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# Investigation of pork meat in chicken- and beef-based commercial products by ELISA and real-time PCR sold at retail in Kosovo

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**Abstract:** Food adulteration and fraudulent practices are widely observed in the food industry worldwide and are of great concern for Balkan countries. This study aims at investigating the level of undeclared pork meat in commercial beef and chicken meat products sold in Kosovo by implying one commercial enzyme-linked immunosorbent assay (ELISA) and two confirmatory real-time polymerase chain reaction (PCR) approaches [ready-to-use real-time PCR and real-time PCR with primers specific for pork mitochondrial deoxyribonucleic acid (DNA)]. In supermarkets in the capital city, Prishtina, 62 meat products were randomly sampled, and the three methods were applied. Additionally, these three approaches were evaluated for their practicability, reproducibility, and cost. The results showed that pork was present in 32% of beef- and 8% chicken-based products. ELISA and real-time PCR with pork specific primers showed 100% of reproducibility for beef- and chicken-based products. In contrast, the ready-to-use real-time PCR kit showed 100% reproducibility in chicken-, but only 75% in beef-based samples. ELISA was more rapid than both real-time PCR approaches, but it was more challenging when large numbers of samples were processed. The real-time PCR approach with pork specific primers was the cheapest, while the ready-to-use real-time PCR was the most practical method. Commercial ELISA, in combination with real-time PCR with pork specific primers, provides a reliable and affordable testing methodology that can be implemented for rapid detection and monitoring of pork adulteration in diverse commercial foods.

**Keywords:** food adulteration; animal-based foods; ready-to-use real-time PCR; commercial foods

Meat consumption makes up a significant percentage of the everyday diet. In the Balkan region, it is considered that meat consumption makes up to 44.1% of the diet, and there is an increasing tendency (FAO 2019). Increasing meat consumption has also raised the demand for safer and more controlled meat products, not only for the aspect of pathogen detection but also for authenticity and undeclared meat species.

Adulteration of meat products is common and might be the result of the substitution of expensive meat species by cheaper ones [(economically motivated adulteration (EMA)] or due to cross contaminations during the process

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of meat grinding (Spink and Moyer 2011). It is estimated that food adulteration causes a loss of up to USD 15 billion per year to the food industry worldwide, although the exact impact is expected to be higher (Johnson 2014).

Adulteration of beef and chicken meat products can result in a number of potentially harmful effects related to the exposure to allergens (WHO, HACCP, 1997). In addition, it is a problem because of the prohibition of consuming pork meat in several religious beliefs. Therefore, methods are pivotal which are able to detect even minimal amounts.

The fraudulent practices reported over the years (Ballin et al. 2009; Nau 2013; Hsieh and Ofori 2014) show that there is rising demand from consumers' side for a rigorous food control. A study conducted recently by Wisniewski and Buschulte (2019), aiming at food fraud-tackling, found that 72% of the participants were interested in a tool that could help them detect fraud, preferring one that would not imply complex laboratory tests.

However, the morphological transformation of beef and chicken meat after processing or even mincing makes it difficult to detect fraud visually; therefore, analytical methods are unavoidable. For the verification of meat composition, two of the most widely used methods are the detection of proteins by enzyme-linked immunosorbent assay (ELISA) or deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR) (Cammà et al. 2012; Ali et al. 2014; Giovannacci et al. 2004). Both methods can be highly specific and sensitive for the detection of adulteration in different raw meat and some processed foods (Perestam et al. 2017). ELISA methodology is often the first choice and preferred in many food safety and inspection services; however, it has some limitations when testing highly processed foods due to the denaturation of proteins (mainly albumins) during processing (Asensio et al. 2008). The PCR method is a more robust method and shows greater sensitivity and therefore, it overcomes the ELISA limitations (Dooley et al. 2004). However, it has also some limitations due to the interferences from vegetable ingredients in processed foods (Piskata et al. 2019). Over the last decade, sophisticated omics (genome, proteome and transcriptome) approaches or droplet digital PCR are gaining interest, not only for multiple species identification but also for the quantification of adulterated meats (Flaudrops et al. 2015; Cai et al. 2017). As a new technology, these methods are expensive and accompanied by high-tech laboratory equipment. Therefore, they are not suitable for routine application of food adulteration monitoring in developing countries.

