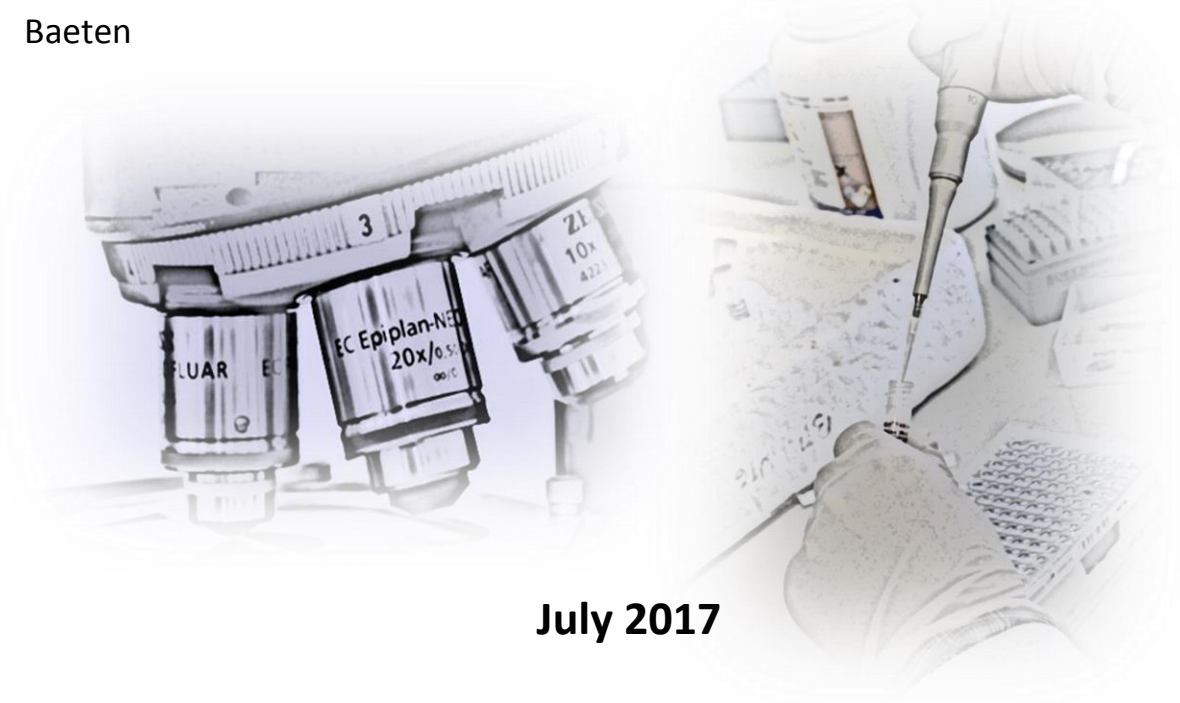
European Union reference Laboratory for animal proteins in feedingstuffs

EURL-AP report on the "Technical zero" to be used with respect to detection of processed animal proteins in feedingstuffs

Authors

Gilbert Berben, Aline Marien, Olivier Fumière, Timur Kaliyev, Véronique Ninane, Viviane Planchon, Marie-Caroline Lecrenier, Pascal Veys, Vincent Baeten



July 2017

Walloon Agricultural Research Centre – CRA-W (Belgium)

ISBN 978-2-87286-104-0
Legal Deposit D/2018/1463/3

Editor :

Centre wallon de Recherches agronomiques

Rue de Liroux, 9

5030 Gembloux (Belgique)

EURL-AP report on the “Technical zero” to be used with respect to detection of animal proteins in feedingstuffs

Abstract

The technical zero is a concept of action limit that DG Sante would like to integrate in the PCR reference methods used for detection of processed animal proteins in feedingstuffs. This report explains how it could be practically addressed for the ruminant PCR method and it shows the potential interests that motivate DG Sante to add this to the legislation.

However, by introducing such an action limit, the risk of transmission of BSE will increase somewhat. This explains why DG Sante asks EFSA to evaluate what is the risk if such an action limit would be adopted. In that perspective, the EURL-AP was requested to provide some useful data to EFSA in order to help to achieve the risk assessment. The report provides such data. Among them one of the main aspects was to solve the problem of conversion of a technical zero expressed in copy numbers of the ruminant PCR target into a probability of presence in terms of mass fraction. The rationale followed to get these data is outlined in detail in the annexes of the report.

List of abbreviations

BLG : beta-lactoglobulin

BSE : Bovine spongiform encephalopathy

Co ID_{50} : Cattle oral Infectious dose

DNA : Deoxyribonucleic acid

EFSA: European Food Safety Authority

ELISA : Enzyme-linked immunosorbent assay

EURL-AP : European Union Reference laboratory for animal proteins in feedingstuffs

GM : Genetically modified

JRC : Joint Research Centre

LOD : Limit of detection

PAP : Processed animal proteins

PCR : Polymerase chain reaction

QRA : Quantitative risk assessment

SOP : Standard operating procedure

TSE : Transmissible spongiform encephalopathy

Table of content

1. Aim

To detect processed animal proteins (PAP) in feedingstuffs there are presently two official methods : light microscopy and PCR (polymerase chain reaction). Both methods are said to have a limit of detection below 0.1 % (expressed in mass fractions) but in most of the cases it is even far below 0.1 %. The use of PCR was required with the partial lifting of the feed ban that allowed use of non-ruminant PAP in aquafeed (European Commission, 2013). Once that PAP can be used in feed, light microscopy (which mainly focusses on bone spicules) is generally no longer useful while PCR can help to detect if the PAP does not contain ruminant material.

A ruminant PCR method was validated by the EURL-AP and its implementation requires the use of a cut-off in order to determine if an amplification signal has to be interpreted as a positive or as a negative result (Benedetto *et al.,* 2014).

Even though it is not frequent in aquafeed, sometimes authorized products (e.g. milk products) can lead to positive results with the ruminant PCR method (which analytically is correct) but thereby be wrongly interpreted as due to the presence of unauthorized ruminant material. This frequency is susceptible to increase if lifting the feed ban would be enlarged for feeding of other farmed animals like pig and poultry. That is the reason why DG Sante would like to introduce a kind of action limit that would be the technical zero.

This document first aims to give evidence of the interest of such an approach by illustrating its usefulness through practical examples. It also shows how to achieve such a technical zero at laboratory level. However, as the introduction of an action limit might increase the risk of propagation of TSE (all depends also at what value the technical zero is set), EFSA is asked to reiterate their quantitative risk assessment (QRA) (EFSA, 2011) to evaluate what is precisely the increase in risk that would be associated to such an action limit and thereby allow the risk managers to integrate or not this new concept in the legislation. The document tries to identify some aspects that might help to achieve this goal, in particular it provides figures that in our opinion could be helpful to EFSA for their risk assessment process in this specific area.

2. Some background information

Before handling the concept of technical zero to be integrated in a new version of the quantitative risk assessment on use of PAPs in feed, it is important to highlight some particular background aspects making understanding of what follows easier.

PCR

PCR is a genetic amplification technique that enables detection of well-defined DNA targets (i.e. a piece of DNA with some well-defined features relating to its sequence in nucleotides). The technique

can be quantitative in the sense that the amount of DNA targets detected can be estimated. Nevertheless, it is impossible to link the quantity of copy numbers obtained in that way to the mass of PAP that it represents. This is due to the fact that according to the rendering process used, the remaining DNA in the sample is more or less degraded.

In what follows, the concept of "copy number of the ruminant target" in a sample that results from a PCR analysis will be widely used. It has to be stressed that this type of information is only of interest if the method to establish this parameter is done according the harmonized way that was set up by the EURL-AP which includes in particular the DNA extraction step on a well-defined test portion as well as the official ruminant PCR method to be performed afterwards on the obtained extract. It is absolutely crucial to stick to that, otherwise the copy numbers that are obtained cannot be compared between labs and no valid interpretation of the results can be done. Just to take one example, the DNA extraction method to be used is defined because it has its own recovery rate. If another method would be used the obtained result in copy numbers might be different.

Limit of detection

There are several definitions possible for the limit of detection (LOD). One that is commonly accepted for a qualitative method can be defined according to Codex Alimentarius as the "concentration at which a positive sample yields a positive result at least 95% of the time" (CCMAS, 2010).

