Original scientific paper
UDC 614.31:637.523/.524(497.115)

DETECTION OF INGREDIENTS IN SALAMI AND SAUSAGES FROM DIFFERENT BRANDS SOLD IN KOSOVO MARKET BY PCR

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Abstract

Food adulteration is a major problem throughout the world and its detection is of economic, health and ethical interest. Declaration of ingredients in animal-based food products is of particular importance to consumers' safety and is a legally guaranteed right. In Kosovo so far there has been no study that analyzes domestic products in this regard. Therefore, this study has the purpose of identifying the ingredients in salami and sausages declared as "chicken" and sausages declared as "beef" sold in Kosovo market.

Samples were collected at random from different brands in the Prishtina region. A total of 22 samples are included in this study. 200 mg from each sample were used as the initial material to extract the genomic DNA. Using the Qiagen food authentication portfolio (DNeasy mericon Food Kit and mericon PCR assays) we successfully extracted the genomic DNA and then amplified the target in a Roter-Gene Q. Mericon assays for target detection use a real-time PCR-based protocol by implying two different dyes (FAM dye for target detection and MAX dye for internal control to control the inhibition). Samples declared as "chicken" have been tested for the swine and ruminants specific DNA, while those declared as "beef" were tested for the presence of swine and sheep sequences.

The first results show a discrepancy between the declaration and the actual ingredients in few products. Overall the undeclared swine meet in all products we tested is surprisingly low, whereas the undeclared ruminant meet seems to be more highly present.

Although the mericon PCR assays are designed for the target-specific detection a confirmation with other methods is necessary to ensure a transparent and fair labeling of the ingredients.

***Key words**: Ingredients authentication, Sausages, Salami, Food safety, PCR.*

1. Introduction

In every developed country, food security is a routine question of food control and safety for meat products where the possibility of fraud is most likely, especially in the developed and middle-developed countries. For this reason food safety and food quality is gaining high attention of consumers and of all food industry actors.

One of the main food quality-related issues is the authentication of food content, as food products may be adulterated, and similar but cheaper ones may substitute highly valuable species, partially or entirely. Consequently a detailed and reliable labelling of food has become inevitable and is important not only for economic reasons, but also for safety, health and religion reasons. In the case of pork, food manufacturers may choose to use porcine derivatives because they are cheap and readily available [1]. Allergic individuals and those who hold religious beliefs that specify allowable intake of certain species [2], have a special interest in proper labelling. Proper labelling is also important to help fair-trade and determining the species which meat originated from is an integral part of food regulatory control with respect to economic fraudulence.

In addition, determination of the species of the meat origin components in meat products is an important task in: food hygiene, food control, food codex and veterinary forensic medicine [3].

Visual differentiation of similarly pigmented meats (especially if they have been frozen in blocks) is almost impossible. This situation has prompted research to

find methods for the detection of the meat origin in food products. Currently, ELISA (enzyme-linked immunosorbent assays) and polymerase chain reaction (PCR) methods are routinely used for the species authentication. Molecular biology-based methods use techniques such as PCR [4-8], and restriction-enzyme fragment length polymorphism (RFLP) [9-13], to identify species-specific nucleotide sequences or variations within the mitochondrial DNA are widely distributed, in routine laboratories for meat specification for the basis of species recognition.

ELISA can also be used as a fast qualitative analysis systems on the production site or as quantitative method, however it has a limitation of reproducibility [14]. Yet the PCR remains one of the most reliable, fast and very sensitive methods that can detect up to 0.1% of raw pork in poultry [15].

Considering that food authentication is a major concern worldwide and the fact that Kosovo as a developing and post-war country potentially can have high fraud prevalence for meat products, this study aims at examining for the first time the fraud prevalence in some domestic salami and sausages. For this purpose we implied the Qiagen food authentication portfolio and the reverse transcription - polymerase chain reaction (RT-PCR) was performed on the highly effective thermocycler Rotor-Gene® Q (Qiagen).

2. Materials and Methods

2.1 Sample collection

Samples were collected at random from different supermarkets in the region of Prishtina. In total, 8 different processed salami and 16 different packaged sausages have been tested. Salami samples were declared as either only "chicken" or "chicken and mechanically detached meat (MDM)", whereas the sausages were all sold as only "beef" contains and unprocessed meat.

