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Review

Genetically modified animals: Options and issues for traceability and enforcement

A. Lievens*[,1], M. Petrillo[1], M. Querci and A. Patak

Molecular Biology and Genomics Unit, European Commission – Joint Research Centre, Institute for Health and Consumer Protection, Via Enrico Fermi 2749, TP201, I-21027 Ispra, VA, Italy (e-mail: antoon.lievens@ec.europa.eu)

The past two decades have witnessed the rise of commercial crops that have been genetically modified for an increased suitability in extensive cultivation. Currently, a substantial body of research is being carried out in order to produce Genetically Modified (GM) animals that may similarly yield improvements in animal breeding, genetics and reproduction. Here, we attempt a comprehensive review of the existing trails at animal modification with commercial applications and aimed at a deliberate release onto the market. In addition, we investigate detection and quantification options within the frame of food/feed control and traceability on the European market.

Introduction

Over the past 30 years, biotechnological developments have allowed scientists to alter the genetic make-up of bacteria, plants, and animals. Initially, these modifications have served the purpose of basic research (the study of gene function and genetic mechanisms), but these techniques quickly became promising tools from agricultural point of view since they allow the addition of novel traits to organisms which may increase their suitability for use in extensive mono-cultures (*e.g.* animals with better neonatal survival, or plants with herbicide resistances and insect tolerances). The first genetically modified organisms (GMOs) for agricultural use were introduced in 1996 and currently, more than 170 million hectares of GM crops are being cultivated worldwide (James, 2010, 2011) and while genetically modified (GM) animals are not yet in farms or on the market, their introduction is foreseen for the near future (Ledford, 2013).

Since the creation of the first GM livestock (Hammer *et al.*, 1985) effort has been made to modify several aspects of farm animals to improve their cultivation. Amongst the most targeted traits are: animal health (increased neonatal survival (Bleck, White, Miller, & Wheeler, 1998; Tong *et al.*, 2011; Wheeler, Bleck, & Donovan, 2001), disease resistance (Denning *et al.*, 2001; Lyall *et al.*, 2011; Richt *et al.*, 2007), growth rate (Devlin, Sakhrani, Tymchuk, Rise, & Goh, 2009; Nottle *et al.*, 1999; Saunders, Fletcher, & Hew, 1998), improvement of meat (Lai *et al.*, 2006; Saeki *et al.*, 2004) and milk composition (Brophy *et al.*, 2003; Wu *et al.*, 2012), and increased wool production (Bawden, Sivaprasad, Verma, Walker, & Rogers, 1995; Damak, Sul, Jay, & Bullock, 1996). In addition, the reduction of the impact of animal culture on the environment has been attempted (Phillips, Golovan, Meidinger, & Forsberg, 2006). Table 1 includes further examples of animal modifications. Fig. 1 shows an example of the effects of growth rate modification (AquAdvantage®Salmon, currently considered for approval for the American market).

Another branch of animal modification is molecular farming, also known as 'pharming', in which biopharmaceuticals are manufactured in transgenic animals (Kind & Schnieke, 2008). More than recombinant cell cultures, animals are attractive bioreactors: they have the correct metabolic pathways, are reproducible, easily maintained, and do not require expensive infrastructure (Dyck, Lacroix, Pothier, & Sirard, 2003). Production of these recombinant proteins usually happens in mammalian milk, since it offers flexible production and relatively straightforward purification, but also egg white and seminal plasma are being used (Dyck *et al.*, 2003). Blood, on the other hand, is usually not able to store high concentrations of recombinant proteins (Houdebine, 2009b). Another medical application of genetic modification aims to improve the

* Corresponding author.
[1] Both authors contributed equally.

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Table 1. Overview of some of the transgenic animals with potential commercial applications (excluding mice and rats). Animals are classified in three categories: 'Livestock', 'Bioreactor', and 'Companion'. Other columns list the main inserted/deleted gene, its origin, and main effect or use. 'ID' is a numerical identifier given to enable straightforward comparison with Table 2. References to literature for each entry can be found in the supplemental material. This table is adapted from (Forabosco, Lohmus, Rydhmer, & Sundstrom, 2013; Pursel & Rexroad, 1993; Rudolph, 1999; Vazquez-Salat, Salter, Smets, & Houdebine, 2012; Wall, 1999) and has been updated & expanded.

Species	Category	ID	Transgene	Origin	Effect/Goal
Cattle	Livestock	1	Lysozyme	Human	Milk composition
		2	PrP	Knockout	Animal health
		3	$\alpha-,\kappa$-Casein	Bovine	Milk composition
		4	Omega-3	Nemateode	Milk composition
		5	Lysostaphin	Bacterial	Mastasis resistance
	Bioreactor	6	Lactoferrin	Human	Prophylactic treatment
Chicken	Livestock	7	alv6 enveloppe glycoprotein	Viral	Disease resistance
		8	short hairpin RNA	Viral	Disease resistance
		9	LacZ	Bacterial	Animal Health
	Bioreactor	10	α-interferon	Human	Hepatitis treatment
Carp	Livestock	11	Growth Hormone	Piscine	Growth rate
		12	Lactorferrin	Human	Disease resistanc
Catfish	Livestock	13	Cercopin B	Insect	Disease resistanc
Fruit Fly	Livestock	14	fsRIDL	Hymnopteran	Pest control
Frog	Bioreactor	15	GFP	Cnidarian	Water purity
Goat	Livestock	16	Lysozyme	Human-Bovine	Animal Health
		17	Monosat. fat. acid	Rat-Bovine	Mastasis resistance
		18	MSP(1)42	Plasmodial	Malaria vaccine
		19	Antithrombin III	Human	Thrombosis/embolism treatment
		20	Tissue plasminogen activator	Human-Mouse	Anti clotting agent
		21	Lactoferrin	Human	Prophylactic treatment
		22	lysosomal acid β-glucosidase	Human	Gaucher disease treatment
		23	Human coagulation factor IX	Human	Haemophilia treatment
		24	Human beta-defensin 3	Human	Milk composition
Pig	Livestock	25	Phytase	E. Coli-Mouse	Feed uptake
		26	Growth hormone	Human-Porcine	Growth rate
		27	cSKI	Chicken	Muscle development
		28	Lysozyme	Human	Piglet survival
		29	Unsat. fat. acid	Spinach	Meat composition
		30	Omega-3	Nematode	Meat composition
		31	α-lactalbumin	Bovine	Piglet survival
		32	Mx1	Murine	influenza resistance
	Bioreactor	33	Factor VIII	Human	Haemophilia treatment
		34	α(1,3)galactosyltransferase	knockout	Human transplantation
		35	N-glycolylneuraminic acid	knockout	Human transplantation
		36	CD59, DAF	Human	Human transplantation
		37	DAF	Human	Human transplantation
		38	hHO-1	Human	Human transplantation
		39	hHO-1,DAF	Human	Human transplantation
		40	β-D Mamose, GnTIII	Human	Human transplantation
		41	Fibrinogen	Human	Tissue sealant
		42	Haemoglobin	Human	Transfusion
		43	Protein C	Human	Blood coagulation
		44	Albumin	Human	Human transplantation
Rabbit	Bioreactor	45	Calcitonin	Salmon	Osteoporosis treatment
		46	Erythropoietin	Human	Anemia treatment
		47	Superoxide dismutase	Human	Blood purification
		48	Interleukin-2	Human	Cancer treatment
		49	Tissue plasmogen activator	Human	Anti Clotting Agent
		50	VP2, VP6	Viral	Rotavirus vaccine
		51	Human Factor VII	Human-Mouse-Chicken	Haemophilia treatment
		52	Growth Hormone	Human	HGH insufficiency treatment
		53	Von Willebrand factor	Human-Bovine	Haemophilia treatment
Salmon	Livestock	54	Growth hormone	Piscine	Growth rate
		55	Growth hormone	Piscine	Growth rate
		56	Lysozyme	Piscine	Animal health
		57	wflAFP-6	Piscine	Cold tolerance
Sheep	Livestock	58	IGF-1	Ovine	Wool growth

