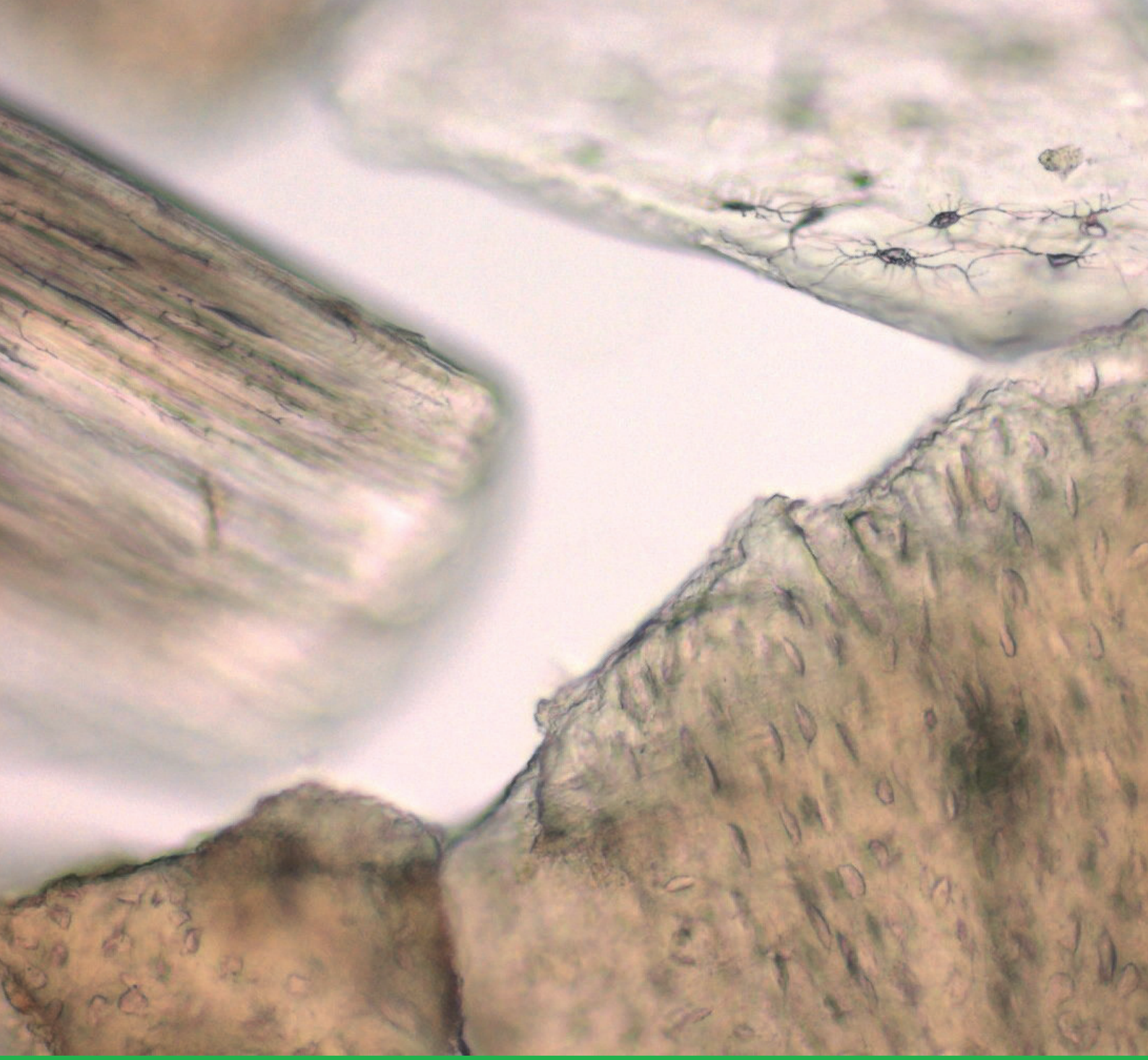

IAG proficiency test animal proteins 2019

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Cover photo: Bone fragments of cod (left), herring (upper right) and chicken (below).

Contents

Summary

The annual proficiency test for the detection of animal proteins in animal feed of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy was organized by Wageningen Food Safety Research, The Netherlands. The aim of the proficiency test was to provide the participants information on the performance of the implementation of the monitoring methods for their local quality systems. A further aim was to gather information about the current practices in the application of the microscopic method. The current 2019 version of the IAG ring test for animal proteins addressed all analytical sections of the methods for microscopy and PCR as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 together with accompanying SOPs.
Three of the four samples used in the proficiency test contained poultry material at the legally required technical limit (0.1% w/w; Regulation (EC) 152/2009), or fish meal at a spike level of 2% (w/w), or both. A fourth sample was left blank. A pig feed, containing 3% (w/w) of bakery by-products and a ruminant feed were used as matrix. None of the samples was labelled as fish feed.
A total of 44 participants subscribed to the proficiency test animal proteins. Two participants did not submit their results and one submitted PCR results only, leaving 41 sets for microscopic evaluation. 18 sets of ruminant PCR results were submitted as well.

Microscopy
All participants were requested to determine the presence or absence of land animal and/or fish, to indicate the type of material found and to describe the method used to achieve these results.
In total eight participants (19.5% of 41 participants) deviated from the official method by applying an incorrect number of determination cycles and/or drawing incorrect conclusions (e.g. "presence" for five particles, "absence" for ten particles). Therefore, all evaluations were based on the actual number of particles reported by all participants.
Incorrect positive results (positive deviations) were expressed in a specificity score and incorrect negative results (negative deviations) were expressed in a sensitivity score. An optimal score is 1.0. The results are analysed in two ways: numbers below threshold (between 1 and 5 particles per determination cycle inclusive) have been considered positive (complying to the zero tolerance) and as alternative considered as negative (for matching the official evaluation).
For all samples several participants did not detect terrestrial animal particles in the presence of fish meal (sensitivity 0.95) in contrast to the optimal result in the absence of fish meal (1.0), or erroneously reported terrestrial animal material when absent (specificity 0.93 and 0.90 in the presence or absence, respectively, of fish material). The absence of fish material in the presence of 0.1% poultry PAP resulted in a specificity score of 0.90. 37 institutes participated in both in the 2018 and 2019 studies. Based on their results an intra-laboratory reproducibility, expressed as concordance between 2018 and 2019 was calculated. Especially for the results representing specificity low concordance was found. This indicates wrong observations seemed incidental in most cases.
The documentation for and training of microscopists for correct identification of particles of animal origin would deserve further attention in order to guard specificity and avoid incidental errors. Evaluation of several aspects of the application of the current microscopic methods would be beneficial for improving harmonization among the laboratories applying the microscopic method.

PCR
In the two samples without addition of ruminant PAP, but still containing the bakery by-products, ruminant DNA was detected by qPCR as far as analysed by the majority of the participants. The list of recognised sources such as milk and milk products, and ruminant gelatine can be extended with bakery by-products, which is important for the recycling of food by-products.

1 Introduction

The monitoring of the presence of animal proteins in feed for prevention of mad cow disease is an important part of the required active monitoring by member states of the European Union. With a long historical track record, microscopic detection of animal by-products is an important method for monitoring. The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy, serves as a platform for exchange of information, knowledge building and quality control. This international group organises annually a proficiency test for animal proteins in feeds for all their members. Wageningen Food Safety Research (WFSR) [1] is managing this proficiency test on behalf of the IAG section Microscopy. Overviews of past results are presented in the annual reports of the ring tests for monitoring animal proteins in feed (latest version: van Raamsdonk et al., 2018).

