Development and full assessment of a Real-Time PCR method for the specific detection of Ruminant DNA in processed animal meal in feed

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- **INTRODUCTION**

- BSE Crisis
- Feed ban for processed animal protein (PAP) according to EU Commission Regulation 1234/2003

- Possible lifting of ban for use of PAP from non-ruminants in non-ruminant feed
- No intra-species recycling (cannibalism)

- Need for validated analytical testing procedures

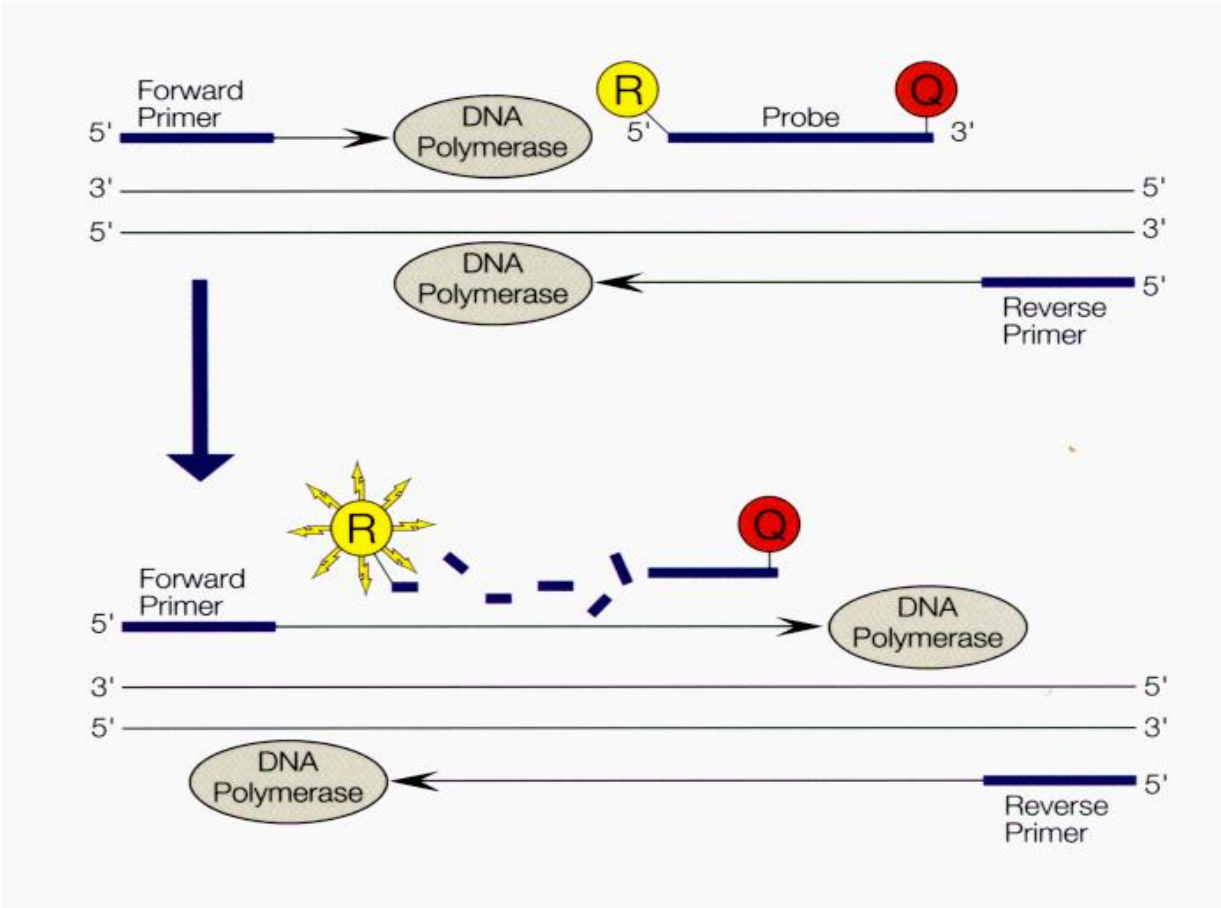
- Real-Time PCR for ruminants, porcine and poultry
- Evaluation of the ruminant specific RT-PCR method