(*continued on next page*)

Table 1 (*continued*)

Species	Category	ID	Transgene	Origin	Effect/Goal
		59	CsK	Bacterial	Wool growth
		60	HTT	Human	Disease model
		61	Visna resistance	Viral	Disease resistance
		62	PrP	knockout	Animal health
		63	GGTA1	knockout	Human transplantation
	Bioreactor	64	Factor IX	Human	Haemophilia treatment
		65	Factor VIII	Human	Haemophilia treatment
		66	α-1-antitrypsin	Human	Cystic fibrosis treatment
Silkworm	Livestock	67	eGFP, DsRed, or mKO	Cnidarian	Silk color
		68	$A2S8_{14}$	Arachnid	Silk strength
	Bioreactor	69	Fibroin	Human	Cell adhesive film
		70	Crp	Canine	Inflammation marker
		71	TRACP5B	Human	Inflammation marker
Tilapia	Bioreactor	72	Insulin	Human	Diabetes treatment
Trout	Livestock	73	Follistatin	Piscine	Muscle development
Zebrafish	Companion	74	GFP or RFP	Cnidarian	Fish color

suitability of animal organs for xenotransplantation, *e.g.* in pigs (Luo, Lin, Bolund, Jensen, & Sorensen, 2012).

Lastly, animals have also been modified to improve, or rather, add 'aesthetic' qualities (Gong *et al.*, 2003; Wan *et al.*, 2002).

Three categories can thus be defined: GM animals for human consumption, as medical bioreactors, and as companion animal. As the European Union (EU) Joint Research Center's Molecular Biology and Genomics Unit we are responsible for the scientific assessment and validation of detection, identification and quantification methods for GMOs (plant, animal, and bacterial) in food and feed and are thus primarily concerned with the first category.

While GM animals are not yet produced on a large scale for human consumption, they are already in US pet shops (Nash, 2004), they are being used for testing water purity (Fini *et al.*, 2007; Thienpont *et al.*, 2011) (also see www.watchfrog.fr), pharmaceuticals from GM animals are currently for sale (Wells, 2010), and at the time of writing, a decision on the approval of GM salmon on the American market by the FDA is under way (April 2015). The EU is the world's largest trading block for food commodities and as a result, once GM animals enter the global market, the likelihood of contamination in the European food chain increases. At least 3 European countries have already developed methods to screen for GM animals in the context of food safety (European Network of GM Laboratories, personal communication).

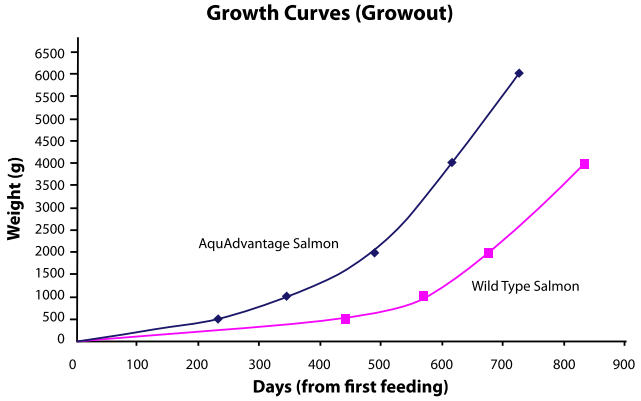

Fig. 1. Effects of growth rate modification in salmon. AquAdvantage®Salmon contains a gene from the Chinook salmon which results in fish with an increased growth rate, thus reaching market size in half the time of conventional salmon. Image courtesy of AquaBounty technologies.

Here we attempt to give an overview of the genetic modifications currently (being) made to animals and the strategies or methods that may be employed in the detection and quantification of their presence in the food chain. We will primarily focus on animals designed for human consumption, bioreactor animals (and pets) are not intended to end up in the food chain as they are usually both rare and valuable. As a consequence, the latter do not pose a traceability problem. Nevertheless, the principles laid out in the following paragraphs are valid for all GM animals.

Genetic modifications in animals

The European Union defines GMOs as Genetically Modified Organisms, such as plants, animals, and micro organisms whose genetic characteristics are modified artificially (*i.e.* not through "natural" breeding) in order to give them a new property. In practice this boils down to inserting or deleting information in the nuclear DNA of (a) single cell(s). The latter are then grown/multiplied to ultimately form a complete organism carrying the changes in each somatic cell.

Transgenesis of animals can be achieved using different techniques: DNA microinjection, retroviral vectors, intracytoplasmic sperm injection, and somatic cell nuclear transfer (for an overview see (Houdebine, 2009a; Kues & Niemann, 2011)). The success rate (live births) of each technique depends on the species, but is usually in the

range of 1–3%. Not all techniques allow for targeted insertion and most are limited in the amount of base pairs they can add to the target genome.

The first generation of these GMOs is hemizygous (only one copy of the gene is present in an otherwise diploid organism) as opposed to heterozygous (two different alleles are present at a gene locus) or homozygous (identical alleles are present). For breeding purposes, homozygous organisms are preferred since their offspring is genetically homogeneous (guaranteed to pass on a trait). In plants transition from hemi-to homozygous is a rather straightforward process of (longterm) inbreeding. In farm animals, more elaborate and time consuming breeding programs are required.