The current version of the microscopic method, together with an official method for DNA identification of ruminant material by means of PCR, was implemented by Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009: EC, 2013a, and its corrigendum EC, 2013b). A combined application of the microscopic and PCR methods is installed. Guidance is implemented in a Standard Operational Procedure (SOP) "operational schemes v3.0", developed by European Union Reference Laboratory (EURL). Other SOPs supporting the new method include details of the microscopic and PCR procedures, slide preparation among them. A Limit of Detection (LOD[2]) of five particles per determination cycle is set. In the view that the IAG proficiency test is intended to monitor the technical performance of the participants, no filtering of results below a certain threshold is applied. As alternative besides this basic choice for evaluation, the LOD of five particles per cycle is applied as well. This alternative approach is applied for comparison with other tests. Although the IAG proficiency test has a primary focus in microscopy, the participants were invited to submit their PCR results as well.

For qualitative detection methods sensitivity (frequency of false negatives) and specificity (frequency of false positives) are important indicators for the performance of the method. Although specific elements of a method can be installed for improving these two indicators, there is a statistical relationship. In statistical terms, a decrease of a type I error (false positives) would imply an increase of a type II error (false negatives) or vice versa (Lindenmayer & Burgman, 2005). An important issue is the correct and precise discrimination between terrestrial animal material and fish material, which are currently the major categories for monitoring. Confusion among particles of these two categories (see van Raamsdonk et al., 2017b), or with plant material or minerals will contribute to specificity and sensitivity scores lower than one. The design of the current test is identical to that of last year's test (van Raamsdonk et al., 2018). This allows to calculate the intralaboratory reproducibility over the combined dataset of 2018 and 2019, expressed as concordance (van der Voet and van Raamsdonk, 2004).

The current 2019 version of the IAG ring test for animal proteins includes four samples with basic spike levels. The samples contain ruminant material at the legally required technical limit (0.1% w/w; Regulation (EC) 152/2009), or fish meal at a spike level of 2% (w/w), or both. A fourth sample was left blank. The final intention is, as in previous years, to provide the participants data on the performance of their own way of implementation, and to document the application of the two enforcement methods.
In this report the proficiency test for animal proteins 2019 is presented.

[1] Formerly RIKILT. WFSR started 1 June 2019 as the merger of RIKILT and the food safety laboratory of the Dutch Food Safety Authority.

[2] The term Limit of Detection suggests that findings below that level (here: 5 particles) are unreliable or even cannot be achieved properly. Instead, the issues of the presence of individual particles at low levels after microscopic examination might be related to possible lab contamination or to erroneous identification (specificity). Since the term LOD is originally defined in the framework of chemical analysis related to technical limitations instead of solving contamination or specificity issues, the term "threshold" is used here in the report.

2 Methods

2.1 Materials

The IAG ring test for animal proteins 2019 was based on two feeds, produced in 2003 in the framework of the European Union project STRATFEED. Two samples were produced from a ruminant feed, containing cornglutenfeed (22%), palmkernelmeal (16%), beetpulp (13%), citruspulp (10%), molasse (10%), coconutmeal (9%), rapeseedmeal (8%), wheat (8%), corn (2%), soybeanmeal (1.2%), magnesiumoxyde (0.4%), vegetable fat (0.2%), vitamin/mineral mix (0.2%). The second feed used was a pig feed, with a composition consisting of tapioca (40%), soybeanmeal (15.9%), rapeseedmeal (12%), wheatglutenfeed (10.0%) and wheat bran (2%), palmkernelmeal (6%), beetpulp (4%), bakery by-products (3%), molasse (2%), vegetable fat (1.8%), barley (1%), animal fat (1.0%), limestone (0.6%), lysine (0.3%), salt (0.2%) and vitamin/mineral mix (0.2%). This is the same pig feed as used in 2018.
The IAG proficiency test for animal proteins 2019 was combined with the IAG proficiency test for botanical composition (sample 2019-B).
The design of the proficiency test animal proteins allowed to apply the full analytical part of the method for the detection of animal proteins as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 (EC, 2013a), its corrigendum (EC, 2013b) and the accompanying SOPs. The samples were chosen to be fit for detection by microscopy. The results to be obtained by PCR can be assumed to be influenced by the presence of dairy products in the pig feed. None of the samples was indicated as feed for aquaculture. The choice and order of the methods was part of the study. The composition of the four samples is listed in Table 1.

Table 1 *Composition of the samples in the NRL-IAG proficiency trial animal proteins 2019.*

Label	Sample type	Content
2019-A	pig feed	0.1% (w/w) poultry MBM
2019-B	ruminant feed	Blank
2019-C	ruminant feed	2% (w/w) fish meal A
2019-D	pig feed	0.1% (w/w) poultry MBM and 2% (w/w) fish meal B

A poultry MBM was chosen for the terrestrial PAP in order to test the performance of the PCR test in absence of a ruminant MBM, but in the presence of a legal version of ruminant material. The poultry MBM showed no signal for the EURL-AP ruminant PCR test. Two fish meals (A and B) were used for spiking. Fish meal A consisted of material originating from Peru and Chili with a high content of particles with visible lacunae. Fishmeal B originated from Denmark and Norway with a lower frequency of bone particles with visible lacunae. Both materials were examined in the WFSR regular control program and found to be negative for land animal material.

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order: 2019-B - 2019-A - 2019-C - 2019-D. All samples were prepared in a laboratory that is located at a distance from the WFSR microscopy laboratory. A sample size of 50 grams was chosen which was sufficient for applying all three determinations as mentioned for the full method in Regulation (EC) 152/2009.
Jars for sample 2016-A were filled with 50 grams of the pure feed, closed and set aside. The other samples were produced by step-wise dilution of the dedicated contaminants down to a level of either 2% or 0.1%. The production scheme is presented in Figure 1.

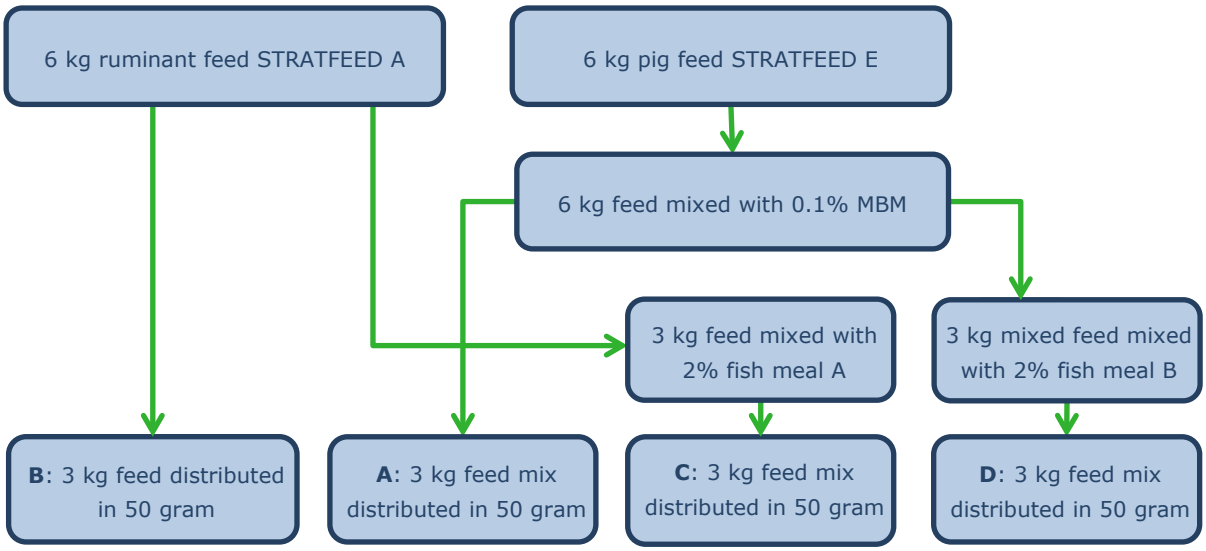

Figure 1 *Flow diagram to produce the samples in order from left to right.*

2.3 Homogeneity study

Two WFSR microscopists examined independently all basic materials and five jars of all four samples according to the procedure of Regulation (EC) 152/2009. PCR was carried out according to the EURL-AP protocol and SOPs for ruminant with IRMM plasmids and use of Diagenode D600 Master mix. The Cut-off value (Cq = 35.11) was established using the official procedure.