In Kosovo, as a developing country, the Food and Veterinary Agency of Kosovo and the National Institute of Public Health are responsible for conducting and monitoring food safety and quality. However, food adulteration data are not available yet from neither institution.

Looking for a suitable testing methodology for efficient monitoring of adulteration of pork meat in commercial meat products sold at the retail market in Kosovo, we have *i*) investigated the incidence of adulterated pork meat in beef- and chicken-based meat products and *ii*) evaluated the usage of two commercial kits (one for protein analysis by ELISA and one for nucleic acids analysis by QIAGEN mericon ready-to-use real-time PCR authenticity pig kit) and one non-commercial real-time PCR assay with pork specific primers.

## MATERIAL AND METHODS

**Sample collection.** Beef- and chicken-based meat products were randomly collected from markets in different regions of Kosovo, representing common products and with different compositions. In total, 62 different processed chicken- and beef-based meat products were collected between December 2018 and January 2019 [Figure 1A and Table S1; for Table S1 see electronic supplementary material (ESM)]. All chicken-based meat samples shared the common fact that the meat content was either 100% chicken meat or 100% chicken and mechanically deboned meat (MDM). All beef-based meat samples were decelerated as 100% beef meat.

**Serum albumin extraction.** The extraction of serum albumin was performed following the Enhanced Extraction Kit instructions (Bio-Check, United Kingdom). In brief, test portions were prepared for each sample separately, and 0.35 g were used for the extraction. The clear liquid layer above the pellet and below any fatty layer was used for ELISA testing.

**ELISA assay procedure.** The semi-quantitative ELISA was run on duplicates according to the manufacturer's protocol (Species-Check$^{TM}$ ELISA Kit; Bio-Check, United Kingdom). One negative control (NC) and two positive controls (PC) [a low detection control (LC) and a high detection control (HC)] supplied by the kit were applied together with the samples. For all tested samples and controls, the optical density (OD) was read at 450 nm using a microtiter-plate reader (HumaReader HS REF 16670; Human, Germany). Samples were declared to be positive with low detection if they contained 0.02–0.1% (w/w) of pork meat, positive with moderate detection if they contained 0.1–0.5% (w/w) of pork

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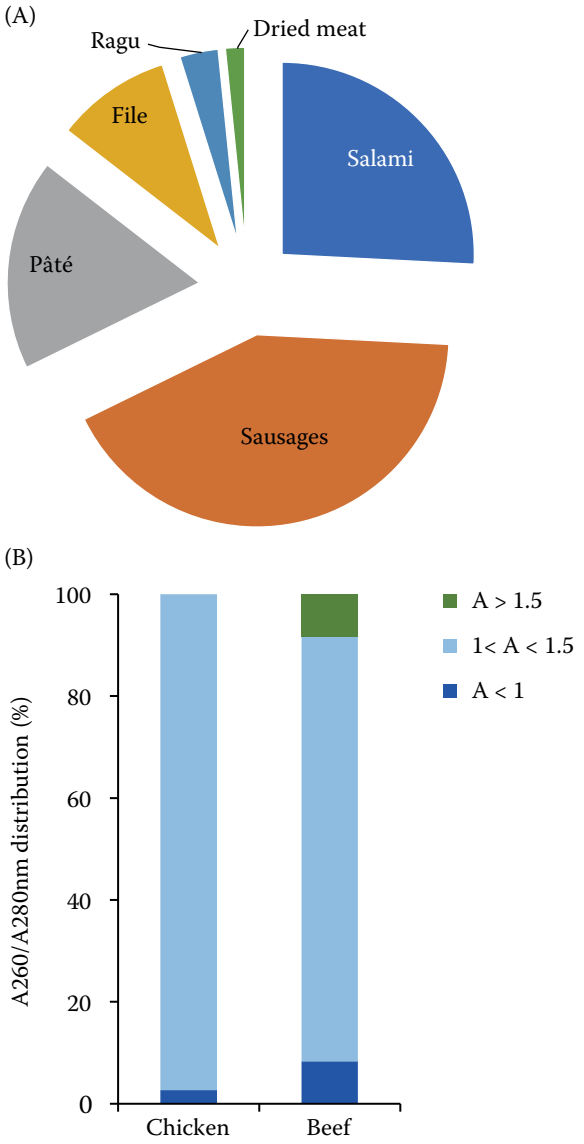


Figure 1. (A) Food samples analysed in this study [sausages (26), pâté (11), file (6), ragu (2), dried meat (1), salami (16)], and (B) absorbance distribution of chicken- and beef-based food products.

