EURL-AP inquiry on PCR analyses performed on feed or feed material for aquaculture in the European Union for the second halfyear of 2013

Report

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Summary

This report is a follow up on the use of the ruminant PCR test that enabled from 1st June 2013 on the reintroduction of non-ruminant processed animal proteins in feed for aquaculture. Due to a certain frequency of rapid alerts with respect to detection of ruminant DNA in aquafeed or feed material intended for aquafeed and the resulting complaints of the industrial sectors related to the activities of production of the by-products or production of aquafeed, it was checked if really the method gives rise to an abnormal rate of false positive results. Industry claimed that up to 60% of feed material analysed came out positive.

In order to get more insight in this question, two approaches were used :

- Checking by enquiry what is the rate of positive results obtained by the NRLs. The rationale for this being that we know the number of positive cases through the rapid alert system but we ignore what is the total number of samples analysed for that parameter in the NRLs.
- To carry out analysis on samples related or not to those linked with the RASFF to try to understand what is the cause of the unexpected positive result.

The main result of the enquiry as well from the figures collected from the NRLs as from analyses carried out at EURL-AP or on demand of EURL-AP is that the PCR method is not too sensitive and it is not to be blamed for the positive results obtained. When the PCR test was positive on aquafeed or a feed material intended for aquafeed, it generally resulted from contamination by ruminant material of porcine blood products or by-products.

Keywords : enquiry, false positive results, contamination, porcine blood product

1. Introduction

Since the 1st of June 2013 Commission Regulation EU/56/2013 authorises the reintroduction of non-ruminant processed animal proteins in feed for aquaculture. This decision was subrogated to the availability of a validated DNA-based method allowing detection ruminant material that might be present in feed. The method that can be used is described in the revised Annex VI of Commission Regulation EC/152/2009 as modified by Commission Regulation EU/51/2013 published on the 16th of January 2013.

The implementation of this PCR method in combination with the light microscopic method depends on the type of feed being tested and follows operational protocols in accordance with standard operating procedures (SOP) established by the European Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) and published on its website.

A prerequisite of the success of the proposed combinatory approach relies on the strict following of the related SOP.

During the first months after this reintroduction, some notifications were recorded on the RASFF portal of the Commission. The related industry alerted on the fact that the frequency of alerts was too high and may contribute to a potential damageable image of the aquaculture sector. It was also claimed that when they performed the analysis up to 60% of the feed material analysed had a positive outcome with the ruminant PCR test. In order to clarify the situation, the EURL-AP in agreement with DG Sanco conducted a survey through the network of NRL to acquire data and figures related to the analyses of fish meals and ingredients intended to fish feeding over the period from the 1st of June until the 31st of December 2013. The collaboration of the NRLs was on a voluntary basis under conditions of anonymity.

The request for information was diffused through the network on the 19th of November 2013 and inquiry forms had to be sent back to the EURL-AP on the 10th of January. [Annex 1]

Next to that it was also tried to get more insight analytically on what is the origin of some of the unexpected positive results that had been found.

2. Operational scheme for PAP detection in feedingstuffs

The SOP entitled ‘operational protocols for the combination of light microscopy and PCR version 2.0 [Annex 2] is a binding document detailing the operational protocols that shall be followed according to the final destination of the feed materials.

In the case of feed materials or feed intended for aquaculture animals, the second protocol (as illustrated in **Figure 1**) must be followed.