Table 2 *Results of the homogeneity study. Sediment amounts are based on 10 grams. Microscopy: five replicates. PCR: Cq values for two replicates for ruminant.*

		Microscopy		PCR				
Sample		MBM	fish	1x	10x	1x	10x	result
2019-A	0.1% (w/w) poultry MBM	present	absent	29.02	31.17	28.11	30.13	present
2019-B	Blank	absent	absent	34.08	36.36	32.07	35.11	present
2019-C	2% (w/w) fish meal A	absent	present	34.06	36.33	34.02	36.01	present
2019-D	0.1% poultry MBM + 2% fish meal B	present	present	28.97	31.10	27.30	29.58	present

The microscopic results were correct in all cases (Table 2). The results for the PCR analysis will be discussed together with the results of all participants.
The microscopy research group and the PCR research group of WFSR did not participate in the further laboratory analysis of this proficiency test.

2.4 Organization of the proficiency trial

All IAG members, all NRLs, participants of former proficiency tests and a series of putative interested laboratories were informed about the proficiency test for 2019 by means of mailing using distribution lists. Until the beginning of March, a total of 44 participants for the proficiency test animal proteins were listed. One participant applied exclusively for PCR. Participants outside Europe were informed to be aware of possible problems with custom regulations. The sets of four samples with an accompanying letter (see Annex 1) were sent to all participants on Tuesday 12th of March 2019. All participants were informed the next day: on Wednesday 13th of March an E-mail message was sent to all participants for supplying the file containing a sheet with instructions on reporting (see Annex 2) and the electronic report forms (see Annex 3 and 4).

The closing date for reporting results was fixed at Monday April 18th. Due to the late availability of the report form the final date postponed to Monday April 23rd. Notwithstanding this several requests were received to extent the period for analysis. Results received after the date at which the evaluation of the results was started were ignored. The analysis of the results was carried out between 8th and 18th of May.

Since the new Regulation (EC) 152/2009 as amended by Regulation (EC) 51/2013 is fully operational for both microscopy and ruminant PCR, the reporting form was designed to accommodate both types of results. The report form consisted of four elements:

- Laboratory and sample numbers.
- Results of the microscopy analysis for up to three analyses. Depending on the results of this first determination the cells for the second determination were made active and depending on the sum of first and second determination the cells for the third and last determination were made active. The final line consisted of a sum of particles found.
- Results of ruminant EURL-AP PCR method.
- The final conclusion of the participant.

The report form was interactive. The decision rule to make the set of cells active for the second determination was made as follows:

IF [#terr.an. IS between 1-5] OR [#fish IS between 1-5] THEN second determination

The decision rule for the third determination was based on the sum after two determinations. The text of the method requires to perform an extra determination when the number of particles is exceeding 10 particles (5 on average per determination cycle). In those cases that a second or third determination cycle is necessary based on an excess for one of the two types (either fish or terrestrial animal), detection of the other type is carried out as well.

2.5 Analysis of results

As in every analytical method, several types of results exist, such as duplicate results, intermediate results and final results (conclusion). Since none of the samples was indicated as aquafeed, light microscopy is the only method for reaching the final conclusion, as stated in the SOP "Operational schemes v3.0". It is the intention of the proficiency test to establish primarily the analytical capability of the participants. Therefore, in those cases where the final conclusion as provided by a participant violates with the actual number of particles encountered, that number is used as basis for the evaluation.

The results are analysed in two ways: numbers below threshold (between 1 and 5 inclusive) have been considered positive and as alternative considered as negative. The choice to consider these number positive was based on the principle that any particle correctly identified as of animal origin is apparently present. This approach fits to the legal principle of zero tolerance and it allows a way to compare the present results with those of previous years.

For binary results (yes/no, positive/negative, etc.) standard statistics are accuracy, sensitivity and specificity. The accuracy is the fraction of correct results, either positive or negative. The sensitivity is the ability of the method used, to detect the contaminant when it is present, whereas the specificity is the ability to not detect the contaminant when it is absent. The following equations have been used to calculate the statistics:

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

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

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

where PA is the number of correct positive identifications (positive agreements), NA the number of correct negative identifications (negative agreements), PD the number of false positives (positive deviations) and ND the number of false negatives (negative deviations). The statistics are presented as fractions. Accuracy (specificity or sensitivity) has been calculated for each sample type.
As criterion for a good or excellent score a threshold of 0.95 for either sensitivity or specificity was applied.

The experimental design of the set of four samples in the current study is identical to that of the 2018 test (van Raamsdonk et al., 2018). This made it possible to calculate the within-laboratory reproducibility over these 2 years. In proficiency testing based on quantitative data repeatability and reproducibility can be calculated based on commonly applied procedures (ISO, 1994: ISO 5725-2:1994). Comparable statistics have been developed for qualitative data (Langton et al., 2002; ISO, 2003; van der Voet and van Raamsdonk, 2004). Accordance is the chance of finding identical results in pairs of replicates of the same treatment in the same laboratory under repeatability conditions. This is equivalent to repeatability for quantitative results. Concordance is the chance of finding the same result for the same treatment in two different laboratories. ISO (2003) as well as Langton et al. (2002) presented calculation models based on sampling without replacement. Nevertheless, in comparison to accordance a calculation based on the same principle, i.e. sampling with replacement, will be used in this study. Then concordance is calculated from the chance of finding a pair of replicates from any laboratory with identical results, either 1 (p) or 0 (1 – p):

$$CON = \overline{p}^2 + (1 - \overline{p})^2$$

where $\overline{p}$ is the average fraction of replicates with result 1 for all labs. The closer the value of concordance to one the better the between lab reproducibility. The strategy followed in this study can be indicated as application of a random model, whereas the strategy of Langton et al. (2002) can be indicated as applying a fixed model. This approach was followed in a bench mark study for performance of the microscopic method organised in 2003 (van Raamsdonk and van der Voet, 2003).

3 Results

A total of 44 participants subscribed for the proficiency test animal proteins. One participant did not submit their results and one participant reported results for three of the four samples without further information. Of the remaining 42 participants, one applied exclusively PCR results, leaving 41 sets of microscopic results, accompanied with PCR results in 17 cases. The participants originated from 19 countries: 14 member states of the European Union, and five other countries (Serbia, Norway, Peru, Thailand and Switzerland). The list of participants is presented in Annex 5. Five member states have been involved with three or more participating laboratories: Germany (15 labs), the Netherlands (5), France (3) and Belgium (3).
All results were received by E-mail, in most cases by means of a scan and the original report file. Not in all cases the original Excel file was submitted although clearly requested. In all those cases that a participant sent in several versions of the report sheet the most recent version was used. All full and correct reports were included. The draft report was finalised at May 28th.
The full results are presented in the Tables of Annexes 6, 7 and 8.