A – absorbance

meat and positive with high detection if they contained > 0.5% (w/w) pork meat.

**DNA extraction and purification.** To extract the genomic DNA, the DNeasy® mericon® Food Kit protocol (QIAGEN, Germany) was used. This kit is in accordance with ISO-21571:2005 and utilises a cetyltrimethylammonium bromide (CTBA) extraction protocol. The manufacturer's instructions were followed with some modification: to achieve a better homogenisation, a TissueLyser LT (QIAGEN, Germany) with one stainless steel bead (3 mm) per tube was used. The DNA concentration and purity were evaluated by measuring the ratio A260/A280nm as well as A260/A230nm using the Biophotometer/Hellma® TrayCell (Eppendorf BioPhotometer plus UV/Vis; Eppendorf/Hellma Analytics, Germany). All samples were measured in triplicates.

**Real-time PCR with the commercial mericon kit.** The mericon Ingredient Authentication Assay (mericon Pig Kit, ID: 292015; QIAGEN, Germany) was used to perform the real-time PCR. The assay is supplied with an internal control (IC), allowing for monitoring of PCR inhibition and PC. The reactions were carried out on a Rotor-Gene® Q 72 well disc (QIAGEN, Germany) with a final reaction volume of 20 µL as described previously (Gecaj et al. 2018). All food samples were run as duplicates. The samples were determined to be positive if both duplicates had a cycle threshold (Ct) value between 24–38 and a Ct value between 30–32 for the corresponding ICs. The results state either the presence or the absence of pork meat.

**Real-time PCR with designed primers.** The PCR assay with designed primers was carried out on a Rotor-Gene® Q 72 well disc (QIAGEN, Germany) using the SYBR Green dye [HotStarTaq® Plus DNA polymerase, real-time PCR buffer and deoxynucleoside triphosphate (dNTP)]. PCR amplifications were performed in a total volume of 20 µL consisting of 10 µL SYBR Green dye, 0.6 µL of each primer (with the final concentration of 0.15 µM) and 5 µL of the extracted genomic DNA. We used the primer set developed by Dooley et al. (2004) and confirmed by Nikzad et al. (2017) to be highly specific for the simultaneous detection of pork and beef for halal authentication. The primer set used in this study targets the cytochrome b (Cytb) gene and was synthesised by Sigma-Aldrich (Germany) (Table 1).

All samples were run in triplicates with the following conditions: denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30 s, 60 °C

Table 1. Porcine specific primers

| Specie | Primer sequence (5' → 3') | Target gene | Reference |
|---|---|---|---|
| Porcine | F: ATGAAACATTGGAGTAGTCCTACTATTTACC<br>R: CTACGAGGTCTGTTCCGATATAAGG | Cytb | (Nikzad et al. 2017) |

Cytb – cytochrome b; F – forward; R – reverse

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for 30 s, and 72 °C for 30 s and a final extension step at 72 °C for 5 min. The qualitative results were considered to be positive if all triplicates had a Ct value lower than 40. The results were also reported as the presence or absence of pork. In addition, to assess the detection limit, standard food matrices consisting of 1, 5, 10, 25, 50, and 75% pork meat in either chicken- or beef-based meat products were tested (Figure S1; see ESM).

**Confirmation of the amplified constructs**. The PCR products amplified were evaluated on the microfluidal electrophoresis (Bioanalyzer 2100; Agilent, USA). The Agilent DNA 1000 assay was used for the confirmation, and according to the manufacture's instruction 9.0 µL of freshly prepared gel-dye-mix was loaded on the DNA chip. The results were considered positive if a clear band was visible for mericon pig-assay at 88 base pare (bp) (Gecaj et al. 2018; Manovi et al. 2019) and for the real-time PCR with designed primers at 149 bp (Dooley et al. 2004; Nikzad et al. 2017).