Insertion of genetic material

When expression of novel traits or production of proteins is the intended goal, the DNA coding for these properties has to be added to the host genome. To attain reliable expression the transgene must be accompanied by: a promoter, enhancers, insulators introns, and a terminator (Houdebine, 2009a). For GM crops this scheme is well established and many plants that express new proteins have been realized. For example, the expression of insecticidal proteins yielding an increased innate protection from insect damage thus reducing the need for the application of pesticides (Nelson & Alves, 2014). In animals, one of the main applications of protein expression is the production of pharmacologically important proteins for harvest (animals as bioreactors, see Table 1 for examples). Excretion of biopharmaceuticals in milk, for instance, can be achieved using promoters from milk protein genes (*e.g.* whey acidic protein), whereas for expression in egg, the promoter of the ovalbumin gene can be used. Other applications of protein expression focuss on improving the fitness of animals in high-density conditions or to alter meat composition (animals as livestock, see Table 1 for examples).

In addition to the introduction of new traits, the insertion of genetic material can also be used to 'silence' genes: either by expressing short synthetic oligonucleotides with complementary to the sequence of the mRNA of the targeted gene, preventing its translation, or by expressing intracellular antibodies against the target protein (intrabodies). Another strategy is to overexpress a gene coding for an inactive version of the targeted protein.

Deletion of genetic information

Sometimes the removal of a gene (and thus its product) may be advantageous: *e.g.* protein PrP which may cause prion diseases when its misfolded forms are propagated (cf. Bovine spongiform encephalopathy (Richt *et al.*, 2007)), myostatin which acts as a downregulator for muscle growth (cf. Belgian Blue Cattle (Grobet *et al.*, 1997)), or α(1,3)galactosyl transferase which may cause hyperacute rejection in xenotransplants (Kuwaki *et al.*, 2005; Lutz *et al.*, 2013; Yamada *et al.*, 2005).

Deletions or 'knockouts' can be achieved through homologous recombination or by use of a recombinase driven system like Cre-LoxP or Flp-FRT. The latter systems operate by adding a recombinase site to both ends of the target fragment, the recombinase can then be synthesized under the direction of a cell specific promoter, making it possible to delete a DNA fragment only in a chosen cell type.

Current GM practice

Detection and quantification strategies

For most of the members of the European Network of GMO Laboratories (ENGL) the workflow surrounding GM sample processing includes of the following steps: **(I)** analysis of the meta information surrounding a sample in order to decide which screening methods to used (eg. only markers related to a single crop or a full screening), **(II)** run these screening methods and if all are negative –stop the testing, **(III)** in case of positive results, identify possible events and run the event specific tests, possibly quantifying any positive result in the same run, **(IV)** if no event specific tests show a positive result, declare the sample being contaminated with an un-authorised GMO.

Current EU practice in GM quantification is to evaluate a sample's GM content on a per ingredient base (*i.e.* the percentage GM is expressed, for each ingredient (species), as fraction of the total amount present in the sample). This approach allows for a straightforward implementation in a (real time) PCR set-up. It is, however, not the only option available: quantification of GM-material could also be expressed as the fraction of the total material of the entire group of ingredients/components (e.g. all plant material in a food product). Another option could be GM material as fraction of the total material of the food or feed product. For plant-derived materials, the latter two options currently not deemed feasible on the basis of DNA.

Amplification methodology

At present, (real time) PCR-based analysis is the method of choice for the routine analysis of food and feed samples for their GMO content (Bonfini, Kay, Heinze, & Van den Eede, 2002; Holst-Jensen, 2009; Zel *et al.*, 2012). These methods are DNA based and are thus applicable for the detection of all (GM) organisms. They consist of targeting specific DNA sequences (between 60 and 200 base pairs long) for enzymatic amplification, revealing the presence (amplification) or absence (no amplification) of the target sequence. By following the amplification process fluorometrically (in real time) quantification of the initial amount of target sequence becomes possible.

Traditionally, three levels of specificity are distinguished within GMO-detection methods:

Screening methods target DNA sequences that are frequently inserted into GMOs, *e.g.* promoters, terminators, sequences of genes conferring certain tolerances or resistances. These methods allow the detection of a broad range of genetically modified organisms, but they do not allow to

identify with absolute certainty which GM event(s) is (are) present in the sample.

Construct specific methods target two or more adjacent genetic elements in a transgene construct by amplifying their junction. These methods are more informative in terms of presence or absence of specific events, but cannot distinguish between different events containing the same (or similar) constructs.

Event specific methods target the host-transgene DNA junction. As a consequence, these methods are highly specific and amplify only a single event. They are usually applied in downstream confirmation of GM positive samples.

Detection, identification and quantification of GM animals

In the following paragraphs we list several aspects of the method design, sample analysis, and interpretation of results involved in the establishment of a GM animal detection and quantification platform. Many of these topics are not confined to the GM animal field, we will therefore also summarize the existing approaches in the field of GM plant detection/quantification in each section. However, not all of the strategies and assumptions currently employed in GM plants may transfer directly to animals. Possible challenges will be identified where appropriate.

Detection and quantification targets

The different type of DNA modifications can be evaluated for their capability to yield event-specific methods (also see Fig. 2):

Insertions

Insertions are the most straightforward group of modifications to detect. Insertion of a foreign sequence into the host genome inevitably generates two unique junctions. Even if the donor organism is the same species, the junctions at the insertion sites are wholly unique to the GMO and specific detection is possible. In addition, targeting genes or genetic elements inside the insertion may yield construct specific and screening methods. The success of such screening strategies will largely depend on the origin of the inserted elements and whether its presence in the food chain may cause false-positive screening results.

Deletions

Deletions may not be detected by screening or construct specific methods. However, similar to insertions, the removal of sequences usually creates a new, unique, junction that may be targeted for amplification, thus rendering the event detectable. Theoretically, exceptions are possible if the deletion took place in a high-repeat region, this might create an undetectable (*i.e.* non-unique) junction region. However, it should be stressed that this is a statistically highly unlikely scenario. In addition, a deletion that is hard to detect is usually not interesting from the point of GM production because it may complicate screening for successful deletion in the early stages of the production process when the modification is attempted on a large number of cells.

Recombinase

Recombinase mediated deletions form a more heterogeneous group of modifications. Depending on the nature of the construct, the resulting organism may or may not be mosaic for the deletion. In the latter case targeting either the pre- or post-deletion border may not suffice to ensure detection and both sites should be screened for in parallel. Another possibility is targeting the insertion of the recombinase gene. But again, depending on the case, this may not yield event-specific detection. In research it is common to have two lines of mutants: one containing the recombinase-gene, the other containing the deletion-targeted genes. It is the offspring of these two lines which then form the functional mutants. It is thus possible to generate many different GM animals whose genomes all