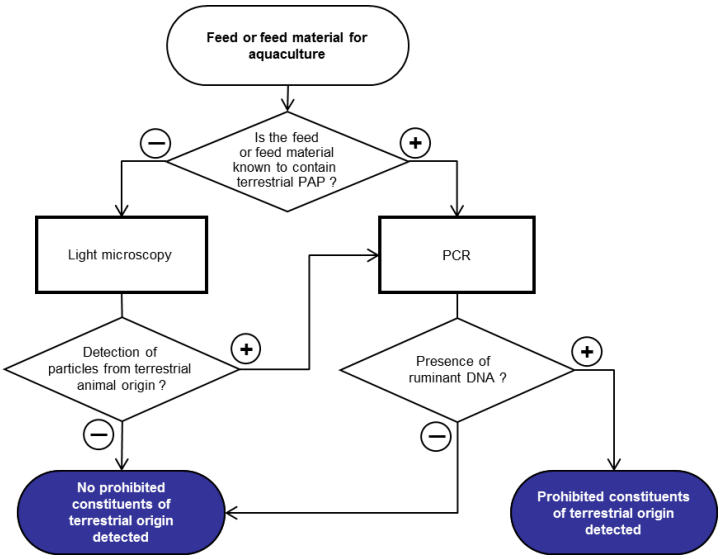

Figure 1 – Flow chart on how to analyse feed and feed material for aquaculture.

3. Survey results

The rate of participation to the survey reached 63% (15 NRLs out of 27) which is beyond expectations.

A list of participating Member States is mentioned in Annex 3.

The results are summarised in **Table 1.**

Table 1 : Summary of data provided by the NRLs that participated to the survey

Member state	Total nb official analyses	Methods used		Positive ruminant DNA detection		
		Microscopy	PCR	Total	fish feed	fish feed ingredients
#1	6	2	4	3	3	0
#2	52	52	1	1	1	0
#3	16	16	1	1	1	0
#4	3	3	1	1	1	0
#5	17	17	0	0	0	0
#6	0	0	0	0	0	0
#7	11	0	11	0	0	0
#8	0	0	0	0	0	0
#9	43	43	0	0	0	0
#10	69	54	57	35	15	20
#11	0	0	0	0	0	0
#12	20	20	4	2	2	0
#13	163	163	18	0	0	0
#14	43	43	13	10	10	0
#15	72	72	0	0	0	0
TOTAL	***515***	***485***	***110***	***53***	***33***	***20***

Among the participants 3 did not perform any analysis on aquaculture intended materials. For the 12 other Member States the number of official analyses varies from 3 to >150.

Investigations by light microscopy are largely predominant to those by PCR (485 *vs* 110). This reflects the fact that either the samples to be analysed are not presenting information on the possibility of containing terrestrial PAP or that analyst still prefer a first analysis by light microscopy. In only one Member State (#7) analyses are performed directly by PCR which delivers indirect evidence of declaration or labelling.

From the 110 PCR analyses about half of them were positive. From this pool of positive ruminant DNA detection, 33 concerned fish feeds and 20 ingredients intended for aquaculture. It has to be noted that 66% of positive ruminant DNA detection comes only from one Member State (#10).

The explanations delivered by the participating NRLs related to the positive cases in the survey were:

- Use of porcine blood meal (for 3 samples restricted to one supplier)
- Cross contamination of feed (1 sample, derived from results that were positive but close to the cut-off value for the used PCR platform)
- Contamination by whey powder and skimmed milk (1 sample)
- Use of greaves from bovine origin
- Blood meal from porcine origin and bovine origin (several samples)

This number of positive ruminant DNA detection (53) has to be tempered by comparing it to the number of recorded posts on the RASFF for the 1st June – 31st December 2013 period. Only 13 notifications were recorded (**Table 2**).

From the 13 notifications made on the RASFF platform regarding the detection of ruminant DNA, 7 were performed on compound feeds or feed ingredients which were not intended to aquaculture (protein concentrate for dairy cows, compound feed for bovines, porcine blood powders not referred as to be used for aquaculture, poultry meals). According to the SOP operational scheme the use of PCR detection of ruminant DNA had not to be applied. A single microscopic analysis is prescribed. Only six (eventually extended to ten, as the blood products could be used in aquafeed) relevant and justified notifications related to the disclosure of ruminant DNA (in green in **Table 2**) are thus to be taken into account.