When validating a method it is not required to determine exactly the LOD but this performance parameter can be set as being below or equal to a certain value (CCMAS, 2010). For the light microscopy and PCR methods approved up to now for the detection of PAP, the LOD is equal or below 0.1 % of PAP in feed expressed as mass fractions. This does not mean that a level of PAP in feed lower than 0.1 % (in mass fraction) will not be detected by the method. In fact in most cases it will still be detected. As such, this is not a problem for the practical implementation of the method in a regulatory framework. The important point is that when two different laboratories perform the analysis on a same sample, they arrive to similar results and conclusions.

The consequence of this however is that the developed ruminant PCR method is rather sensitive as this was a requirement if one expects that method is able to detect 0.1 % PAP in feed material or in compound feed whatever the origin of the PAP in terms of process it had undergone. This means that with most of the PAP available on the market, the LOD is in fact much lower than 0.1 % in mass fraction.

Cattle oral Infectious dose (Co ID50)

This is a key parameter used in the previous quantitative risk assessment of EFSA (EFSA, 2011). It corresponds to the oral dose in mass which enables to infect 50 % of cattle in an experimental test. An essential aspect deriving from this parameter is that EFSA absolutely needs to have data expressing the contamination of ruminant feed by ruminant PAP in mass fraction and not in copy numbers.

3. What is a technical zero?

There is not yet a legal definition for a technical zero to be applied to the PCR methods considered by the EURL-AP. It could however be defined as a threshold below which no action should be taken mainly because of the high probability that the positive results are linked to the presence of traces of authorized constituents of animal origin.

4. How to implement the technical zero?

The implementation of a technical zero for ruminant PCR is not a big issue when taking into account what is the present-day SOP for the ruminant PCR (EURL-AP, 2014). Indeed the method as it is applied now is considered as qualitative in the sense that it results in a positive/negative response. However as this requires also to determine a cut-off, the method is, if not completely quantitative, at least semi-quantitative because it makes it possible to check if the signal obtained is larger or smaller than a cut-off. To achieve this requires the use of a calibration curve that can be built up thanks to the existence of European reference material consisting of certified amounts of plasmids (reference material ERM-AD482 ruminant pDNA calibrant - JRC-IRMM, 2015) bearing the target on which the PCR is focused (the set consists of three vials at different concentrations of such plasmids). This same reference material could also be used to define a technical zero and thus to compare if a sample provides a signal with a higher or lower copy number than the technical zero.

This however also means that the technical zero can only be expressed in copy numbers of the target for the considered PCR method.

5. Examples of the interest of the use of a technical zero

Before the implementation of the new legislation lifting the ban on the use of non-ruminant PAP in aquafeed (European Commission, 2013), it was checked by the EURL-AP on aquafeed from several producers in Europe what would be the result of applying the ruminant PCR method on aquafeed that was supposed to be free of terrestrial PAP as the material was collected before the new regulation 56/2013 was in force. Amazingly, 14 out of 58 aquafeeds under analysis (Table 1 – samples of lines 1 to 58) delivered generally late but nevertheless positive signals with the ruminant PCR test. Specificity of this PCR method had however been thoroughly checked and it was almost impossible that any lack of performance at that level was the cause of the problem. Sometimes presence of milk or dairy products could explain the signal. Nevertheless, that accounted only for a minority of aquafeed in which a positive ruminant PCR result occurred. It appeared with all the verifications that were performed that the unexplained positive results only could arise from the use of pig blood (which is an authorized product) but contaminated by ruminant blood (which is unauthorized). Even industry finally admitted that this was the explanation. However the levels of detected ruminant targets were indeed not that high.

Table 1 : Results obtained on 73 aquafeeds (compound feed with fish meal) analysed by light microscopy, PCR and ELISA methods.
Legend: + : positive result; - : negative result; N.D. = not done – The result between brackets for the PCR porcine target refers to a result obtained through a different PCR test than the common one used at EURL-AP.

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
1	DQ-08-0697-025	Trout feed	Fish meal of Chili 32 %, soybean, fish oil 15 %, pig blood powder 10 %, wheat, maize gluten, minerals	+	-	-	-	-	-	+	N.D.	N.D.	/
2	DQ-08-0697-026	Carp feed	Brazilian soybean oilcake (48% crude proteins), wheat, fish meal 15 %, maize gluten, minerals	+	-	-	-	-	-	+	N.D.	N.D.	/
3	DQ-08-0697-027	Feed for carps, koï, goldfish	Brazilian soybean oilcake (48% crude proteins), flour, fish meal of chili 5%, soybean, fish oil 1.8 %, minerals, chalk, anti-oxidants	+	-	+	(+)	-	-	+	-	-	12
4	DQ-08-0697-028	Juvenile sturgeon feed	Fishmeal of Chili 40 %, soybean, maize gluten, fish oil, wheat, vitamin E	+	-	-	-	-	-	+	N.D.	N.D.	/
5	DQ-08-0697-029	Sturgeon grow-out feed	Fish meal of Chili 40 %, soybean, wheat, fish oil, maize gluten, minerals	+	-	-	-	-	-	+	N.D.	N.D.	/
6	DQ-08-0697-030	Fry crumbs	Fish meal (herring) 78.5 %, flour 15 %, fish oil 4.5 %, additives	+	-	-	-	-	-	+	N.D.	N.D.	/
7	DQ-08-0697-031	Fry crumbs	Fish meal (herring) 78.5 %, flour 15 %, fish oil 4.5 %, additives	+	-	-	-	-	-	+	N.D.	N.D.	/
8	DQ-08-0697-032	Fry crumbs	Fish meal (herring) 78.5%, flour 15 %, fish oil 4.5 %, additives	+	-	-	+	+	-	+	-	-	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
9	DQ-08-0697-033	Fry crumbs	Fish meal (herring) 78.5 %, flour 15 %, fish oil 4.5 %, additives	+	-	-	+	+	-	+	-	-	/
10	DQ-11-0174-001	Aquafeed	Unknown	+	-	-	-	-	-	+	N.D.	N.D.	/
11	DQ-11-0174-031	Aquafeed	Unknown	+	-	-	-	-	-	+	N.D.	N.D.	/
12	DQ-11-0212-003	Non-floating feed for trouts	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
13	DQ-11-0212-004	Trout feed	Unknown	+	-	-	+	+	-	+	N.D.	N.D.	/
14	DQ-11-0212-011	Complete feed for sturgeons	Fish meal, products of terrestrial animals, fat, oil, oilcake, cereals, yeast, minerals	+	-	+	+	-	-	+	-	-	136
15	DQ-08-0622-002	Carp feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
16	DQ-08-0622-003	Trout feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
17	DQ-08-0622-005	Trout feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
18	DQ-08-0622-006	Fish feed	Unknown	+	-	-	-	-	-	+	N.D.	N.D.	/
19	DQ-08-0622-007	Fish feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	ruminant target
20	DQ-08-0622-008	Trout feed	Unknown	+	-	+	+	-	-	+	-	-	17
21	DQ-08-0622-009	Fish feed	Unknown	+	- (4part.)	+	-	-	-	+	-	-	31
22	DQ-08-0622-010	Trout feed	Unknown	+	-	+	+	+	-	+	+ (0.3 ppm)	+ (>5 ppm)	19
23	DQ-08-0622-011	Fish feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
24	DQ-08-0622-013	Fish feed	Unknown	+	-	-	-	-	-	+	+ (1 ppm)	+ (>5 ppm)	/
25	DQ-08-0622-015	Trout feed	Unknown	+	-	-	+	+	-	+	N.D.	N.D.	/
26	DQ-08-0622-016	Trout feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
27	DQ-08-0622-017	Fish feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
28	DQ-12-0402-001	Complete fish feed	Fish trimmings meal, fish oil, peas, sunflower extract	+	- (1part.)	-	-	-	-	+	N.D.	N.D.	/
29	DQ-12-0402-002	Complete fish feed	Fish meal, fish oil, sunflower extract, wheat, soybean oilcake, Faba beans, maize gluten, distiller's dried grains with soluble, vegetal oil, yeasts and like products, grain flour	+	-	-	-	-	-	+	N.D.	N.D.	/
30	DQ-12-0402-003	Complete salmon feed	Fish meal, fish oil, wheat, soya, vitamins and minerals, *Phaffia* yeast	+	- (1part.)	+	-	-	-	+	-	-	12