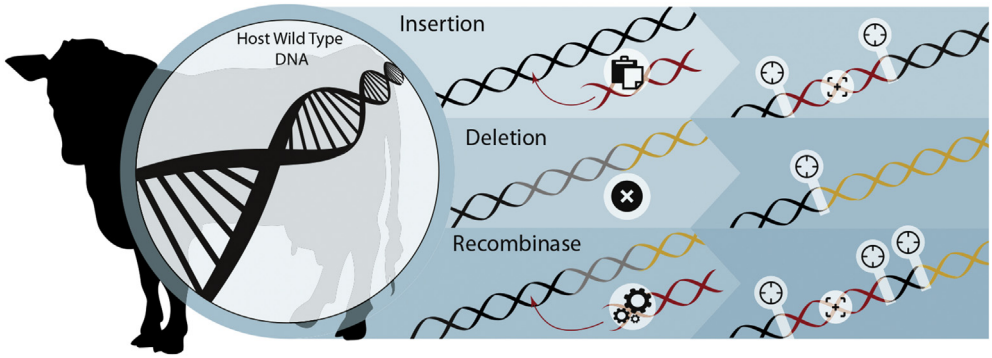

Fig. 2. Schematic overview of detection targets. DNA is represented in different colours for explanatory purpose. Insertions are represented in red, deletions as the omission of grey coloured stretches of DNA, orange is used to help indicate the new junction after deletion. Depending on the type of modification (insertion, deletion and deletion by recombinase) there are different options for event specific identification (circular cross-hairs) or construct specific detection (square cross-hairs). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

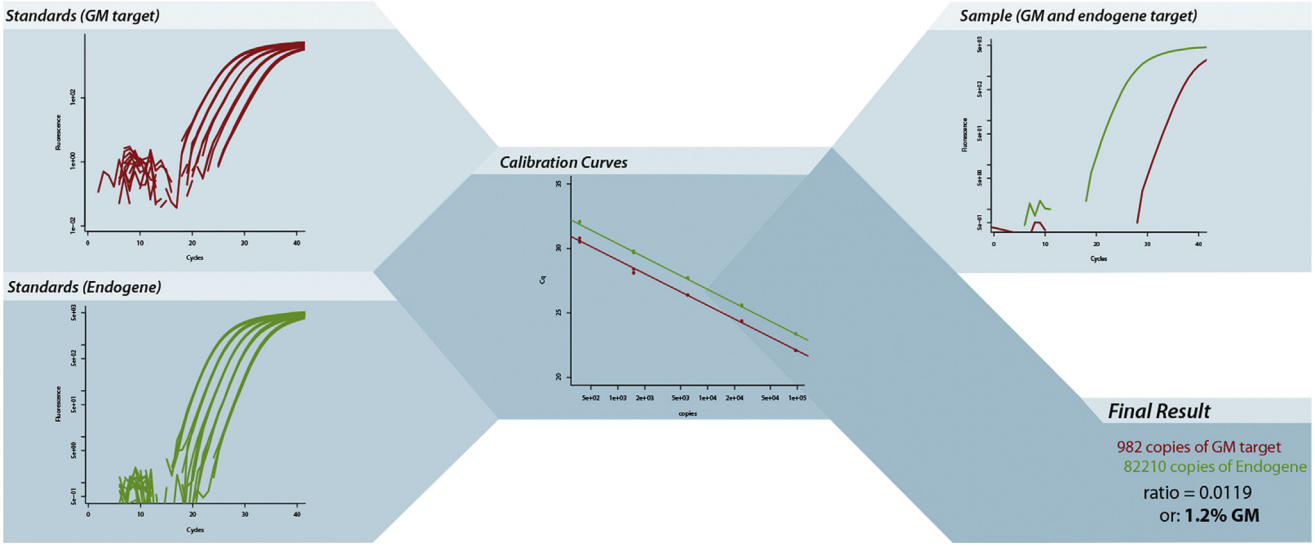

Fig. 3. GMO quantification strategy. Real time PCR detection is carried out for two targets: a wild type endogene and an event specific transgene. Both reactions are performed on dilution series of a reference material (calibration curves) and on the actual sample. The calibration curves then allow the calculation of the GM content of the sample.

contain the same identical junctions flanking the recombinase gene. However, it is currently unclear if this practice will be applied in livestock.

Quantification

Quantification involves the parallel detection of two targets: a wild type endogene and an event specific transgene (both present in one copy per haploid genome). In addition, calibration curves for both genes are constructed using reference materials. The whole of results then allows accurate quantification of the GM content of the sample (see Fig. 3). In current GM quantification strategies, the GM-unique junction regions are used for the purpose of quantification. And as explained in the above, similar targets are expected to be present in GM animals. However, in some cases, difficulties may be caused by the number of transgene copies per haploid genome. Plants are usually homozygous for the transgene construct, containing exactly one transgene target per haploid genome copy. However, for animals this may not always be the case: as mentioned above, certain recombinase-mediated deletion strategies employ two parental lines of GM animals to create a double hemizygous offspring as functional GM. All of this can be coped with when making the calculations, provided the information is available. One remaining drawback is that, in such cases, the limit of quantification is doubled since there are only half as many PCR targets available.

Availability of sequence information

The availability of sequence information is of paramount importance in order to design detection methods and to exclude cross-reactivity. Current EU legislation foresees the producer/applicant to provide this information when applying for market access, along with specific identification and quantification methods for the event under evaluation. The latter are in turn validated by EU-Reference Laboratory for GM Food and Feed (EU-RL GMFF). For a review of the current EU GMO policy see Davison (2010), Devos *et al.* (2013)).

The sequence information the producer/applicant has to provide is the complete sequence of the insertion site, *i.e.* the sequence of the transgenic insert (usually called event sequence) and its flanking genomic sequences (referred to as the 5- and 3-prime flanking regions). This information allows the *in silico* verification of both the method's specificity and its possible cross-reactivity with previous or future detection methods for other events. Moreover, if multiple transgenic insertion sites were created, they should all be provided, together with their flanking regions.

For the current generation of GM animals, in spite of the high number of publications reporting successful transformations, very little sequence information was found to be readily available. For this reason, starting from the references shown in Table 1, a literature and patent-database search was carried out. In a few cases the main publication itself provided useful sequence information, but in most cases we had to resort to tracing the origin of the inserted sequence mentioned by its GenBank sequence id, gene or construct name and references to other publications. While the ultimate goal is always to find the full event sequence, also partial sequences like the sole flanking regions or the inserted gene or construct can prove very useful. The result of this research is presented in Table 2 .

In practically none of the cases there was a well-defined reference or pointer to the actual sequence used. Most publications report the sequence used as a construct that is the

result of a previous work and thus described in another publication. However, the latter publication then usually cites another publication that in turn cites another one and so on. In some cases the chain of references goes back more than thirty years, to periods when sequencing was very difficult and the reliability of the reported sequence information is highly doubtful.

Even the exact extra-genic gene that has been used is in many cases difficult to retrieve: often its sequence was derived as cDNA from reverse transcription-PCR experiments and used directly, in some occurrences without even sequencing it. In other cases, the sequence was obtained before the availability of the complete genome of the donor animal or host animal and as a consequence no similarity comparison was made in order to verify the presence of single nucleotide polymorphisms or non-synonym variants in the inserted sequence.