## RESULTS AND DISSCUSION

**DNA quantity and purity.** The amount of extracted DNA was in average 94.5 ng $\mu L^{-1}$ in chicken- and 70.4 ng $\mu L^{-1}$ in beef-based food samples. The quantities obtained in the present study agree with previous studies, which report a similar DNA yield using the DNeasy mericon Food Kit (QIAGEN, Germany) for extraction from different food matrices (Piskata et al. 2017). During food preparation, the ground meat is subjected to different treatments procedures e.g. temperature and high pressure, which are known to degrade the DNA (Bauer et al. 2003). The higher yield in chicken-based food samples may be related to the fact that in heat-processed foods such as sausages, the heat leads to cell membrane break and allows more DNA to be released (Musto 2011). Furthermore, many processed foods like sausages usually do not only consist of muscle tissue but also of liver, heart, or kidney tissue. Those tissues have a higher density of cells and, therefore, a higher amount of DNA (Laube et al. 2007).

Impurities or carryovers during the extraction step can interfere later on with the PCR or lead to PCR inhibition and false detection of fraud (Di Pinto et al. 2017). Food samples tested in this study show acceptable purity as judged by the A260/A280nm ratio that ranges between 1–1.5. As shown in Figure 1B, over 97% of chicken-based food samples have a ratio between 1–1.5 and 83% of beef-based food samples. The ratio we obtained is not optimal; however, most of the processed foods usually contain undeclared chemicals that cannot be completely removed during the extraction step; therefore, it is acceptable for the purpose of PCR used here (Piskata et al. 2019).

**ELISA: Pork positive commercial samples.** Overall, 3% of the chicken-based food samples show a moderate level of adulteration with pork and 5% have a low level of adulterated pork, while 4% of beef-based food samples show moderate and 28% a low level of adulterated pork (Figure 2, Tables S2A and S2B; see ESM). The number of chicken-based food samples which are positive for pork is low but comparable to our previous study. In contrast, we found a higher number of adulterated beef-based food samples compared to our previous study (Gecaj et al. 2018).

**Real-time PCR.** In this study, we used the primer set Forward/Reverse to amplify a 149 bp long fragment of the mitochondrial Cytb gene. Studies using the same primers (Dooley et al. 2004; Nikzad et al. 2017; Gecaj et al. 2018) demonstrated that the primer set was very specific, did not show any primer dimerisation, or cross-reactivity with other species' DNA. Additionally, pork DNA could be detected as low as 0.1% in binary meat mixtures and could be implied for simultaneous detection of bovine and porcine DNA. In our study, we used the same primer set in a SYBR Green dye-based real-time PCR approach. In the PCR three chicken samples (Figure 3A) and eight beef samples (Figure 3B) (ID: 42, 49, 53, 54, 57, 58, 59, and 60) are clearly confirmed to contain undeclared pork. A single band corresponding to the