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
31	DQ-12-0402-004	Complete salmon feed	Fish oil, fish meal, sunflower meal, wheat, soybean oilcake, wheat gluten, beans, vitamins and minerals, L-lysine, DL-methionine, single cell pigment (astaxanthin of *Paracoccus carotinifaciens*)	+	-	-	-	-	-	+	N.D.	N.D.	/
32	DQ-12-0402-005	Bass and bream feed	Fish meal, tuna meal, rework (10 %), rapeseed oil, premix vitamins and minerals, spraydried bloodmeal (7 %), soybean oil cake, maize gluten, rapeseed oilcake, field peas, fish oil, monocalcium phosphate, methionine	+	-	+	+	-	-	+	-	-	20
33	DQ-12-0402-006	Sturgeon feed	Fishmeal, tuna meal, recycling (7 %), rapeseed oil, premix vitamins and minerals, soybean oilcake, wheat gluten, rapeseed oilcake, groundnut oilcake, whole wheat, fish oil, monocalcium phosphate, methionine, lysine	+	-	-	-	-	-	+	N.D.	N.D.	/
34	DQ-12-0402-007	Trout feed	Soybean protein, rapeseed oilcake, wheat gluten, wheat, fish meal, fish oil, Faba beans, sunflower seed oilcake, mono-ammonium phosphate	+	-	-	-	-	-	+	N.D.	N.D.	/
35	DQ-12-0402-008	Trout feed	Soya protein, rapeseed oilcake, wheat, fish oil, fish meal, sunflower seed meal, Faba beans, wheat gluten, mono-ammonium phosphate	+	-	-	-	-	-	+	-	-	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
36	DQ-12-0402-009	Salmon feed	Soya protein, fish meal, rapeseed oil, wheat gluten, fish oil, wheat, Faba beans, mono-ammonium phosphate, yeasts and like products, sun flower seed meal	+	-	-	-	-	-	+	N.D.	N.D.	/
37	DQ-12-0402-010	Salmon feed	Soybean protein, rapeseed oil, wheat, fish oil, fishmeal, wheat gluten, Faba beans, sunflower seed meal, mono-ammonium phosphate	+	-	-	-	-	-	+	N.D.	N.D.	/
38	DQ-12-0402-011	Complete fish feed (growing rainbow trout)	Fish meal, soybean meal, fish oil, wheat gluten, wheat, maize gluten, rapeseed, sunflower seed, soya protein concentrate, rapeseed oilcake, vitamins, lysine, minerals, methionine	+	-	-	-	-	-	+	N.D.	N.D.	/
39	DQ-12-0402-012	Carp out-grow feed	Soya meal, wheat, fish meal, sunflower seed, maize gluten, flavour, fish oil, rapeseed oil, vitamins, mono ammonium phosphate, anti-fungal/anti-oxidant, yeasts, minerals, algae, betaine, astaxanthin	+	-	-	-	-	-	+	-	-	/
40	DQ-12-0402-013	Complete fry feed	Fish meal, wheat gluten, fish oil, soybean oilcake, maize gluten, wheat, fish protein hydrolysed, Faba beans, vitamins, yeasts, minerals, permitted flavour, lysine, methionine	+	-	-	-	-	-	+	N.D.	N.D.	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
41	DQ-12-0402-014	Complete fish feed	Fish meal, wheat gluten, fish oil, maize gluten, peas, soybean oilcake, premix vitamins and minerals	+	-	-	+	-	-	+	N.D.	N.D.	/
42	DQ-12-0402-015	Sea bream feed	Amino acids, distiller's dried grains with solubles, fishmeal, fish oil, maize gluten, premix oligo vitamins, rapeseed oilcake, soya meal, soya oil, wheat	+	- (1part.)	+	+	-	-	+	-	-	117
43	DQ-12-0402-016	Sea bream feed	Amino acids, fishmeal, fish oil, linseed oil, maize gluten, premix oligo vitamins, soya meal, soya oil, wheat flower, wheat gluten	+	- (1part.)	+	+	-	-	+	-	-	44
44	DQ-12-0402-017	Complete fish feed	Amino acids, Faba beans, fishmeal, fish oil, linseed oil, maize gluten, premix oligo vitamins, soybean oilcake, soya oil, wheat flower	+	-	+	+	-	-	+	-	-	563
45	DQ-12-0402-018	Complete trout feed	Amino acids, Faba beans, fish meal, fish oil, haemoglobin (10%), linseed oil, other micro-ingredients, premix oligo vitamins, soybean oilcake, soya oil, soya concentrate, sunflower oilcake, wheat, wheat gluten	+	-	+	+	-	-	+	-	-	67
46	DQ-12-0402-019	Fish feed	Fish meal, fish oil, other micro-ingredients, premix oligo vitamins, wheat, wheat flower, wheat gluten	+	-	-	+	-	-	+	N.D.	N.D.	/
47	DQ-12-0402-020	Bass feed	Amino acids, Faba beans, fishmeal, fish oil, maize gluten, premix oligo vitamins, soybean oilcake, soya oil, sunflower meal, wheat	+	-	-	+	-	-	+	N.D.	N.D.	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
48	DQ-12-0402-021	Fish feed	Amino acids, Faba beans, fish meal, fish oil, other micro-ingredients, premix oligo vitamins, soybean oilcake, soya oil, wheat, wheat flower, wheat gluten	+	-	+	+	-	-	+	-	+ (0.6 ppm)	38
49	DQ-12-0402-022	Sturgeon feed	Distiller's dried grains with solubles, fish meal, fish oil, guar protein meal, haemoglo-bin (10%), linseed oil, premix oligo vitamins, rapeseed oilcake, soybean oilcake, soya oil, sunflower meal, wheat, wheat gluten	+	-	+	+	-	-	+	-	-	154
50	DQ-12-0402-023	Fish feed	Rapeseed oil, soya protein, wheat, fishmeal, haemoglobin (11 %), fish oil, rework (5 %), Faba beans, wheat gluten, roasted soya bean, monocalcium phosphate, lysophospholipids, premix methionine, vitamins, antioxidant, ethoxyguin	+	-	-	+	-	-	+	N.D.	N.D.	/
51	DQ-12-0402-024	Fish feed	Fish meal, fish oil, wheat, wheat gluten, rework (3 %), Faba beans, cod-liver oil, premix, lecithin, L-carnitine	+	-	-	+	-	-	+	N.D.	N.D.	/
52	DQ-12-0402-025	Complete fish feed	Fish meal, soya protein, fish oil, hydrolysed fish protein, wheat gluten, Faba beans, wheat, yeast and like products	+	-	-	-	-	-	+	N.D.	N.D.	/
53	DQ-12-0402-026	Complete fish feed	Fish meal, fish oil, wheat gluten, hydrolysed fish protein, Faba beans, soya protein, sunflower seed oilcake, wheat	+	-	-	-	-	-	+	N.D.	N.D.	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
54	DQ-12-0402-027	Atlantic salmon feed	Rapeseed oil, sunflower, North Atlantic fish meal, wheat, soya protein, fish oil, South American fishmeal, maize gluten meal, wheat gluten, additives, rework (2 %)	+	-	-	-	-	-	+	N.D.	N.D.	/
55	DQ-12-0402-028	Fish feed	Unknown	+	-	-	+	-	-	+	-	-	/
56	DQ-12-0402-029	Fish feed	Unknown	+	-	+	-	-	-	+	-	+ (4ppm)	22
57	DQ-12-0402-030	Fish feed	Unknown	+	-	-	+	-	-	+	N.D.	N.D.	/
58	DQ-12-0402-031	Fish feed	Unknown	+	-	-	-	-	-	+	N.D.	N.D.	/
59	DQ-15-0719-004	Atlantic salmon feed	Soya protein concentrate, vegetable oil, fish oil, fish meal, Faba beans, wheat gluten, maize gluten meal, wheat, mono-ammonium phosphate, monocalcium phosphate, yeast	+	-	-	-	-	-	+	N.D.	N.D.	/
60	DQ-15-0719-005	Complete Atlantic salmon feed	Fishmeal, wheat, maize gluten meal, soya protein concentrate, fish oil, vegetable oil, wheat gluten, Faba beans, mono-ammonium phosphate	+	-	-	-	-	-	+	N.D.	N.D.	/
61	DQ-15-0719-006	Complete Atlantic salmon feed	Fishmeal, fish oil, organic soya (bean) expeller, organic wheat, organic peas, mono-ammonium phosphate, yeast by product	+	-	-	-	-	-	+	N.D.	N.D.	/
62	DQ-15-0719-007	Complete fish feed	Fishmeal, fish oil, wheat gluten, protein concentrate extracted from pea, maize starch, yeast, lecithin, vitamins, minerals	+	-	-	+	-	-	+	N.D.	N.D.	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
63	DQ-15-0719-008	Complete fish feed	Fishmeal, fish oil, wheat gluten, protein concentrate extracted from pea, maize starch, wheat gluten, yeast, lecithin, vitamins, minerals	+	-	-	+	-	-	+	N.D.	N.D.	/
64	DQ-15-0719-009	Fish feed	Unknown (label no longer readable)	+	-	-	+	-	-	+	N.D.	N.D.	/
65	DQ-15-0719-010	Complete tilapia feed	Fish meal, wheat, wheat gluten, soybean oilcake, fish oil, krill meal, yeast, pepper	+	-	-	+	+	-	+	N.D.	N.D.	/
66	DQ-15-0719-011	Complete trout feed	Fish meal, soybean oilcake, wheat, fish oil, corn gluten, haemoglobin powder, palm oil, wheat gluten	+	-	-	+	-	-	+	N.D.	N.D.	/
67	DQ-15-0719-012	Complete fish feed	Fish meal, wheat, soybean oilcake, fish oil, maize gluten, wheat gluten, krill meal, yeast, concentrated fish meal juice	+	-	-	+	+	-	+	N.D.	N.D.	/
68	DQ-15-0719-013	Fish feed	Fish meal, protein concentrate from pea, fish oil, wheat gluten, maize starch, lecithin, yeast, vitamins, minerals	+	-	-	-	-	-	+	N.D.	N.D.	/
69	DQ-15-0719-014	Complete trout feed	Poultry meal, wheat, soya, fish meal, rapeseed oil, maize gluten, wheat gluten, soybean oilcake, palm oil, fish oil	+	+	-	+	+	-	+	N.D.	N.D.	/
70	DQ-15-0719-015	Complete fish feed	Fish meal, wheat, wheat gluten, roasted defatted soybean oilcake, fish oil, soya protein concentrate	+	+	+	+	+	-	+	N.D.	N.D.	15
71	DQ-15-0719-016	Complete fish feed	Fish meal, wheat, roasted defatted soybean oilcake, fish oil, wheat gluten	+	+	-	+	+	-	+	N.D.	N.D.	/