Based on a total of 515 analyses on fishmeals or fish feed ingredients, as collected from this survey, the six to ten notifications from the RASFF barely represents 1.2 - 1.9 % of problematic cases. Considering (1) that the survey only gathered data from only 15 participants and that some large Member states did not contributed to the inquiry and (2) that the notifications are mandatory on the RASFF, it is reasonable to conclude that the ~2% is a far overestimated percentage. A real picture on the situation of ruminant DNA detection in fish feeds or fish feed ingredients in the EU would reveal to be significantly lower than 2% and hence to be considered as a background noise within the sector.

Table 2 : Notifications from RASFF of presence of ruminant DNA in feed, feed materials and feed premixtures for the period from 1st of June 2013 to 31st of December 2013

date	reference	product type	notification type	notification basis	notified by	origin	subject	distribution	action taken	distribution status
compound feeds										
9/10/2013	2013.1346	feed	information for follow-up	official control on the market	Latvia	from Lithuania	presence of ruminant DNA in protein concentrate for dairy cows from Lithuania	Latvia	recall from consumers	no distribution from notifying country
23/10/2013	2013.1408	feed	information for follow-up	official control on the market	Latvia	from Lithuania	presence of ruminant DNA (bovine) in compound feed for dairy cows from Lithuania	Latvia	recall from consumers	no distribution from notifying country
28/11/2013	2013.1563	feed	information for follow-up	official control on the market	France	from Netherlands	presence of ruminant DNA in compound feed for fish from the Netherlands	France	withdrawal from the market	no distribution from notifying country
20/12/2013	2013.1709	feed	information for follow-up	official control on the market	Cyprus	from Italy	presence of ruminant DNA in fish feed from Italy	Cyprus	no action taken	no distribution from notifying country
feed materials										
22/08/2013	2013.1152	feed	information for follow-up	official control on the market	Czech Republic	from Italy	fragments of bones of land animals (presence of land animals components, chicken DNA, ruminant DNA) in tuna meal from Italy	Spain, Czech Republic	use for other purpose than food/feed	distribution to other member countries
19/09/2013	2013.1276	feed	information for follow-up	official control on the market	France	from Spain	presence of ruminant DNA in porcine blood powder from Spain	France	no action taken	information on distribution not (yet) available
9/10/2013	2013.1349	feed	information for attention	official control on the market	Netherlands	from Belgium	presence of ruminant DNA in pork hemoglobin powder from Belgium	Germany, Netherlands	no action taken	product (presumably) no longer on the market
23/10/2013	2013.1411	feed	information for follow-up	company's own check	Latvia	from Lithuania	presence of ruminant DNA in compound feed from Lithuania	Latvia	recall from consumers	no distribution from notifying country
14/11/2013	2013.1496	feed	information for attention	official control on the market	France	from Netherlands	presence of ruminant DNA in fish meal from the Netherlands	France		product (presumably) no longer on the market
28/11/2013	2013.1571	feed	information for attention	official control on the market	Greece	from Portugal	presence of ruminant DNA in complete feed for fish from Portugal	Spain, Greece	no action taken	product (presumably) no longer on the market
11/12/2013	2013.1640	feed	information for follow-up	official control on the market	Czech Republic	from Italy	bone fragments (mammalian and fish) and presence of ruminant DNA in poultry meal from Italy	Czech Republic	use for other purpose than food/feed	no distribution from notifying country
27/12/2013	2013.1734	feed	information for follow-up	official control on the market	France	from Germany	presence of ruminant DNA in pork blood powder from Germany	France	withdrawal from the market	no distribution from notifying country
feed premixtures										
14/11/2013	2013.1495	feed	information for follow-up	official control on the market	France	from Italy	presence of ruminant DNA in fish feed from Italy	France	informing authorities	distribution restricted to notifying country

