Development of an analytical method to detect plant proteins in meat products

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Abstract - New analytical methods are necessary to monitor authenticity and integrity of meat and meat products. One possibility to prove the addition of plant proteins in meat products is to detect specific fragments of plant deoxyribonucleic acid (DNA). A convenient method is multiplex quantitative polymerase chain reaction (qPCR). With this method up to five protein sources can be simultaneously determined and quantified. For establishing a multiplex qPCR five steps are obligatory. In this contribution a duplex qPCR was developed to quantify the concentration of oat and barley proteins in emulsified type sausages. The detection range varies from 0.0005 to 0.1% plant protein in emulsified type sausages. The resulting limit of quantification is sensitive enough to quantify allergenic substances like gluten (≤ 20 ppm).

Key Words - Emulsified type sausage, foreign plant protein, multiplex qPCR.

I. INTRODUCTION

Meat and meat products are valued goods. To ensure the authenticity and integrity of meat, new analytical methods are necessary.

In the meat industry, foreign proteins are occasionally added to meat products. On the one side, this might be due to food technological processes and on the other side to reduce the production costs. By adding foreign proteins, the water content can be higher while the protein to water ratio stays constant. Thus, the overall meat content of the product is lower and the production costs are simultaneously decreased.

Adding undeclared foreign protein is food fraud to the disadvantage of the consumer. Within the EU as well as in Germany the regulations are strict for adding non meat protein to meat products [1, 2]. Additionally, a potential health risk exists for allergic persons. After consuming specific plants or with plant protein adulterated meat products sensitive persons can suffer from allergic reactions. In the worst case, this can lead to an anaphylactic shock [3, 4]. At the moment, 14 ingredients are listed that can elicit hypersensitivity [2]. One of them is gluten which is a storage protein in cereals. In around 1% of the population the consumption of gluten can trigger the symptoms of celiac disease [5].

Foreign proteins like gluten can originate from various plants. And subsequently, the origin of the protein sources can be determined by the differences of their deoxyribonucleic acid (DNA) sequences. As DNA is a relatively stable molecule, it can be detected even after heat treatment at high temperatures [6]. And therefore it is a good target for screening the composition of processed food. A molecular biological approach for detecting varying DNA sequences is quantitative polymerase chain reaction (qPCR). By use of multiplex qPCR assays several protein sources can be simultaneously analyzed. In this way time and expenses can be minimized.The aim of this contribution is the development of a duplex qPCR method to detect oat and barley in emulsified type sausages in parallel.

II. MATERIALS AND METHODS

Bioinformatics

The Primer-BLAST software from NCBI [7] was applied to theoretically check the specificity of the published primers.

Isolation of DNA

Plant samples were either obtained from the Federal Research Centre for Cultivated Plants (Julius Kühn-Institute), the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) or bought in local stores. Dried samples were ground in a Tube Mill control (IKA). For the isolation of DNA the Wizard® Plus Minipreps DNA Purification System (Promega) was used and the concentration was measured with a NanoDrop 2000c (ThermoScientific).

Reference material

The protein content was analyzed with a Vapodest 50sC (Gerhardt) by the Kjeldahl method [8]. Emulsified type sausages were prepared with 0 - 0.1% plant protein content after Hoffmann *et al.* [9]. The metal cans (200 g) were heat treated as normal cans.

Quantitative PCR

All experiments were performed on a Rotor-Gene™ 6000 (Qiagen). The assays were performed using QuantiTect Multiplex PCR NoRox Kit (Qiagen) and primers and probes were synthesized by Eurofins (Table 1). The following cycler conditions were used: 15 min at 95°C, 45 cycles of 1 min at 94°C and 1 min at 60°C. All samples were analyzed in duplicates or triplicates and for the limit of detection (LOD) in sextet. For the calculations a C value of 5.5 pg for barley [10] and of 12.85 pg for oat [11] were used.

Table 1 Sequences of primers and probes

plant	name	sequence 5'-3'	reference
oat	OAT-F	AGCCAGATCGCAGGAAAGAC	[12]
	OAT-R	ATGCATTGGCGAGGACATC	
	OAT-P	TCCATCCTACGTGCATTGCCCGT	
barley	hor-F	AGACAAGGCGTGCAGATCG	[13]
	hor-R	GACCCTGGACGAGCACACAT	
	hor-P	CCTCAGCCGCAACAGGTGGGTC	

III. RESULTS AND DISCUSSION

Five steps are normally necessary to develop a multiplex qPCR method for surveillance authorities to monitor authenticity and integrity of meat and meat products. The five steps system is graphically depicted in Fig. 1.

The initial step was to find suitable literature on quantification of grain DNA. Several convenient publications were found, especially for oat [12] and barley [13]. Subsequently, bioinformatic tools were used to check the theoretical specificity of primers and probes. The analyses revealed that all primers were highly specific and not cross-reactive.