acknowledgement

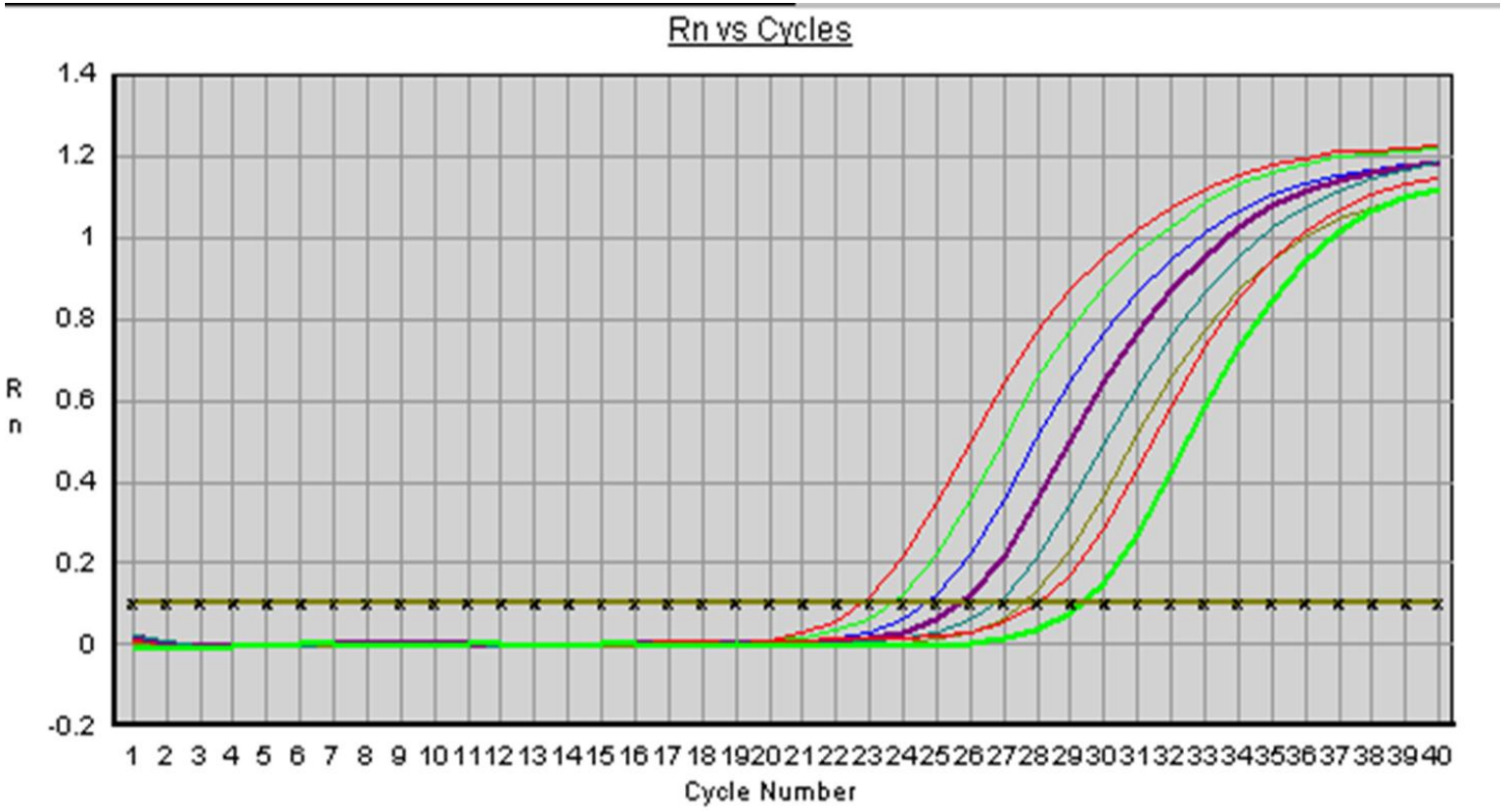
The Evaluation of the TNO Triskelion PCR method for the detection of ruminant DNA was performed by, and in close cooperation with the European Union Reference Laboratory for Animal Proteins in feeding stuffs as part of the Walloon Agricultural Research Centre

Principle of Real-Time PCR

(Polymerase Chain Reaction)



REAL - TIME QUANTITATIVE PCR (TAQMAN)



