of PCR analyses. So the deviations recorded are due to analytical PCR problems only for lab 2 (1 ND) and lab 8 (2 PD). So globally, it can be concluded that the performance of the network is still quite good.

Only 2 non-EU participants returned PCR results. One performed excellently and the other one was considered as underperforming but this evaluation has to be nuanced as it is due again to an absence of PCR results consecutive to negative microscopic results.

Acknowledgment

We are grateful to the EURL-AP technical staff for their preparation work and the efforts made to meet the ISO 17043 requirements: M. Collard, J. Hulin and J. Maljean. We also thank the participants for their fruitful collaboration.

References

[1] EU. 2017. Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation). Official Journal of the European Union L 95, 7/4/2017: 1-142.

[2] EU. 2011. Commission Regulation (EU) No 208/2011 of 2 March 2011 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council and Commission Regulations (EC) No 180/2008 and (EC) No 737/2008 as regards lists and names of EU reference laboratories. Official Journal of the European Union L 58, 3/3/2011: 29–35.

[3] EU. 2013. Commission Regulation (EU) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed. Official Journal of the European Union L 20, 23/01/2013: 33-43.

[4] EU. 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Official Journal of the European Union L 54, 26/2/2009: 1-130.

[5] EURL-AP. 2015. EURL-AP Standard Operating Procedure Operational protocols for the combination of light microscopy and PCR (Version 3.0) *download from* : eurl.craw.eu/img/page/sops/EURL-AP%20SOP%20operational%20schemes%20V3.0.pdf

[6] EU. 2013. Commission Regulation (EU) No 56/2013 of 16 January 2013 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Official Journal of the European Union L 21, 24/1/2013: 3-16.

[7] Veys P, Fumière O, Marien A, Baeten V and Berben G. 2016. Combined microscopy-PCR EURL-AP Proficiency Test 2015: Final version. CRA-W, Gembloux, Belgium.

[8] Fumière O, Veys P, Marien A, Baeten V and Berben G. 2017. Combined microscopy-PCR EURL-AP Proficiency Test 2016: Final version. CRA-W, Gembloux, Belgium.

[9] Veys P, Fumière O, Marien A, Baeten V and Berben G. 2018. Combined microscopy-PCR EURL-AP Proficiency Test 2017: Final version. CRA-W, Gembloux, Belgium.

[10] ISO 13528, Statistical methods for use in proficiency testing by interlaboratory comparison.

[11] EU. 2001. Commission Regulation (EC) No 999/2001 of the European Parliament and the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Official Journal of the European Union L 147, 31.5.2001: 1–40.

[12] Fumière O., Marien A. and Berben G. 2014. EURL-AP PCR Proficiency Test 2014: Final version. CRA-W, Gembloux, Belgium.

[13] Fumière O., Marien A. and Berben G. 2013. EURL-AP PCR Proficiency Test 2013: Final version. CRA-W, Gembloux, Belgium.

Annex 1

List of participants (Laboratories that do not belong to the NRL network are *in italics*).

Country	Institute Name
Australia	*Biosecurity Sciences Laboratory*
Austria	Austrian Agency for Health and Food Safety
Belgium	Federal Agency for the Safety of the Food Chain
Bulgaria	National Diagnostic Research Veterinary Medical Institute
Botswana	*Botswana National Veterinary Laboratory*
China	*China Agricultural University*
Croatia	Croatian Veterinary Institute
Cyprus	Cyprus Veterinary Services
Denmark	The Danish Plant Directorate
Estonia	Veterinary and Food Laboratory
Finland	Finnish Food Safety Authority
France	DG for Fair Trading, Consumer Affairs and Fraud Control-Laboratory Directorate Rennes
Germany	Federal Institute for Risk Assessment
Greece	Feedstuffs Control Laboratory
Hungary	Central Agricultural Office-Directorate Food and Feed Safety-Central Feed Investigation Lab.
Ireland	Department of Agriculture and Food Microscopy Laboratory - Seed Testing Station
Italy	National Reference Centre for the Surveillance and Monitoring of Animal Feed
Japan	*Food and Agricultural Materials Inspection Center*
Latvia	Institute of Food Safety, Animal Health and Environment "BIOR"
Lithuania	National Food and Veterinary Risk Assessment Institute
Luxemburg	Agroscope Liebefeld-Posieux Research Station (Switzerland)
Netherlands	RIKILT Institute of Food Safety, Wageningen UR
Norway	*LabNett AS and Institute of Marine Research*
Peru	*Inspectorate Services Peru SAC*
Poland	National Veterinary Research Institute
Portugal	Laboratorio Nacional de Investigaçao Veterinaria
Romania	Hygiene Institute of Veterinary Health
Serbia	*Institute of Veterinary Medicine of Serbia*
Slovakia	State Veterinary and Food Institute
Slovenia	Veterinary faculty - National Veterinary Institute - Institute of Food Safety, Feed and Environment - Department of Environment, Animal Nutrition, Welfare and Hygiene
Spain	Laboratorio Arbitral Agroalimentario
Sweden	National Veterinary Institute, Department of Animal Feed
United Kingdom	Animal and Plant Health Agency

Annex 2

Announcement letter

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval building
Chaussée de Namur 24, B – 5030 GEMBLOUX

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e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Announcement of the EURL-AP proficiency test 2018/01 for the determination of Processed Animal Proteins (PAPs) in feed

Introduction

The use of processed animal by-products as ingredient for animal feedingstuffs within the European Union is regulated by the TSE Regulation (Regulation EC N°999/2001), as amended. In particular, Article 7 imposes a prohibition to use processed animal proteins in the feeding of farmed animals (extended feed ban).