3.1 Application of the method and reporting

Five participants (12.2% of 41 participants: 9, 21, 25, 37, 46) carried out an incorrect number of determination cycles for one or more samples. In four out of five cases more determination cycles were applied than requested by the official method. In total five participants (12.2%: 15, 21, 25, 37, 45) included incorrect interpretations of the encountered number of particles (e.g. "presence" for five particles, "absent" for 10 particles).
Incomplete or non-conclusive reports were submitted by a range of participants. The errors include missing results for one sample, use of a wrong sample number, report of fictional numbers of particles, and/or missing final conclusions for one or for all samples. In one occasion four final conclusions were drawn without a logical relationship with the observed numbers of particles, but three of these conclusions matched the correct composition. In terms of proficiency test procedures some participants only submitted a PDF file without the Excel file, empty Excel files, other file names than desired were used, or reports were overlooked for submission. Eleven participants (26.8% of 41 participants) were asked to clarify or complete their results. Participants were not contacted in all cases where the number of cycles and/or the number of declared particles could be evaluated without further information. If the second submitted version still contained any erroneous evaluation of their own results, this was accepted as such.
The official method includes basically several steps: the analytical procedure including the determination of the number of cycles, the drawing of the conclusion and filing the report. the latter part, the use of the official texts for reporting, is excluded from the procedure in this proficiency test (see Annex 2). An evaluation of the final conclusion as reported would combine the analytical and a part of the "administrative" procedure. This evaluation would include the wrong interpretations of five participants and would be hampered by the missing conclusions of three other participants. Based on these issues, and in the view that the analytical performance of the participants should be the primary focus, the numbers of particles as reported are chosen as basis for the evaluation of the results. This approach also fits in the strategy to consider all results below the threshold as positive. As in previous years, the results with all results below threshold as negative will be shown as well.

3.2 Microscopic procedure

An inventory of nine different parameters was added to the report sheet of the actual results of the four samples. These results are shown in Annex 6 and summarised in Table 3. The main purpose of this inventory was to provide benchmark information for the individual participants for comparison

with the general application of the method. Although this has to be considered additional information only, a proficiency test with a random set of participants provides a good opportunity to collect meta-data on the application of the method. The current results provide the opportunity to discuss some parameters of the microscopic method.
The results as presented in Table 3 generally show a good application of the method, except for the number of determinations applied and for the embedding agent. Differences with previous years will be presented in the next chapter (Discussion).
The minimum share of the total amount of sediment declared to be used was 6% for one sample (participant 38), although one participant (35) declared to have used 1% of the sediment material for all samples. In general, the portions of the sediment material used were exceeding 15%. The amounts of sediment extracted from the samples ranged from approx. 50 to 350 mg. One participant (41) extracted only 40 to 70 mg from every sample, whereas another participant (1) reported sediment amounts between 811 and 1737 mg per sample.

Table 3 *Inventory of parameters for microscopic detection and their application. Pink cells indicate deviations from the official method. *: different types of glassware are in use, which could be summarised of glassware as "champagne glass". The correct indication needs further examination.*

Parameter	parameter state	number of participants	amount
Correct application of the number of determinations	yes	36	
	no; too many determinations	4	
	no; insufficient determinations	1	
Extra milling step (<1.0 mm)	no	31	
	yes	9	
type of glassware	chemical sedimentation funnel	19	
	conical glass with cock	8	
	champagne glass	6	
	beaker (flat bottom)	4	
	other	3	
sedimentation agent	TCE	38	
	Chloroform	1	
	TCE/Petroleumether	1	
use of staining of sediment	no	23	
	yes	17	
use of binocular for examination at lower magnifications	yes	26	
	no	13	
size of cover glass used	small (e.g. 20 x 20 mm)	31	
	medium	3	
	large (e.g. 26 x 50 mm)	5	
share of the total sediment used for examination	minimum		6% (1%)
	maximum		100%
embedding agent for the sediment	glycerine / glycerol	16	
	paraffin oil	12	
	immersion oil	8	
	Norland Adhesive	4	
	other (water, glycerol:water mixture, mineral oil)	0	

3.3 Microscopic detection

The results of the application of the microscopic detection, expressed exclusively on the basis of declared numbers of particles, are presented in Table 4; full results are listed in Annex 7. The amount of added material, 0.1% (w/w) of terrestrial animal material and/or 2% (w/w) of fish material, would theoretically be sufficient for the application of one determination cycle at all times to reach a

conclusive result. Twenty-two participants reported the correct maximum number of particles possible according the report form (20) after one cycle in all cases (Annex 8). Four additional participants reported 16-19 particles after one cycle for one single sample combined with 20 particles in all other cases. Four participants reported average numbers of particles below 16 but well over the threshold of 5 particles per cycle for all samples. It can be noted that the participants reporting less than the maximum number possible in the report form (20) but still higher than 5 particles primarily report this lower number for terrestrial animal material in the presence of fish meal. One participant reported correct results in terms of presence or absence but based on numbers of particles up to 60 dues to the analysis of three determinations per sample. The remaining 10 participants declared one or more errors.
The total overview of results shows good results for sensitivity. Some suboptimal values for specificity (below 0.95) were calculated from the data. Four participants (1, 21, 37, 46) and one participant (1), respectively, reported particles of terrestrial animals or fish material in the blank sample 2019-B. Up to 58 particles in this blank were declared by one participant. Participants 5 and 26 did not detect terrestrial animal material in the presence of fish.

Table 4 *Sensitivity and specificity scores for the detection of animal proteins by the* ***microscopic*** *method of four samples (top row: values below the threshold considered positive; bottom row in italics: values below the threshold considered negative). Abbreviations: n: number of participants. Capitals A to D: sample indication.*

		Terrestrial animal				Fish			
		A	B	C	*D*	A	B	C	*D*
n		0.1%	0%	0%	*0.1%*	0%	0%	2%	*2%*
41	specificity		0.90	0.93		0.90	0.98		
			0.98	*0.98*		*0.95*	*0.98*		
	sensitivity	1.0			*0.95*			1.0	*1.0*
		0.95			*0.88*			*0.98*	*0.98*

The results were stratified according to several of the parameters as presented in Table 2. No significant differences were found among the different states for each of these parameters.

3.4 Detection by PCR

Participants were invited to perform DNA analysis targeted for ruminants (EURL-AP Method) and to submit their results together with the results for microscopy. Although none of the feeds was indicated as feed for aquaculture, 17 participants reported results for the two samples (-A and D) which were found positive after microscopic analysis. Eleven of them also reported results for one or both of the samples B and C. The overall results are shown in Table 5. Full results are shown in Annex 9. Ruminant material was not added to any of the materials. The positive signal for the samples 2019-A and 2019-D is based on the presence of 3% bakery by-products in the pig feed. A range of participants reported positive signals for the ruminant feed as well (2019-B and 2019-C). This feed was produced in a special pilot plant, intended to be animal protein free, in the framework of the project STRATFEED.

Table 5 *Results for DNA analyses (PCR) for four samples. Target: ruminant. *: results based on the presence of 3% bakery by- products in the matrix.*

		Ruminant			
		A	B	C	*D*
		0% *	0%	0%	*0% **
	specificity		0.78	0.70	
	sensitivity	0.89			*0.89*
	n	18	9	10	*18*

4 Discussion

4.1 Specificity and sensitivity

The design of the current version of the IAG proficiency test was to mimic the design of that of last year (van Raamsdonk et al., 2018). The pig feed used for two samples (2019-A and 2019-D) was used in 2018 for all four samples. Also, the contamination levels (0.1% for terrestrial PAP and 2% for fish meal) and their combination were identical. This makes it possible to compare the results for both microscopy and for PCR, and within laboratory reproducibility can be calculated since 37 institutes participated in both years.
The level of detection of the microscopic method is much lower than the legal technical limit of 0.1% (Engling et al., 2000; Veys et al., 2010; van Raamsdonk et al., 2014), which was used as spike level in the current design, and the hypothesis was that a vast majority of participants would be able to report the maximum number of particles accepted in the report form (20 particles per type per cycle). Accepting 16 or more particles as close to the maximum score, 26 participants were able to report this for all relevant samples, which is 63% of all participants. At the other end of the spectrum, 10 participants (24%) account for all errors influencing the sensitivity and/or specificity scores. A comparison with the 2018 data (Table 6) reveals that the sensitivity is at a comparable level. The specificity in a specific sample, however, is considerably higher than accounted in 2018 (no terrestrial animal material in a fish meal contaminated feed: 0.93 versus 0.84), but lower than the result obtained in the same composition of the comparable experimental design in 2009 (0.98).