2.2 DNA extraction and purification

The extracting procedure was performed using the DNeasy® mericon® Food Kit (QIAGEN, Hilden, Germany). The standard protocol (200 mg) with some modification according to manufacturer description was used. In brief, 200 mg from each food sample was placed in 2 mL Eppendorf tube and homogenized with 1mL Food Lysis Buffer and 20 ul Proteinase K solution. For consistent homogenization this step was performed in a TissueLyser LT (QIAGEN) using one Stainless Steel Bead (5 mm) per tube.

Homogenized solution was incubated in a thermomixer for 30 min. at 65 ^{0}C with constant shaking (1000 rpm). Food samples were immediately cooled on ice and centrifuged for 5 min. at 2,500 x g. 700 uL of the clear supernatant were transferred into the microcentrifuge tube containing 500 uL chloroform, vortexed and centrifuged for further 15 min. at 14,000 g.

After the phase separation, 350 uL of the aqueous phase was mixed with equal amount of binding buffer (PB). This solution (700 uL) was transferred into the QIAquick spin column placed in a 2 mL collection tube and centrifuged at 17,000 x g for 1 min. Prior to the elution the DNA was washed with 500 uL buffer AW2 (with Et-OH). The purified DNA was eluted with 150 uL buffer EB. The DNA concentration and purity were evaluated by measuring the ratio A260nm/A280nm using the Biophotometer (Eppendorf).

2.3 RT-PCR

The ready-to-use mericon animal identification assays from QIAGEN were used to perform the RT-PCR. Each assay contains target-specific primer and probes, which are highly specific and can detect down to 10 target copies in a reaction. In addition assays are provided with an internal control (IC) to monitor the PCR inhibition. The reaction was carried out on the Rotor-Gene® Q (QIAGEN). The provided Multiplex PCR Master Mix is suitable for both multiplex PCR technology (Factor MP) and fast cycling (Q-Bond®).

The PCR reaction final volume was 20 uL consisting of 10 uL reconstituted mericon assay (mixture of PCR assay and Multiplex PCR Master Mix) and either 10 uL of food sample DNA, 10 uL of dissolved positive control DNA or in case of negative PCR control 10 uL of QantiTect Nucleic Acid Dilution Buffer was added. For target amplification we adopted the following cycling protocol: initial PCR activation step (5 min. at 95 ^{0}C, activation of HotStarTaq Plus DNA polymerase) and the 3- step cycling of denaturation 15 s. at 95 ^{0}C, annealing 15 s. at 60 ^{0}C and extension for 10 s. at 72 ^{0}C. The number of cycle was 45. Food samples were run as either duplicates (salami) or triplicates (sausages). Food samples The PCR mericon-assay uses two different dyes reported dyes FAM for the target detection and MAX are implied for the detection of internal control in the food samples.

2.4 Detection of amplified constructs

The PCR products were evaluated on the Bioanalyzer 2100 (Agilent). The amplified targets of positive control from the individual assays (pork, ruminant and sheep) were analyzed using the Agilent DNA 1000 assay. The assay did effectively evaluated the amplicon length by showing a clear peak specific to each PCR-assay and the DNA run was confirmed as successful by showing the 13 peaks of DNA 1000 ladder (15 - 1500 bp).

3. Results and Discussion

3.1 Results

DNA was successfully extracted from 22 different food samples. The extracted DNA was of good quality and purity. The average amount of extracted DNA from food samples was 350 ng and the A260/A280 ratio ranged 1.6 - 2.0.

All positive samples from either assay (pig, ruminants or sheep) tested in this study showed a clear amplification curve with cycle threshold (C_T) value of 24 - 38 (Figure 1, left panel). As documented by the corresponding internal control (IC) C_T values that were from 30 to 32 (Figure1, right panel), there were no signs of PCR inhibition observed in the tested samples. According to the manufacturer description a cycle threshold greater than 33 indicates PCR inhibitor that could lead to false results [16]. Analysis of 24 food samples, of which 8 were salami (light processed) and 16 sausages (raw meat), verified the presence of pig DNA in 2 salami samples and in 2 of the sausages (Table 1). The presence of ruminant specific sequences in the salami samples was more prevalent and 8 out of 8 tested salami samples resulted as positive. Sheep specific sequence was detected in only two out of 5 tested samples. The result outcome was determined as **"+"** if both the amplification curve of the IC and sample were clearly above the threshold and as **"-"** if the amplification of IC did occur and however the amplification curve for sample was missing. The outcome was determined as "PCR failure" in case of missing amplification curve for both the sample and its corresponding IC. Over all the number of pig positive samples was low. To exclude any assay sensitivity obstacle, the pig mericon assay was tested in different food matrices with 10%, 90% and 100% pig meat content in either chicken or beef (Figure 2). The amplification curve for the specific pig sequence in the analyzed food matrices was clearly above the threshold and excluding thus any obstacles related to assay sensitivity.