Commission Regulation (EU) No 51/2013 of 16 January 2013, amending Annex VI of Regulation (EC) No 152/2009, imposes the methods of analysis for the determination of constituents of animal origin for the official control of feed.

Objectives

The first objective of the present proficiency test is to assess the performance of the NRLs to detect the presence of PAPs in feed by the reference methods using light microscopy and PCR as stated in Regulation EC 152/2009 as amended by Commission Regulation EU 51/2013 and related SOPs.

The second objective is to assess the correct application of the legal conditions for the choice of the analytical method with respect to the SOP Operational protocols for the combination of light microscopy and PCR (Version 3.0)

The organizer team

The test will be coordinated by the European Union Reference Laboratory for Animal Proteins in feedingstuffs (EURL-AP).

Test material

Samples containing typical compound feed fortified with processed animal proteins (PAPs) will be prepared. The EURL-AP will endorse the homogeneity of the samples. Nevertheless, each laboratory participating to the test is sole responsible to reach appropriate homogeneity for the sample sub-portions taken for analysis.

Each participant will receive a maximum of 7 samples, each of about 50g. According to legislation some samples will have to be analysed by microscopy only, by PCR only and by combining both microscopy and PCR.

General outline of the exercise

- The light microscopic and PCR methods to use are described in Annex VI of Commission Regulation EC 152/2009 and related SOPs. These methods, alone or combined, shall be applied for the analyses.
- The EURL-AP will provide participants with an Excel file for reporting the results of the proficiency test analyses.
- Each participating laboratory will be assigned a unique code and only the organizer of the study knows the key to this code. After completing the test each laboratory will get a report including its results and lab code.
- The participation in this proficiency study is mandatory and free of charge for national reference laboratories within Member States of the European Union.

European Union Reference Laboratory for Animal Proteins in feedingstuffs

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Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : **7 September 2018**
- Sending of the sample boxes and communication of the instructions : **19 October 2018**

> ***By default, samples will be sent to the NRL microscopy contact person referred on the intranet. You are asked to check if this person is still your contact and to inform the organizer from any change.***

- Deadline for returning of results to the organizer : **16 November 2018**

Further information

- Refer to the address and coordinates mentioned in the heading,

or

- Dr Pascal VEYS
  EURL-AP NRL Network Manager

  ☎32 (0) 81 62 03 75

  32 (0) 81 62 03 88
  E-mail: p.veys@cra.wallonie.be

or

- Dr Olivier FUMIERE
  Head of EURL-AP Molecular biology team

  ☎32 (0) 81 62 03 51

  32 (0) 81 62 03 88
  E-mail: o.fumiere@cra.wallonie.be

Annex 3

Excel result report form

Proficiency Test Microscopy-PCR 2018/01

Laboratory identification

Laboratory code : 1

Responsibility agreement : No

"Yes" means you have read carefully the "Instructions" worksheet and its accurate application through the present study.

Report

Lab code	1	1	1	1	1	1	1
Sample rank	1st	2nd	3th	4th	5th	6th	7th
Sample N°							
Method of analysis	**Light microscopy**						
Light microscopy analyses							
Terrestrial animal particles							
details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*							
Fish particles							
details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*							
Analyses performed on							
Number of determinations							
Free comment *Example : presence of unusual fragments,...*							
Method of analysis	**PCR**						
PCR analyses							
Ruminant DNA							
Cut-off at 15 copies of the PCR platform used (in cycles)							
Copy number at the cut-off of the PCR platform used (in copies)							
Dilution 1 (e.g. 1 fold)							
Ct value replicate 1							
Ct value replicate 2							
Dilution 2 (e.g. 10 fold)							
Ct value replicate 1							
Ct value replicate 2							
Comment *Example : PCR inhibition,...*							

Annex 4

Gross results of participants for microscopy (in numerical order of lab ID).

Laboratory identification code : **1**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	**394**	Present	bones	Present	bones, muscles	Sed. + Flot.	1
6	**485**	Present	milk	Present	bones	Sed. + Flot.	1
4	**583**	Absent		Absent		Sed. + Flot.	1
1	**674**	Present	bones	Present	bones, cartilage, muscles	Sed. + Flot.	1
2	**793**	Not to perform		Not to perform			
3	**1976**	Present	bones	Present	bones, cartilage, muscles	Sed. + Flot.	1
5	**2774**	Absent		Present	bones	Sed. + Flot.	1

In the 7th sample No 2774, in the sediment > 5 shell particles were also detected.

Laboratory identification code : **2**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	**1535**	Absent		Absent		Sed. + Flot.	2
5	**1598**	Absent		Present	bons	Sed. + Flot.	1
6	**2165**	Absent		Present	bons,cartilages,scales	Sed. + Flot.	1
3	**2312**	Present	bons	Present	bons, cartilages,scales	Sed. + Flot.	1
2	**2473**	Not to perform		Not to perform			
1	**3418**	Present	bons	Present	bons, cartilages,scales	Sed. + Flot.	1
1	**4930**	Present	bons	Present	bons, cartilages,scales	Sed. + Flot.	1

Laboratory identification code : **3**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	**1528**	Present	Terrestrial bone	Present	Fish bone, skin, otolith, muscle, cartilage, gill.	Sed. + Flot.	1
2	**1633**	Not to perform		Not to perform			
5	**2326**	Absent		< LOD	Fish bone, cartilage, muscle, scale, gill, skin.	Sed. + Flot.	2
4	**2487**	Absent		Absent		Sed. + Flot.	1
6	**2557**	Absent		Present	Fish bone, scale, gill, muscle fibres, otholith, skin.	Sed. + Flot.	1
1	**3138**	Present	Terrestrial bone	Present	Fish bone, otolith, scale, muscle, cartilage, skin.	Sed. + Flot.	1
1	**3474**	< LOD	Terrestrial bone	Present	Fish bone, muscle, otolith, scale, gill	Sed. + Flot.	2

where muscle and cartilage were seen in samples containing fish and terrestrial they have classified have fish.