Development of Ruminant Specific RT-PCR

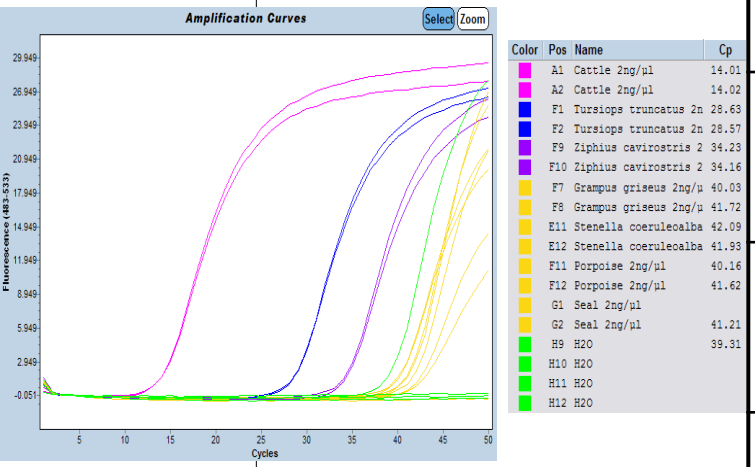
- 1: DNA sequence from the bovine alpha subunit precursor of the acethylcholine receptor
- 2: Highly abundant DNA: sensitivity
- 3: Amplicon size of RT-PCR target is 85 bp: small
- 4: RT-PCR product is cloned into a plasmid: calibrant

Full Assessment of the RT-PCR Method

- 1: Specificity
- 2: Efficiency
- 3: Robustness
- 4: Limit of Detection
- 5: Sensitivity

Specificity of Ruminant specific RT-PCR

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Cattle	14.01 14.02	14.02	+	Human	50 50	50	-
Sheep	14.21 14.30	14.26	+	Pig	41.21 50	45.61	-
Goat	14.21 14.28	14.25	+	Donkey	45 50	48	-
Dolphin	28.63 28.57	28.60	+	Horse	43.70 50	46.85	-
Whale	34.23 34.16	34.20	+	Rabbit	39.34 40.30	39.82	-
0.05 % ruminant in feed	30.49 30.39	30.44	+	Chicken	50 50	50	-



Color	Pos	Name	Cp
	A1	Cattle 2ng/µl	14.01
	A2	Cattle 2ng/µl	14.02
	F1	Tursiops truncatus 2n	28.63
	F2	Tursiops truncatus 2n	28.57
	F9	Ziphius cavirostris 2	34.23
	F10	Ziphius cavirostris 2	34.16
	F7	Grampus griseus 2ng/µ	40.03
	F8	Grampus griseus 2ng/µ	41.72
	E11	Stenella coeruleoalba	42.09
	E12	Stenella coeruleoalba	41.93
	F11	Porpoise 2ng/µl	40.16
	F12	Porpoise 2ng/µl	41.62
	G1	Seal 2ng/µl	
	G2	Seal 2ng/µl	41.21
	H9	H2O	39.31
	H10	H2O	
	H11	H2O	
	H12	H2O	

Efficiency & Robustness

Device	LightCycler LC480		ABI7500	
Master Mix	Diagenode	Eurogentec	Diagenode	Eurogentec
Efficiency	95.49 %	97.21 %	94.78 %	97.67 %

- Acceptance criterion for the efficiency value is between 80 % and 120 %

PCR equipment	LC480 and ABI7500				
PCR reagents	Universal Mastermix (Diagenode s.a.) and qPCR MasterMix for probe assay ROX (Eurogentec s.a.)				
Annealing temperature	59, 60 and 61 °C				
Primer concentration	Minus 30 %	Standard	Standard	Standard	Standard
Probe concentration	Standard	Minus 30 %	Standard	Standard	Standard
PCR volume	Standard	Standard	Standard (20 µl mix + 5 µl DNA)	Standard + 1 µl Mastermix (21 µl mix + 5 µl DNA)	Standard – 1 µl Mastermix (19 µl mix + 5 µl DNA)

- Rate of positive results always above 95 % !