Table 6 *Results for detection of material of terrestrial animals and of fish in feed samples based on sediments of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and WFSR (2008-2019) on behalf of the IAG section Microscopy. Results have been communicated in the framework of the annual meetings of this Section. Results indicate specificity in the case of the blank, and sensitivity in the case of the other sample types.*

Detection of:	Land animals						Fish		
Content: fish	0	2-5%	2%	0	2%	0	0	0	0
year land animal	0	0	0.1%	0.1%	0.05%	≤0.05%	0	0.1%	≤0.05%
2003 (n=29)	0.86			1.0					
2004 (n=30)	0.93					0.97	0.97		0.93
2005 (n=42)			0.95	0.95				0.76	
2006 (n=43)	0.98		1.0				0.93		
2007 (n=45)		0.89	0.93						
2008 (n=45)	0.93			0.98		0.96	0.98	0.91	0.84
2009 (n=49)	0.96	0.98		1.0			0.96	0.88	
2010 (n=53)	0.96		0.98		0.91		0.98		
2011 (n=56)	1.0					0.98	0.98		0.91
2012 (n=53)	0.94			0.98		0.98	0.94	0.96	0.92
2013 (n=53)	0.94	0.98		0.94 [1)]		1.0	0.96	0.94	0.96
2014 (n=52)	0.96		0.94				0.96		
2015 (n=42)	0.95			0.93			0.88	0.90	
2016 (n=45)	0.96		0.96 0.91				0.98		
2017 (n=36)	0.89 0.94					0.91 [2)]	0.94 0.97		
2018 (n=43)	0.91	0.84	0.95	1.0			0.93	0.95	
2019 (n=41), current results	0.90	0.93	0.95	1.0			0.98	0.90	

1) TCP used as contaminant for land animal material

2) 0.01% of bone meal representing 0.03% MBM

There are several strategies to compare the results of two comparable datasets, developed in two subsequent years. Overall accuracy can be calculated. Concordance, as measure of reproducibility for qualitative methods, is a parameter which is based on the analysis of replicates established in the same laboratory under reproducible circumstances. Besides the performance in terms of absence or presence of targets, the implementation of the method can be compared as well.
Overall performance, calculated from the eight scores on sensitivity and specificity in both years, shows a slight improvement: 0.9477 in 2018 versus 0.9573 in 2019. Several situations pertaining to specificity need further notion: absence of terrestrial animal material in blanks (0.90), absence of terrestrial animal material in the presence of fish meal (0.93), and absence of fish in the presence of terrestrial animal material (0.90). to gain further insight, concordance was calculated from 37 replicates per sample/target combination, shown in Table 7.

Table 7 *Values for the quality parameter concordance, representing intralaboratory reproducibility for qualitative methods.*

	Target:	**Terrestrial animal**				**Fish**			
n	**Terrestrial animals**	**0.1%**	**0.1%**	**0%**	***0%***	**0.1%**	**0.1%**	**0%**	***0%***
	Fish	**2%**	**0%**	**2%**	***0%***	**2%**	**0%**	**2%**	***0%***
37	concordance	0.947	1.0	0.693	*0.807*	1.0	0.807	1.0	*0.898*

The report of blood material by one participant in 2018 was not replicated in 2019. Since this situation does not represent reproducibility circumstances, the results of this participant are excluded from the comparison among years. Reproducibility includes the replication of a wrong result as well. The total collection of pairs of replicates obtained in 2018 and 2019 show in only three occasions a duplication of a wrong observation (total number of pairs is 296). This has only a very minor effect on the final results as presented in Table 7, which means that the figures primarily indicate the share of pairs of correct results in both years as fraction of all pairs of results obtained in both years for every sample type. The same combinations of contaminants (fish and/or terrestrial animal) as indicated for specificity show notable values for concordance: absence of terrestrial animal material in blanks (CON=0.807), absence of terrestrial animal material in the presence of fish meal (CON=0.693), and absence of fish in the presence of terrestrial animal material (CON=0.807). As in 2018, a majority of participants (close to 75%) has shown to be capable to implement and maintain the microscopic method.
The results indicate that the suboptimal performance for specificity is almost exclusively caused by incidental erroneous identification of non-animal particles; two of the three pairs of incorrect results were achieved for a specificity score. Still specificity needs further attention for avoiding these incorrect observations. The use of a threshold will mimic a few wrong identifications as long as the number of them is below that threshold, but automatically the sensitivity of the method will decrease.

4.2 Application of the microscopic method

The current microscopic method was introduced in 2013 (EC, 2013a). The share of five participants (12.2%) applying an incorrect number of cycles fits in the range of shares as achieved in previous years (2014: 33% of the total participants applied incorrect number of cycles, 2015: 12%, 2016: 13%, 2017: 6%; 2018: 23%; Table 6; van Raamsdonk et al., 2014, 2015, 2016, 2017; 2018). These participants include both public as well as private laboratories. The report form of the IAG proficiency tests provides guidance when results have been entered by changing colour for the cycles still to perform (Annex 4). A comparable active report form could be extended to routine analyses.
The embedding agent used can be supposed to influence the appearance of animal particles, especially those in the sediment. The expertise of a technician, achieved after years of training, is presumably based on one or a few specific types of embedding agents. This assumption is likely to be reflected in the numbers of participants using different types of embedding agents (Table 8). In general, the different steps in a visual method can be organised in three type of elements: technical actions (e.g.

grinding, sieving, preparing the slides, types of equipment used for observations), aspects related to personal preferences for optimal performance (e.g. embedding agent, aperture of condenser, use of polarisation), and administrative actions (e.g. deciding on the number of observation cycles, drawing conclusions from the observations, reporting). Where harmonisation is necessary for the technical and administrative actions (the procedure), the specific circumstances for the observations should meet the precise skills of the observer (the expertise). Precisely the importance of the expertise of the technician for the identification of particles is one of the principal differences between visual methods at one side and chemical analytical methods at the other.

Table 8 *Comparison between some parameter distributions in the IAG proficiency studies between 2008 and 2018. *: number of cycles since 2014.*

parameter	parameter choice	2008	2009 -2017	2018
correct number of cycles *			67.3% - 94.3%	87.8%
share of the total sediment used	minimum	4%	0.2%-3%	6% (1%)
for examination	maximum	100%	100%	100%
embedding agent for sediment	glycerine / glycerol	8	10-25	16
	paraffin oil	18	12-23	12
	immersion oil	8	7-14	8
	Norland Adhesive	0	2-7	4
	chloral hydrate	3	1-0	0
	other (e.g. Depar 3000, water)	8	5-0	0

4.3 PCR

Although the design of the current proficiency test was assumed to be relatively straightforward for the microscopic method, the interpretation of the ruminant PCR results appeared to be more difficult. The pig feed used to produce the samples 2019-A and 2019-D was identical to the feed used for all samples in the 2018 design. This year's results (0.89 and 0.89; Table 4) are highly comparable to the results for the two samples that were not spiked with the ruminant PAP in the 2018 test (0.90 and 0.89; Table 5 in van Raamsdonk et al., 2018). The combination of a PAP of non-ruminant terrestrial origin, encountered microscopically, and a ruminant PCR signal resulting from a legal ingredient, would in practice result in the reporting of the presence of a ruminant PAP. Such a situation would complicate the monitoring of the species-to-species ban, which would be the only enforcement rule for non-ruminant terrestrial animal feeds after lifting the extended feed ban (van Raamsdonk et al., 2019).