4. Analytical results

Some of the RASFF results were only reported after confirmation by the EURL-AP but not all. For several of these latter samples that were not checked by the EURL-AP, it was attempted to get part of the sample analyzed at EURL-AP level after notification in order to try to find out if it was or not a false positive result as claimed by operators. If in most cases official results were confirmed it also happened that on some porcine haemoglobin products the outcome for the ruminant target was negative at EURL-AP and not necessarily because the sample was in a grey zone where indeed repeats may sometimes have different results as at least on one sample the result was clearly negative. This means the proficiency of the labs may be a problem but that is why the EURL-AP 2014 PCR proficiency test will integrate such kind of samples, the implementation of the method is still recent and this kind of problem will progressively disappear.

More in depth analysis were carried out on some samples received from the NRL-network to try to find out what was exactly the origin of the positive result with the ruminant PCR test. Some indeed argued that it might be a contamination by milk proteins. However this can be checked by use of ELISA tests targetting casein and β-lactoglobulin. These tests were outsourced at another institute (CER, Marloie, Belgium). Except in one case where extremely low levels of casein was detected in a fishfeed sample (about 0.6 ppm) all other samples although positive for the ruminant PCR tests gave negative results with these ELISA tests targetting milk proteins. It is therefore difficult to claim that the PCR results are false positives due to contamination by whey and skimmed milk. In fact all indicates (but it is difficult to bring a direct evidence) that generally the origin of the ruminant DNA is linked to porcine blood products or by products contaminated by ruminant material. In the mean time at least two producers admit that indeed there was a contamination of the porcine blood products by ruminant material which confirms that the results obtained by PCR correctly detect unauthorized products.

5. Conclusions

Contrary to what has been claimed by some industrial operators, the implementation of the PCR method used to detect ruminant DNA does not result in a lot of false positive results and hence is not too sensitive. However as it is a new method that is a bit more tricky than usual PCR methods, the EURL-AP has to check the proficiency of the laboratories applying this technique in order to get correct results.

As a consequence, the EURL-AP does not see any need for a revision of the prescribed cut-off values at present. Nevertheless, the EURL-AP is open to any possible substantial improvement of the detection methods if desired, provided the minimum detection level of PAPs at 0.1% w/w is scientifically demonstrated by changing any parameter of the method. As revealed by this survey, the EURL-AP strongly recommends laboratories in charge of the analyses to respect scrupulously the operational schemes as they are published and not to perform unneeded PCR tests.

Finally it seems that most of the unexpected positive results that were found arise from the fact that porcine blood products or by-products were contaminated by ruminant material and PCR was able to detect this.

Acknowledgment

We are grateful to the participants of the enquiry for their fruitful collaboration.

Annex 1

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Request for information

Gembloux, 19 November 2013

Dear Colleagues,

Since the 1 June 2013, Commission Regulation EU/56/2013 authorises the reintroduction of non-ruminant processed animal proteins in feed for aquaculture.

After a first 7 months period since this reintroduction, the EURL-AP team wishes to establish at small survey on the results obtained by the NRL network on the detection of animal proteins in fish feed and fish feed ingredients. Some notifications were transferred on the RASFF portal of the Commission but the frequency of notifications vs. the total number of analyses carried out by the NRL network remains unknown. Therefore we would like to ask you some statistical input *on voluntary basis*.

The information we want to collect is the following (**for the period 1 June – 31 December 2013**):

1. Total number of official analyses performed on fish feed or fish feed ingredients : ……..
   a. Total number of microscopic analyses : ……..
   b. Total number of PCR analyses (ruminant target) : ……..
2. Number of positive cases[1] : ……..
   a. Number of positive cases on fish feed : ……..
   b. Number of positive cases on fish feed ingredients : ……..
3. If available, potential explanation of the cause of the positive cases :
   a. ……
   b. ……
   c. ……
   d. ……
   e. ……

The delivered information shall be pooled together and used anonymously. If you are willing to participate, simply fill the present document, scan it and send it to secretary@eurl.craw.eu by the *10 January 2014* the latest. Simultaneously to the sending of the document, feel free to ask any question you might have on the subject into the email.

