Quantitative Detection of raw sheep in Meat Products by a TaqMan Real-Time PCR Assay

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Abstract—In the present work, we propose a normalised real-time quantitative PCR assay to determine the sheep content in raw meat. For the development of the method, the mixtures meats contain known amounts of sheep meat in pork from 20% to 100% can be successfully in-house validated by a normalised calibration model with high linear correlation (R^2=0.994) and PCR efficiency (E=77%). The method also showed that the limit of detection of sheep in sheep-pork mixtures was 0.8% (W/W). Thus, this methodology could serve as a fast and sensitive method for quantitative detection of sheep for meat species verification, avoiding fraudulent practices.

Keywords—real-time PCR; quantitative detection; raw; Sheep; adulteration

I. INTRODUCTION

Transparency in meat speciation is an ever increasing demand and is essential for the protection of consumers'heath, religious credence and hard earned wealth[1,2]. Food manufacturers are not only responsible for the safety but also for the authenticity of their products. Since economically advantageous adulteration is a permanent temptation for producers the authorities have to face recurring violation of legal regulations. Recently the detection of horsemeat in a number of processed beef products across Europe led to a series of product recalls. Similar incidents had been occurred in the Chinese market: Fox were posing as donkey meat sold in supermarkets was exposed by Shandong Province Exit Inspection and Quarantine in 2013. Such an event was not the case, making fake sheep meat with a fox, fake dog meat, ham were not uncommon.

At present, A number of analytical analysis methods have been proposed for the verification of the origin of animal products in order to guarantee its traceability, such as electronic nose coupled to gas chromatography−mass spectrometry[3], electrophoretic, chromatographic[4], immunological techniques[5], Fourier transform infrared spectroscopy[6], SYBR green real-time PCR[7] and TaqMan probe realtime PCR[8].

Among these methods, TaqMan real-time PCR assays combined with species-specific primers and TaqMan probe are particularly promising because in addition to specific primers, additional species-screening is provided through the specifically-designed TaqMan probe, significantly enhancing the specificity and reliability of the assay[9].

In this work, a TaqMan real-time PCR approach is proposed as a simple, fast, sensitive and reliable method for sheep meat detection and quantification. The method was validated using blind mixtures applied to quantify the presence of sheep meat in sheep-pork meat mixtures available commercially.

II. MATERIALS AND METHODS

A. Samples

Reference samples were prepared in the laboratory with sheep and pork muscles from a local retail market. Immediately after purchase, both meats were cut and the outside portions rejected. The samples were then minced separately and reference binary mixtures containing 100%, 80%, 60%, 40% and 20% (W/W) of sheep in pork meat were prepared to a final weight of 10 g.

To validate the methodology, blind validation mixtures containing 79.89%, 48.32%, 31.74% and 21.30% (w/w) of sheep meat in pork-sheep mixture. To avoid contaminations, all samples and mixtures were minced and homogenised separately in a knife mill using different knives and different blender containers, previously treated with DNA decontaminator solution.

All samples were stored at -20°C to avoid enzymatic degradation of the DNA before the extraction procedure.

B. DNA extraction

DNA was extracted using the CTAB method described by Barbara Druml[10] with minor modifications. Briefly, 15 mL CTAB extraction buffer (2% (w/v) CTAB, 0.02 M EDTA, 0.1 M Tris, 1.4 M sodium chloride, adjusted to pH8.0 with 4 M hydrochloric acid, autoclaved) and 10 μL proteinase K solution were added to 1 g food meat mixture and incubated at 56℃ under shaking in an incubator (SW22 Julabo) overnight.