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result					ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	Sheep	Fish	BLG	Casein	
72	DQ-15-0719-017	Complete fish feed	Poultry meal, fish meal, wheat, roasted defatted soybean oilcake, fish oil, spray dried haemoglobin powder, maize gluten, wheat gluten	+	+	-	+	+	-	+	N.D.	N.D.	/
73	DQ-15-0719-018	Fish feed	Fish meal, maize starch, fish oil, wheat gluten, protein concentrate from pea, vitamins, minerals	+	-	-	-	-	-	+	N.D.	N.D.	/

If a technical zero would have been applied (let us consider it here at the level of 300 copies), most samples (13 out of 14) – as well those with milk as those supposed with pig blood supposed to be contaminated with ruminant blood – would have been considered as negative.

An additional set of 15 aquafeed samples was analysed more than one year after the implementation of regulation 56/2013 (Table 1 – samples from lines 59 to 73). Only one out of these fifteen samples gave a positive ruminant signal but with an outcome in copy numbers that would be below all levels of technical zero suggested in this document.

A global overview of the distribution of copy numbers in the positive samples of Table 1 is provided by Figure 1.

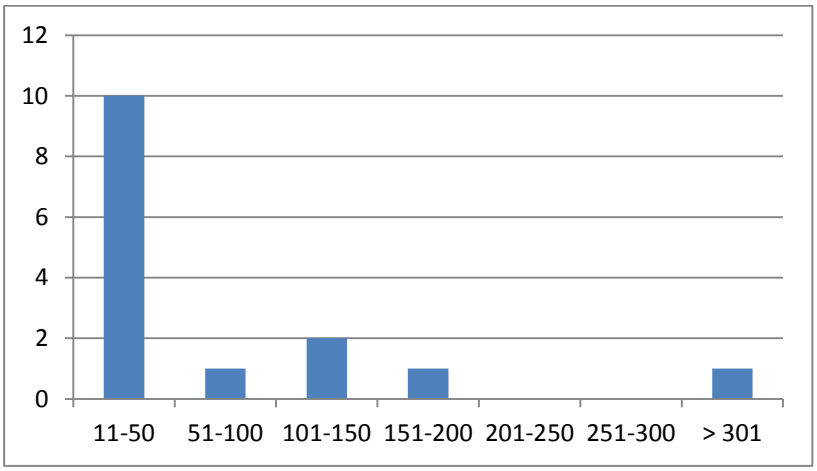


Figure 1 – Frequency of distribution of the copy numbers of the ruminant target found in the positive samples of the aquafeed listed in Table 1.

DG Sante asked the EURL-AP to check what would be the frequency of interference of the official PCR methods if pig PAP was authorized for reintroduction in poultry feed. Therefore, poultry feed originating from several Member States, with a special focus on feed in which feed material of animal origin was present, were analysed by microscopy and PCR (using the ruminant, pig and poultry targets) and by ELISA methods targeting milk proteins (beta-lactoglobulin and casein). The results are reported in Table 2. No wrong interpretation happened with the poultry PCR method even though some of the poultry feed contained egg products (mainly egg shells) but these products are not an important source of poultry DNA. On the other hand, problems are more frequent with the ruminant PCR method because a significant number of the poultry feed do contain milk or whey powder generally not as a feed material but as a carrier for feed additives. This presence is sufficient to trigger a positive PCR result with the ruminant PCR test but the copy numbers detected are rather low and introduction of a technical zero would make it possible to consider most of these samples (12 out of 15) as below an action limit (considering a technical zero at the level of 300 copies of the ruminant target). If the technical zero would be set at 200, 150 or 100 copies, then the samples below the action limit would respectively be : 11 out of 15, 10 out of 15 and 9 out of 15 (Figure 2).

Table 2: Results obtained on 26 poultry feed analysed by light microscopy, PCR and ELISA methods.

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result			ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	BLG	Casein	
1	DQ-16-0229-001	Poultry feed for free range chickens	Maize, dehulled soybean oilcake (GM), wheat, animal fat (porcine and bovine), calcium carbonate, wheat glutenfeed, monocalcium phosphate	-	-	+	-	-	+ (4.8ppm)	+ (1.1ppm)	588
2	DQ-16-0229-002	Feed for laying hens	Maize, dehulled soybean oilcake (GM), calcium carbonate, wheat, rice bran, animal fat, roasted soybeans, alfalfa, dicalcium phosphate, sodium chloride, sodium bicarbonate	-	-	-	-	-	-	-	
3	DQ-16-0229-003	Poultry feed for free range chickens	Maize, wheat, soybean oilcake (GM), wheat by-product, calcium carbonate, soybean oil, premix, sodium chloride, lysine, methionine, essential oils	-	-	-	-	-	-	+ (0.5ppm)	
4	DQ-16-0229-004	Pellets for turkey	Wheat, brewer's grains (maize), maize, soybean oilcake, maize germ, rapeseed oilcake, calcium carbonate, monocalcium phosphate, sodium chloride	-	-	+	-	-	+ (1.3ppm)	+ (2.6ppm)	117
5	DQ-16-0229-005	Feed for laying hens	Maize, wheat, soybean oilcake, sunflower seed oilcake, calcium carbonate, barley, sorghum, roasted soybean, peas, calcium carbonate of seashells, limestone, maize germ, monocalcium phosphate, palm oil, beet molasses, sodium chloride, wheat glutenfeed	-	-	+	-	-	+ (3.5ppm)	+ (3.8ppm)	59
6	DQ-16-0229-006	Cereals for laying hens	Maize, wheat, roasted soybean, peas, sorghum, linseed, barley, sunflower seeds, calcium carbonate of seashells, spinach seeds, oat, paddy rice	-	-	-	-	-	-	-	