Another facet of the current generation of GM-animals that became apparent during this literature review, is that the number of integrated genes is often very high. In some cases it is even extremely high. Examples are the cattle carrying the cattle α- and κ-casein gene, where 84 extra copies of the bovine beta-casein gene are predicted to be present (Brophy *et al.*, 2003), and the case of the trout with more than 200 transgenes copies arrayed head-to-tail spread over four or five different insertion loci (Wu, Sun, Wang, Wang, & Zhu, 2005).

Species markers

A crucial point in the current identification strategies using real time PCR, is the requirement for highly specific endogene markers that show no cross-reactivity with other species and are able to detect all (commercial) varieties and/or sub-species of the given organism. On this basis, many PCR based detection and identification strategies for animal ingredients have already been published (see Table 3 for examples) and the identification of (non-GMO) animal ingredients in processed foods by means of PCR has been gaining importance, most notably in the field of food authentication.

Most existing strategies in this field rely on the identification of animal species by targeting their mitochondrial DNA (see Table 3). For achieving high sensitivity, the latter is an ideal target as it is present in many copies per cell and since mitochondria may remain intact during certain forms of processing (Unseld, Beyermann, Brandt, & Hiesel, 1995). Both of these properties increase the probability of detection in highly processed or severely inhibited DNA samples. However, this also means that these methods are at best semi-quantitative since the number of mitochondria per cell may vary widely between cells and tissues.

Nevertheless, identification alone is often suffices when the presence or absence of a given species is relevant. This is, for example, the case for allergens (*e.g.* hazelnut in cake) and food fraud (*e.g.* horse meat in beef burgers). In addition, the presence of certain ingredients may be essential for consumers in order to choose certain foods over others in a reflection of lifestyles (*e.g.* vegan, vegetarian) or religious practices (*e.g.* kosher, halal). In this regard, sequencing in combination with bioinformatics is a versatile tool for detecting highly specific sequences in relevant species that may serve as PCR targets for detection and identification, especially highly conserved regions (HCR).

The above underscores the difficulty of obtaining correct sequence information in the absence of reliable, confidential, producer/applicant provided sequences. Although it seems unlikely that any of these 'first generation' research focussed GM-animals will find their way onto the market, we could scarcely find any mention of follow-up or of the fate of these animals.

In addition to a GM-specific target, the DNA-based quantification of a sample's GM-content requires an endogenous (wild type) target sequence that is both species specific (no cross reactivity with other ingredients) and preferable present in a single copy per haploid genome. Such targets are typically found in HCR of the genome regions, but the identification of such sequences and the verification of their suitability for quantification purposes will require significant effort.

Availability of reference materials

Another crucial point in the quantification of GM content using standard real-time PCR is the availability of certified reference materials (CRM), without which the measurement uncertainty dramatically increases. Generally speaking, since quantification is done by comparison to a standard, a quantification result is only as good as the standard that has been used. Materials of high purity and well-defined GM content (either in target copy numbers or weight percentage) are paramount in the process of efficiently enforcing a labelling threshold.

For GM plants, most reference materials are currently provided by the European Commission's Institute for Reference Materials and Methods (IRMM, Belgium) and the American Oil Chemists' Society (AOCS, Boulder IL). As long as no alternative approaches are developed, quantification of GM animal material in the food chain without the availability of similar reference materials will be difficult. However, there are several technologies that may provide absolute quantification in the absence of CRMs (e.g. digital PCR) but none of these are currently routinely employed in control laboratories across Europe.

Equivalence of DNA ratio and mass percentage

The current quantification approach of the GM (plant) content of food and feedstuffs is based on the assumption of equivalent DNA extraction from all sample ingredients. In other words: the percentage of GM target relative to a species specific target as observed in DNA copies is equivalent to the weight/weight percentage of GM in the ingredient of the original sample. Even though there are many factors that may complicate this assumption (cell size

Table 2. Overview of the sequence availability of the GM animals listed in Table 1. 'ID' is a numerical identifier given to each example to enable straightforward comparison with Table 1. The table indicates whether the junctions between host genome and transgene constructs (so-called event sequence) are currently available or not. In addition, the table lists the accession number(s) of the inserted sequence(s), the sequences' origin (Sequence type), and any relevant patents found.

Species	ID	Involved gene	Event sequence available?	Accession number(s)	Sequence type	Relevant patent	Comments
Cattle	1	hlz, Human lysozyme	No	X14008.1	Genomic	CN-1891821-B	Multiple copies were inserted
	2	Prp, cattle prion protein	No	BTU63637; AY221099	Genomic	US-2011-0231943-A1	–
	3	CSN2, cattle β-casein; CSN3, cattle κ-casein	No	NM_181008.2; NM_174294.2	Genomic	–	Multiple copies were inserted (up to 84 for CSN2)
	4	C. elegans mfat-1 n-3 fatty acid desaturase	No	NM_001028389	cDNA	CN-103074347-A	Humanized mfat-1 gene present in the patent
	5	S. aureus lysostaphin	No	AR649720.1	Genomic	US-6875903-B2	Modified gene never sequenced
	6	hLf, Human lactoferrin	Yes[1]	U95626	Genomic	CN-1873001-A	Contains multiple copies of the incomplete target gene and the vector backbone
Chicken	7	alv, avian leukosis virus	No	AF257657.1	cDNA	US-H001065-H	–
	8	decoy hairpin RNA expression cassette	Yes[1]	–	Synthetic	–	One insertion site on chromosome 2
	9	lacZ, beta-galactosidase from retrotransposon vector	No	AF062997.1	Genomic	–	beta-galactosidase from a retrotransposon vector
	10	TPD IFN-α 2b, recombinant transgenic poultry derived interferon-α 2b	No	GZ791535.1	Synthetic	US-8372956-B2	Codon optimized cDNA derived from human
Carp	11	hGH, Human growth hormon gene, with introns 2, 3 and 4 deleted	Partially[1]	–	Genomic	–	Multiple copies were inserted (more than 200) in 4–5 different loci
	12	hLf, Human lactoferrin	No	U95626	cDNA	–	Plasmid used in transformation is pCAgcGH
Catfish	13	HCECB, cecropin B	No	EA072598	Synthetic	US-7183079-B2	
Fruit Fly	14	Cctra, Ceratitis capitata transformer intron	No	CS802232.1	Genomic	WO-2007091099	the sequence from CS802232.1 is present in the patent, but it is identical to a sequence provided as supplemental material in (Fu *et al.*, 2007)
Frog	15	eGFP, enhanced green fluorescent protein; chicken lysozyme gene promoter region	No	X98408.1	cDNA and genomic	US 8476484 B2	X98408.1 corresponds to the chicken lysozyme gene promoter region
Goat	16	hlz, Human lysozyme; bovine kappa casein	No	NA	cDNA	US-7199281-B2	–
	17	scd, rat stearoyl CoA desaturase	No	NM_031841.1; X14710.1	cDNA	US-244874-B2	Expression controlled by the bovine β-lactoglobulin promoter (X14710.1)
	18	msp-1, merozoite surface protein-1	No	AX686827.1	cDNA	WO-2002058727-A2	MSP-1 sequence present in the patent

