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Recent Patents for Detecting the Species of Origin in Animal Feedstuff, and Raw and Processed Meat Products

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Abstract: The value of the traceability and labeling of food is attributable to two main aspects: health safety and/or product or process certification. The identification of the species related to meat production is still a major concern for economic, religious and health reasons. Many approaches and technologies have been used for species identification in animal feedstuff and food. The early methods for meat products identification include physical, anatomical, histological and chemical. Since 1970, a variety of methods were developed, these include electrophoresis (i.e. isoelectrofocusing), chromatography (i.e. HPLC), immunological techniques (i.e. ELISA), Nuclear Magnetic Resonance, Mass Spectrometry and PCR (DNA and RNA based methods). The recent patents on species detection in animal feedstuffs, raw meat and meat processed products, listed in this work, are mainly based on monoclonal antibodies and PCR, especially RT-PCR. The new developments under research are looking for more sensible, specific, less time consuming and quantitatively detection methods, which can be used in highly processed or heated treated meat food.

Keywords: Contaminant, DNA, fraud, meat, processed, species identification.

INTRODUCTION

The importance of foods' traceability and source/product labeling is mainly attributable to health safety issues and/or product/process quality certification. Although recent technological advances have led to more sensitive techniques for traceability of foods, mislabeling of food products, intentional or unintentional, is still a major concern in world commerce. The identification of species origin of raw meat and meat products is central to protect both producers and consumers from trade fraud [1]. There are three levels at which specific controls for food traceability may be established. These levels relate to different stages in the food chain production, and include feedstuffs for animal breeding, fresh or minimally processed meat, and highly processed meat products.

***Feedstuffs for animal breeding*:** with Bovine Spongiform Encephalopathy (BSE) emerging in 1986, there was a generalized alert about animal feeding due to its potential implications on human health. As a consequence, several countries either prohibited or heavily controlled the use of artificial foods with products of mammalian origin as a source of proteins in animals' feeding rations [2]. Furthermore, species identification in animal feedstuff can help in detecting adulterations [3], genetically modified components, potential presence of toxins (incorporated through some specific seeds), or important specific nutrients (e.g., omega 3 from fish sources).

***Fresh meat or minimum processed meat products*:** Tests for the characterization of fresh meat and minimally processed food products can be used for a variety of reasons, including health, economic, and religious. Usually, adulteration is done for economic reasons, using meat sources of lower economic value or wild species, such as using horse meat or wild boar in beef products [4]. On the other hand, some religious traditions do not allow eating meat from selected species, like pork for Muslims and Jewish or beef for Hindus. Other communities may have special feeding restrictions due to cultural trends. For example vegetarians do not eat any meat, and most occidental cultures prefer not to eat meat from animals that can be considered pets (e.g., meat from dogs or cats). Furthermore, there are individual predispositions that can generate allergic reactions to specific meat proteins, which in some cases may be fraudulently added into the food [5].

***Highly processed meat products*:** Highly processed products lead to major disruptions of tissue structures and molecular denaturalization and degradation of proteins and DNA). Therefore, traceability and identification techniques confront major challenges associated with the alteration of original products by food processing. These challenges make highly processed meat products more prone to be subjected to adulteration or fraud. This has been confirmed by previous

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studies, which have detected a higher adulteration rate in cooked meat products over raw meat [6]. Furthermore, highly processed products may have complex composition mixtures which may also need to be tested or controlled [7].

Many strategies and techniques have been commonly used for identification of species source in both animal feedstuff and food. Early methods for meat product identification include characterization of physical and anatomical features (e.g., size of muscles, color, odor, texture and/or vertebrae number in carcasses), histological characters (e.g., length, size and density of the muscle fibers) and characterization of chemical compounds (e.g., myoglobin or carotenes) (reviewed in [8]). Some of the early methods were claimed to detect animal contamination in meat, like patent US5213830 [9], which describes a UV absorption based method to detect worms in fish products. Since 1970, methods based on the characterization of the protein fraction present in meat were developed. These included protein electrophoresis [10, 11], chromatography [12], and immunological techniques [13, 14]. The development of the Polymerase Chain Reaction (PCR) led to the rapid expansion of DNA based methods for species detection in organic products. PCR based methods used multiple amplification strategies, including Randomly Amplified Polymorphic DNA (RAPD) [15], species-specific primers [7], Restriction Fragment Length Polymorphisms (RFLP) [16,17], Real time PCR (RT-PCR), [18] and others [19].