A minority of participants reported a positive signal for ruminant material in samples 2019-B and 2019-C, which were assumed to be free of ruminant DNA (Table 5). The WFSR results for these two samples (Table 2) show Cq values for the 10x dilutions at or after the cut-off, but the initial conclusion is presence of ruminant DNA. The Cq values for the pig feed (2019-A and 2019-D) indicate signals arising approximately five cycles earlier than those for the ruminant feed (2019-B and 2019-C). These ruminant feed results close to the cut-off value could indicate a not intended very low level of contamination with ruminant DNA, which would clarify the different results of the participants. The results are finally evaluated in the view of the "consensus rule" as applied by FAPAS for GMO detection, explained in FAPAS (2010). This means that the final result of absence for ruminant material as reported by the majority of the participants would be correct.

5 Conclusions and recommendations

5.1 Conclusions

The design of the samples in the current proficiency test should allow the participants to demonstrate their performance under legal requirements (0.1% w/w): two samples with terrestrial animal material spiked at the legal technical requirement for monitoring methods in the presence and absence of fish material (2% w/w), and two additional samples without terrestrial animal material with the same conditions for fish material. The two samples containing fish material were intended to mimic feed for aquaculture. The design of this year's study is identical to that of the test of 2018.
The total overview of results for the microscopic method shows some suboptimal values for specificity. Four participants and one participant, respectively, reported particles of terrestrial animals or of fish material in the blank sample 2019-B. The intra-laboratory reproducibility, expressed as concordance, calculated from pairs of results for identical samples, shows lower values for the combination of contaminations sowing specificity issues. These results indicate that varying sets of participants reported errors in identification of particles. Numbers of particles less than the maximum number possible in the report form per cycle (20) pertain primarily to terrestrial animal material in the presence of fish meal.
The detection of 3% of bakery by-products, part of the pig feed, by most participants adds to the complicating situation that presence of authorised ruminant material containing DNA results in positive signals. The list of recognised sources such as milk and milk products, and ruminant gelatine can be extended with bakery by-products, which is important for the recycling of food by-products.

5.2 Recommendations

- The documentation for and training of microscopists for correct identification of particles of animal origin would deserve further attention in order to guard specificity.
- Evaluation of several aspects of the application of the current microscopic methods would be beneficial for improving harmonization among the laboratories applying the microscopic method.

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Annex 1 Introduction to the test

Test 2019-A: animal proteins in feed

The IAG ring test animal proteins in feeds is designed to apply both the microscopic method and the PCR ruminant method. The procedures to be followed are described in Annex VI of Regulation (EC) 152/2009 from the European Union, amended by (EC) 51/2013, and the related SOPs. All the documentation can be found on the website of the EURL AP: http://eurl.craw.eu/index.php?page=187.
The jars contain 50 gram of feed, which is sufficient for carrying out three cycles of the microscopic method and/or for carrying out the PCR analysis, if necessary according to the SOPs. Take care to homogenise the content of each vial before taking the amount for analysis. The samples are prepared in such a way that you can start with the procedure as described in "EURL-AP SOP operational schemes", followed by the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). If more than 16 fragments per category are found in any cycle, just choose "16" from the drop-down list.
Based on the average number of particles found, you have to make the decision whether each of the two types is absent in a sample (zero particles on average), below threshold (between 1 and 5 particles on average) or present (6 or more particles on average).
In addition to the work flow as presented in the paragraphs 2.1.3.4 until and including 2.1.4.3, it is mandatory to weight the sediment BEFORE and AFTER the analysis as performed in every determination.
All results can be entered in the report form with "animal proteins" in the name, which will be send to you separately.

Annex 2 Basic instructions for the test procedure

IAG ring test 2019 animal proteins

Instructions for the IAG ring test

1 You have received a box with an introduction letter and four vials containing 50 grams of possibly contaminated animal feed. Please report the receipt of your package as soon as possible by E-mail to the address mentioned below.

2 The samples have to be analysed according to Annex VI of Regulation (EC) 152/2009 from the European Union, modified by (EC) 51/2013. The consolidated version and the SOPs can be found on the EURL website. The sample design allows to carry out the PCR ruminant analysis, but follow the SOPs carefully! **Take care to homogenise the content of each vial before taking the amount for analysis**.
The samples are prepared in such a way that you can start with the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The sample amount allows you to analyse three determinations of 10 grams as indicated in paragraph 2.1.4.3. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle and a final conclusion.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, horn, skin, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). If more than 16 particles are found in any category, please enter the value 16.
The report form is interactive: if the results in the first determination cycle make it necessary to perform a second or third analysis according to the requirements of the Regulation, additional cells will turn pink.
The final conclusion, according to Regulation (EC) 152/2009, can be reported in three ways, depending on the average number of particles found per category:
= Zero particles: animal proteins **absent**. If the first determination reveals no particles in any category, a second determination is not necessary.
= More than 5 particles on average per determination: **present**.
= Between 1 and 5 particles on average: sample is positive but a risk of a false positive result cannot be excluded. For the sake of the framework of the current report form the term 'suspect' has to be chosen.

Click here for the Regulation and connected SOPs

3 Reporting consists of the following steps:

3a Please fill in the questionnaire on the page "Procedure".
Most of the cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list.

Your unique lab number is mentioned in the introduction letter, enclosed in the box.
All the fields with a drop-down list have to be completed.

3b Please enter your results in the fields at page "Results". Your unique lab number automatically shows up after you have entered it at the page Procedure. Enter yourself the four unique labels of the vials.

All fields with a drop-down list have to be completed. Please add the exact sediment weight in milligrams, without a decimal sign, of the total amount just before analysis and the remaining amount just after analysis.

4 After completing the two forms "Procedure" and "Results", they have to be sent to the organisers in two ways:

4a Save the Excel file by using "Save as …", add your unique lab code to the end of name (replace the ## signs with your lab number). The forms have to be sent by E-mail as Excel file and as a scan (*.PDF) to leo.vanraamsdonk@wur.nl AND to microscopie.WFSR@wur.nl.

4b Results will be included in the final analyses and report only if both forms are send in by electronic mail, and after the proper receipt of the requested fee.

5 Direct any questions to leo.vanraamsdonk@wur.nl

6 Closing date is Friday April 18th, 2019.

WFSR Wageningen UR, the Netherlands

Annex 3 Report form for procedure details

Please complete at least all the cells with a drop-down list	select your choice from a drop-down list	type in your answer if necessary
IAG ring test 2019 animal proteins		
Please select your unique lab number	**-- select --**	
Have you read the ring test instructions?	-- select --	
Did you apply PCR ruminant detection method?:	-- select --	
Did you apply grinding before performing the detection procedure?	-- select --	
Indicate your glassware for sedimentation	-- select --	
if other, please specify		
Describe your sedimentation agent	-- select --	
if other, please specify		
Did you apply staining of the sediment (e.g. alizarin staining) as standard procedure?	-- select --	
Did you examine at lower magnifications (using a binocular)?	-- select --	
Indicate the size of cover glass	-- select --	
Please describe your embedding agent for the sediment material	-- select --	
if other, please specify		
Did you use the expert system ARIES for identification of particles?	-- select --	

Annex 4 Report form: results

Please complete all the cells which are pink coloured. Additional cells will turn pink depending on your results. If more than 16 particles were found in any category, please enter the value 16.