After centrifugation at 5000g (Centrifuge 5415 R, Eppendorff) for 5 min, 500 μL of the supernatant was transferred into a 2 mL Eppendorff tube The supernatant was decanted and 500 μL of phenol/chloroform/iso-amylalcohol (25:24:1, v/v/v) was added,. After vortexing for 30 s, the mixture was centrifuged at 10,000g for 5 min. 600 μL of the aqueous phase was transferred into a new 2

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mL Eppendorff tube containing 600 μL chloroform/iso-amylalcohol (24:1, v/v) and carefully mixed. After incubating the mixture at room temperature for at least 5 min a further centrifugation step was carried out (10,000g, 10 min). 400 μL of the aqueous phase was transferred into a new 1.5 mL Eppendorff tube that already contained 320 μL of isopropanol. The mixture was carefully mixed and incubated in the freezer at -20 ℃ for at least 1 h. After centrifugation at 10,000g for 10 min, the supernatant was decanted. The precipitated nucleic acid was washed with 500 μL of 70% (v/v) ethanol and centrifuged at 21 000 g for 5 min. The nucleic acid was resuspended in 100 μL 0.1×TE buffer (10 mM Tris－HCl, 1 mM EDTA; pH 8.0).

The extractions were performed in duplicate for each sample and binary mixtures. All extractions included a blank extraction for the control of reagents and contaminations during extraction procedure.

C. DNA quantification and purity

The concentration and the quality of the extracted DNA were determined by measuring its absorbance at 260 nm ($\boldsymbol{A}_{260}$) and 280 nm ($\boldsymbol{A}_{280}$) with a spectrophotometer (DS-11 Spectrophotometer DeNovix). The DNA concentration was calculated according to the following equation: c[ng/μL] = $\boldsymbol{A}_{260}$× 50×dilution factor. The ratio $\boldsymbol{A}_{260}/\boldsymbol{A}_{280}$ was used to assess the purity of the extracted DNA. The DNA extracts were stored at -20℃.

D. Oligonucleotide primers/probe

The oligonucleotide primers and probes used in this work are presented in Table 1. The primers were synthetised by Life Technologies (Shanghai).

TABLE I. OLIGONUCLEOTIDE PRIMERS AND PROBES USED IN THE PCR AMPLIFICATIONS TARGETING THE CYTB AND THE 12S RRNA GENES.

Target gene	Primer/probe	Sequence(5'-3')	Reference
Cytb	Sheep(F)	ACA CAA CTT CTA CCA CAA CCC	[11]
	Sheep(R)	AAA CAA TGA GGG TAA CGA GGG	
	CytbP	(FAM)-ACA CCG AAA CAA AAT ACT CCT TGA GAA ACA-(TAMRA)	
12S rRNA	12S(F)	CAA ACT GGG ATT AGA TAC CCC ACT A	[12]
	12S(R)	ATC GRT TMT AGA ACA GGC TCC TCT AG	
	12SP	(VIC)-CAC CGC CAA GTC CTT TGR GTT TTA RGC-（none）	

E. Real-time PCR analysis

Real-time PCR reactions were carried out in a total volume of 15μl in strip tubes or in 96 well plates, depending on the ABI 7500 Fast Real-time PCR System (Applied Biosystems), using the following conditions: 95 °C for 30s, 40 cycles at 95°C for 5s and 60°C for 34s, with collection of fluorescence signal at the end of each cycle. Data were collected and processed using the 7500 Fast System Software V1.4.2. To develop a robust quantitative method that can be applied to processed meat products, the construction of a normalized calibration curve was proposed using the real-time PCR cycle threshold (Ct) values from the amplification of the binary reference mixtures targeting Cytb and the endogenous control (12S rRNA). For that system, the application of ΔCt method to construct a calibration model was used by calculating: $\Delta Ct = Ct_{Cytb} - Ct_{12s\ rRNA}$

where Ct_{cytb} and $Ct_{12srRNA}$ are the cycle thresholds for soybean and eukaryotic systems, respectively. The calibration curve was then obtained by plotting the calculated ΔCt vs. the logarithm of sheep percentage of five concentration levels.

