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SHORT COMMUNICATION

Occurrence of mislabeling in meat products using DNA-based assay

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Abstract Considering that the authentication of food contents is one of the most important issues for the food quality sector, and given the increasing demand for transparency in the meat industry followed the horsemeat scandal in Europe, this study investigates processed-meat products from Italian markets and supermarkets using the mitochondrial *cytochrome b* gene qualitative PCR identification system in order to verify any species substitution or mislabeling. The results revealed a high substitution rate among the meat products, highlighting a mislabeling rate of 57 %, and consequently, considerable discordance with the indications on the labels, which raises significant food-safety and consumer-protection concerns.

Keywords Meat products · Species identification · Mislabeling · DNA-based method

Introduction

Food safety and quality is increasingly the focus of attention on the part of the food industry and of consumers. One of the main food quality-related issues is the authentication of food contents, as food products may be adulterated, and highly valuable species may be substituted, partially or entirely, by similar but cheaper ones. Food authentication is a major concern not only in order to prevent commercial fraud, but also to assess the safety risks arising from the undeclared introduction of any food ingredient that might be harmful to human health, such as potentially allergenic or toxic compounds, or others that might cause problems for the diets of certain consumers, such as vegetarians or religious groups (Ortea et al. 2012).

Species identification is a major concern, due to increased consumer awareness regarding food composition and to the need to verify labeling statements. Processed-meat products are susceptible targets for fraudulent labeling due to the economic profit that results from selling cheaper meats as partial or total replacements for high-value ones (Mafra et al. 2008; Singh and Neelam 2011; Soares et al. 2013). In addition, the increasing demand for meat products in general may lead to deliberate adulteration along the food chain, by substituting high-quality species with lower-quality counterparts. Also, there is increasing evidence that even meat can cause allergic reactions in sensitized patients. Indeed, a prevalence of beef, pork and chicken allergies has been reported (Tanabe et al. 2007).

As stated in the introductory statement to Directive 2000/13/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to the labeling, presentation and advertising of foodstuffs, "the prime consideration for any rules on the labeling of foodstuffs should be the need to inform and protect the consumer". Therefore, verification of declared components in food products is essential for the protection of consumer health (Fajardo et al. 2010; Ali et al. 2012) but also to ensure fair trade and compliance with legislation (Ballin et al. 2009; Nakyinsige et al. 2012; Spink and Moyer 2011).

Considering that the authentication of food contents is one of the most important points concerning food quality, and given the increasing demand for transparency in the meat industry following the horsemeat scandal in Europe, this study investigates processed-meat products from Italian markets and supermarkets using the mitochondrial *cytochrome b* gene qualitative PCR identification system to verify any species

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substitution or mislabeling and consequently whether the contents match the labels.

Materials and methods

Reference materials

VERYfinder Swine Pure DNA Extract - HEAT TREATED (Generon, Italy), VERYfinder bovine DNA Extract - HEAT TREATED (Generon, Italy), VERYfinder poultry DNA Extract - HEAT TREATED (Generon, Italy) and VERYfinder equine DNA Extract - HEAT TREATED (Generon, Italy) were used as positive controls.

Experimental design

In order to assess the detection limit for the *cytochrome b*-PCR assay, PCR assays were carried out on binary extracted DNA mixtures, containing either bovine, pork or horse DNA ranging from 0.5 to 5 % in chicken DNA, as well as binary extracted DNA mixtures, containing either bovine, chicken or horse DNA ranging from 0.5 to 5 % in pork DNA. In addition, the *cytochrome b*-PCR assay was verified on DNA mixtures from heat-treated (121 °C/20 min) sausages - containing either bovine, pork or horse meat ranging from 0.5 to 5 % in chicken sausage, as well as binary meat mixtures, containing either bovine, chicken or horse meat ranging from 0.5 to 5 % in pork sausage – specifically prepared from chicken, pork, bovine and horse fresh muscle samples.

Sampling

A total of 72 packaged meat products, including 36 processed chicken sausage samples, 12 processed pork sausage samples, 12 pâté samples and 12 meat patties, manufactured using chicken, pork and bovine meat or a mixture of two thereof were purchased from different dealers, markets and supermarkets. In particular, the chicken sausage samples were labeled as chicken only, the pork sausage samples as pork, the pâté samples as a mixture of bovine and pork, and the meat patties as pork only. The sausage samples and meat patties were also labeled as containing mechanically separated meat EFSA (2013). The samples were stored at −20 °C until processing.