Limit of Detection (LOD-6 approach)

	LC480 - Master mix Diagenode Cut-off (1 copy) : 39.874				
	Run 1		Run 2		
copies/5µl	Ct	Ĉt	Ct	Ĉt	
20	35,22	35,49	35,72	35,40	
20	36,11		35,60		
20	34,65		35,29		
20	35,72		35,24		
20	35,78		34,99		
20	35,45		35,56		
10	35,89	36,31	35,71	35,87	
10	36,29		35,82		
10	36,29		36,17		
10	36,25		35,79		
10	36,58		35,73		
10	36,56		36,02		
5	36,51	37,05	38,16	39,52	
5	37,31		36,99		
5	36,85		37,46		
5	36,79		36,95		
5	37,95		50		
5	36,86		37,54		
2	39,67	38,45	37,57	38,00	
2	38,97		37,32		
2	37,81		38,77		
2	39,31		38,85		
2	37,44		37,05		
2	37,49		38,46		

Sensitivity of Method in Feed

Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with cut-off at			
					1 copy (38.693)	5 copies (36.284)	10 copies (35.248)	15 copies (34.642)
0.1 % cattle at 133 °C in pig meal	1	3x	25.76 25.65 25.58	25.66	+	+	+	+
0.1 % sheep at 133 °C in fish meal	1	3x	32.12 31.82 31.60	31.85*	+	+	+	+
0.1% sheep at 133 °C in feed	1	3x	50 50 50	50	-	-	-	-
		30x	31.88 31.81 31.73	31.81	+	+	+	+
0.1 % MBM in feed	1	3x	34.54 33.72 33.64	33.97*	+	+	+	+
		30x	31.45 31.47 31.32	31.41	+	+	+	+

Discrimination between chicken and porcine DNA

				Real time PCR		
Species				**Ruminant**	**Porcine**	**Poultry**
Reference samples of CCL-Research						
Pork soft	pre-pressure cooking	133°C	8)	-	+ 24,9	- 40
		159°C		-	± 38,1	- 40
	post-pressure cooking	133°C		-	+ 26,9	- 40
		159°C		-	+ 36,2	- 40
	defatted post-pressure cooking	133°C		-	+ 32,8	+ 35,8/36,7
		159°C		-	- 40	- 40
Pork bones	pre-pressure cooking	133°C	9)	-	+ 25,0	- 40
		159°C		-	+ 35,4	- 40
	post-pressure cooking	133°C		-	+ 28,8	- 40
		159°C		-	- 40	- 40
	defatted post-pressure cooking	133°C		-	+ 32,4	- 40
		159°C		-	- 40	± 40
Chicken soft	pre-pressure cooking	133°C	6)	-	- 40	+ 29,7
		159°C		-	- 40	+ 25,9
	post-pressure cooking	133°C		-	- 40	+ 31,0
		159°C		-	- 40	+ 36,4
	defatted post-pressure cooking	133°C		-	- 40	+ 35,6
		159°C		-	- 40	- 40
Chicken bones	pre-pressure cooking	133°C	7)	-	- 40	+ 21,7
		159°C		-	- 40	+ 31,0
	post-pressure cooking	133°C		-	- 40	+ 26,4
		159°C		-	- 40	+ 22,6
	defatted post-pressure cooking	133°C		-	- 40	+ 30,7
		159°C		-	- 39,4	- 40

Traceability of Bovine and Chicken DNA in pig feed

			Real time PCR		
Species			Ruminant	Porcine	Poultry
Pig feed %	Bovine %	Chicken %			
95	0	5	-	- 39,6	+ 28,4
99	0	1	-	- 40	+ 29,1
100	0	0	-	- 40	- 40
0	0	100	- *	- 40 *	+ 25,7
95	5	0	+	- 40	- 40
99	1	0	+	- 40	- 40
99,5	0,5	0	+	- 40	- 40
99,7	0,3	0	+	- 40	- 40
99,8	0,2	0	+	- 40	- 40
99,9	0,1	0	+	- 40	- 40
90	5	5	+	- 40	+ 27,6
94	1	5	+	- 40	+ 27,5
94,5	0,5	5	+	- 40	+ 27,2
94,7	0,3	5	+	- 40	+ 25,0
94,8	0,2	5	+	- 40	+ 26,7
94,9	0,1	5	+	- 40	+ 26,8
0	5	95	+	- 40	+ 22,4
0	1	99	+	- 40	+ 22,2
0	0,5	99,5	+	- 40	+ 21,5
0	0,3	99,7	+	- 40	+ 22,0
0	0,2	99,8	+	- 40	+ 21,4
0	0,1	99,9	+	- 40	+ 21,6

Conclusions

- A validated RT-PCR method for the specific detection of at least 0.1 % ruminant derived material in feed is available.
- This method will facilitate the re-introduction of non-ruminant PAP in (aqua) – feed.

- In order to prevent intra-species recycling, RT-PCR methods for the discrimination between poultry and porcine DNA have been developed.

- Many thanks to:

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