We thank you in advance for your collaboration

Dr Gilbert Berben
EURL-AP Director

Dr Pascal Veys
EURL-AP NRL Network Manager

[1] By positive cases it is meant ruminant DNA presence is reported.

Walloon Agricultural Research Centre

Wallonie

RÉGION WALLONNE

Annex 2

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EURL-AP Standard Operating Procedure

Operational protocols for the combination of light microscopy and PCR

Experts for edition and revision	
Version 1.0	**Last major revision**
Alessandro BENEDETTO Gilbert BERBEN Hermann BROLL Olivier FUMIÈRE Geneviève FRICK Christoph HALDEMANN Lotte HOUGS Jette MÅRTENSSON Inge PARADIES-SEVERIN Ingrid SCHOLTENS Igor UJČIČ VRHOVNIK Pascal VEYS	NRLs-AP network Pascal Veys

1. SCOPE AND PURPOSE

The purpose of the SOP is to present the operational protocols combining light microscopy and PCR for the detection of constituents of animal origin in feed materials and compound feed. **This SOP is a binding complement to point 1 of Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission Regulation (EU) No 51/2013.**

The current SOP details 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 (feed ban). The final destination of the compound feed or feed materials determines the operational protocol which has to be followed.

Taking into consideration the current European Union legislation regarding the feed ban, the two following protocols for the detection of constituents of animal origin shall be applied depending on the type of feed or feed material being tested.

2. SUMMARY

This SOP details 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 (feed ban). The final destination of the feed or feed materials determines the operational protocol which has to be followed. Two operational protocols are described: one for feed or feed material intended for farmed animals others than aquaculture and fur animals*, and a second for feed or feed material intended for aquaculture animals.

3. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS

NA

4. DEFINITIONS

Abbreviations used :

- SOP : standard operating procedure
- NA : not applicable
- PCR : polymerase chain reaction
- PAP : processed animal proteins

5. HEALTH AND SAFETY WARNINGS

NA

* *Feed and feed ingredients intended for fur animals are not subjected to EU feed ban regulations. It is only referred to them as they are also farmed animals.*

6. EQUIPMENT AND MATERIALS

NA

7. STEP BY STEP PROCEDURE

7.1. Sample preparation

NA

7.2. Protocol 1: for feed or feed material intended for farmed animals other than aquaculture and fur animals (Figure 1)

For this protocol analysis by light microscopy is sufficient to detect the presence of prohibited constituents of animal origin.