IAG ring test 2019 animal proteins

lab number				
sample number				
First determination				
weight of sediment before analyses (in mg)				
weight of sediment after analyses (in mg)				
sediment % used for analyses	-		-	
	land	fish	land	fish
Result of first determination cycle	- select -	- select -	- select -	- select -
Second determination				
	-		-	
	land	fish	land	fish
Third determination				
	-		-	
	land	fish	land	fish
Total number of particles per category	0	0	0	0
PCR results				
Ruminant (EURL method)				
	land	fish	land	fish
Final conclusion	- select -	- select -	- select -	- select -
Type of particles				
Comment, if necessary				

Annex 5 List of participants

Austrian Agency for Health and Food Safety-AGES	Austria
Laboratorium ECCA nv	Belgium
LFSAL	Belgium
FLVVT	Belgium
Croatian Veterinary Institute	Croatia
Danish Veterinary and Food Administration	Denmark
Inovalys-Nantes	France
Laboratoire Départemental d'Analyse & de Recherche	France
S.C.L. Laboratoire de Rennes	France
LUFA-Speyer	Germany
LUFA Nord-West	Germany
SYNLAB Umweltinstitut GmbH, Lebensmittelinstitut Jena	Germany
WESSLING GmbH	Germany
CVUA-RRW	Germany
SGS Germany GmbH	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
Landeslabor Berlin-Brandenburg	Germany
LUFA Rostock	Germany
LTZ Augustenberg	Germany
Thüringer Landesanstalt für Landwirtschaft Jena	Germany
Futtermittelinstitut Stade (LAVES)	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
Equine Centre	Ireland
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
Ministero delle politiche agricole alimentari e forestali, Laboratorio di Modena	Italy
Nutreco Nederland BV - Masterlab	Netherlands
ForFarmers	Netherlands
Eurofins Food Testing Rotterdam BV	Netherlands
CCL - Nutricontrol	Netherlands
TLR	Netherlands
Nofima AS	Norway
Synlab StjØrdal	Norway
SGS del Perú S.A.C.	Peru
World Survey Services SAC	Peru
Cargill Poland	Poland
Lab. Regional de Veterinária	Portugal
Scientific Veterinary Institute "Novi Sad"	Serbia
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	Slovenia
Dirección General de Produccion Agropecuaria, Laboratorio Agrario Regional	Spain
National Veterinary Institute, SVA	Sweden
Agroscope (ALP), Swiss Research Station	Switzerland
CPF (Thailand) Public Company Limited	Thailand

Annex 6 Details of procedures applied, microscopic method

lab nr	prior grinding	glassware	agent	staining	binocular	size	embedding	ARIES
1	no	chemical sedimentation funnel	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
2	no	beaker (flat bottom)	TCE	no	no	large (22 x 50 mm)	glycerine	no
3	no	other	TCE	no	no	small (20 x 20 mm)	glycerine	no
4	no	beaker (flat bottom)	TCE	no	yes	medium	paraffin oil	no
5	no	special conical glass with cock	TCE	yes	no	small (20 x 20 mm)	glycerine	no
6	no	special conical glass with cock	TCE	no	yes	small (21 x 26 mm)	glycerine	no
7	yes	chemical sedimentation funnel	TCE	yes	no	small (20 x 20 mm)	glycerine	no
8	no	special conical glass with cock	TCE	no	no	small (21 x 26 mm)	immersion oil	no
9	no	conical champagne glass	TCE	no	yes	small (20 x 20 mm)	immersion oil	no
10	no	conical champagne glass	TCE	no	no	small (20 x 20 mm)	glycerine	no
11	no	chemical sedimentation funnel	TCE	no	no	medium	paraffin oil	no
15	no	chemical sedimentation funnel	TCE	yes	yes	medium	immersion oil	no
16	no	chemical sedimentation funnel	TCE	no	yes	small (20 x 20 mm)	glycerine	no
17	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	paraffin oil	no
18	no	special conical glass with cock	TCE	yes	yes	large (26 x 50 mm)	paraffin oil	no
19	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
20	no	conical champagne glass	TCE	no	no	small (20 x 20 mm)	immersion oil	no
21	yes	chemical sedimentation funnel	TCE	yes	no	small (21 x 26 mm)	paraffin oil	no
22	no	chemical sedimentation funnel	TCE	no	yes	small (20 x 20 mm)	Norland adhesive 65	no
23	no	other	TCE	no	no	large (26 x 50 mm)	paraffin oil	no
24	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
25	no	conical champagne glass	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
26	no	beaker (flat bottom)	TCE	no	yes	small (20 x 20 mm)	immersion oil	no
27	yes	chemical sedimentation funnel	TCE	no			paraffin oil	
29	yes	special conical glass with cock	TCE	no	yes	small (20 x 20 mm)	glycerine	no
30	no	special conical glass with cock	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
31	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	Norland adhesive 65	no

lab nr	prior grinding	glassware	agent	staining	binocular	size	embedding	ARIES
32	no	conical champagne glass	TCE	no	yes	large (22 x 50 mm)	immersion oil	no
33	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
34	no	conical champagne glass	TCE	no	yes	small (20 x 20 mm)	glycerine	no
35	no	beaker (flat bottom)	chloroform	no	yes	small (20 x 20 mm)	immersion oil	no
36	no	chemical sedimentation funnel	TCE	yes	no	small (20 x 20 mm)	glycerine	no
37	yes	special conical glass with cock	TCE/PE	yes	yes	small (20 x 20 mm)	glycerine	no
38	yes	chemical sedimentation funnel	TCE	no	no	small (20 x 20 mm)	immersion oil	no
39	no	chemical sedimentation funnel	TCE	yes	no	small (20 x 20 mm)	Norland adhesive 65	no
40	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
41	no	other	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
43	yes	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	Norland adhesive 65	no
44	yes	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
45	yes	chemical sedimentation funnel	TCE	yes	yes	large (22 x 50 mm)	paraffin oil	yes
46	no	special conical glass with cock	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no

Annex 7 Results: presence of animal proteins, microscopic detection

lab nr		sample number				land				fish			
	PCR					A	B	C	D	A	B	C	D
1	yes	178	172	131	524	present	suspect	absent	present	present	present	present	present
2	yes	199	431	117	335	present	absent	absent	present	absent	absent	present	present
3	yes	157	578	208	531	present	absent	absent	present	absent	absent	present	present
4	no	213	571	320	489	present	absent	absent	present	absent	absent	present	present
5	no	192	501	250	293	suspect	absent	absent	absent	absent	absent	present	present
6	no	150	214	180	167	present	absent	absent	present	absent	absent	present	present
7	no	171	249	152	230	present	absent	absent	present	absent	absent	present	present
8	yes	220	200	124	384	present	absent	absent	present	absent	absent	present	present
9	no	290	466	285	160	present	absent	absent	present	absent	absent	present	present
10	no	122	144	348	146	present	absent	absent	present	absent	absent	present	present
11	no	143	361	201	608	present	absent	absent	present	absent	absent	present	present
15	no	206	130	166	209	present	absent	suspect	present	absent	absent	present	present
16	yes	227	165	334	153	present	absent	absent	present	absent	absent	present	present
17	no	129	585	215	433	present	absent	absent	present	present	absent	present	present
18	no	283	333	488	202	present	absent	absent	present	absent	absent	present	present
19	yes	374	228	292	377	present	absent	absent	present	absent	absent	present	present
20	yes	248	123	313	195	present	absent	absent	present	absent	absent	present	present
21	yes	430	221	145	223	present	suspect	suspect	suspect	suspect	absent	present	present
22	no	395	151	474	412	present	absent	absent	present	absent	absent	present	present
23	yes	360	319	411	342	present	absent	absent	present	absent	absent	present	present
24	no	458	438	362	363	present	absent	absent	present	absent	absent	present	present
25	no	493	529	390	174	suspect	absent	absent	suspect	absent	absent	suspect	suspect
26	no	542	389	376	125	present	absent	present	absent	absent	absent	present	present
27	no	437	606	397	440	present	absent	absent	present	absent	absent	present	present
29	no	521	207	369	475	present	absent	absent	present	absent	absent	present	present
30	no	605	158	425	251	present	absent	absent	present	absent	absent	present	present
31	no	598	116	306	468	present	absent	absent	present	absent	absent	present	present