	19	rhAT, recombinant Human antithrombin cDNA	No	–	cDNA	EP-0256302-A2	cDNA derived from a modified pBAT6 plasmid (sequence in patent)
	20	LAtPA, Human longer acting tissue plasminogen activator	No	–	–	–	LAtPA is mentioned as being a modified version the human version and is under control of the murine whey acid promoter (WAP)
	21	hLf, Human lactoferrin	No	X53961	cDNA	–	Multiple copies are inserted.
	22	hGCase, Human beta-glucosidase	No	M16328	cDNA	–	–
	23	hFIX, Human coagulation factor IX	No	–	cDNA	–	–
	24	HBD3, Human beta defensin 103B	No	–	Genomic	CN 101591672 B	Patent refers to transgenic cows, but sequence n.1 is the HBD3 described in the article
Pig	25	AppA, E. coli phytase	Yes	AR986122	Genomic	US-7115795-B1	Expression controlled by the murine parotid secretory protein (PSP) promoter/ enhancer
	26	Porcine growth hormone	No	–		US-5573933-A	Human metallothionein-IIA promoter fused to porcine growth hormone. Sequence may be extracted from the patent.
	27	c-ski, chicken c-ski protein gene	No	M28517.1	Genomic	US-6218596-B1	–
	28	hlz, Human lysozyme	No	X14008.1	Genomic	CN 1891821 B	cDNA with 2 copies of chicken β-globin insulator and one copy of the goat β-casein promoter. Two different GM-pigs were produced: one described in (Tong *et al.*, 2011) where hLZ expression levels were low; a second one described in (Lu *et al.*, 2014) that used the same construct of (Yang *et al.*, 2011), successful for GM-cattles.
	29	FAD2, spinach fatty acid desaturase	No	AB094415	cDNA	US-2006-0282907-A1	Construct contains 2 mouse AP2 promoter regions. In the patent a slightly different sequence from spinach is reported.

(*continued on next page*)

Table 2 (*continued*)

Species	ID	Involved gene	Event sequence available?	Accession number(s)	Sequence type	Relevant patent	Comments
	30	hfat-1, humanized Caenorhabditis elegans gene	No	DJ417997.1	Genomic	US-2007-0274952-A1	Expression controlled by the chicken βactin promoter.
	31	alpha-LA, bovine alpha-lactalbumin gene		EA061936.1	Genomic	US-7169963-B2	
	32	mouse Mx1 cDNA	No	–	cDNA		The authors published the sequence of Sus scrofa Mx1 gene (X54328.1, X54329.1, X54330.1)
	33	Human factor VIII	No	–	cDNA	US-5880327-A	
	34	GGTA1, sus scrofa alpha-galactosyltransferase 1	No	NM_213810.2	cDNA	–	Sequence knockout, actually no new gene inserted
	35	CMAH, sus scrofa monophosphate-N-acetylneuraminic acid hydroxylase	No	NM_001113015.1	cDNA	–	Sequence knockout, actually no new gene inserted
	36	Human membrane cofactor protein (MCP); Human decay accelerating factor (DAF); Human CD59 complement regulatory protein	No	–	cDNA	US-6166288-A	The promoter sequences are reported in the patent (SEQ ID 3 and SEQ ID 4)
	37	hDAF, Human decay accelerating factor	No	AB025019.1	Genomic	US-6825395-B1	Also known as CD55 gene. In the patent the porcine promoter is reported as SEQ ID 1, that is a fragment derived from AB025019.1
	38	hHO-1, Human heme oxygenase-1	No		cDNA	US-7378569-B2	–
	39	hDAF, Human decay accelerating factor (hDAF); hHO-1, Human heme oxygenase-1 (hHO-1)	No	NM_000574; NM_002133	cDNA	US-7378569-B2	–
	40	GnT-III, Human N-acetylglucosaminyltransferase III	No	–	Genomic	US-7378569-B2	In the patent the authors also described the GnTIII gene under the control of the DAF promoter
	41	Human fibrinogen A chain, Human heterologous fibrinogen B chain and human gamma chain	No	–	cDNA	US-7435869-B2	–

	42	Mutated human alpha globin; mutated human beta globin	No	–	Genomic	WO-1993-025071-A1	In the patent, it is stated that the inserted human genes carry one or more mutations to increase the level of human haemoglobin dimers in transgenic pigs
	43	Human protein C	No	–	cDNA	US-5589604-A	–
	44	Human albumin; green fluorescence protein	No	–	cDNA	–	–
Rabbit	45	sCT, salmon calcitonin	No	–	Synthetic	US-6211427-B1	In the article the authors describe a human alpha lactalbumin joined by an enterokinase cleavable linker to salmon calcitonin
	46	Human erythropoietin	No	Z48305.1	Synthetic	–	Z48305.1 record corresponds to B.taurus gene for beta-lactoglobulin variant B. In fact in the article the authors describe a bovine beta-lactoglobulin variant B gene with an in-frame inserted human erythropoietin cDNA.
	47	Human EC-SOD, extracellular superoxide dismutase	No	BC014418.1	cDNA	US-6025540-A	Sequence SEQ ID 1 of the patent is part of sequence Genbank: BC014418.1
	48	hIL2, Human interleukin-2	No	–	Genomic	–	–
	49	htPA, Human tissue plasminogen activator	No	–	cDNA	CU-22365-A1	–
	50	VP2, VP4, VP6 and VP7 retroviral proteins	No	–	Synthetic	US-2010-0028371-A1	In the patent, several mutagenized sequence versions of the VP cDNAs are present.
	51	Human factor VII	No	M13232; M13438; U78775; J00440	cDNA	US-2011-0059510 -1	1 integration site mapped on the q26-27 telomere region of chromosome 7q
	52	hGH, Human grow factor	No	M13438	Genomic	–	cDNA with ovine alpha-S1 casein (bCSN1S1) promoter and bovine growth hormone (bGH) polyadenylation signal sequences

(*continued on next page*)

Table 2 (*continued*)