Laboratory identification code : **4**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	1066	Absent		Present	bones, muscle fibres, cartilage, scales, gills	Sed. + Flot.	1
2	1521	Present	TMB positive reaction only. No other particles found	Present	bones, muscle fibres, cartilage, scales, gills	Sed. + Flot.	2
3	1584	Present	bones,muscle fibres,	Present	bones,muscle fibres, cartilage	Sed. + Flot.	1
1	1738	Absent		Present	bones,muscle fibres, cartilage	Sed. + Flot.	1
5	1990	Absent		Absent		Sed. + Flot.	2
4	2599	Absent		Absent		Sed. + Flot.	1
6	2725	Absent		Present	bones,muscle fibres, cartilage	Sed. + Flot.	1

n. 1521 TMB positive reaction

Laboratory identification code : **5**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
5	254	Absent		Present	Fishbones, muscles	Sed. + Flot.	1
6	429	Present	Milk powder	Present	Fishbones,cartilages,gills,scales, muscles	Sed. + Flot.	1
4	695	Absent		Absent		Sed. + Flot.	1
3	2032	Present	Bones	Present	Fishbones, cartilages,muscles	Sed. + Flot.	1
2	2081	Not to perform		Not to perform			
1	4202	Present	Bones	Present	Fishbones, cartilages, gills, muscles	Sed. + Flot.	1
1	4650	Present	Bones	Present	Fishbones, cartilages, gills, muscles	Sed. + Flot.	1

Laboratory identification code : **6**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
5	534	Absent		Present	fishbones, scales	Sed. + Flot.	1
4	1591	Absent		Absent		Sed. + Flot.	1
1	338	Present	bones, muscle	Present	fishbones, scales, cartilage, muscle	Sed. + Flot.	1
1	2802	Present	bones, muscle	Present	fishbones, scales, muscle	Sed. + Flot.	2
3	2648	Present	bones, muscle	Present	fishbones, scales, muscle	Sed. + Flot.	1
6	1213	Present	milk powder	Present	fishbones, scales, gills, muscle	Sed. + Flot.	1
2	1353	Not to perform		Not to perform			

Laboratory identification code : **7**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	135	Absent		Absent		Sed. + Flot.	1
2	177	Not to perform					
3	296	Present	bones	Present	bones, muscle	Sed. + Flot.	1
1	1010	Present	bones	Present	bones, muscle	Sed. + Flot.	1
1	1122	Present	bones	Present	bones, muscle	Sed. + Flot.	1
6	1269	Absent		Present	bones, scales, gills, muscle	Sed. + Flot.	1
5	2102	Absent		Present	bones (few), starfish endoskeleton, 2 muscle fibers	Sed. + Flot.	1

1010: few and atypical terrestrial animal bone fragments; 1269: presence in the sediment of undetermined light AR colored globuli + probable presence of milk product in the flotate; 2102: presence of shell grit, "normal" fish bones are very few and show few typical lacunae

Laboratory identification code : **8**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	58	Present	bones, cartilage, muscles.	Present	fishbones, cartilage, muscles, otoliths, teeth, scales, gills.	Sed. + Flot.	1
5	702	Absent		< LOD	As far as was discernible using a light microscope, no more than 5 particles derived from fish were detected on average per determination in the submitted sample. The particles were identified as fishbones and muscle. This low level presence, being below the limit of detection of the microscopic method, means that a risk of false positive result cannot be excluded.	Sed. + Flot.	2
4	751	Absent		Absent		Sed. + Flot.	1
1	1458	Present	bones, cartilage, muscles.	Present	fishbones, cartilage, muscles, otoliths, teeth, scales, gills.	Sed. + Flot.	1
3	2144	Present	bones, muscles.	Present	fishbones, muscles, otoliths, teeth, scales, gills.	Sed. + Flot.	1
6	2221	Absent		Not to perform		Sed. + Flot.	1
2	2697	Not to perform		Not to perform			

More than 5 particles derived from fish were detected in sample No 2221. Particles were identified as fishbones, muscles, teeth, scales and gills. However, sample was a milk replacer and milk replacers containing fishmeal and intended only for unweaned ruminants are authorised.

Laboratory identification code : **9**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	506	Absent		Present	fish bone, gills, scales	Sed. + Flot.	3
4	639	Absent		Absent		Sed. + Flot.	3
5	814	Absent		Absent		Sed. + Flot.	3
2	905	Not to perform		Not to perform			
6	1773	Absent		Present	fish bones, gills, scales	Sed. + Flot.	3
1	2130	Absent		Present	fish bones, gills, scales	Sed. + Flot.	3
3	2256	Present	bones	Present	fish bones, gills, scales	Sed. + Flot.	3

Laboratory identification code : **10**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	170	Present	bones	Present	bones	Sed. + Flot.	2
1	842	Present	bones, cartilages of unknown	Present	bones, cartilages of unknown	Sed. + Flot.	2
4	1647	Absent		Absent		Sed. + Flot.	1
5	1822	Absent		Present	bones, cartilages of unknown	Sed. + Flot.	2
3	1864	Present	bones, cartilages of unknown	Present	bones, cartilages of unknown	Sed. + Flot.	2
6	2333	Absent		Present	bones, cartilages of unknown	Sed. + Raw	1
2	2641	Not to perform		Not to perform			