lab nr		sample number				land				fish			
	PCR					A	B	C	D	A	B	C	D
32	yes	339	305	418	307	present	absent	absent	present	absent	absent	present	present
33	yes	591	340	558	419	present	absent	absent	present	absent	absent	present	present
34	yes	486	592	439	552	present	absent	absent	present	absent	absent	present	present
35	no	388	284	530	391	present	absent	absent	present	absent	absent	present	present
36	yes	549	312	460	426	present	absent	absent	present	absent	absent	present	present
37	no	577	375	551	398	present	suspect	absent	present	absent	absent	present	present
38	yes	500	396	579	517	present	absent	absent	present	absent	absent	present	present
39	yes	332	347	593	461	present	absent	absent	present	absent	absent	present	present
40	yes	402	487	516	370	present	absent	absent	present	absent	absent	present	present
41	no	311	417	572	496	present	absent	absent	present	absent	absent	present	present
43	yes	416	557	600	580	present	absent	absent	present	absent	absent	present	present
44	no	570	424	404	601	present	absent	absent	present	absent	absent	present	present
45	no	423	550	523	405	present	absent	absent	suspect	suspect	absent	present	present
46	no	556	410	194	594	present	present	absent	present	absent	absent	present	present

Annex 8 Results: reporting details and evaluation, microscopic detection

Explanation:
Blue cells: incorrect number of determination cycles; pink cells: incorrect findings (false positives or false negatives).
Codes for evaluation:
A: participants with correct indications of 19 or 20 particles, found after one determination cycle.
B: participants with correct indications of 6 to 18 particles per cycle for one or more samples, found after any number of determination cycles.
C: participants with a low sensitivity (lower than or equal to 5 particles) for one or more samples.
D: participants with one or more false positive indications.
E: participants with incorrect number of determination cycles for at least one sample.

lab	%sed				#det. cycles				#terr				#fish				EVAL
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
2	27%	50%	33%	26%	1	1	1	1	20	0	0	20	0	0	20	20	A
3	18%	39%	29%	17%	1	1	1	1	20	0	0	20	0	0	20	20	A
4					1	1	1	1	20	0	0	20	0	0	20	20	A
6	55%	78%	50%	50%	1	1	1	1	20	0	0	20	0	0	20	20	A
7	23%	44%	35%	29%	1	1	1	1	20	0	0	20	0	0	20	20	A
8	29%	47%	44%	23%	1	1	1	1	20	0	0	20	0	0	20	20	A
10	13%	15%	15%	10%	1	1	1	1	20	0	0	19	0	0	20	20	A
11	35%	48%	49%	21%	1	1	1	1	20	0	0	20	0	0	20	20	A
16	43%	72%	33%	21%	1	1	1	1	20	0	0	20	0	0	20	20	A
18	100%	100%	100%	100%	1	1	1	1	20	0	0	20	0	0	20	20	A
19	66%	70%	61%	62%	1	1	1	1	20	0	0	20	0	0	20	20	A
20	18%	32%	27%	14%	1	1	1	1	20	0	0	20	0	0	20	20	A
22	34%	24%	23%	27%	1	1	1	1	20	0	0	16	0	0	20	20	A
23	25%	22%	30%	16%	1	1	1	1	20	0	0	20	0	0	20	20	A
24	45%	97%	85%	43%	1	1	1	1	20	0	0	20	0	0	20	20	A
27	34%	45%	26%	23%	1	1	1	1	20	0	0	17	0	0	20	20	A

lab	%sed				#det. cycles				#terr				#fish				EVAL
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
30	12%	28%	15%	11%	1	1	1	1	20	0	0	20	0	0	20	20	A
31	50%	96%	43%	27%	1	1	1	1	20	0	0	20	0	0	20	20	A
32	33%	100%	73%	29%	1	1	1	1	20	0	0	20	0	0	20	20	A
33	44%	88%	60%	41%	1	1	1	1	20	0	0	20	0	0	20	20	A
34	14%	36%	24%	15%	1	1	1	1	20	0	0	20	0	0	20	20	A
36	26%	32%	26%	32%	1	1	1	1	20	0	0	20	0	0	20	20	A
38	10%	19%	13%	6%	1	1	1	1	20	0	0	20	0	0	20	20	A
39	100%	100%	100%	100%	1	1	1	1	20	0	0	16	0	0	20	20	A
40	89%	100%	88%	67%	1	1	1	1	20	0	0	20	0	0	20	20	A
43	86%	100%	100%	77%	1	1	1	1	20	0	0	20	0	0	20	20	A
41	43%	75%	67%	50%	1	1	1	1	16	0	0	16	0	0	16	16	B
44	54%	82%	55%	36%	1	1	1	1	9	0	0	10	0	0	8	14	B
35	1%	1%	1%	1%	1	1	1	1	11	0	0	8	0	0	20	20	B
29	38%	56%	51%	35%	1	1	1	1	16	0	0	6	0	0	16	16	B
9	42%	53%	34%	41%	2	1	2	3	32	0	0	20	0	0	40	60	B E
15	71%	55%	61%	65%	1	1	2	1	20	0	4	20	0	0	40	20	D
25	100%	100%	100%	100%	1	1	1	1	5	0	0	5	0	0	5	5	C E
26					1	1	1	1	20	0	20	0	0	0	20	20	D
45	37%	71%	55%	41%	1	1	1	1	20	0	0	9	7	0	20	20	C D
17	64%	44%	55%	57%	1	1	1	1	16	0	0	16	8	0	20	16	D
5	64%	67%	52%	49%	2	1	1	1	9	0	0	0	0	0	10	20	C
37	18%	47%	14%	11%	2	2	2	2	10	7	0	9	0	0	32	25	C D E
21	18%	21%	17%	12%	2	2	2	2	13	1	5	10	1	0	23	15	C D E
46	65%	57%	36%	60%	2	3	3	2	40	58	0	31	0	0	60	20	D E
1	35%	56%	43%	28%	1	2	1	1	20	1	0	20	20	20	20	20	D

Annex 9 Results: PCR ruminant

lab nr	sample				ruminant			
	A	B	C	D	A	B	C	D
1	178	172	131	524	present	absent		present
2	199	431	117	335	present	absent	present	present
3	157	578	208	531	present			present
8	220	200	124	384	present	present	present	present
16	227	165	334	153	present			present
19	374	228	292	377	present	absent	absent	present
20	248	123	313	195	present		absent	present
21	430	221	145	223	present	present	present	present
23	360	319	411	342	present	absent	absent	present
28	318	291	495	314	present	absent	absent	present
32	339	305	418	307	present	absent	absent	present
33	591	340	558	419	present			present
34	486	592	439	552	present			present
36	549	312	460	426	present			present
38	500	396	579	517	absent		absent	absent
39	332	347	593	461	absent			absent
40	402	487	516	370	present	absent	absent	present
43	416	557	600	580	present			present

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