DNA extraction and purification

Aliquots of each sample (25 mg) were subjected to DNA extraction and purification using the DNeasy® Tissue Kit (QIAGEN, Hilden, Germany). Briefly, aliquots (25 mg) of each meat sample added to 180 μl ATL lysis buffer and 20 μl of Proteinase K (20 mg/ ml) were incubated at 56 °C for 2 h. After adding 200 μl AL Buffer, the solution was mixed thoroughly by vortexing and incubated at 70 °C for 10 min. The resulting mixture was then added with 200 μl ethanol (96–100 %) and mixed by vortexing to yield a homogenous solution and transferred into the DNeasy® Mini spin column sitting in a 2 ml collection tube. The DNA, adsorbed into the QIA amp silica-gel membrane during subsequent centrifugation steps at 6,000 g for 1 min, was washed using 500 μl AW1 and 500 μl AW2 washing buffers. Finally, the DNA was eluted with 200 μl of AE Elution Buffer (QIAGEN, Hilden, Germany). The DNA concentration and purity were established by evaluating the ratio A260nm/A280nm using a Beckman DU-640B Spectrophotometer.

Oligonucleotide primers

The oligonucleotide primers used in this study, described by Matsunaga et al. (1999) and synthesized by PRIMM Srl (Milan, Italy), were the common forward primer SIM (5'-GACCTC CCAGCTCCATCAAACATCTCATCTTGATGAAA-3') and reverse primers, chicken primer C (5'-AAGATA CAGATGAAGAAGAATGAGGCG-3'), bovine primer B (5'–CTAGAAAAGTGTAAGA CCCGTAATATAAG-3'), pork primer P (5'-GCTGATAGTAGATTTGTGATGACC GTA-3'), and horse primer H (5'-CTCAGATTCACTCGAC GAGGGTAGTA-3') (Table. 1).

PCR assay

The PCR reactions were performed in a final volume of 25 μl, using 12.5 μl of HotStarTaq Master Mix 2× (QIAGEN, Hilden, Germany), containing 2.5 units of HotStarTaq DNA Polymerase, 1.5 mM of $MgCl_2$ and 200 μl of each dNTP. Then, 0.25 μM of each oligonucleotide primer and 2 μl of DNA were added. The amplification profile involved an initial denaturation step at 95 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. The positive and negative controls for the extraction and PCR were included. The PCR reactions were processed in a Mastercycler Personal (Eppendorf, Milan, Italy). All reactions were performed in duplicate. The sequence analysis was carried out in order to confirm the specificity of the PCR assay. Sequencing reactions were performed by PRIMM Srl (Milan, Italy).

Detection of amplified products

PCR amplified products were analyzed by electrophoresis on 1.5 % (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1× TBE buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with ethidium bromide. A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf).

Table 1 Oligonucleotide primers

Primer	Sequence (5'–3')	Length of amplicon	References
CF	5'GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3'	-	(Matsunaga et al. 1999)
Bovine	5'-CTAGAAAAGTGTAAGACCCGTAATATAAG-3'	274	(Matsunaga et al. 1999)
Chicken	5'-AAGATACAGATGAAGAAGAATGAGGCG-3'	227	(Matsunaga et al. 1999)
Pork	5'-GCTGATAGTAGATTTGTGATGACCGTA-3'	398	(Matsunaga et al. 1999)
Horse	5'-CTCAGATTCACTCGACGAGGGTAGTA-3'	439	(Matsunaga et al. 1999)

Results

The limit of detection (LoD) for the *cytochrome b*-PCR assay, defined as the lowest DNA concentration that produced amplification, carried out on binary extracted DNA mixtures, corresponded to 1 % for each species. The PCR-assay sensitivity was then confirmed on binary DNA mixtures from heat-treated sausages.

DNA was successfully extracted from all 72 samples of processed-meat products. All of these extractions resulted in PCR products clearly visible as single bands of expected size (227 bp chicken, 398 bp pork, 274 bp bovine, 439 bp horse) on agarose gel. All positive and negative controls, which were run alongside each separate PCR, gave the expected results.

The results, reported in Table 2, revealed a high species substitution rate among the meat products, highlighting 41/72 (57 %) mislabeling cases. In particular, 20/36 chicken sausage samples resulted pork- and bovine-positive, while 9/12 pork sausage samples resulted bovine-positive. Further, 5/12 meat pâté samples labeled as pork and bovine also resulted chicken-positive. The remaining 7/12 meat patties samples labeled as pork resulted bovine-positive. All 72 samples of processed-meat products resulted horse-negative. The sequence analysis then confirmed the specificity of the amplified products.

Discussion

The global incidence of food misdescription and adulteration is increasing, and the international food trade is frequently disrupted by disputes over food safety and quality requirements (Di Pinto et al. 2013; Doosti et al. 2014). The recent controversies surrounding the horse meat scandal have forced the authorities to enforce stringent regulations on food adulterations (Premanandh 2013).