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result			ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	BLG	Casein	
7	DQ-16-0229-007	Broilers starter feed	Maize, wheat, soya feed, wheat bran, brewer's grains (maize), sunflower seed oilcake, calcium carbonate, beet molasses, wheat gluten feed, sodium chloride, monocalcium phosphate, palm oil	-	-	+	-	-	+ (1ppm)	+ (6ppm)	33
8	DQ-16-0229-008	Broilers feed	Wheat, brewer's grains (maize), maize, soybean oilcake, maize germ, soybean oil, calcium carbonate, monocalcium phosphate, sodium chloride	-	-	+	-	-	+ (0.9ppm)	+ (3.9ppm)	32
9	DQ-16-0437-001	Poultry feed	Unknown	-	-	+	-	-	-	+ (0.5ppm)	26
10	DQ-16-0437-002	Poultry feed	Unknown	-	-	+	-	-	-	+ (0.5ppm)	23
11	DQ-16-0437-003	Poultry feed	Unknown	-	-	-	-	-	-	-	
12	DQ-16-0475-001	Poultry feed	Wheat, roasted soybean, crude palm oil, soybean oil, methionine, lysine, poultry product, monocalcium phosphate, oyster shells, chalk, sodium chloride, feed enzymes, buckwheat flour, soybean oilcake, acid whey powder, maize	-	1 part	+	-	-	+ (>5ppm)	+ (3.7ppm)	909
13	DQ-16-0516-001	Turkey feed	Maize, wheat, dehulled soybean oilcake, animal fat, dehulled sunflower seed oilcake, dicalcium phosphate (inorganic), dicalcium carbonate (ground limestone rocks), sodium bicarbonate, sodium chloride	-	-	+	-	-	-	-	25
14	DQ-16-0516-002	Hens feed	Maize, dehulled soybean oilcake, wheat, calcium carbonate (from limestone rocks), roasted soybeans, dehulled sunflower seed oilcake, animal fat, calcium carbonate (ground limestone), dicalcium phosphate (inorganic), sodium bicarbonate, sodium chloride	-	-	-	-	-	-	-	

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result			ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	BLG	Casein	
15	DQ-16-0516-003	Broilers feed	Maize, dehulled soybean oilcake, wheat, animal fat, sorghum, dehulled sunflower seed oilcake, roasted soybeans, egg shells, dicalcium phosphate (inorganic), sodium bicarbonate, sodium chloride, sodium butyrate	-	-	+	-	-	+ (1.5ppm)	-	92
16	DQ-16-0516-004	Broilers feed	Maize, dehulled soybean oilcake, wheat, roasted soybeans, animal fat, sorghum, dehulled sunflower seed oilcake, dicalcium phosphate (inorganic), calcium carbonate (ground limestone), sodium bicarbonate, sodium chloride, sodium salt of butyric acid	-	-	+	-	-	-	-	13
17	DQ-16-0516-005	Broilers feed	Maize, dehulled soybean oilcake, wheat, animal fat, sorghum, dehulled sunflower seed oilcake, egg shells, dicalcium phosphate (inorganic), sodium chloride	-	-	+	+	-	+ (0.25ppm)	-	231
18	DQ-16-0516-006	Feed for turkey (> 16 weeks)	Maize, wheat, dehulled soybean oilcake, animal fat, dehulled sunflower seed oilcake, dicalcium phosphate (inorganic), dried egg shells, sodium bicarbonate, sodium chloride	-	-	+	-	-	+ (1.5ppm)	-	470
19	DQ-16-0613-001	Broilers starter feed	Wheat, soybean oilcake, maize, wheat flour, extruded soyabean, wheat middlings, sunflower seed oilcake, rapeseed oilcake, barley, calcium carbonate, premix of additives, dicalcium phosphate, sodium bicarbonate, sodium chloride	-	-	-	-	-	-	-	
20	DQ-16-0613-002	Broilers feed	Wheat, barley, extruded soyabean, dehulled sunflower seed oilcake, maize, wheat middlings, wheat bran, calcium carbonate, premix of additives, sodium chloride, sodium bicarbonate	-	-	-	-	-	-	-	

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result			ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	BLG	Casein	
21	DQ-16-0628-001	Feed for broilers till age 12 days	Wheat, maize, rapeseed oilcake, soybean oilcake, sunflower oilcake, extruded full-fat soya, full-fat rapeseed, rapeseed oil, limestone, vitamins and minerals premix, monocalcium phosphate, lysine, threonine, methionine, sodium chloride, sodium bicarbonate, choline chloride, veterinary drugs, feed enzymes	-	-	-	-	-	-	-	
22	DQ-16-0628-002	Feed for laying hens	Maize, wheat, soybean oilcake, limestone, extruded full-fat soya, sunflower oilcake, rapeseed oilcake, monocalcium phosphate, vitamins and minerals premix, sodium chloride, sodium bicarbonate, methionine, lysine, threonine, feed enzymes	-	-	-	+	-	-	-	
23	DQ-16-0628-003	Feed for broilers from 35 days	Wheat, maize, rapeseed oilcake, soyabean oilcake, sunflower oilcake, full-fat rapeseed, rapeseed oil, limestone, lysine, vitamins and minerals premix, sodium chloride, monocalcium phosphate, methionine, sodium bicarbonate, choline chloride, feed enzymes, threonine	-	-	-	-	-	-	-	
24	DQ-16-0628-004	Fattening turkeys starter feed	Maize, soyabean oilcake, extruded full-fat soya, sunflower oilcake, limestone, monocalcium phosphate, lysine, vitamins and mineral premix, rapeseed oil, methionine, yeast, sodium chloride, sodium bicarbonate, choline chloride, veterinary drugs, threonine, feed enzymes	-	-	-	-	-	-	-	

#	ID CRA-W	Type of sample	Composition	Microscopy		PCR Result			ELISA		Copy numbers ruminant target
				Fish	Terrestrial	Rumi-nant	Por-cine	Poultry	BLG	Casein	
25	DQ-16-0639-001	Feed for game grower	Wheat, wheat bran and dried distiller's grain of wheat, barley, soybean oilcake, extruded rapeseed, sunflower oil cake, veterinary drugs, fish meal, vitamins and mineral premix, methionine, yeast by-product, choline chloride, feed enzymes, limestone, dicalcium phosphate, sodium chloride, sodium bicarbonate, vegetal fat	+	-	+	-	-	+ (4.3ppm)	+ (0.6ppm)	37
26	DQ-16-0639-002	Feed for farmyard layer	Wheat, wheat bran and dried distiller's grain of wheat, barley, biscuit meal, soybean oil cake, sunflower oil cake, premix, lysine, methionine, limestone, dicalcium phosphate, sodium chloride, vegetal fat	-	-	+	-	-	+ (1ppm)	+ (3.9ppm)	191

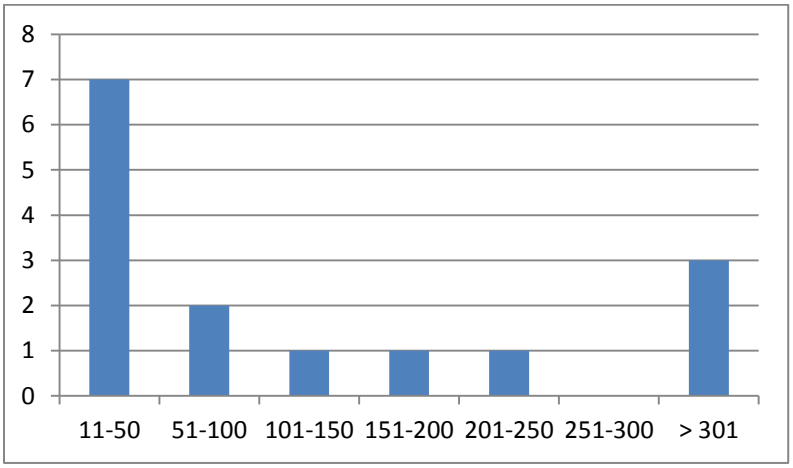


Figure 2 – Frequency of distribution of the copy numbers of the ruminant target found in the positive samples of the poultry feed listed in Table 2.

6. Influence of a technical zero on the quantitative risk assessment

Looking at the parameters under consideration in the last quantitative risk assessment carried out by EFSA (EFSA, 2011) with respect to the risk of BSE by accidental transfer of ruminant PAP to the animal ration of ruminants leads to the conclusion that a technical zero would not change the statements of this assessment. Nevertheless, it seems logical to imagine that introducing a higher action level can have an influence on the risk. The reason however why this has no impact on the former analysis as it was carried out by EFSA is because to grasp this risk enhancement requires an additional scenario for the contamination pathways.