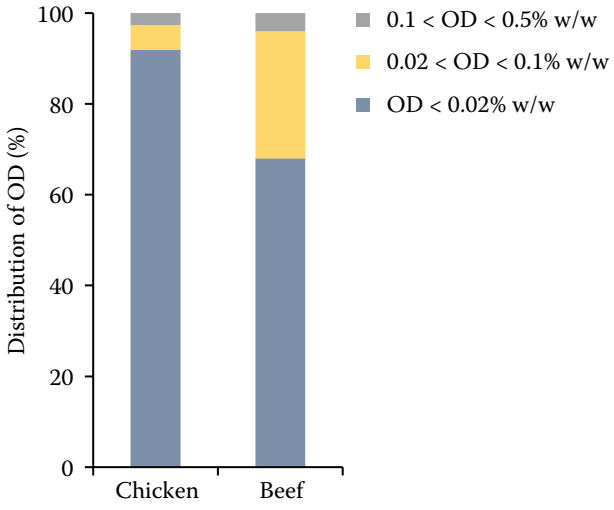


Figure 2. ELISA screening and distribution of average replicate OD values

ELISA – enzyme-linked immunosorbent assay; OD – optical density

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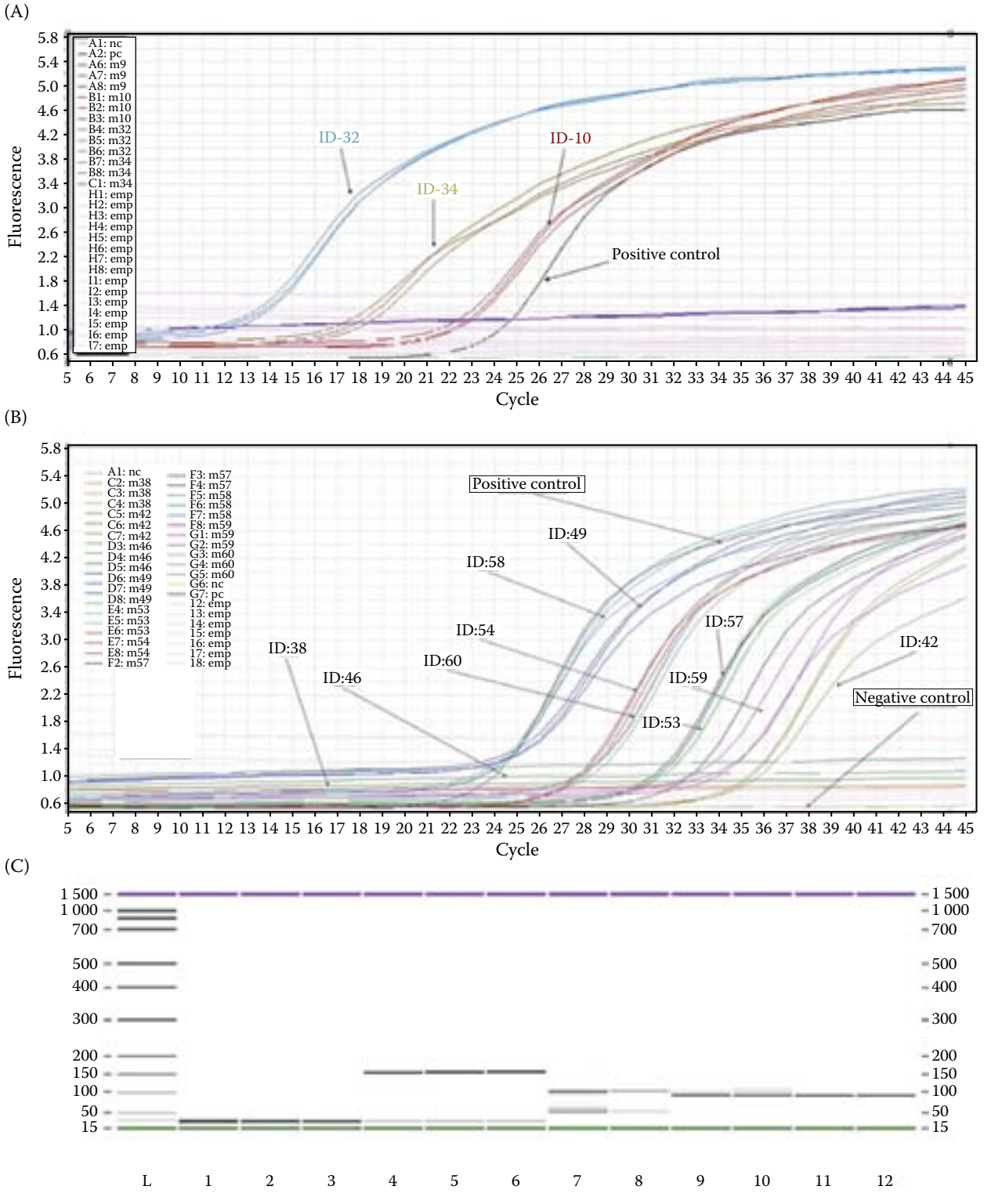