Species	ID	Involved gene	Event sequence available?	Accession number(s)	Sequence type	Relevant patent	Comments
	53	vWF, Von Willebrand factor	No	–	Genomic	US-6255554-B1	Expression controlled by the ovine alpha-S1 casein (bCSN1S1) promoter and bovine growth hormone (bGH) polyadenylation signal sequences
Salmon	54	Salmon growth hormone	Partially[1]	AR093231.1	Genomic	US-5998697-A	Multiple copies inserted in one locus. Flanking sequence in (Uh, Khattra, & Devlin, 2006)
	55	Salmon growth hormone	Yes[1]	AY594644	Genomic	EP-0578653-B1	Flanking sequence published in (Yaskowiak, Shears, Agarwal-Mawal, & Fletcher, 2006) and also present in patent
	56	lyzII, trout lysozyme II	No	X59491	cDNA	–	In (Fletcher *et al.*, 2011) the sequence of the full construct is reported in Fig. 2
	57	AFP, antifreeze protein	Partially[1]	AR222424	cDNA	US-6429293-B1	Flanking sequence is reported in Fig. 1C of the article.
Sheep	58	IGF-1, ovine insulin-like growth factor 1	No	–	cDNA	AU-1996-050613-A	The IGF-1 is from ovine origin and it was linked to the mouse ultra-high-sulfur keratin promoter
	59	cysK, S.typhimurium cysteine synthase A	No	M21450.1	Genomic	US-5360742-A	
	60	HTT, Human huntingtin	No	FJ457100	cDNA	US-2009-0304595-A1	In the patent a pig model is described.
	61	Visna virus	No	M10608.1	Genomic	–	–
	64	hFIX, Human coagulation factor IX	No	K02402.1	Genomic	US-6344596-B1 WO-1990-005188-A1 WO-1988-000239-A1	At least three different transgenic sheep carrying hFIX been created by different authors. The authors of the patent US-6344596-B1 are not the same of the cited references. Patent WO 1990–005188 A1 refers to (Clark *et al.*, 1989). In (Schnieke *et al.*, 1997) a PGKneo marker gene is also described as inserted.
	65	hFVIII, Human coagulation factor VIII	No	–	cDNA	WO-1996-009377-A1	More than one transgenic sheep carrying hFVIII have been created by different authors. The authors of the patent US-6344596-B1 are not the same of the cited references.

	66	AAT, Human α-1-antitrypsin	No	A01395.1	Synthetic	WO-1990-005188-A1	–
Silkworm	67	eGFP, enhanced green fluorescent protein	No	–	cDNA	US-7459599-B2	The patent is referred to the initial invention
	68	$A2S8_{14}$, spider silk protein	No	JB815849.1	cDNA	US-2013-0212718-A1	In the patent, there are also other sequences that have been used in the different experiments.
	69	Synthetic silkworm fibroin gene	No	–	Synthetic	–	The gene described here is a synthetic gene obtained by fusing part of silkworm fibroin gene with DNA fragments coding for active sites of collagen or fibronectin proteins
	70	CRP, canine C-reactive Protein	No	–	cDNA	US-8865966-B2	Sequence available in the patent (SEQ ID No 6)
	71	TRACP5, Human tartrate-resistant acid phosphatase 5	No	GZ833686.1	Synthetic	US-8426674-B2	No publication, only patent
Trout	72	FST, follistatin	No	FJ185129.1	cDNA	–	
Tilapia	73	Humanized tilapia insulin	No	AR252279	Synthetic	US-6476290-B1	Sequence available in the patent (SEQ ID Nos 6 and 7)
Zebrafish	74	GFP, green fluorescent protein	No	GX731578	Genomic	US-8378169-B2	GX731578 refers to the MLC2f promoter region, that is the most important part of the GM cassette, as it allows tissue-specific expression of fluorescent proteins.

[1]To be reconstructed *in silico*.

Table 3. Examples of animal species-specific PCR markers used in food authentication. The species, location and identity of the PCR targets, and amplicon size are listed along with the respective references. References to literature for each entry in the table can be found in the supplemental material. Note that most detection methods use mitochondrial DNA targets.

Species	Target origin	ID	Gene	Amplicon size (bp)
Chicken	Mitochondrial	75	D-loop	256
	Mitochondrial	76	ND5/Cytochrome b	117
	Mitochondrial	77	Cytochrome b	106
Cow	Mitochondrial	78	D-loop	513
	Mitochondrial	79	12S	252
	Mitochondrial	80	Cytochrome b	120
	Mitochondrial	81	12S	256
Donkey	Mitochondrial	82	ND2	145
	Mitochondrial	83	ND2	183
Duck	Mitochondrial	84	D-loop	292
Emu	Mitochondrial	85	Cytochrome b	229
Goat	Mitochondrial	86	12S	117
	Mitochondrial	87	12S	326
Haddock	Nuclear	88	transferrin	72
Halibut	Nuclear	89	5S	368
Horse	Mitochondrial	90	ATPase 8/6	153
	Mitochondrial	91	ATPase 8/6	147
	Mitochondrial	92	Cytochrome b	76
Ostrich	Mitochondrial	93	Cytochrome b	543
Pig	Mitochondrial	94	D-loop	835
	Mitochondrial	95	ND5	227
	Mitochondrial	96	Cytochrome b	131
	Mitochondrial	97	ND5	115
Pigeon	Mitochondrial	98	D-loop	401
Seagull	Mitochondrial	99	ND2	94
Sheep	Mitochondrial	100	12S/16S	172
	Mitochondrial	101	t-glu/Cytochrome b	119
	Mitochondrial	102	Cytochrome b	95
Sole	nuclear	103	ITS1	116
	nuclear	104	5S	187
Tuna	Mitochondrial	105	16S	63
	Mitochondrial	106	16S	130
Wallaroo	Mitochondrial	107	Cytochrome b	205

may vary between tissues, fruits and seeds may be (partially) haploid, *etc*.) a large body of practical experience currently justifies its use (Berdal & Holst-Jensen, 2001; Corbisier *et al*., 2007; Trapmann, 2006; Trapmann, Corbisier, Schimmel, & Emons, 2010) and its existence as an official recommendation from the European Commission (EC 2004/787/EC, 2004).

In animals, there are again several biological facts that complicate the assumption of equivalence between DNA ratio and mass percentage, the most straightforward relating to the nature of muscle tissue. During embryonic development, myoblasts fuse to form multinucleated myofibers. Postnatal growth of skeletal muscle happens mainly by increases in length and width of the muscle fibres and not by an increase of the number of muscle fibres (Rehfeldt, Fiedler, Dietl, & Ender, 2000) (and therefore, in the number of cell nuclei). Yet, there is significant postnatal DNA accumulation in muscle tissue (Allen, Merkel, & Young, 1979) in so-far that there appears to be a quite linear relation between the number of nuclei and the muscle mass (Trenkle, DeWitt, & Topel, 1978), relating to the large population of satellite cells that are associated with muscle tissue (Mauro, 1961; Moss & Leblond, 1971).

Transferability of the observed DNA percentages to ingredient weight ratios thus seems likely but will have to be confirmed and validated before this assumption can be put to practice in quantifying food and feed for their GM animal content. As an additional complication, 'fat' is often regarded as a separate ingredient for certain food products. On DNA basis however, the distinction between different tissues is not readily made and in practice it may only be possible to distinguish between ingredients if they originate from different (animal) species.