The study reveals a high probability of incorrect species declaration in meat products and insufficient labeling information for sausages, pâté and meat patties. Thus this study confirms that such fraudulent misdescriptions, with various undeclared species in ready-to-cook meat products, and adulteration of meat products with an unindicated mixture of meats, are widespread problems (Di Pinto et al. 2005; Ballin 2010; Doosti et al. 2014). It is important that information on the presence of different animal species, should be given to enable consumers, particularly those suffering from a food allergy or intolerance, to make informed choices. The results of this study showed a significant presence of bovine DNA (36/72 positive samples), probably due to the addition of non-fat dry milk powder in order to increase overall yield, to improve taste and to improve binding qualities, indicated exclusively on the labels of the meat pâté. By contrast, the chicken and the pork sausages and the meat patties failed to report the addition of milk powder on their labels, although the results showed the presence of bovine in 20/36 chicken, 9/12 pork sausages, and 7/12 meat patties. Also the presence of different animal species in these products could be due to the fact that meat from different animal species is processed in the same meat plants and the presence may be caused by the unintentional and incidental commingling of trace amounts of one type of meat or meat products with another during processing and handling. Whether deliberate or unintentional, moreover, the effects of meat product misdescription are similar, and include consumer deception, potential health risks and the inability of individuals to choose products on the basis of their religious and ethical beliefs.

Therefore, whether deliberate or unintentional, the presence of different animal species where the meat constitutes an ingredient of another food must be indicated as "… meat' preceded by the name (s) of the animal species from which it comes" in accordance with Reg. EC 1169/11 (Annex VII, Part B, comma 17). That said, EC regulations do tolerate accidental contamination exclusively for products containing GMOs (Reg. EC 1829/2003, Reg. EC 1830/2003).

Given that the authentication of species in meat products is crucial to protect the consumer and has various implications: (i) economic, since it leads to unfair competition among producers; (ii) religious, since the consumption of certain species is not allowed in some religions; (iii) ethical, reflecting lifestyles such as vegetarianism; and (iv) health concerns (Soares et al. 2013), regular monitoring to counteract fraud is a requirement if the authorities are to ensure safe, unadulterated and quality food.

As described in Regulation CE 1169/11, identification of origin of food ingredients is of prime importance for consumer safety, particularly when products are found to be faulty.

Table 2 PCR results of meat products

Samples no	Product type	Labelled as	Bovine	Chicken	Pork	Horse
1	Sausage	Chicken	+	+	+	-
2	Sausage	Chicken	+	+	+	-
3	Sausage	Chicken	+	+	+	-
4	Sausage	Chicken	-	+	-	-
5	Sausage	Chicken	-	+	-	-
6	Sausage	Chicken	+	+	+	-
7	Sausage	Chicken	+	+	+	-
8	Sausage	Chicken	-	+	-	-
9	Sausage	Chicken	+	+	+	-
10	Sausage	Chicken	-	+	-	-
11	Sausage	Chicken	+	+	+	-
12	Sausage	Chicken	+	+	+	-
13	Sausage	Chicken	+	+	+	-
14	Sausage	Chicken	-	+	-	-
15	Sausage	Chicken	-	+	-	-
16	Sausage	Chicken	-	+	-	-
17	Sausage	Chicken	+	+	+	-
18	Sausage	Chicken	-	+	-	-
19	Sausage	Chicken	-	+	-	-
20	Sausage	Chicken	-	+	-	-
21	Sausage	Chicken	+	+	+	-
22	Sausage	Chicken	-	+	-	-
23	Sausage	Chicken	+	+	+	-
24	Sausage	Chicken	-	+	-	-
25	Sausage	Chicken	+	+	+	-
26	Sausage	Chicken	+	+	+	-
27	Sausage	Chicken	-	+	-	-
28	Sausage	Chicken	+	+	+	-
29	Sausage	Chicken	-	+	-	-
30	Sausage	Chicken	-	+	-	-
31	Sausage	Chicken	+	+	+	-
32	Sausage	Chicken	-	+	-	-
33	Sausage	Chicken	+	+	+	-
34	Sausage	Chicken	+	+	+	-
35	Sausage	Chicken	+	+	+	-
36	Sausage	Chicken	+	+	+	-
37	Sausage	Pork	+	-	+	-
38	Sausage	Pork	-	-	+	-
39	Sausage	Pork	+	-	+	-
40	Sausage	Pork	+	-	+	-
41	Sausage	Pork	+	-	+	-
42	Sausage	Pork	-	-	+	-
43	Sausage	Pork	+	-	+	-
44	Sausage	Pork	+	-	+	-
45	Sausage	Pork	+	-	+	-
46	Sausage	Pork	+	-	+	-
47	Sausage	Pork	-	-	+	-
48	Sausage	Pork	+	-	+	-
49	Pâté	Pork- Bovine	+	+	+	-
50	Pâté	Pork- Bovine	+	+	+	-
51	Pâté	Pork- Bovine	+	-	+	-
52	Pâté	Pork- Bovine	+	+	+	-
53	Pâté	Pork- Bovine	+	+	+	-
54	Pâté	Pork- Bovine	+	-	+	-
55	Pâté	Pork- Bovine	+	-	+	-
56	Pâté	Pork- Bovine	+	-	+	-
57	Pâté	Pork- Bovine	+	-	+	-
58	Pâté	Pork- Bovine	+	-	+	-
59	Pâté	Pork- Bovine	+	+	+	-
60	Pâté	Pork- Bovine	+	-	+	-
61	Meat patties	Pork	+	-	+	-
62	Meat patties	Pork	-	-	+	-
63	Meat patties	Pork	+	-	+	-
64	Meat patties	Pork	-	-	+	-
65	Meat patties	Pork	-	-	+	-
66	Meat patties	Pork	+	-	+	-
67	Meat patties	Pork	-	-	+	-
68	Meat patties	Pork	+	-	+	-
69	Meat patties	Pork	+	-	+	-
70	Meat patties	Pork	+	-	+	-
71	Meat patties	Pork	-	-	+	-
72	Meat patties	Pork	+	-	+	-