Figure 3. Positive chicken and beef samples tested with the pork specific primers: (A) amplification curves for chicken food products positive for pork, (B) amplification curves for beef food products positive for pork, (C) gel image of PCR amplicons

PCR – polymerase chain reaction; L – ladder; lanes 1, 2, 3 – negative control; lanes 4, 5, 6 – positive samples (ID: 34, 53, 60) for real-time PCR with pork specific primers; lanes 7, 8 – negative control; and lanes 9, 10, 11, 12 (88 bp) – positive samples ready-to-use maricon PCR; the bands in lanes 7 and 8 correspond to the internal control (IC)

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149 bp long PCR product and shown in Figure 3C confirms this result (lines 4–6).

In the literature, the detection limit for this assay is given as low as 0.1% pork in binary meat mixtures (Soares et al. 2013; Gecaj et al. 2018).

In addition, it was shown that the primer set was specific not only for the products tested by Nikzad et al. (2017) but also for diverse other commercial foods (Table S1; see ESM).

**Mericon food authenticity pig kit.** The amplification curves of chicken (ID: 10, 32, and 34) cross the threshold between cycles 20 and 34 (Figure 4A). The mericon pig kit re-confirmed only two out of eight beef food samples that were screened positive for pork

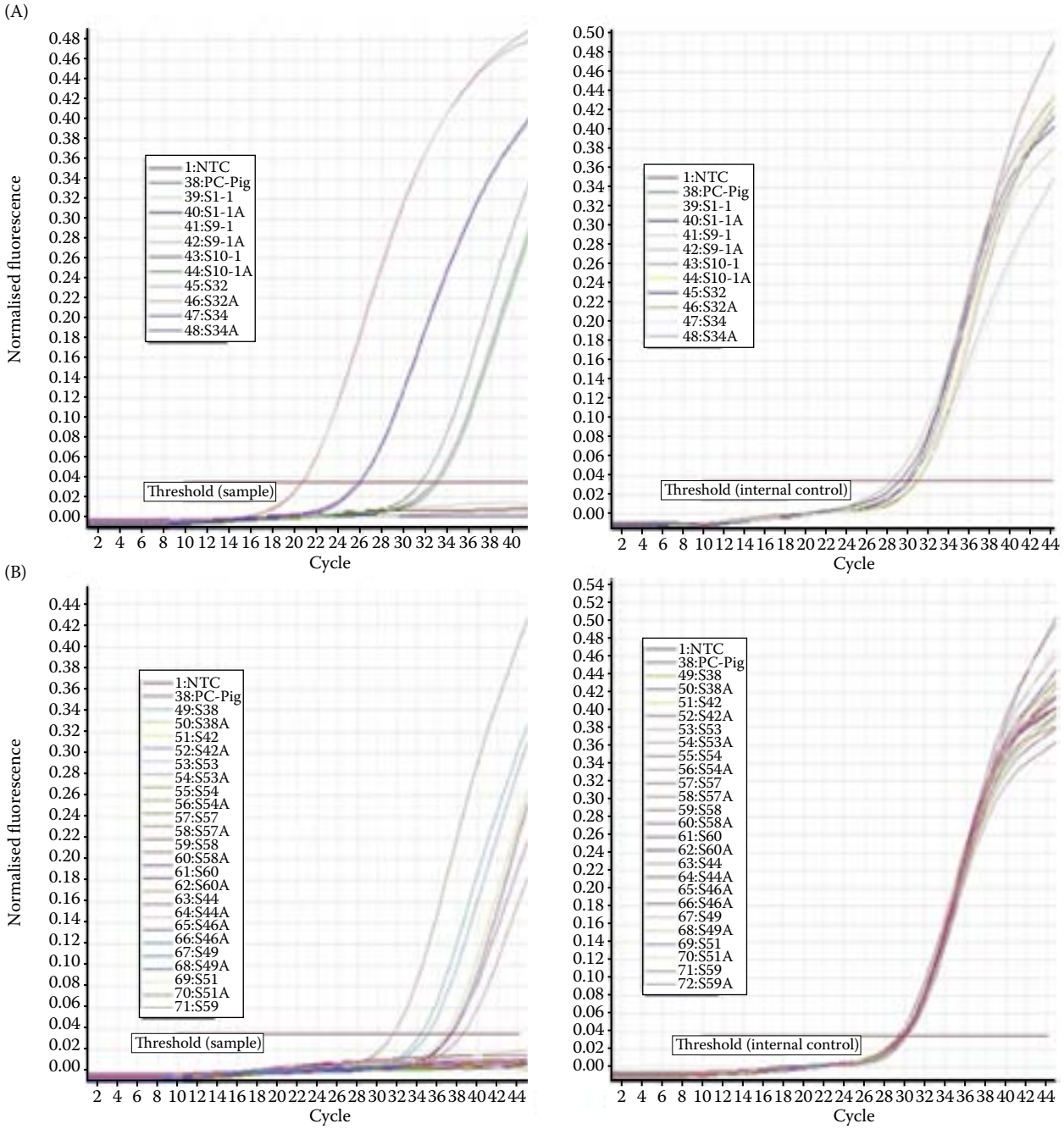


Figure 4. Positive chicken and beef samples tested with the mericon pig commercial kit: (A) amplification curves for chicken food products positive for pork (left panel) and their corresponding ICs (right panel), (B) amplification curves for beef food products positive for pork (left panel) and their corresponding ICs (right panel)

ICs – internal controls

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Table 2. Comparison of ELISA, *maricon* real-time PCR and real-time PCR with pork specific primers in commercial samples for the detection of undeclared pork matter

| Measure | ELISA commercial kit* | PCR commercial kit* | PCR pork specific primer** |
|---|---|---|---|
| Agreement among duplicates/triplicate (%) | 100[a,b] | 100[a]/75[b] | 100[a,b] |
| Sample preparation and extraction – sample/12 samples | 1.5 min/18 min | 0.9 h/1.4 h | 0.9 h/1.4 h |
| Total time for the given methodology – 12 samples (h) | 1.7 | 2.55 | 4.9 |
| Cost – extraction and assay per sample (EUR) | 12 | 18 | 7 |