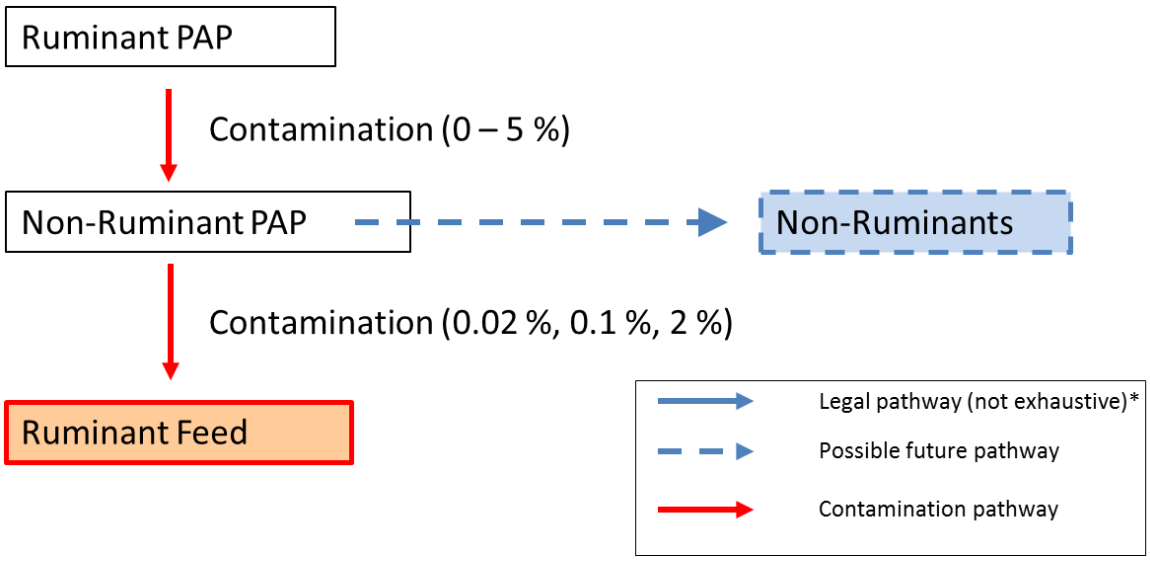


Figure 3 – Pathways of contamination considered in the EFSA QRA (EFSA, 2011) (* pathway not represented in this part of the figure).

Figure 3 takes over a part of the scheme for the contamination pathways considered in the EFSA document (EFSA, 2011) at page 10.

The new completed scheme we propose is illustrated at Figure 4.

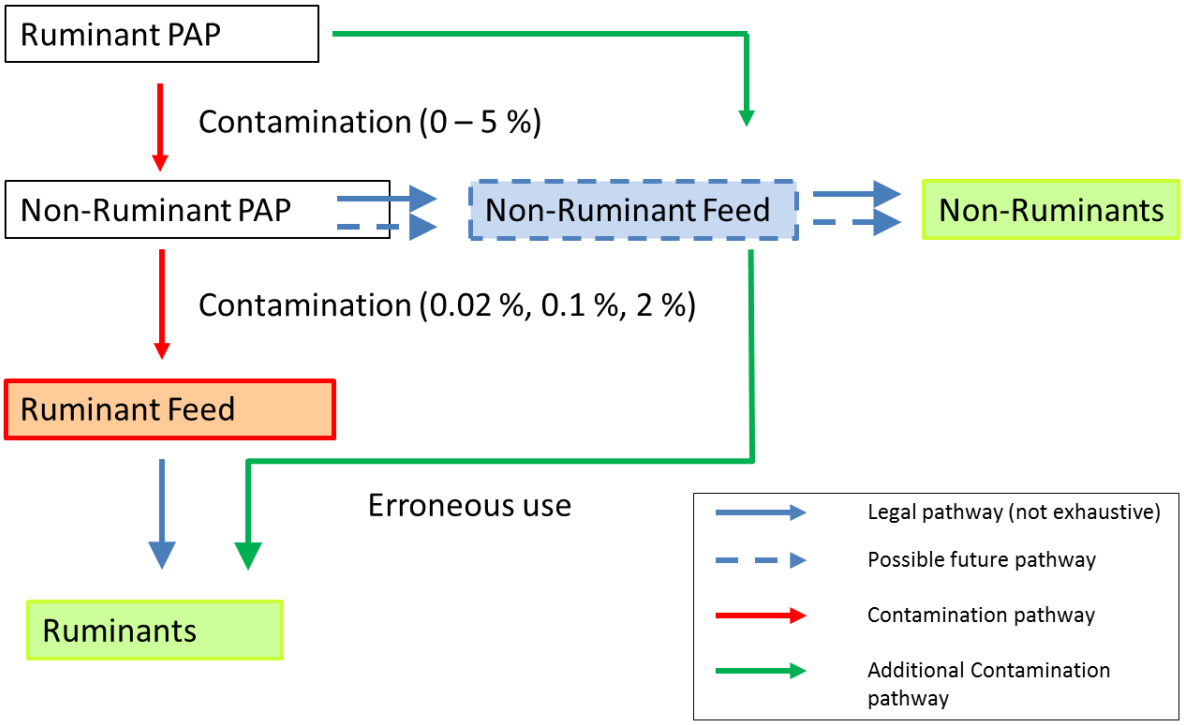


Figure 4 - New scheme proposed to complete the pathways of contamination of Figure 1.

This new scheme considers that there might be a risk that non-ruminant feed may by error be used, be it in small quantities, to feed ruminants. Due to the introduction of a technical zero, this feed might contain ruminant PAP. The same holds true for the non-ruminant PAP, due to the introduction of a technical zero. The background noise of ruminant material in this feed material might increase compared to what it is presently without a technical zero. Nevertheless, the contamination rate that was considered in the EFSA QRA (up to 5 %) is in fact already larger than the quantities that would generally be tolerated by referring to a technical zero. Moreover as the technical zero does not apply to ruminant feed, its introduction as an action level should have no influence on the assumptions made by EFSA for the contamination rate of ruminant feed by non-ruminant PAP. This means that the contamination pathways under consideration in the previous QRA are not influenced by the introduction of a technical zero. Conversely, when non-ruminant feed is erroneously used to feed ruminants, the fact of using an action limit enhances the risk that this might be a pathway to feed ruminants with unauthorized ruminant material. The rationale behind this is that without a technical zero, the probability that there might be unauthorized ruminant material in a non-ruminant feed

(presently only aquafeed) is small as it can be estimated based on data of counter-analysis (see Annex II) that more than 95 % of the aquafeed batches contain less than 0.1 % of unauthorized ruminant PAP (in mass fraction). By introducing a technical zero, the regulation pressure to limit some contamination will inevitably be lower and as a consequence the contamination level will increase slightly. Even though the main reason for the introduction of a technical zero is primarily to enable the use of authorized products, it cannot be impaired that in a worst case scenario the ruminant signals found by PCR below the technical zero might exclusively originate from unauthorized ruminant material. Moreover, extending the lifting of the feed ban on non-ruminant PAP in other feed than just aquafeed also increases the risk resulting from the confusion in the use of for instance pig feed or poultry feed to feed ruminants. Therefore, it seems that the main additional risk that can arise from a technical zero is linked to this additional pathway of contamination. We leave to EFSA to estimate the risk of this contamination route. In the next section we will nevertheless try to facilitate the work of EFSA to perform this additional risk assessment that results from the introduction of a technical zero by providing some helpful data which should allow keeping the same rationale of assessment as what was done previously.

7. Data of interest to assess the risk along the additional contamination pathway.

To achieve the quantitative risk assessment as done previously by EFSA (EFSA, 2011), it is important to be able to express what is the quantity in mass of PAP that could enter into ruminant feed or the ruminant ration. Doing this with reference to the technical zero is trickier because of the fact that the technical zero is not directly related to a quantity in mass of PAP. Nevertheless linking the risk to the technical zero is important because this is the level where the tolerance will be, which means that one might expect that the overall background noise with respect to use of the PCR method might increase up to that level (worst case). In order to allow EFSA to be able to do that with mass fractions we made an estimate of the probability of the mass of ruminant PAP that this represents. To that purpose several levels were considered for the technical zero : 300 copies, 250 copies, 200 copies, 150 copies and 100 copies. It is therefore suggested to perform the risk assessment first with the highest proposed technical zero (300 copies). Would this have as outcome a too high risk[1], then the other levels may be considered. However as the additional contamination pathway added should most probably not represent an event with a large occurrence, the chance is high that the risk will not be enhanced that much by using the highest proposed technical zero. To deliver the figures provided in the tables 3A, 3B, 3C, 3D and 3E, a set of assumptions had to be done. This is explained in detail in annex I. It is important that EFSA also evaluates the rationale that was followed to build up these tables as it is a rather theoretical approach on some points (especially the way the distribution was chosen).