Labeling is the primary means of communication between producers and consumers. Although labeling policies differ widely in character, material facts and allergen information are expected to be part of standard labeling practice (Premanandh 2013). However, legitimate information on authenticity is generally lacking. Traceability, defined in Regulation EC/178/2002 as the ability to trace and follow food, feed and ingredients through all stages of production, processing and distribution. facilitates the withdrawal of foods and provides consumers with targeted and accurate information concerning the implicated products.

This study, therefore, demonstrates the need to adopt and carry out stringent control measures, as well as to assess compliance with labeling requirements. European regulations on the traceability and labeling of food products require the food chain to be traceable, so as to inform consumers via compulsory product labeling and in order to create a safety net based on the traceability at all stages of production and distribution to the marketplace.

The study further proves DNA-based molecular investigations to be one of the most powerful tools for assessing species identity, food traceability, safety and fraud. In recent decades,

many analytical PCR-based methods for qualitative detection and quantitative determination of different species in mixed-meat products have been developed (Lopez-Andreo et al. 2006; Reid et al. 2006; Karlsson and Holmlund 2007; Soares et al. 2013). Although PCR quantification applied to food analysis still raises various analytical problems, such as lack of certified reference standards and effect of food technologies (Sakalar et al. 2012), qualitative PCR analysis targeted mitochondrial DNA is suitable for monitoring mixed-meat products in official food safety controls (Matsunaga et al. 1999; Ballin 2010; Ghovvati et al. 2009; Linacre 2012; Amaral et al. 2014). Specifically, the species-specific DNA detection assay described can easily be performed as an initial screening tool, thus achieving considerable savings on analytical costs. In addition, the detection limit of this particular assay on reference sausage samples (1 %) showed the method to be sensitive, reliable and thus suitable for carrying out initial screening.

A great effort should therefore be made to create a strong and standardized monitoring program or strategy, and finally, to evoke consumer awareness on several aspects relating to accurate labeling information. Progress in the area of authentication of traded food products requires the use of molecular tools to ensure proper species identification, thus enhancing the application of effective food control regulations and consumer protection (Marín et al. 2013). Considering the high prevalence of meat fraud and mislabeling in meat and meat products, robust analytical tests are required to ensure adherence to regulations and to enforce punitive measures (Ballin 2010).

Conclusions

Considering the widespread distribution of fraudulent misdescription of food contents (Di Pinto et al. 2005; Aida et al. 2007; Cawthorn et al. 2013; Doosti et al. 2014), intensive and continuous monitoring is strongly recommended in order to ensure that consumers can make conscious choices. Therefore, the food control authorities may upgrade their systems to identify food sources and monitor quality to ensure that proper processing has taken place and labeling information reflects actual contents. Moreover, authentication of species in meat products usually involves testing procedures to confirm the species reported on the label and the presence of other suspected species. In addition, adventitious traces of meat have become a major concern for regulators when formulating significant legislation. Any adventitious or low-level presence linked to the unintentional and incidental presence of trace amounts of one type of meat or meat products with another during processing and handling may be regulated and controlled by the authorities with frequent monitoring procedures at all levels starting from primary production and processors all the way to the end of the supply chain (Premanandh 2013).

Enforcing European legislation guidelines would be associated with the development and application of reliable labeling implementation plans and appropriate traceability systems in order to guarantee an efficient food safety system. In summary, a continuous monitoring scheme along with improved detection methodologies and stringent sanctions on defaulters may help to minimize authentication problems in future.

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