The amplification of kit positive controls has been confirmed on Bioanalyzer and each assay/kit amplified a specific fragment sequence detected as a peak fluorescence shown both as electropherograms (Figure 3 a, b, c, left) and as gel image next to the ladder (Figure 3 a, b, c, right). Specific sequence for each positive control was: pig 88 bp, sheep 98bp and ruminants showed two specific fragments 99 and 110 bp.

Table 1. Summary of the tested products and the result outcome: "+" if both the sample and IC amplification curve are above the threshold; "-" if the amplification curve of IC occurred above the threshold, but the amplification curve for sample was absent

Sample ID	***Product type***	***Labeled as***	***Pig***	****) Ruminant***
A2, A2.1	Salami	"chicken", MDM	Not tested	**"+"**
B, B1	Salami	"chicken"	"-"	"+"(§)
B2, B2.1	Salami	"chicken"	"-"	**"+"**
C2, C2.1	Salami	"chicken", MDM	Not tested	**"+"**
E, E1	Salami	"chicken"	"-"	Not tested
G, G1	Salami	"chicken"	"-"	"+"(§)
G2, G2.1	Salami	"chicken"	"-"	**"+"**
H, H1	Salami	"chicken"	"-"	"+"(§)
H2, H2.1	Salami	"chicken"	"-"	**"+"**
J, J1	Salami	"chicken", MDM	**"+"**	Not tested
L, L1	Salami	"chicken"	**"+"**	"+"(§)
L2, L2.1	Salami	"chicken"	"+" (§)	**"+"**
Sample ID	***Product type***	***Labeled as***	***Pig***	*****) Sheep***
A1, A2	Sausages	"beef"	**"+"**	Not tested
B1, B2	Sausages	"beef"	"-"	Not tested
C1, C2	Sausages	"beef"	**"+"**	Not tested
D1, D2	Sausages	"beef"	"-"	Not tested
E1, E2	Sausages	"beef"	"-"	Not tested
F1, F2	Sausages	"beef"	"-"	Not tested
G1, G2	Sausages	"beef"	"-"	Not tested
H1, H2	Sausages	"beef"	"-"	Not tested
I1, I2	Sausages	"beef"	"-"	Not tested
J1, J2	Sausages	"beef"	"-"	Not tested
L1, L2	Sausages	"beef"	"-"	Not tested
M4.1	Sausages	"beef"	Not tested	**"+"**
M5.1	Sausages	"beef"	Not tested	"-"
R4.1	Sausages	"beef"	Not tested	**"+"**
R5.1	Sausages	"beef"	Not tested	"-"
R6.1	Sausages	"beef"	Not tested	"-"
Total positive (%)			**23.5**	***) 100, **) 40**

MD - Mechanically deboned meat.
(§) Same samples, tested with both pig and ruminant assay, consequently are count only one time as positive "+".