F. Amplification efficiency(E)

The amplification efficiency of the real-time PCR assay was determined by analyzing 4 DNA extracts from sheep that had been diluted serially (1:10 to 1:100000). The final concentration of sheep DNA ranged from 50.75 ng/μL to 5.075 pg/μL. The amplification efficiency (E) was calculated from the slope of the standard curve: E[%] = [10(-1/(slope)) -1] ×100.

G. Limit of detection (LOD)

The limit of detection (LOD) of the real-time PCR assay was determined according to the European Network of GMO Laboratories guidelines. The DNA was isolated from a model meat mixture containing 20% (w/w) sheep in pork. After adjusting the concentration to 50 ng/μL the DNA extract was diluted 1:5, 1:25 and 1:125. Each of the diluted extracts was analyzed in 10 replicates. The LOD was defined as the lowest concentration that yielded an increase of the fluorescence signal within 37 cycles in at least 9 out of 10 replicate measurements.

III. RESULTS AND DISCUSSION

A. Amplification efficiency

For calirbration, the amplification efficiency of DNA extracts from sheep were analyzed by both real-time PCR assay, the Cytb gene and the reference gene (12S rRNA). The concentration of sheep DNA was in the range from 50.75 ng/μL to 5.075 pg/μL. The amplification curves and the standard curve obtained by plotting the Ct value against the logarithm of the DNA concentration are shown in Fig. 1A and Fig. 1B, respectively. For Cytb, a linear relationship (R^2=0.9958), the slope of the calibration curve was -3.2861, corresponding to an amplification efficiency of 101.5%. while for 12S rRNA, showed the similar dynamic equation: a linear relationship (R^2=0.9997), the slope of the calibration curve was-3.3093, corresponding to an amplification efficiency of 100.5%.

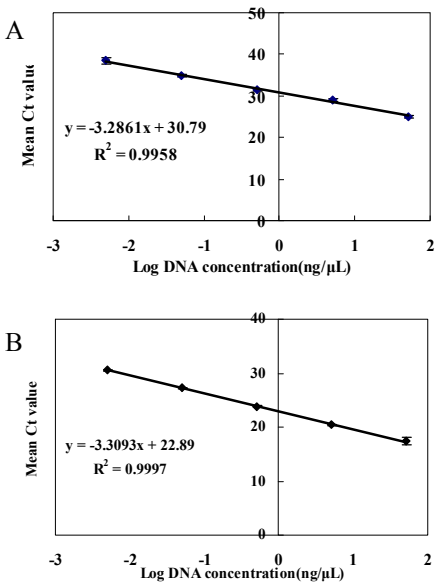

Figure 1. The amplification efficiency of the target gene (Cytb) and the endogenous control (12srRNA) .a.cyctb; b.12SrRNA

B. *Real-time PCR calibration curve and linearity*

The normalised calibration curve obtained by plotting the calculated ΔCt vs. the logarithm of sheep percentage of five concentration levels is presented in Fig. 2. Therefore, the estimation of sheep was determined by the equation:

$$\text{Sheep\%} = 10^{\frac{1.3077-\Delta Ct}{4.0057}}$$

This approach allows the estimation of added sheep meat at concentrations of 20% to 100% with a high correlation coefficient (R^2 = 0.994). The calculation of PCR efficiency is of major relevance to assess the performance of real-time quantitative PCR assays, being calculated from the slope of the calibration curve using the following expression: $E\%=[10^{(-1/slope)}-1]\times 100$.

The calculated value shows that a considerably high PCR efficiency was achieved for the normalised calibration curve (77%) which is particularly important for a robust and precise quantitative PCR assay[13].