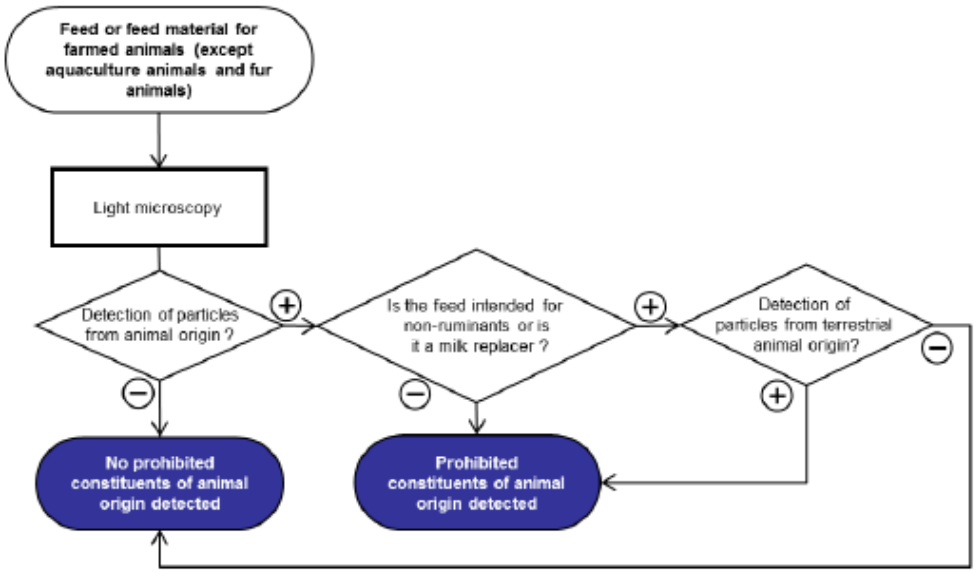

Figure 1. Operational protocol for the analysis of feed or feed material intended for farmed animals other than aquaculture animals and fur animals (e.g. feed for ruminants, pigs, poultry, horses, rabbits,…).

7.3. Protocol 2: for feed or feed material intended for aquaculture animals (Figure 2)

For feed or feed material intended for aquaculture animals (Figure 2), either the light microscopy or the PCR method may be performed in the first instance depending on the composition of the feed.

Light microscopy shall be applied in the first instance when the composition of the feed is unknown or when the feed is not supposed to contain PAP. If the feed is known to contain PAP, as indicated for instance from the declaration or the labelling, the PCR method shall be applied at first.

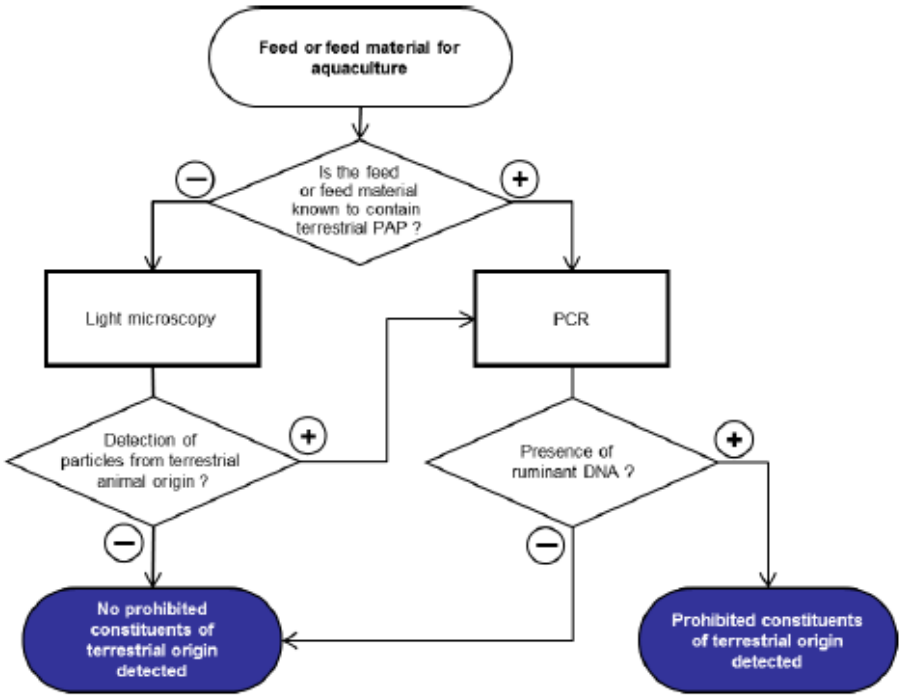

Figure 2. Operational protocol for the analysis of feed or feed material intended for aquaculture animals.

8. INTERPRETATION OF RESULTS

NA

9. REFERENCES

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, L20, 23.1.2013, 33-43.

Version	Publication date	Application date	Repeal date
2.0	29.04.2013	04.05.2013	
1.0	03.04.2013	03.05.2013	04.05.2013

Annex 3

List of participating NRLs.

Austria
Belgium
Bulgaria
Cyprus
Denmark
Finland
France
Greece
Ireland
Italy
Luxemburg
Poland
Portugal
Slovakia
Slovenia