Laboratory identification code : **11**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
6	93	Absent		Present	muscles, fishbones	Sed. + Flot.	2
1	730	Present	bones	Present	muscles, fishbones	Sed. + Flot.	2
3	1136	Present	bones	Present	muscles, fishbones	Sed. + Flot.	2
2	1241	Not to perform		Not to perform			
5	1878	Absent		Present	fishbones	Sed. + Flot.	2
1	1962	Present	bones	Present	muscles, fishbones	Sed. + Flot.	2
4	1983	Absent		Absent		Sed. + Flot.	2

Laboratory identification code : **12**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	191	Absent		Absent		Sed. + Flot.	1
2	345	Not to perform		Not to perform			
5	1038	Absent		Present	Bones	Sed. + Flot.	2
3	1248	Present	Bones	Present	Bones, cartilages	Sed. + Flot.	1
6	1829	Absent		Present	Bones	Sed. + Flot.	1
1	3866	Present	Bones	Present	Bones, cartilages	Sed. + Flot.	1
1	4314	Present	Bones	Present	Bones, cartilages	Sed. + Flot.	1

Laboratory identification code : **13**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
2	9	Not to perform		Not to perform			
3	1080	Present	bones	Present	fish bones, cartilages, muscle fibers	Sed. + Flot.	1
4	2263	Absent		Absent		Sed. + Flot.	1
5	2494	Absent		Present	fish bones, muscle fibers	Sed. + Flot.	1
1	2634	Present	bones	Present	fish bones, cartilages, muscle fibers	Sed. + Flot.	1
6	2781	Absent		Present	fish bones, cartilages, scales, gills and muscle fibers	Sed. + Flot.	1
1	4538	Present	bones	Present	fish bones, muscle fibers	Sed. + Flot.	1

Laboratory identification code : **14**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
6	205	Absent		Present	fishbones,mucles,scales	Sed. + Flot.	2
5	478	Absent		Present	fishbones, muscles,scale,otholit	Sed. + Flot.	2
4	975	Absent		< LOD	3fish bones,2muscles	Sed. + Flot.	2
2	1185	Not to perform		Not to perform			
3	1752	Present	bones and muscles	Present	fishbones,scale,gill,muscles	Sed. + Flot.	2
1	1850	< LOD	3bones,and 2 feather	Present	fishbones,scales,muscles,otholit	Sed. + Flot.	2
1	2354	Present	Bones and muscles	Present	fishbones,scales,muscles,otholit	Sed. + Flot.	2

we have found 3 fragments of insects in the sample number 4

Laboratory identification code : **15**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	303	Absent		Absent		Sed. + Raw	1
6	317	Absent		Present	Fishbone, Scales, Gills & Muscle	Sed. + Raw	1
5	758	Absent		Present	Fishbone	Sed. + Raw	1
1	954	Present	Bone	Present	Fishbone Muscle	Sed. + Flot.	1
3	1192	Present	Bone	Present	Fishbone, Cartilage, Gills and Muscle	Sed. + Flot.	1
2	2193	Not to perform		Not to perform			
1	3754	Present	Bone	Present	Fishbone, Gill, Cartilage, Muscle	Sed. + Flot.	1

Laboratory identification code : **16**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	23	Absent		Absent		Sed. + Flot.	1
1	114	Present	bones, no diff. between MBM- and FM muscle fibres possible	Present	fishbones, scales, muscle fibres, no diff. between MBM- and FM muscle fibres possible	Sed. + Flot.	3
5	646	Absent		Present	fishbones, scales, muscle fibres, it can't be excludet, that the muscle fibres found only derive from fish meal	Sed. + Flot.	1
2	681	Not to perform		Not to perform			
1	1402	Present	bones, no diff. between MBM- and FM muscle fibres possible	Present	fishbones, scales, muscle fibres, no diff. between MBM- and FM muscle fibres possible	Sed. + Flot.	2
3	1416	Present	bones, no diff. between MBM- and FM muscle fibres possible	Present	fishbones, scales, muscle fibres, no diff. between MBM- and FM muscle fibres possible	Sed. + Flot.	1
6	2109	Absent		Present	fishbones, scales, muscle fibres, it can't be excludet, that the muscle fibres found only derive from fish meal	Sed. + Flot.	1

For sample nr. 646 and 2109 fragments of insects were found

Laboratory identification code : **17**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	184	Present	bones, meat	Present	fish bones, cartilage, fish scales, gills, otolith, meat	Sed. + Raw	1
6	597	Present	whey powder, positive test for blood	Present	meat, fish bones, cartilage, fish scales, gills, fish teeth	Sed. + Raw	1
2	961	Present	Haemoglobin	Present	meat, fish bones, cartilage, fish scales, gills	Sed. + Raw	1
4	2207	Absent		< LOD	bone, gill	Sed. + Raw	3
5	2270	Absent		Present	<LOD for fish bone, but lot of starfish fragments	Sed. + Raw	1
1	3082	< LOD	bones, feather*	Present	fish bones, cartilage, fish scales, gills, otolith, meat	Sed. + Raw	3
1	3642	< LOD	bones	Present	fish bones, cartilage, fish scales, gills, otolith, meat	Sed. + Raw	3