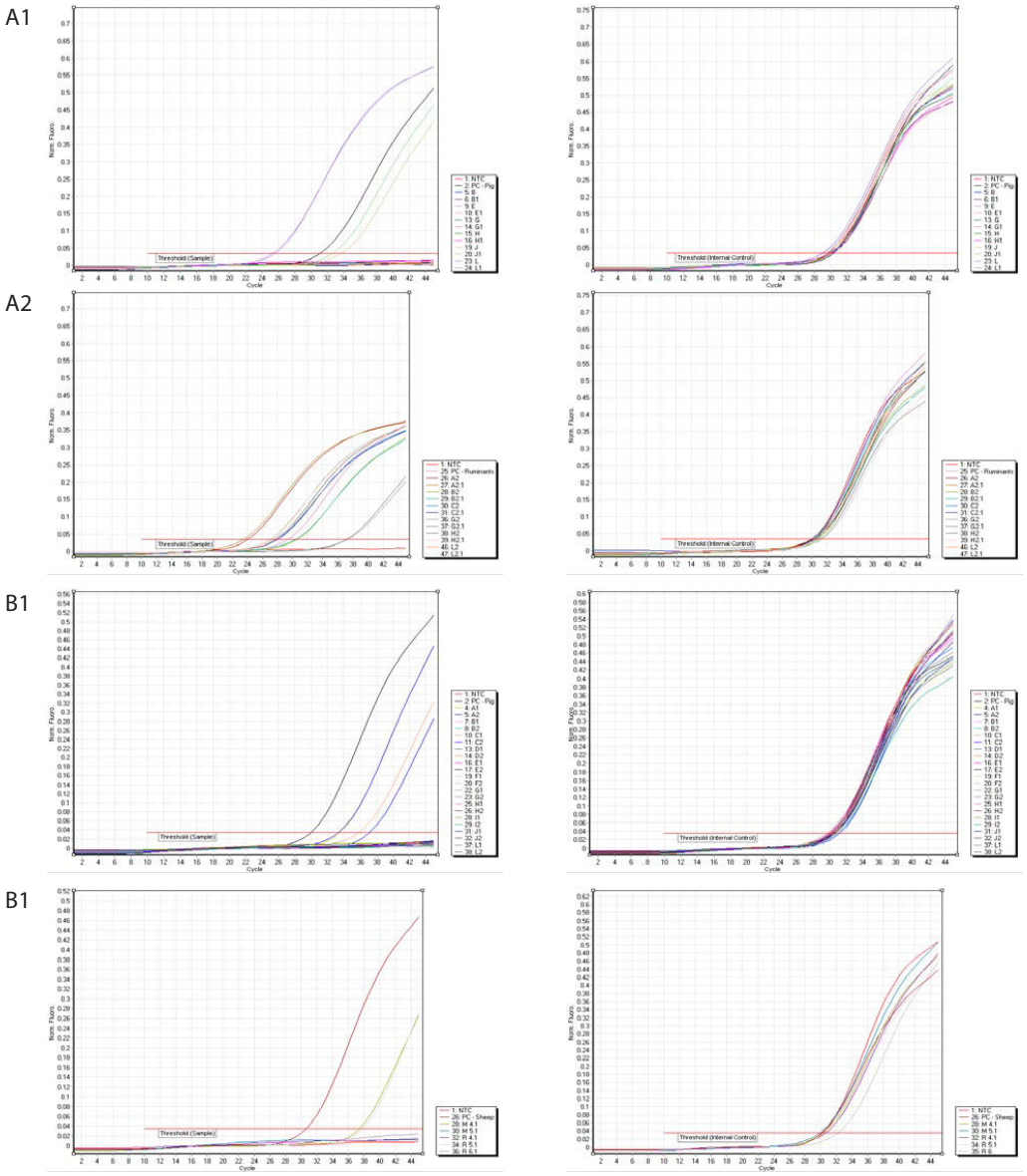

Figure 1. Amplification curves for salami food samples positive for the presence of:
A1) pig specific target sequences (left) and their corresponding internal controls (right), A2) ruminant specific sequences (left) and their corresponding internal controls (right).
Sausage food samples positive for the presence of:
B1) pig specific target sequences (left) and their corresponding internal controls (right), B2) sheep specific target sequences (left) and their corresponding internal controls (right)