GM animal product derivatives

Next to samples that are either pure meat or have meat as their main ingredient, there are a large number of processed food products that contain animal derivatives (see Table 4 for a list of common ingredients derived from animals). Although the DNA content of food has been of increasing interest to control authorities worldwide, data that show which food or food fractions still contain DNA are not readily available. As a consequence, the degree to which a single animal derivative ingredient is detectable by DNA based methods in a sample (if at all) is currently not known and is likely to differ between products and ingredients. In such cases, the detection of GM animal content and its quantification may be more difficult and/or require specific DNA extraction methods or entirely different approaches.

PCR inhibition and DNA degradation

Many problems of sensitivity and repeatability in the field of real-time PCR have to do with the quality of the

Table 4. Common processed food ingredients from animal origin. Ingredients marked with a* may also be derived from plants.

Ingredient	Origin
Albumin	Egg whites
Casein (caseinate)	Milk protein
Gelatin	Bones, cartilage, tendons, and skin
Glucose (dextrose)*	Animal tissues and fluids
Glycerides (mono-, di-, and triglycerides)	Animal fat
Isinglass	Air bladder of freshwater fish
Lactose (saccharum lactin, D-lactose)	Milk sugar
Lecithin*	Animal tissues and egg yolks
Lutein*	Egg yolks
Oleic acid (oleinic acid)	Tallow
Pepsin	Pig stomach
Stearic acid (octadecanoic acid)	Tallow
Vitamin A (A1, retinol)*	Egg yolks, fish liver oil
Vitamin D3	Fish liver oils

extracted DNA and its ability to sustain an efficient PCR reaction. Maximal reaction efficiency is rarely guaranteed due to the possible presence of so-called PCR inhibitors (Bessetti, 2007) which limit reaction efficiency. These inhibitors generally act by direct interaction with DNA or interference with the polymerases (Opel, Chung, & McCord, 2010). In addition, DNA polymerases have ionic requirements that can be the target of inhibition.

These problems are not limited to GM animal samples and strongly depends on where in the processing chain the sampling takes place since the prime source of inhibitors are the samples themselves. In most cases these chemicals are co-extracted with the DNA and additional purification steps are required to reach a sufficient level of DNA purity. Another source of inhibitors are the reagents used during sample processing and DNA extraction. These include high concentrations of KCl, NaCl and other salts, ionic detergents (sodium deoxycholate, sarkosyl, SDS), alcohols (ethanol, isopropanol), phenol, etc. (Bessetti, 2007).

In addition to inhibitors, processing of foodstuffs further reduces the DNA quality. Chemical and physical treatment of the products (heat, pH, excessive grinding, etc.) result in random fragmentation of the DNA (Kakihara, Matsufuji, Chino, & Takeda, 2006). This reduction in the average length of the DNA molecules makes their isolation more challenging (Kakihara *et al*., 2006) and may cause loss of amplification targets, thus decreasing sensitivity of detection and inducing bias in the quantification results (Godalova, Bergerova, & Siekel, 2013).

Although there are no direct reasons to suspect the situation will be significantly different in animal samples compared to other food samples, an assessment of which methods work best on which matrices may be necessary to validate the applicability of existing protocols. Especially during the initial stages of testing for GM animals, the adoption of specific extraction methods and the design of standard operating protocols may slow the pace of the laboratory work.

Conclusion

We have counted at least 74 (on-going) attempts at animal modification, of which about half are designed for use as livestock. Amongst the latter, at the majority are intended for direct release onto the food market (as meat or meat product) as opposed to animals for wool or milk production. The number of GM animals for which we were able to find (partial) sequence information was 56. Of all the genetically modified animals listed, there is currently only one that is actually being brought into production (AquaAdvantage® salmon).

These figures are testament of the increasing scientific activity related to GM animals and of the development of a growing number of early commercial products in all of the categories defined (*i.e*. livestock, bioreactor, companion). Incidental contamination of the food chain with GM animal material or derived products will become increasingly likely, more so once the commercial farming of GM animals begins.

Detection and identification of GM materials (be it plant, animal or microbial) in food and feed samples is, in principle, no problem as it can be assumed that every genome contains relatively short (50–500 bp) characteristic sequences by which it can be distinguished from others and that can be readily amplified using standard PCR approach. Current methodology for the control of genetically modified organisms should be therefore be transferable and perform comparably on animal derived products. However, techniques for detection and identification of sub-species, varieties, and specific tissues are not readily available in control laboratories, but DNA targets for such tests may be become available through the expansion of the genome databases and on-going bioinformatic analysis efforts.

Accurate quantification of GM animal products, on the other hand, requires a number of prerequisites to be fulfilled. The assumption of equivalence between DNA target ratio and weight percentage should be validated and certified reference materials must be available. In addition, quantification on a per ingredient basis may complicated by the classification of different parts of the animal as separate ingredients. There are, however, several alternative quantification strategies such as quantification on a ‘per species’ base, or on a ‘per total animal content’ base. Although the latter may complicate the assumption of DNA/weight equivalence. Lastly, there are some particular genetic constructs that may compromise accurate quantification (mosaic animals, hemizygote animals, etc.).

Several of the challenges involved in the appearance of GM animal derivatives on the international market may be tackled by the further incorporation of novel techniques into the routine testing work flow. Most notably, digital (droplet) PCR and Next Generation Sequencing (NGS) have the power to transform the current practice of quantification based on standard curves using well-defined targets. For instance, taxonomic profiling and classification involves the computational analysis of (NGS) random shotgun sequencing results from DNA isolated directly from a sample for its species composition and abundance. Current efforts for developing such algorithms are mainly focussed on bacterial samples (Darling *et al*., 2014; Dröge, Gregor, & McHardy, 2014; Gregor, Dröge, Schirmer, Quince, & McHardy, 2014), but the principles used for binning the sequences can be applied to higher species as well. Similarly, a suitable dilution of the sample DNA run in carefully constructed ddPCR multiplex conditions may simultaneously characterize and quantify a sample for its species composition. Although in the latter case, one can only find the species one is looking for, whereas NGS analysis is usually only limited by the content of the databases to which the results are compared.

In summary, at the base of a good enforcement systems lies **(I)** correct, representative sampling and **(II)** analytical

methods that allow reliable detection and quantification. The latter is facilitated by the availability of correct sequence information which allows the construction of powerful screening methods and the prevention of cross-reactivity of the detection methods that are to be installed. **(III)** Another pivotal point is the availability of reference materials. These allow validation of the detection methods and the reliable quantification of GM-positive samples. We see no issues as far as the detection of GM animals is concerned. However, their quantification faces two challenges: one relates to the search for single copy nuclear endogenes, the other relates to the cases in which the animals are either mosaic or not homozygous for the transgene. Whether the latter are truly problematic can only be decided on a case-by-case basis through thorough examination of their complete sequence information.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tifs.2015.05.001.

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