**3082 had 2 feather like fragments*

Laboratory identification code : **18**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
2	121	Present	blood particles	Present	fishbones, otoliths, scales, muscles	Sed. + Raw	1
1	282	Absent		Present	fishbones, otoliths, cartilage, muscles	Sed. + Raw	1
5	1094	Absent		Present	starfish	Sed. + Raw	1
4	1423	Absent		Absent		Sed. + Raw	1
6	1941	Absent		Present	fishbones, scales, gills, teeth, muscles	Sed. + Raw	1
3	2424	Present	bones	Present	fishbones, muscles	Sed. + Raw	1
1	2970	Absent		Present	fishbones, muscles	Sed. + Raw	1

Laboratory identification code : **19**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	352	Present	bones	Present	bones, scale, cartilage, muscle	Sed. + Flot.	1
6	989	Absent		Present	bones, scale, cartilage, muscle	Sed. + Flot.	1
1	1234	Present	bones	Present	bones, scale, cartilage, muscle	Sed. + Flot.	1
2	1409	Not to perform		Not to perform			
5	1654	Absent		Present	bones, muscles	Sed. + Flot.	1
4	2319	Absent		Absent		Sed. + Flot.	1
1	2578	Present	bones, blood	Present	bones, scale, cartilage, muscle	Sed. + Flot.	1

Laboratory identification code : **20**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	912	Present	bones, muscle fibres,	Present	fish bones, muscle fibres, gills,	Sed. + Raw	1
6	1549	Absent		Present	fish bones, muscle fibres, gills,	Sed. + Raw	1
2	1913	Not to perform		Not to perform		Sed. + Raw	1
4	1927	Absent		Absent		Sed. + Raw	1
1	2466	Present	bones, muscle fibres,	Present	fish bones, muscle fibres, gills	Sed. + Raw	1
5	2662	Absent		Absent		Sed. + Raw	1
1	3362	Present	bones, muscle fibres,	Present	fish bones, muscle fibres, scales	Sed. + Raw	1

sample N 1927 and sample N 2662: presence a lot of insect parts

Laboratory identification code : **21**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	247	Absent		Absent		Sed. + Flot.	1
6	541	Absent		Present	fishbones, scales, muscle, gill, cartilage	Sed. + Flot.	1
3	744	Present	bones, muscle, cartilage	Present	fishbones, muscle, cartilage, skin, scales, gill	Sed. + Flot.	1
2	849	Not to perform		Not to perform			
5	2718	Absent		Absent		Sed. + Flot.	1
1	3250	Present	bones, muscle, cartilage, blood	Present	fishbones, muscle, cartilage, skin, scales, gill	Sed. + Flot.	1
1	4090	Present	bones, muscle, cartilage	Present	fishbones, muscle, cartilage, skin, scales, gill	Sed. + Flot.	1

Mouse hair in sample 3250

Laboratory identification code : **22**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
5	198	Absent		Present	scale, fish bones, muscle fibres	Sed. + Flot.	1
2	569	Not to perform		Not to perform			
4	919	Absent		Absent		Sed. + Flot.	1
3	968	Present	bones	Present	scales, fish bones, muscle fibres , gills	Sed. + Flot.	1
6	1661	Absent		Present	muscle fibres, fish bones	Sed. + Flot.	1
1	3026	Present	bones, blood	Present	scales, fish bones, muscle fibres , gills	Sed. + Flot.	1
1	4706	Present	bones, blood	Present	scales, fish bones, muscle fibres , gills,	Sed. + Flot.	1

Presents of mites in samples: 968,3026,4706

Laboratory identification code : **23**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	464	Present	bones, muscle fibers cartilage	Present	scales, otolithes, muscle fibers, cartilage	Sed. + Raw	1
5	982	Absent		Present	fish bones, scales	Sed. + Raw	1
2	1073	Not to perform					
1	1178	Present	bones, muscle fibers, cartilage	Present	fish bones, scales, cartilage, muscle fibers	Sed. + Raw	1
4	1815	Absent		Absent		Sed. + Raw	1
1	2186	Present	bones, muscle fibers, cartilage	Present	fish bones, scales, cartilage, muscle fibers, cartilage	Sed. + Raw	1
6	2389	Absent		Present	scales, fish bones, muscle fibers, cartilage	Sed. + Raw	1

464 = 2018-2139, 982=2018-2140, 1073=2018-2141, 1178=2018-2142, 1815=2018-2143, 2186=2018-2144, 2389=2018-2145,

Laboratory identification code : **24**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	72	Present	bones	Present	fishbones, gills, scales	Sed. + Flot.	1
6	653	Absent		Present	fishbones, gills, scales	Sed. + Flot.	1
2	737	Not to perform		Not to perform			
4	863	Absent		Absent		Sed. + Flot.	1
1	1626	Present	bones	Present	fishbones, gills, scales	Sed. + Flot.	1
5	1934	Absent		Absent		Sed. + Flot.	1
1	2410	Present	bones	Present	fishbones, gills, scales	Sed. + Flot.	1

Laboratory identification code : **25**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	415	Absent		Absent		Sed. + Flot.	1
3	576	Present	Cartilage, bones	Present	Cartilage, bones otholit, muscle	Sed. + Flot.	1
5	590	Absent		Present	Cartilage, bones gills	Sed. + Flot.	2
1	1290	Present	Cartilage, bones	Present	Cartilage, bones otholit, muscle	Sed. + Flot.	1
2	1297	Not to perform		Not to perform			
6	1885	Absent		Present	Bones cartilage scales gills, muscle	Sed. + Flot.	1
1	3306	Present	Cartilage, bones	Present	Cartilage, bones otholit, muscle	Sed. + Flot.	1