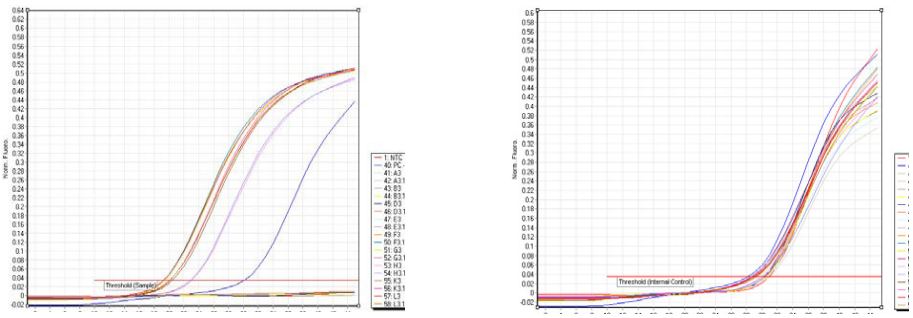

Figure 2. Amplification curves for different pig food matrices to confirm the mericon pig assay sensitivity. Left: food matrices with 100% pig (F3 and F3.1), food matrices with 90% pig: 10% chicken (G 3and G3.1), food matrices with 90% pig: 10% beef (H3 and H3.1), food matrices with 10% pig: 90% chicken (K3 and K3.1), 10% pig: 90% beef (L3 and L3.1). Right panel: internal controls for the tested food matrices. Samples labeled from A3- E3.1 are pig free food matrices and their amplification curves remains under the threshold.

