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Evaluation of a loop-mediated isothermal amplification (LAMP) method for rapid on-site detection of horse meat

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ABSTRACT

Detection of horse DNA by loop-mediated isothermal amplification (LAMP) seems one of the most promising methods to meet the criteria of fast, robust, cost efficient, specific, and sensitive on-site detection. In the present study an assessment of the specificity and sensitivity of the LAMP horse screening assay was made and outcomes were compared with the EURL-AP (European Union Reference laboratory for Animal Proteins in feeding stuffs) qPCR method. The specificity was tested with DNA samples from seven other species. The sensitivity of the LAMP assay was subsequently challenged with different percentages of horse DNA in cattle DNA and different percentages of horse meat in cattle meat. Both qPCR and LAMP were able to reliably detect horse DNA or meat below 0.1%, but LAMP was able to do so in less than 30 min. The DNA of other species did not result in a response in the LAMP horse assay. These features show that the LAMP method is fast, specific, and sensitive. Next, 69 processed meat samples were screened for the presence of horse DNA. The results showed that the LAMP horse assay, combined with a simple and fast on-site DNA extraction method, results in similar outcomes as the EURL-AP qPCR method and is thus a promising screening assay to be used outside the laboratory. Only samples that are screened on-site as suspect in the LAMP horse assay, need to be brought to the laboratory for confirmation with the more laborious EURL-AP qPCR reference method.

1. Introduction

The presence of undeclared horse meat in food products is primarily a fraud issue and damaging the meat industry's reputation. In 2013 there was a series of incidents in Europe, starting in January when the Food Safety Authority of Ireland (FSAI) detected horse DNA in some beef burger products. In the months to follow, many more products were found to be positive for undeclared horse meat and lots of products were withdrawn from the market (Food Safety Authority of Ireland (FSAI), 2013a, 2013c; Walker, Burns, & Burns, 2013). The incident had a huge impact on the food industry and the faith of consumers in meat products (O'Mahony, 2013). Besides the authenticity issue, undeclared horse meat might be contaminated with phenylbutazone (PBZ). PBZ is a non-steroid anti-inflammatory drug (NSAID) that is used as a painkiller for horses suffering from musculoskeletal disorders such as rheumatoid and arthritic diseases (Fodey et al., 2014). PBZ treated animals are banned from the food chain, as PBZ causes severe adverse effects in humans, including suppression of white blood cell production (agranulocytosis) and aplastic anaemia (Dodman, Blondeau, & Marini, 2010; Lees & Toutain, 2013a, 2013b). In addition to PBZ, undeclared horse meat might be contaminated with harmful micro-organisms such as *Toxoplasma gondii* and *Trichinella* (Aroussi et al., 2015; Boireau et al., 2000; Food Standards Agency (FSA), 2015; Pozio, 2015).

At present, methods that are most often used are protein-, fat-, enzyme-, or DNA-based (Di Giuseppe, Giarretta, Lippert, Severino, & Di Maro, 2015; Fumière et al., 2009; Lin et al., 2014; Mahajan, Gadekar, Dighe, Kokane, & Bannalikar, 2011; Masiri et al., 2017; Walker et al., 2013). The method described by Masiri et al. for instance, is a semi-quantitative lateral flow immunoassay capable to detect raw and cooked horse meat residues. However, in general DNA based methods are preferred to detect undeclared horse meat in food products (Food Safety Authority of Ireland (FSAI), 2013b; Food Standards Agency (FSA), 2013a).

European Regulation in this area is primarily linked to more general regulations on the labelling of food products. Directive

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2000/13/EC (The European Parliament and the council of the European Union, 2007) states that consumers should not be misled: the label should provide the information that allows consumers to know the true nature of the product including information of the species from which meat products have been obtained. In Recommendation 2014/180/EU (relating to monitoring) it was stated that monitoring should be performed on the basis of a real-time PCR (qPCR) method that allows the detection of the presence of, e.g. horse meat material down to the level of 1% (w/w fraction), based on the comparison with a standardised control sample as provided by the European Union Reference Laboratory for Animal Proteins in feedingstuffs (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2014). In line with this recommendation, a qPCR method has been developed and validated and is also available (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2013).

Later on, several other qPCR tests were developed to detect horse DNA in meat products (Meira et al., 2017; Nixon, Wilkes, & Burns, 2015; Pegels, García, Martín, & González, 2015; W.; Wang, Zhu, Chen, Xu, & Zhou, 2015). The method described by Wang et al. for instance, uses a PCR followed by a visual detection of the formed product. This visual detection takes about an additional 30 min, but the method has not only been developed for horse, but also seven other animal species. However, the disadvantage of qPCR methods is that they can only be applied in the laboratory and not on-site. For enforcement purposes, it would therefore be of added value to have additional screening methods that are specific, robust, fast, easy to perform, cheap, and that can be used by inspectors on-site, to select samples that can be further analysed in the laboratory.