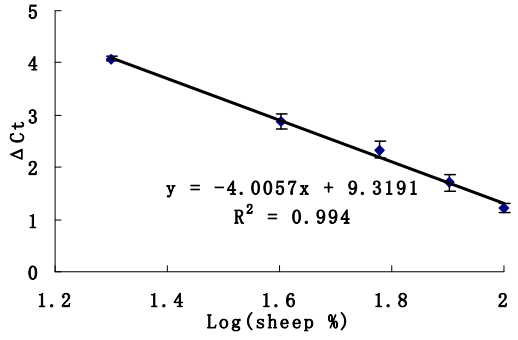

Figure 2. Normalised calibration curve for the estimation of the sheep meat in sheep-pork meat by real-time PCR, using eukaryotic amplification as a reference gene and the ΔCt method (mean values of independent assays and the bars are the standard deviations, n=4).

C. *Limit of detection*

The LOD of the real-time PCR assay were determined by analyzing serially diluted DNA extracts from a model meat mixture containing 4% (w/w) sheep in pork. The concentration of sheep obtained ranged from 4% (w/w) to 0.16% (w/w). The Ct values are summarized in Table 2. The LOD, defined as the lowest concentration that resulted in an increase of the fluorescence signal within 37 cycles in at least 9 out of 10 replicates, was found to be 0.8% sheep. The limit of 37 cycles is set in routine analysis in the AGES in order to lower the probability of obtaining false positive signals, e.g. due to cross-reactivity.

TABLE II. DETERMINATION OF LOD WITH A SERIALLY DILUTED DNA EXTRACT FROM A MODEL MEAT MIXTURE CONTAINING 4%SHEEP IN PORK

content of sheep(%)	*Ct value*		*Mean Ct Value*	*std*
4	32.85	32.17	32.71	0.60
	32.30	32.36		
	32.53	32.73		
	32.30	33.73		
	32.32	33.83		
0.8	34.86	35.98	35.64	0.92
	34.89	35.87		
	34.67	37.05		
	34.76	36.16		
	35.13	36.88		
0.16	37.65	37.58	37.93	0.60
	37.74	37.37		
	38.14	37.32		
	38.02	38.57		
	37.61	39.26		

D. *In-house assay validation*

To validate the proposed real-time PCR methodology, binary mixtures prepared with known amounts of sheep in sheep-pork meat mixtures were analysed as blind samples. The respective Ct values for sheep and endogenous control of amplified blind mixtures were used to calculate Δ Ct and estimate the amount of sheep based on the proposed standard calibration curve presented in Fig. 2. Table 3 shows the predicted and actual values of sheep meat percentage for the validation mixtures of 79.89, 48.32, 31.74 and 21.30%.

The close agreement between the average values obtained for the tested concentrations is evidenced by the high proximity between true and predicted values. The low coefficients of variation, from 0.11% to 0.38% in the range of tested concentrations, evidence the high repeatability of the technique. This is indicated by the low standard errors, demonstrating the trueness of the proposed technique for estimating the level of addition of sheep in meat products in the range 20 – 100%.

TABLE III. RESULTS FOR THE VALIDATION OF THE REAL-TIME PCR QUANTITATIVE ASSAY.

Samples	*Sheep meat (%)*		*SD*	*CV(%)*	*Error*
	Actual	*Mean predicated*			
A	79.89%	72.79%	0.0920	0.13	-0.0888
B	48.32%	46.46%	0.0506	0.11	-0.0385
C	31.74%	32.37%	0.1028	0.32	0.0198
D	21.30%	19.93%	0.0767	0.38	-0.0642

SD—standard deviation
CV—coefficient of variation
Error—(mean value－true value)
Values are the mean of independentassays(n=4)

IV. CONCLUSION

In the present work, we propose a real-time PCR method based on the use of specific TaqMan probes that proved to be a powerful tool, highly specific, sensitive and accurate for sheep detection and quantification in pork meat product. The method was successfully in-house validated, as evidenced by the low standard errors and high repeatability obtained in the analysis of blind mixtures.

In conclusion, this work highlights the importance of inspection programmes in the sector of meat products avoiding fraudulent practices and protecting the health of allergic consumers. Thus, the relevance of the proposed methodology was demonstrated as a useful tool for authentication/control, allowing the specific detection and quantification of sheep in meat products.

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