Laboratory identification code : **27**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
6	373	Absent		Present	fish bones, scales, cartilage	Sed. + Raw	1
5	1318	Absent		Present	fish bones	Sed. + Raw	1
3	1920	Present	bone fragments	Present	fish bones, muscle fibres	Sed. + Raw	1
2	2361	Not to perform		Not to perform			
1	2522	Present	bone fragments	Present	fish bones, muscle fibres	Sed. + Raw	2
4	2543	Absent		Absent		Sed. + Raw	1
1	4034	Present	bone fragments	Present	fish bones, muscle fibres	Sed. + Raw	3

Laboratory identification code : **29**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	2	Present	Bones	Present	Fishbones, muscles	Sed. + Flot.	1
6	709	Absent		Present	Fishbones, scales, muscles	Sed. + Flot.	1
5	1150	Absent		Absent		Sed. + Flot.	1
3	1360	Present	Bones	Present	Fishbones, muscles	Sed. + Flot.	1
2	1689	Not to perform		Not to perform			
4	2095	Absent		Absent		Sed. + Flot.	1
1	3922	Present	Bones	Present	Fishbones, muscles	Sed. + Flot.	1

Laboratory identification code : **30**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
6	1437	Absent		Present	Bones, Scales	Sed. + Raw	2
1	1682	Present	Bones	Present	Bones	Sed. + Raw	2
2	1745	Absent		Present	Bones, Scales	Sed. + Raw	2
4	1759	Absent		Absent		Sed. + Raw	2
5	1766	Absent		Present	Bones	Sed. + Raw	2
3	1808	Present	Bones	Present	Bones	Sed. + Raw	2
1	4874	Present	Bones	Present	Bones, Scales	Sed. + Raw	2

Laboratory identification code : **31**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
5	366	Absent		< LOD		Sed. + Raw	3
3	800	Present		Present		Sed. + Raw	1
4	1479	Absent		Absent		Sed. + Raw	3
2	1577	Not to perform		Not to perform			
6	2053	Absent		Present	Fish shells detected	Sed. + Raw	3
1	2242	< LOD		Present		Sed. + Raw	3
1	4146	Absent		Present		Sed. + Raw	3

Laboratory identification code : **32**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
4	**527**	Absent		Absent		Sed. + Flot.	1
3	**688**	Absent		Present	fishbones	Sed. + Flot.	1
5	**870**	Absent		Present	fishbones	Sed. + Flot.	1
1	**898**	Absent		Present	fishbones	Sed. + Flot.	1
6	**1325**	Absent		Present	fishbones	Sed. + Flot.	1
2	**1969**	Present	bones	Present	fishbones	Sed. + Flot.	1
1	**4258**	Absent		Present	fishbones	Sed. + Flot.	1

Lots of unusual fragments were detected in the sediment of No.1325, whose figure and color were very similar to the terrestrial bones. However, no characteristic lacuna was detected. So, the result was given as "absent".

Laboratory identification code : **33**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
2	**1017**	Absent		Present	bones, scales, gills	Sed. + Flot.	1
4	**1367**	Absent		Absent		Sed. + Flot.	1
5	**1374**	Absent		Absent		Sed. + Flot.	1
6	**1997**	Absent		Present	bones, scales, gills	Sed. + Flot.	1
1	**2298**	Present	bones	Present	bones, scales	Sed. + Flot.	2
3	**2536**	Present	bones	Present	bones	Sed. + Flot.	1
1	**4482**	Absent		Present	bones, scales	Sed. + Flot.	1

Laboratory identification code : **35**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
5	**310**	Absent		Not to perform		Sed. + Flot.	
2	**1129**	Present		Not to perform		Sed. + Flot.	
3	**1304**	Present		Not to perform		Sed. + Flot.	
4	**2431**	Absent		Not to perform		Sed. + Flot.	
6	**2613**	Absent		Not to perform		Sed. + Flot.	
1	**3698**	< LOD		Not to perform		Sed. + Flot.	
1	**3978**	Present		Not to perform		Sed. + Flot.	

Annex 5

Gross results of participants for PCR (in numerical order of lab ID).

Laboratory identification code :	1

Cut-off at 15 copies of the PCR platform used (in cycles)	34.60
Copy number at the cut-off of the PCR platform used (in copies)	12.15

Sample type	Sample N°	Ruminant DNA	Comment
1	394	Absent	
1	674	Absent	
2	793	Present	
3	1976	Present	
4	583	Not to perform	
5	2774	Not to perform	
6	485	Not to perform	

Laboratory identification code :	2

Cut-off at 15 copies of the PCR platform used (in cycles)	33.37
Copy number at the cut-off of the PCR platform used (in copies)	10.55

Sample type	Sample N°	Ruminant DNA	Comment
1	3418	Absent	
1	4930	Absent	
2	2473	Present	
3	2312	Absent	Inhibition
4	1535	Not to perform	
5	1598	Not to perform	
6	2165	Not to perform	

Laboratory identification code :	3

Cut-off at 15 copies of the PCR platform used (in cycles)	35.90
Copy number at the cut-off of the PCR platform used (in copies)	10.76

Sample type	Sample N°	Ruminant DNA	Comment
1	3138	Absent	No inhibition observed
1	3474	Absent	No inhibition observed
2	1633	Present	No inhibition observed
3	1528	Present	No inhibition observed
4	2487	Not to perform	
5	2326	Not to perform	
6	2557	Not to perform	