Loop-mediated isothermal amplification (LAMP) is a very powerful and specific DNA-based detection method that can be used on-site. This method uses the specific properties of six primers and a polymerase with strand displacement activity that can be used at the same temperature as the primer annealing temperature (Nagamine, Hase, & Notomi, 2002; Notomi et al., 2000; Jenny; Tomlinson, 2013). Studies have shown that LAMP methods can be even more specific than qPCR and immunoassays (Bühlmann et al., 2013; Kang, Kim, Han, Moon, & Koh, 2014; JA; Tomlinson, Dickinson, & Boonham, 2010; Y.; Wang et al., 2014). LAMP is not only very specific, but also fast, as it is a simplified amplification reaction that does not need the temperature cycles as those in qPCR methods and therefore the LAMP reaction is performed within 30 min.

On-site detection consists of three parts: sampling, sample preparation, and the measurement or sample analysis. On-site DNA isolation and purification is quite a challenge, but not needed for LAMP meat methods since a simple procedure based on KOH lysis turned out to work well for LAMP methods applied on meat materials. A fast and specific LAMP method for horse was recently developed and brought to the market in combination with a portable incubator/reader (Genie II from Optigene).

In the present study, a more in depth assessment of the sensitivity and specificity of this horse LAMP method was made and when applied in routine sample testing, results were critically compared with outcomes obtained with the EURL-AP qPCR reference method (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2013).

2. Materials & methods

2.1. Chemicals

DNA of different animal species, i.e. buffalo (*Bubalus bubalis*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), pig (*Sus scrofa*), grasshopper (*Locusta migratoria*), red deer (*Cervus elapus*), and cattle (*Bos taurus*), was isolated by either the Cethyltrimethylammonium Bromide (CTAB) isolation procedure (Murray & Thompson, 1980) or the Wizard® magnetic DNA purification system for food (Promega, Madison, USA) according to the manufacturer's protocol with some minor adjustments, i.e. 100 mg of starting material was used instead of 200 mg, 100 μl of MagneSil® PMPs was added instead of 50 μl, and 900 μl of 70% (v/v) EtOH was used to suspend the magnetic beads instead of 1 ml. The species identity of all animal samples was confirmed by DNA barcoding (Staats et al., 2015).

2.2. Animal meat samples

Smoked horse meat and cattle meat were obtained from a local supermarket. Horse meat steak was obtained from the national monitoring program of the Netherlands Food and Consumer Product Safety Authority (NVWA), verified by a veterinarian inspector (100% horse) and used to prepare a processed in-house made 1% (w/w) horse reference sample according to the EURL-AP recommended protocol for preparation of a 1% (w/w) reference standard (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2014) and freeze-dried afterwards. Two reference 1% (w/w) horse meat mixture samples were obtained from Walloon Agricultural Research Centre (CRA-W) (2014) and 69 processed meat samples were provided by the NVWA. These 69 processed meat samples were varying from meat shreds to whole pieces of meat, and from raw meat to cooked sausages.

2.3. Preparation of "horse meat in cattle meat" meat mixtures

Different percentages of horse meat steak in cattle meat (w/w) were prepared. For this, 45 g of cattle meat was minced and mixed with 5 g of horse meat steak using a Moulinex blender to obtain 10% (w/w) horse meat in cattle meat. Further dilutions were made by mixing 5 g of a diluted mixture with 45 g cattle meat, thus resulting in samples containing 100%, 10%, 1%, 0.1%, and 0.01% of horse meat steak in cattle meat (w/w).

2.4. DNA extractions for qPCR

DNA was isolated using the CTAB isolation procedure according to ISO 21571:2005 Appendix A.3 (International Organisation for Standardisation, 2005) or the Wizard® magnetic DNA purification system for Food (Promega, Madison, USA) according the manufacturer's protocol with some minor adjustments, as mentioned in Section 2.1. Serial dilutions (100%–0.01%) of horse DNA were prepared in both water (w/v) and cattle DNA (w/w).

At RIKILT Wageningen UR, DNA was extracted from horse meat steak and eight of the 69 processed meat samples, according to the CTAB isolation protocol (n = 2). DNA was extracted from the "horse meat in cattle" meat mixtures, smoked horse meat, our in-house made 1% (w/w) horse reference sample and CRA-W's two reference 1% (w/w) horse meat mixtures, using the Wizard® magnetic DNA purification system for Food (n = 2). At the NVWA, DNA from reference samples and 69 processed meat samples was isolated using the Wizard® magnetic DNA purification system for Food according to the manufacturer's protocol (n = 2). In all cases, DNA quality was checked and concentrations were measured on either the NanoDrop ND-1000 spectrophotometer (RIKILT) or NanoDrop 2000 spectrophotometer (NVWA) (Thermo Fisher Scientific Inc.).

2.5. qPCR

At RIKILT Wageningen UR, the qPCR for the detection of horse