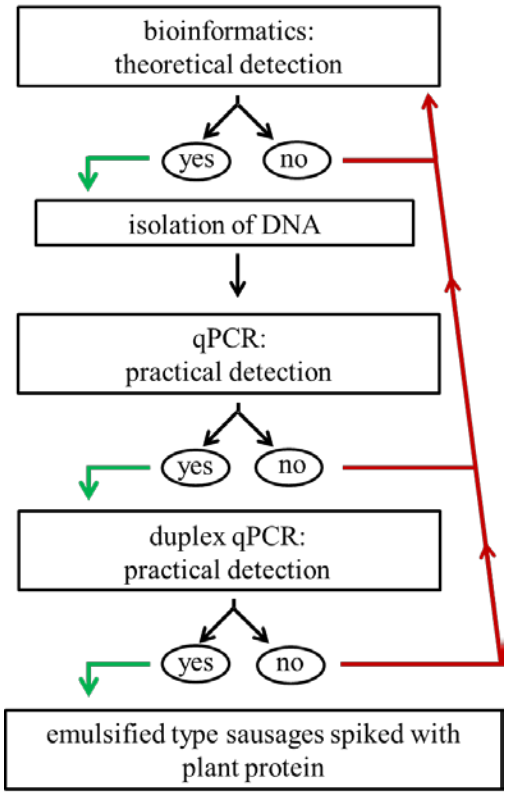

Figure 1. Graphical overview of the five steps for establishing a duplex qPCR surveillance method.

In the second step, DNA was isolated from oat, barley and other plants from the *Pocaceae* family. These DNA samples of closely related plants were at first used as templates in singleplex qPCR assays in the third step. DNA of oat, barley, wheat and rye were used as templates and no false positive signals were detected in both systems (data not shown). Additionally, data on efficiency, R^2 and LOD were elaborated. For the oat system, the efficiency was 0.96, R^2 0.996 and the LOD between 0.1 and 1 copy of DNA per reaction. For the barley system the respective values were 1.00, 0.991 and the LOD between 0.1 and 2 copies of DNA per reaction. In Fig. 2 the standard curve is exemplarily shown for barley. All in all, this implies that the singleplex qPCRs are stable, have a high sensitivity and are specific for oat, respectively barley.

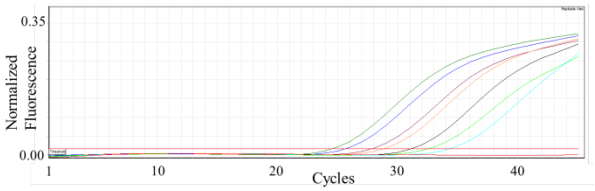

Figure 2. Standard curves for barley. A serial dilution from 10 – 0.01 ng DNA per reaction was used. All data sets were performed in triplicate.

In the fourth step, the robustness of the duplex qPCR was compared to the singleplex ones. Therefore, low concentrations of oat DNA were analyzed together with high concentrations of barley DNA and vice versa. The Cq-values of low concentrations were afterwards compared to the singleplex values. No differences were obtained and the presence of barley DNA did not interfere with the oat system and vice versa (Fig. 3).

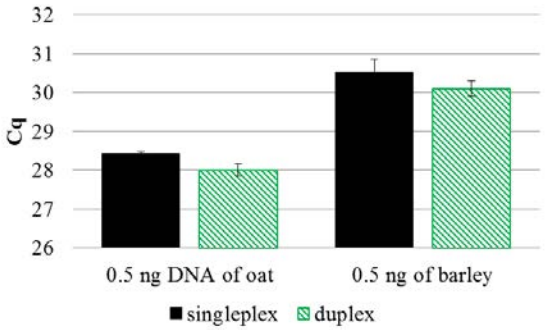

Figure 3. Comparison between singleplex and duplex qPCR for both, the oat and barley system. All data sets were derived in triplicate and error bars indicate the standard deviation.

Additionally, possible cross-reactivity of the primers was practically checked. This means, that DNA from wheat, rye, rice, maize, sorghum, panicum, durum, foxtail millet, spelt and triticale was tested with the duplex qPCR assay. No signals were detected and consequently, the developed duplex qPCR assay was specific for oat and barley (data not shown).

In the last and final step, the detection of oat and barley in emulsified type sausages was tested. Therefore, spiked sausages were prepared with 0 – 0.1% oat, respectively barley protein. The efficiency values were 1.15 for oat and 1.04 for barley, the R^2 values 0.978 and 0.989, respectively (Fig. 4). Oat and barley can be specifically detected and quantified in emulsified type sausages within a particular range. For gluten as an allergenic substance the limit of detection is set to 20 ppm [14]. Applying duplex qPCR system for oat and barley, these limits were reached.

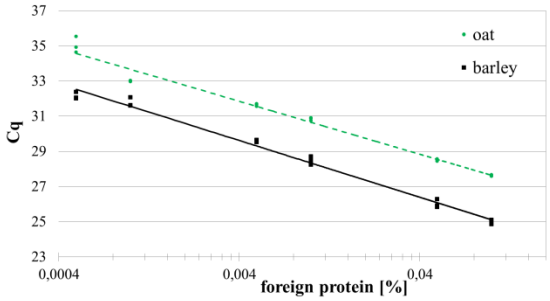

Figure 4. Standard curves of barley and oat. DNA was isolated from emulsified type sausages spiked with 0.0005 – 0.1% oat (green, dotted line), respectively barley (black, straight line) protein. qPCR was run in triplicate.

IV. CONCLUSION

To monitor authenticity and integrity of meat products new methods are necessary. In this short communication it is shown, that existing methods for detection of oat and barley by singleplex qPCR can be combined to a duplex qPCR to quantify simultaneously oat and barley in emulsified type sausages.

ACKNOWLEDGEMENTS

We thank T. Schobert and U. Köstner for technical assistance in the lab and S. Eckl for producing the emulsified type sausages.

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