To date, the United States Department of Agriculture (USDA) recognizes three major methods for identification of species source in fresh meat and meat products; these include Agarose Thin-layer Isoelectrofocusing, Serological techniques and enzyme-linked immunosorbent assay (ELISA) techniques [20]. Although multiple patents for species identification of meat products have been filed, most are aimed at detecting specific antigens based on monoclonal antibodies, or detecting specific DNA or RNA regions based on PCR. In most cases these methods have shown to have the sensitivity and specificity needed for detection of fraudulent meat and/or contamination.

STATE OF THE ART IN MEAT SPECIES DETECTION

One of the main difficulties in species identification is that the marker used must be specific and thus, should have limited variation and be well characterized. In addition, markers should be able to be measured accurately [21]. Prior to the development of molecular techniques, microscopical and anatomical methods required highly trained personal and were time consuming. The early molecular methods included the detection of certain species-specific components. Monoclonal antibodies were a great tool for this purpose and different assays were developed, including agglutination tests [22], ELISA [23, 24] and inmunoelectrophoresis [25]. Other target molecules for species identification included histidine dipeptides (anserine, carnosine and balenine) to differentiate between pork and lamb [26], fatty acids or triglycerides detected by Nuclear Magnetic Resonance (NMR) [27], or specific hemoglobins characterized by High Pressure Liquid Chromatography (HPLC) [28].

Table 1. Recent Patent on Specie Identification in Feedstuff, Raw and Processed Meat

Patent or Application Number	Year	Author	Use	Technology	Gene/Protrine	Kind of Material Used	Species Tested	Contaminant Detection
US5213830	1993	Haagensen, *et al*. [9]	Detection of worms in meat	Specific UV Absorption			Fish	Anisakis and Phocanema (Nematodes)
EP0714515	1997	Ansfield [49]	Detection of beef in feedstuff	Meat protein concentration + Inmunological (Ab)	Heat stable meat proteins	Rendered animal material	beef, pork, lamb, avian (chicken/Turkey)	0.8966 ug/ml (beef), 0.3833 ug/ml (ovine)
US6288215	2001	Hsieh [50]	Specie Identification	MoAb and polyclonal Ab	Heat stable meat proteins	Raw and heated meat	Beef, pork, lamb, horse, deer, chicken, turkey, duck	NS
US6423506 - WO9808371	2002	Hsieh [51,52]	Specie Identification	MoAb and polyclonal Ab	Heat stable meat proteins	Raw and heated meat	Beef, pork, lamb, horse, deer, chicken, turkey, duck	1% w/w
US6692930	2004	Hsieh [53]	Specie Identification	MoAb and polyclonal Ab	Heat stable meat proteins	Raw and heated meat	Beef, pork, lamb, horse, deer, chicken, turkey, duck	0 - 10% depending in the contaminant
US7297500 - WO02065126	2007	Hsieh & Chen [54,55]	Specie Identification	Troponine I MoAb	Troponine I	Raw and heated meat	Beef, pork, lamb, horse, deer, chicken, turkey, duck, geese, ostrich	less than 1%