Laboratory identification code :	4

Cut-off at 15 copies of the PCR platform used (in cycles)	35.57
Copy number at the cut-off of the PCR platform used (in copies)	11.25

Sample type	Sample N°	Ruminant DNA	Comment
1	1066	Not to perform	
1	1738	Not to perform	
2	1521	Present	
3	1584	Present	
4	2599	Not to perform	
5	1990	Not to perform	
6	2725	Not to perform	

Laboratory identification code :	5

Cut-off at 15 copies of the PCR platform used (in cycles)	36.65
Copy number at the cut-off of the PCR platform used (in copies)	9.24

Sample type	Sample N°	Ruminant DNA	Comment
1	4202	Absent	The PCR was repeated at the dilution rates 3x and 30x, none of the test portions delivered positive result
1	4650	Absent	The PCR was repeated at the dilution rates 3x and 30x, none of the test portions delivered positive result
2	2081	Present	
3	2032	Present	
4	695	Not to perform	
5	254	Not to perform	
6	429	Not to perform	

Laboratory identification code :	6

Cut-off at 15 copies of the PCR platform used (in cycles)	32.54
Copy number at the cut-off of the PCR platform used (in copies)	8.18

Sample type	Sample N°	Ruminant DNA	Comment
1	338	Absent	with inhibition
1	2802	Absent	
2	1353	Present	
3	2648	Present	with mild inhibition
4	1591	Not to perform	
5	534	Not to perform	
6	1213	Not to perform	

Laboratory identification code :	7

Cut-off at 15 copies of the PCR platform used (in cycles)	34.33
Copy number at the cut-off of the PCR platform used (in copies)	10.79

Sample type	Sample N°	Ruminant DNA	Comment
1	1010	Absent	no inhibition
1	1122	Absent	no inhibition
2	177	Present	no inhibition
3	296	Present	no inhibition
4	135	Not to perform	
4	2102	Not to perform	
5	1269	Not to perform	

Laboratory identification code :	8

Cut-off at 15 copies of the PCR platform used (in cycles)	36.27
Copy number at the cut-off of the PCR platform used (in copies)	9.20

Sample type	Sample N°	Ruminant DNA	Comment
1	58	Present	PCR result was verified with second PCR apparatus
1	1458	Present	PCR result was verified with second PCR apparatus
2	2697	Present	
3	2144	Present	
4	751	Not to perform	
5	702	Not to perform	
6	2221	Not to perform	

Laboratory identification code :	9

Cut-off at 15 copies of the PCR platform used (in cycles)	34.07
Copy number at the cut-off of the PCR platform used (in copies)	14.34

Sample type	Sample N°	Ruminant DNA	Comment
1	506	Not to perform	
1	2130	Not to perform	
2	905	Present	
3	2256	Present	
4	639	Not to perform	
5	814	Not to perform	
6	1773	Not to perform	

Laboratory identification code :	10

Cut-off at 15 copies of the PCR platform used (in cycles)	35.76
Copy number at the cut-off of the PCR platform used (in copies)	9.09

Sample type	Sample N°	Ruminant DNA	Comment
1	170	Absent	
1	842	Absent	
2	2641	Present	
3	1864	Present	
4	1647	Not to perform	
5	1822	Not to perform	
6	2333	Not to perform	

Laboratory identification code :	11

Cut-off at 15 copies of the PCR platform used (in cycles)	33.06
Copy number at the cut-off of the PCR platform used (in copies)	10.21

Sample type	Sample N°	Ruminant DNA	Comment
1	730	Absent	Inhibition
1	1962	Absent	Inhibition
2	1241	Present	
3	1136	Present	Inhibition
4	1983	Not to perform	
5	1878	Not to perform	
6	93	Not to perform	

Laboratory identification code :	12

Cut-off at 15 copies of the PCR platform used (in cycles)	34.26
Copy number at the cut-off of the PCR platform used (in copies)	11.17

Sample type	Sample N°	Ruminant DNA	Comment
1	3866	Absent	
1	4314	Absent	
2	345	Present	
3	1248	Present	
4	191	Not to perform	
5	1038	Not to perform	
6	1829	Not to perform	

Laboratory identification code :	13

Cut-off at 15 copies of the PCR platform used (in cycles)	37.02
Copy number at the cut-off of the PCR platform used (in copies)	9.84

Sample type	Sample N°	Ruminant DNA	Comment
1	2634	Absent	
1	4538	Absent	
2	9	Present	
3	1080	Present	
4	2263	Not to perform	
5	2494	Not to perform	
6	2781	Not to perform	

Laboratory identification code :	14

Cut-off at 15 copies of the PCR platform used (in cycles)	36.13
Copy number at the cut-off of the PCR platform used (in copies)	11.24

Sample type	Sample N°	Ruminant DNA	Comment
1	1850	Not to perform	
1	2354	Absent	Partial inhibition. Inhibition test OK
2	1185	Present	Partial inhibition
3	1752	Present	Partial inhibition
4	975	Not to perform	
5	478	Not to perform	
6	205	Not to perform	

Laboratory identification code :	15

Cut-off at 15 copies of the PCR platform used (in cycles)	35.87
Copy number at the cut-off of the PCR platform used (in copies)	11.24

Sample type	Sample N°	Ruminant DNA	Comment
1	954	Absent	
1	3754	Absent	
2	2193	Present	
3	1192	Present	
4	303	Not to perform	
5	758	Not to perform	
6	317	Not to perform	

Laboratory identification code :	16

Cut-off at 15 copies of the PCR platform used (in cycles)	37.04
Copy number at the cut-off of the PCR platform used (in copies)	11.29