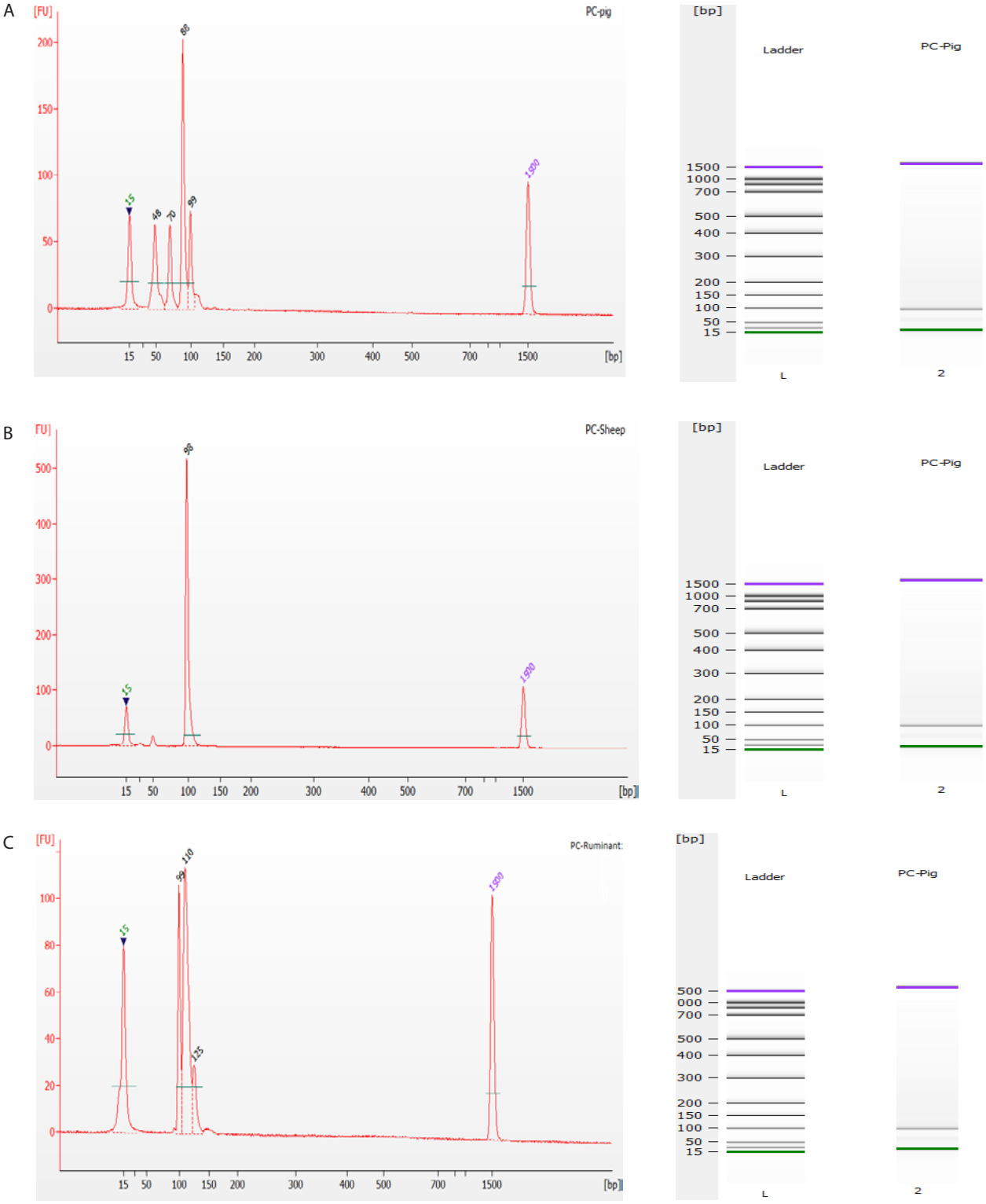

Figure 3. Electropherograms of the positive controls for each of the assays: A) pig specific target sequence length 89 [bp], B) sheep specific target sequence length 98 [bp] and C) ruminant specific target sequences length 99 and 110 [bp]

3.2 Discussion

According to the Food and Agriculture Organization Corporate Statistical Database (FAOSTST), annual levels of meat consumption in Balkan is estimated to be 44.1% [17]. In Kosovo generally meat and dairy products are among the first choice in consumers' bag, consequently the demand for more information from consumers' site concerning the food origin has been increased. Kosovo's regulation (MTI)-No. 03/L-016 on labeling, presentation and advertising of food

products, article 4 and 7 envisages the detailed list of: ingredients and quantities, sustainability, origin and production process for any packed foods. Therefore meat products prepared in this context should be labeled properly regarding the species of origin. In addition to the basic legal protection of consumers against deception, meat species that are not or incorrectly declared can also interfere with religious taboos for Kosovo population.

This is the first study analyzing domestic salami and sausages in regard to fraud and adulteration. The study revealed surprisingly low meet adulteration and substitution when it comes to the pig meet, however the substitution/deception of chicken with ruminant meet in salami was highly prevalent and we detected ruminants meet in all samples we've tested (Table 1). Considering that many of the home made recipes for sausages production do include sheep meet, we also expected to see high prevalence for sheep, however we could only detected sheep meet in 40% of the tested product. As the number of samples was rather low (5 samples), these results should be noted with caution. The ready to use mericon assays, were shown to be highly sensitive and appropriate assay for the detection of species-specific sequences [16]. The assay utilizes a real-time PCR protocol for the target amplification. As shown in the Figure 1, all samples that were detected as positive have their amplification curves C_T values in the desired range of 24 - 40 cycles (Figure 1, left panel).

In addition their corresponding internal controls show clear amplification with C_T value between 28 and 32 cycles confirming that there was no PCR inhibition (Figure1, right panel). The specificity of the assay was in line with the results expected when working with RT-PCR, which is shown to be more accurate, reliable and sensitive compared to conventional PCR methods, especially in the sensitive case of pork meet fraud [18, 19].

Moreover, each assay was confirmed as specific by evaluating the positive control amplicon length on the Bioanalyzer (Figure 3 a, b and c). Although meat products we've tested, sausages and salami, are mostly labeled as produced from one type of meet only, we were able to detect considerable fraud and mislabeling and expressed in percentage it reaches 23.5% in case of pig meet, 40% for sheep and 100 % for ruminants.

As a developing country and according to the EU legislation Reg. CE 1169/11 (Annex VII, Part B, comma17) [20], Kosovo needs to improve and harmonize the food quality and safety regulation to the European Union countries. The institution responsible for conducting food analysis is the kosovarian Food and Veterinary Agency, but additional laboratories may be required to carry out these analyzes.

This study offers a solid information and may serve as starting point to design a striking control plan and a traceability system for food products in Kosovo in order to enhance food quality and safety conforming to European norms.

4. Conclusions

- We can conclude that the meat adulteration is present in the Kosovo meat market. In the 24 samples analyzed in this study we detected high discrepancies between declaration and the actual content.

- The mericon assays for RT-PCR is very reliable and specific option for the detection of food adulteration. The ability to detect down to 10 copies of the target in a reaction ranges it the first assay choice among the DNA based assays. However it still remains costly, considering that samples must be run in at least duplicates for a meaningful outcome.

Acknowledgment

We would like to thank Krenare Bruqi, Rita Panduri and Melisa Jahiu, for their continuous support during the sample collection and preparation.

This study has been supported by the Ministry of Education, Science and Technology, Kosovo under the small research project action foundation: FPVSh: 2-4051-2.

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