(Table 1) coontd……

Patent or Application Number	Year	Author	Use	Technology	Gene/Protrine	Kind of Material Used	Species Tested	Contaminant Detection
US5786144	1998	DeSalle & Birstein [57]	Differentiate species of caviar	PCR (universal primers)	mtDNA	Processed caviar	27 sturgeon species	NS
JP2000210085	2000	Kato, *et al.* [64]	Specie Identification	PCR (universal primers)	Cytochrome B (MtDNA)	meat	Beef, lamb	NS
US20050069890	2005	Mabilat, *et al*. [63]	Specie Identification	Microarray	specie specific multiprimers	various materials	Beef, pork, lamb, goat, rabbit, bare, deer, ostrich, chicken, turkey, goose	1% v/v of DNA
US7141364	2006	Verma & Singh [65]	Specie Identification	PCR (universal primers)	Cytochrome B (MtDNA)	NS	221 wild species	Yes
WO2007119066 - US20090170107	2007 - 2009	Reaney, *et al*. [66,67]	Detection of Animal source in feeds	PCR (specific primers)	16s rRNA	ruminant feed	Beef, pork, lamb, avian, fish	0.1%
WO 2008/056325	2008	Macedo Pereira, *et al.* [68]	Specie Identification	PCR (specific primers)	12s and 16S rRNA	NS	Beef, pork, lamb, goat, horse, mouse, dog, cat, rabbit	NS
US20080085522	2008	Maghen, *et al*. [58]	Specie Identification	PCR (universal primers)	Microsatellite	various materials	Beef, pork, lamb, goat,, venison, horse, chicken, turkey.	NS
US7582452	2009	Sinha, *et al.* [62]	Specie Identification	Real Time PCR	Short Interspersed elements (SINEs)	meat and meat products - feedstuff for cattle	Beef, pork, lamb, horse, deer, chicken, antelope, rabbit, duck, cat, rat, mouse, human	DNA mixtures of pork, beef and chicken
EP1693464 - US7816078 - WO2005040423	2008 - 2010 - 2005	Riviere [61,70,71]	Specie Identification	PCR + secuencing (universal primers)	Citoplasmatic Beta Actin	NS	Lamb, chimpanzee, human, rabbit, dog, cat, bear, horse.	NS
EP1702078 - WO2005061726 - US8158353	2010 - 2005 - 2012	Bulte, *et al.* [59,60,69]	Specie and central nervous tissue detection	Real time PCR	Glial fibrillary acid protein (GFAP) mARN	meat and meat products (including heated samples)	Beef, pork, lamb, goat	NS
US7927841	2011	Sinha, *et al.* [62]	Detection of ruminant or pork material - quantify Beef/Chicken proportions	Real Time PCR	Short Interspersed elements (SINEs)	meat and meat products, feedstuff, cosmetics	Beef, pork, lamb, horse, deer, chicken, antelope, rabbit, duck, cat, rat, mouse, human	Beef 0.005%, pig 0.0005%, chicken 0.05%

NS = Not specified

Other techniques were based on differences in protein structure or expression. As previously mentioned, electrophoretic methods like polyacrylamide gel electrophoresis [29] and isoelectrofocusing [30] were among the early methods developed; however, these methods are still used for species characterization. For example, Vallejo-Cordoba *et al*. [31] have recently shown differences comparing the water soluble and salt soluble protein fractions from bovine and ostrich muscle using sodium dodecyl sulphate polymer-filled capillary gel electrophoresis (CE-SDS). Furthermore, stable proteins during meat aging and proteins that are slightly degraded during food processing could also be used to differentiate meat species by 2D-gel electrophoresis [32]. Additionally, peptides that are not significantly affected by cooking and heat treatment can also be used to quantitatively detect, using Mass Spectrometry, contaminants in feedstuff or highly processed food [33]. For example, Sentandreu *et al*. [33] were able to detect up to 0.5% w/w of beef, pork,

chicken and turkey in cooked products. As compared to newly established molecular methods, the use of proteomic strategies to detect protein/peptide biomarkers has become limited in meat science [34].

DNA based methods were specifically developed because of the stability and ubiquity of the DNA as a marker, but also these analysis are more specific, less expensive and less time consuming than others, including infrared spectroscopy [35], ELISA (reviewed in [36]), hybridizations [37], or capillary electrophoresis [38]. Furthermore, these methods have proven to be sensitive enough for detecting very small quantities of DNA (up to 1pg) even in heat-treated foods or feedstuff [39]. Different type of DNA polymorphisms and multiple techniques have been used for species-specific identification, including RAPD [15], RFLP [40, 41], or Specific Primer PCR [42, 43]. In particular, the use of forensically informative nucleotide sequencing (FINS) by direct sequencing has been broadly used for the characterization of multiple animal species [44, 45]. Those traditional PCR techniques detect the amplification product in the plateau phase of the reaction, while RT-PCR use florescence compounds to detect and screen amplicons during the whole PCR reaction. Considering that there is a quantitative relationship between the amount of DNA in the sample and amount of PCR product at any given cycle number, and that the exponential phase is the optimal point for analyze the data, RT-PCR increase the sensibility and allow quantification [8, 21] Although nowadays DNA based methods are the most widely used approaches in this field [17, 46, 47], because species-specific identification is usually performed using highly degraded DNA from processed foods [48], this method may be prone to false negative results.