[1] Defining if the risk is too high is of course the task of the risk managers. However to avoid to spend too much time to calculation, it seems unnecessary to analyse the other lower values of the technical zero if the outcome of the quantitative risk assessment compared to that of 2011 is not increased by more than 10% in terms of the total BSE infectivity load that could enter in cattle feed per year in the EU.

Table 3 – Probability of the occurrence of ruminant PAP in terms of mass fraction when the amount of ruminant material in non-ruminant feed in terms of copy number is exactly at the technical zero. Data for a technical zero set at different values (A : 300 copies, B : 250 copies, C: 200 copies, D: 150 copies, E: 100 copies).

A. Technical zero set at : 300 copies	
Ruminant PAP mass fraction in non-ruminant feed	**Probability of occurrence**
< 0.1 %	50.0 %
0.1- 0.2 %	30.3 %
0.2%-0.3%	6.9 %
0.3%-0.4%	2.7 %
0.4-0.5%	1.4 %
0.5-0.75%	1.7 %
0.75-1.0%	0.7 %
1.0-3.0%	1.3 %
> 3%	5.0 %

B. Technical zero set at : 250 copies	
Ruminant PAP mass fraction in non-ruminant feed	**Probability of occurrence**
< 0.1 %	61.2 %
0.1- 0.2 %	22.8 %
0.2%-0.3%	5.1 %
0.3%-0.4%	2.1 %
0.4-0.5%	1.1 %
0.5-0.75%	1.3 %
0.75-1.0%	0.6 %
1.0-3.0%	1.0 %
> 3%	4.9 %

C. Technical zero set at : 200 copies	
Ruminant PAP mass fraction in non-ruminant feed	**Probability of occurrence**
< 0.1 %	71.5 %
0.1- 0.2 %	15.7 %
0.2%-0.3%	3.5 %
0.3%-0.4%	1,5 %
0.4-0.5%	0.8 %
0.5-0.75%	1.0 %
0.75-1.0%	0.4 %
1.0-3.0%	0.8 %
> 3%	4.8 %

D. Technical zero set at : 150 copies	
Ruminant PAP mass fraction in non-ruminant feed	**Probability of occurrence**
< 0.1 %	80.3 %
0.1- 0.2 %	9.6 %
0.2%-0.3%	2.3 %
0.3%-0.4%	1,0 %
0.4-0.5%	0.5 %
0.5-0.75%	0,7 %
0.75-1.0%	0.3 %
1.0-3.0%	0.6 %
> 3%	4.7 %

E. Technical zero set at : 100 copies	
Ruminant PAP mass fraction in non-ruminant feed	**Probability of occurrence**
< 0.1 %	87.2 %
0.1- 0.2 %	5.0 %
0.2%-0.3%	1.3 %
0.3%-0.4%	0.6 %
0.4-0.5%	0.3 %
0.5-0.75%	0.4 %
0.75-1.0%	0.2 %
1.0-3.0%	0.4 %
> 3%	4.6 %

How should the figures provided in the Table 3 be used? In the previous QRA, the outcome was based on what is finally the cattle oral infectious dose that will be in the feed of the ruminants or in their ration. This requires to work in terms of mass fraction of PAP as this precisely mentions what is the amount of unauthorized material that arrives in the ruminant feed or ration and it allows to calculate, based on all the assumptions of the assessment, which oral infectious dose it represents. With the technical zero however it is more complex because a given amount of copy numbers does not correspond to a well-defined mass fraction. Nevertheless, with the outlined distribution a calculation remains possible. We will illustrate this with the technical zero set at 300 (Table 3A). In that case, a mass fraction equal or below 0.1% represent 50% of the frequency in the distribution. This means that the calculation could be performed with a mass fraction at 0.1% and that the outcome in terms of animals that would be infected should then be multiplied by the frequency in the distribution which was 50%. A similar calculation could then be done for the range 0.1-0.2 %, by taking a contamination rate at 0.2%, and the outcome in infected animals should then be multiplied by the frequency in the distribution for that range, i.e. 30,3%. The calculation should of course cover the whole distribution and all the results obtained and multiplied by the frequency of their occurrence have then to be merged by addition.

8. Some other considerations about the technical zero

The technical zero as explained here is applicable to the ruminant PCR method. Thanks to the concept of Co ID_{50}, EFSA has the possibility to deliver an opinion on the risk of propagation of BSE that could arise from the introduction of the technical zero for use of the ruminant PCR test. However, the concept of technical zero might be enlarged to other animal species (or groups of species) like pig or poultry. If with the outcome of the risk assessment, it appears that the technical zero can be acceptable for the ruminant PCR test than this concept can be extended to other PCR methods that could be useful for lifting the feed ban (porcine and poultry PCR). It has to be stressed however that the copy numbers to be applied for the technical zero of the other PCR methods is not necessarily 300 copies. This is because these tests do not focus on the same type of target in terms of abundance in the cell.

9. Bibliographical references

Benedetto *et al.* (2014). Detection of ruminant DNA in feed using real-time PCR. EURL-AP Standard Operating Procedure, version 1.1 of 24 June 2014, [15 p.] Available on: http://eurl.craw.eu/img/page/sops/EURL-AP%20SOP%20Ruminant%20PCR%20V1.1.pdf (consulted on 27 June 2017).

Codex Committee on Methods of Analysis and Sampling (CCMAS) (2010). Guidelines on performance criteria and validation of methods for detection, identification and quantification for specific DNA sequences and specific proteins in foods (Rep. No. CAC/GL 74-2010), [77p.]

EFSA (2011). EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on the revision of the quantitative risk assessment (QRA) of the BSE risk posed by processed animal proteins (PAPs). EFSA Journal 2011;9(1):1947. [80 pp.] doi:10.2903/j.efsa.2011.1947.

EURL-AP (2016). Combined microscopy-PCR EURL-AP Proficiency test 2015 (final version). ISBN 978-2-87286-094-4. [40 p.] Available on : http://eurl.craw.eu/img/page/proficiency/EURL-AP%20report%202015%20FINAL.pdf (consulted on 27/06/2017).

EURL-AP (2017). Combined microscopy-PCR EURL-AP Proficiency test 2016 (final version). ISBN 978-2-87286-099-9 [47 p.]. Available on : http://eurl.craw.eu/img/page/proficiency/EURL-AP%20report%202016%20FINAL.pdf (consulted on 27/06/2017).

European Commission (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, 21, 24.1.2013, pp. 3–16.

JRC-IRMM (2015). Certificate of analysis : ERM-AD482. Available on: https://crm.jrc.ec.europa.eu/p/erm-AD482 (consulted on 27/06/2017).

Annex I

Rationale used to determine in terms of probability what ruminant PAP represents in mass fraction when in terms of copy numbers of the ruminant target the sample is exactly at the technical zero.

Trying to convert the unit in copy numbers into mass fraction would require knowing what would be the distribution in copy numbers of a given amount of ruminant PAP taken randomly in the EU. This is complex to establish in practice, the more as the ruminant material that is rendered is not necessarily only consisting of ruminants. Moreover, the amount produced per rendering process should also be known. As data to do this are lacking, a theoretical distribution based on what are reasonable assumptions is made and even if this does not fit completely to reality it seems to us, it can be used for the risk assessment because we provide evidence that it is worse than reality (see annex II).

The distribution we would like to have relates to the copy number of a sample containing 0.1 % in mass fraction of ruminant PAP in feed, in which the PAP would be taken randomly from what is produced annually in the EU. Establishing this with experimental data would be very time consuming, so we will tackle the problem in a more theoretical way making some assumptions with already some evidence to support each of them but more details are provided in annex II.

1°) We consider that this distribution is normal. This is probably the weakest point in the set of hypotheses to outline. However as the copy numbers remaining in a PAP sample depend on multiple factors this is not impossible. Moreover the use of this kind of distribution also has the major advantage that calculations afterwards are easy to perform.