Sample type	Sample N°	Ruminant DNA	Comment
1	114	Absent	4 test portions were analysed: 1 weak positive result, 3 negative results → negative
1	1402	Absent	
2	681	Present	
3	1416	Present	
4	23	Not to perform	
5	646	Not to perform	
6	2109	Not to perform	

Laboratory identification code :	17

Cut-off at 15 copies of the PCR platform used (in cycles)	35.78
Copy number at the cut-off of the PCR platform used (in copies)	9.26

Sample type	Sample N°	Ruminant DNA	Comment
1	3082	Absent	1 of 2 first PCR's were slightly positive, but DNA purification 3 and 4 were negative
1	3642	Absent	
2	961	Present	
3	184	Present	
4	2207	Not to perform	
5	2270	Not to perform	
6	597	Not to perform	

Laboratory identification code :	18

Cut-off at 15 copies of the PCR platform used (in cycles)	36.64
Copy number at the cut-off of the PCR platform used (in copies)	11.28

Sample type	Sample N°	Ruminant DNA	Comment
1	282	Not to perform	
1	2970	Not to perform	
2	121	Present	
3	2424	Present	
4	1423	Not to perform	
5	1094	Not to perform	
6	1941	Not to perform	

Laboratory identification code :	19

Cut-off at 15 copies of the PCR platform used (in cycles)	35.92
Copy number at the cut-off of the PCR platform used (in copies)	10.76

Sample type	Sample N°	Ruminant DNA	Comment
1	1234	Absent	
1	2578	Absent	
2	1409	Present	
3	352	Present	
4	2319	Not to perform	
5	1654	Not to perform	
6	989	Not to perform	

Laboratory identification code :	20

Cut-off at 15 copies of the PCR platform used (in cycles)	33.97
Copy number at the cut-off of the PCR platform used (in copies)	10.23

Sample type	Sample N°	Ruminant DNA	Comment
1	2466	Absent	
1	3362	Absent	
2	1913	Present	
3	912	Present	
4	1927	Not to perform	
5	2662	Not to perform	
6	1549	Present	

Laboratory identification code :	21

Cut-off at 15 copies of the PCR platform used (in cycles)	35.89
Copy number at the cut-off of the PCR platform used (in copies)	10.03

Sample type	Sample N°	Ruminant DNA	Comment
1	3250	Absent	
1	4090	Absent	
2	849	Present	
3	744	Present	
4	247	Not to perform	
5	2718	Not to perform	
6	541	Not to perform	

Laboratory identification code :	22

Cut-off at 15 copies of the PCR platform used (in cycles)	37.30
Copy number at the cut-off of the PCR platform used (in copies)	9.32

Sample type	Sample N°	Ruminant DNA	Comment
1	3026	Absent	
1	4706	Absent	
2	569	Present	
3	968	Present	
4	919	Not to perform	
5	198	Not to perform	
6	1661	Not to perform	

Laboratory identification code :	23

Cut-off at 15 copies of the PCR platform used (in cycles)	37.20
Copy number at the cut-off of the PCR platform used (in copies)	9.10

Sample type	Sample N°	Ruminant DNA	Comment
1	1178	Absent	The presence of amplifiable DNA has been checked with an universal PCR fish
1	2186	Absent	The presence of amplifiable DNA has been checked with an universal PCR fish
2	1073	Present	PCR first intention
3	464	Present	
4	1815	Not to perform	
5	982	Not to perform	
6	2389	Not to perform	

Laboratory identification code :	24

Cut-off at 15 copies of the PCR platform used (in cycles)	31.91
Copy number at the cut-off of the PCR platform used (in copies)	10.32

Sample type	Sample N°	Ruminant DNA	Comment
1	1626	Absent	
1	2410	Absent	
2	737	Present	
3	72	Present	
4	863	Not to perform	
5	1934	Not to perform	
6	653	Not to perform	

Laboratory identification code :	25

Cut-off at 15 copies of the PCR platform used (in cycles)	37.05
Copy number at the cut-off of the PCR platform used (in copies)	9.06

Sample type	Sample N°	Ruminant DNA	Comment
1	1290	Absent	
1	3306	Absent	
2	1297	Present	
3	576	Present	
4	415	Not to perform	
5	590	Absent	
6	1885	Present	

Laboratory identification code :	27

Cut-off at 15 copies of the PCR platform used (in cycles)	35.11
Copy number at the cut-off of the PCR platform used (in copies)	11.43

Sample type	Sample N°	Ruminant DNA	Comment
1	2522	Absent	18S positive control is detected
1	4034	Absent	18S positive control is detected
2	2361	Present	
3	1920	Present	
4	2543	Not to perform	
5	1318	Not to perform	
6	373	Not to perform	

Laboratory identification code :	29

Cut-off at 15 copies of the PCR platform used (in cycles)	37.95
Copy number at the cut-off of the PCR platform used (in copies)	9.86

Sample type	Sample N°	Ruminant DNA	Comment
1	2	Absent	
1	3922	Absent	
2	1689	Present	
3	1360	Present	
4	2095	Not to perform	
5	1150	Not to perform	
6	709	Not to perform	

Laboratory identification code :	31

Cut-off at 15 copies of the PCR platform used (in cycles)	37.75
Copy number at the cut-off of the PCR platform used (in copies)	10.34

Sample type	Sample N°	Ruminant DNA	Comment
1	2242	Not to perform	
1	4146	Not to perform	
2	1577	Present	
3	800	Present	
4	1479	Not to perform	
5	366	Not to perform	
6	2053	Not to perform	