RECENT PATENTS ON MEAT SPECIFIC IDENTIFICATION

Most recent patents for species specific meat detection use DNA or RNA detection through some kind of PCR technique. However, there have been multiple patents filed in the last several years that use specific antibodies (Ab) or monoclonal antibodies (MoAb) to detect the species source of cooked or heat-treated meat and meat products [49-55]. Most of these patents have been filed by the same inventor. Hsieh & Chen [54, 55] developed MoAb for a specific muscle protein (Troponine I) that can distinguish a wide number of domestic animal species, detecting contamination levels up to less than 1%.

With the development of PCR in 1983 and the massive production of *Taq Polymerase* since 1988 [56], multiple PCR-based techniques for food analyses have been developed. One of the earliest patents for species identification based on DNA was released in 1998 [57]. This patent described a method to differentiate caviar species using universal mtDNA primers. In most patents, the suggested originality was the specific primers designed for the PCR amplification. Thus, all possible techniques later described to differentiate that specific amplified region were usually claimed "on the whole".

Different kinds of polymorphisms used for species identification are commonly described in the most recent patents. These include microsatellites [58], Indels [59-61], Short Interspersed Elements (SINEs) [62] and Single Nucleotide Polymorphisms (SNPs). These are described at the nuclear DNA [63], mtDNA [64, 65], rRNA [66-68] and mRNA levels [69]. Techniques can also be categorized based on the type of technology/primers used (PCR or RT-PCR/specific or universal). In this sense, we can distinguish three major groups of techniques: (i) PCR techniques with universal primers, which can detect differences in the amplicon size between different species (e.g., mtDNA or microsatellites; used in patents [58, 64, 65]), or differences in the DNA sequence (as described in patents [70, 71]). (ii) PCR with species specific primers, which detect the presence of organic material from the specific species tested. In this case a positive PCR result implies the presence of species-specific DNA in the sample [66, 68, 72]. (iii) RT-PCR which detect differences in the melting temperature of both, amplicons with different length and/or different sequences. This technique can use both specific and universal primers [59, 61, 69, 73, 74].

Finally it is worth to mention that the detection thresholds of different approaches and techniques may vary significantly. In general, most recently developed (RT-PCR) techniques tend to detect lower amounts of contaminants and more accurate mixture compositions than the other PCR-based methods. Furthermore, a particular test could have differences in the detection threshold between species. This has been shown, for example, in a recent patent [62] that reported differences in the detection level of a RT-PCR method for beef, pig and chicken meat (0.005%, 0.0005%, and 0.05% w/w, respectively).

CURRENT & FUTURE DEVELOPMENTS

DNA and RNA analyses have shown to be useful to detect the presence of a particular species in a wide range of samples, in particular animal feedstuffs, as well as raw and processed meat. RT-PCR has also been used to detect quantitatively, and very precisely, compound mixtures from different species or the presence of contaminants in meat. Furthermore, most recent patents filed in this area also follow this tendency. Significant advances are currently being done to reduce the length of time needed for the testing of samples (high throughput) and the threshold detection limits [75, 76]. These technological advances in addition to recent techniques in whole genome sequencing, are leading to the development of novel and more robust methods for the analyses of food products.

To avoid the disadvantages of DNA technology, other methods are also being developed. Such is the case of lectin chips that are being tested to detect the species of milk and milk products, hence could be used for meat too. This specific sorbent assay, exploit the differential protein glycosylation between species and also tissues [77]. Other technologies under development, like Raman [78], would be able to do the determination/control in the place where the material is sold or exposed. Finally, during the last few years new methods from the "omics" field have been added to the molecular toolbox for food analyses [21]. Proteomics is proving to achieve the required specificity and precision for detecting species products and compound mixtures of organic material [34], Metabolomics have been commonly applied to fruit

and vegetables, leaving its use on meat, seafood, and other related areas still underexplored. Hence, there is a need to develop a food metabolome database to facilitate compound identification and new developments [79].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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