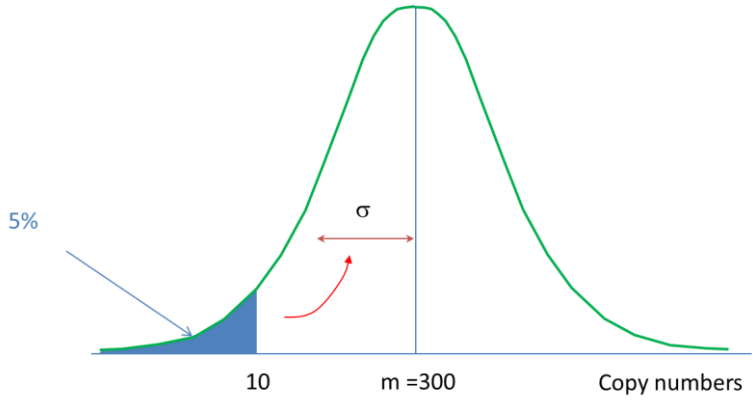


Figure 5 – Defining the distribution in copy numbers for randomly chosen ruminant PAP from the EU present at a content of 0.1 % in mass fraction in feed. The mean is set at 300 copies and the spread (σ : standard deviation) is defined from the fact that what is below 10 copies should represent 5% (the lay-out is not necessarily completely at scale in the figure).

2°) To characterize this normal distribution, we need two parameters: the mean m and the spread σ (Figure 5). The rationale to fix them is the following one:

a) For the mean *m* we make the assumption that a sample of mean complexity for detection is the one that corresponds to the mean occurrence. That is why we set the mean at 300 copy numbers based on the experience of the EURL-AP of what is a sample of mean complexity. This is a conservative assumption as in most cases the mean value at 0.1 % in mass fraction represents a higher number of ruminant targets (see annex II).

b) Concerning the spread σ, as the method is said to have a LOD of at least 0.1 % (generally it is lower), let us take it exactly at 0.1 % for the distribution which means that samples showing less than 10 copies of the target (i.e. at the value corresponding in practice to the cut-off) should represent 5 %. Again this is a conservative assumption as outlined in more detail in annex II.

Thanks to this distribution, it is then possible to find out what is the probability of the representation for given ranges of mass fractions of ruminant PAP in a sample that in copy number of the ruminant target fits exactly to the technical zero. Let us consider the 0.1-0.2 % range in mass fraction and a technical zero set at 300. The probability to get this is represented by the area in yellow outlined in Figure 6 towards the whole area under the curve.

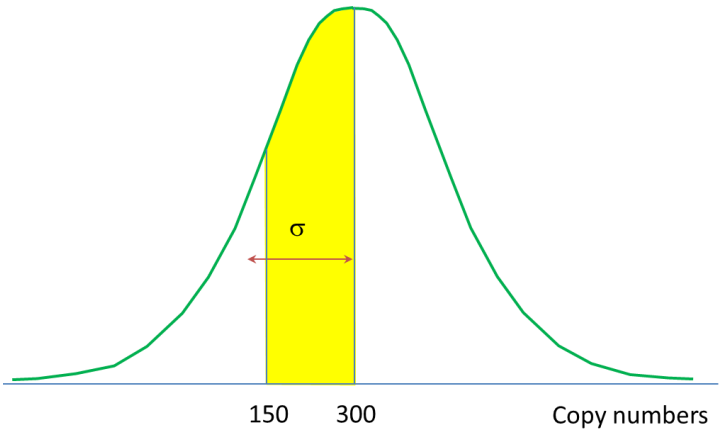


Figure 6 – Calculation of the probability of representation of the mass fraction range of ruminant PAP in feed going from 0.1 % to 0.2 % in a sample for which the result of the ruminant PCR fits exactly to the technical zero (considered here as fixed at 300 copies) taking into account the distribution as set in Figure 5 (the representation is not fully at scale).

The reason why that precise area has to be considered is because for a mass fraction of 0.2 % (= 2*0.1 %) if the technical zero is set at 300 and the sample harbors exactly 300 copies of the ruminant target, it means that the PAP type corresponds in the distribution of Figure 5 to a sample in which there are 150 copies (= 300/2). The other boundary of the area corresponds to 300 copies because in the distribution of Figure 5, a sample at 0.1 % that would be exactly at the technical zero of 300 copies necessarily contains 300 copies. The same rationale holds true for the other calculations given in the Table 3A. Be careful however that if the technical zero is set at 250 (Table

3B), then the calculation has to be adapted but the distribution that was assumed and is represented in Figure 5 would still be the basis for the calculation. In fact for such a technical zero of 250 copies, to calculate the probability of presence of a mass fraction ranging from 0.1 to 0.2 % is given by a ratio of the area in yellow in Figure 7 (i.e. from 125 to 250 copies) towards the whole area below the curve of Figure 7.

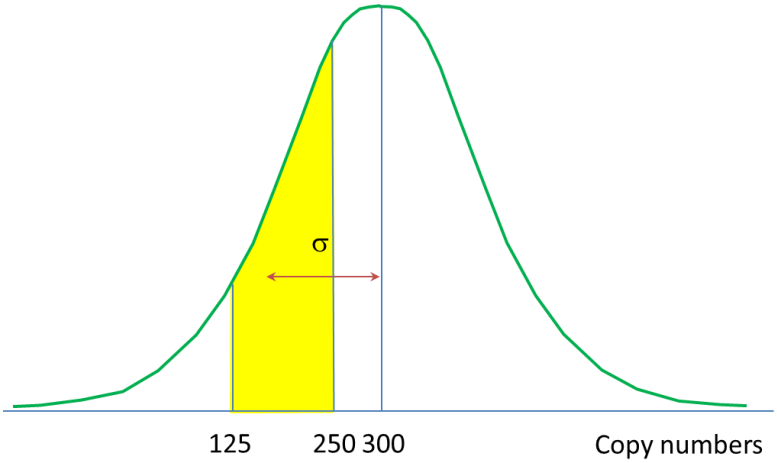


Figure 7 – Same calculation as in figure 4 but with a technical zero set at 250 copies – the distribution to consider is unchanged, it is the same as that in figure 5 (the representation is not fully at scale)

Annex II

Completing the QRA with an additional contamination pathway and considering the contamination level at exactly the technical zero will in fact overestimate the risk. Indeed in a lot of situations where this additional contamination route happens, the contamination level will be below the technical zero. In the assumptions we made, the level is set exactly at the technical zero (of course one cannot exclude that it may sometimes be above). Moreover we consider that the PCR signal obtained always derives from ruminant PAP or other non-authorized ruminant products (e.g. gelatin from ruminants) while in practice part of it may originate from authorized products. In addition to that it should be stressed that even if the distribution of annex I is somewhat theoretical, it nevertheless appears fit for purpose because it is worse than what happens in real life.

Hereafter we provide evidence of the fact that the real world situation is not as worse as that of the theoretical distribution we propose to use for the calculations:

1) The mean of the distribution is in fact higher for real life samples in terms of copy number of the ruminant target. To illustrate this, we can for instance provide figures coming from the proficiency test of APHA for which we have knowledge (after the analysis was done) of the mass fraction of PAP that was used and the copy numbers we obtained for these samples :
    - A sample in which 0.2 % of ruminant PAP was present shows a copy number of the ruminant target of 68 200.
    - A sample in which 0.2 % of ovine PAP was present shows a copy number of the ruminant target of 32 700.

    Even if at first sight about half the copies are expected at 0.1 % in mass fraction (which is not absolutely correct because of the sampling), it nevertheless clearly shows that the copy number of ruminant targets in a feed spiked at 0.1 % is far above 300 (here it is about 50 to 100 times more).

    Similarly considering several samples prepared at the EURL-AP for proficiency tests, the copy number of ruminant targets corresponding to a spiking level of 0.1 % in mass fraction lies within a range from 424 to 798 copies, thus clearly beyond 300 copies (in the distribution of Figure 5, a copy number higher than 476 does not exceed 16 % of probability of occurrence while more than 652 copies does not exceed a frequency of 2.5 %).

    A last evidence that the level of contamination is rather low is that on a total of 54 counter analyses performed at EURL-AP for the NRLs (official and non-official ones), it is only in seven cases that the level detected by PCR goes beyond 300 copies (results for these samples being in a range going from 300 to 10000 copies). All the other ones are below 300 copies of the ruminant target.

2) The spread considered is in fact too narrow because the limit of detection is generally below 0.1 % which means that the area at the left of the boundary of 10 copies as illustrated in Figure 5 is smaller than 5 %. In the 2015 and 2016 EURL-AP proficiency tests (EURL-AP, 2016 and 2017), one of the samples submitted to PCR contained 0.05 % of ruminant PAP in fish feed and was always detected by all the participants.

Acknowledgements

The authors would like to acknowledge Christoph von Holst of JRC-Geel (Geel, Belgium) for the fruitful discussions held with him on the topic of this report.