| Ease of use | moderate | ease | moderate |

*Duplicate samples; **triplicate samples; [a]chicken samples; [b]beef samples; ELISA – enzyme-linked immunosorbent assay; PCR – polymerase chain reaction

when tested with ELISA and real-time PCR with pork specific primer set (Figure 4B). These findings are in line with Nikzad et al. (2017) that also show that the mericon pig kit was not able to detect pork matter in bovine-porcine mixtures when the pork contamination was 0.1% and 1% (w/w). In a recent pilot study was shown that the mericon pig kit was only able to detect pork in beef-porcine standard mixture contaminated with 10% pork (w/w) (Gecaj et al. 2018).

Processed commercial meat products usually contain plenty of ingredients from vegetable sources, which can lead to PCR inhibition and consequently false negative results, and ICs are necessary (Soares et al. 2013). To exclude any false negative results, we checked the amplification of ICs, and as can be seen in Figure 4A and 4B (right panel), the IC is successfully amplified, and clear curves cross the threshold uniformly between Ct = 28–32 for all tested commercial samples. The specificity of the mericon primers used with the kit was checked on the Bioanalyzer 2100 (Agilent, USA), and an 88 bp length band corresponding to a targeted pork DNA (Gecaj et al. 2018) is shown in Figure 3C (lines 7–12). Based on the above results, we can conclude that the presence of pork meat in the commercial beef meat samples tested in this study is below 1% (w/w).

**Reproducibility and practicability.** Of the tested approaches, the commercial ELISA and real-time PCR with pork specific primers showed 100% concordance among replicates for both chicken and beef commercial food samples (Figures 3A and 3B, Tables S2A and S2B; see ESM). The commercial ELISA had the advantage of being less time consuming than the DNA-based methods; however, it was more expensive than the real-time PCR with pork specific primers. Another advantage of commercial ELISA is the inclusion of positive and negative controls, which does not imply extra costs. However, ELISA results usually need to be confirmed with an additional method when analysing highly processed foods (Asensio et al. 2008). Therefore, additional costs should be considered. The mericon commercial kit had the advantage of being less time consuming and easy-to-follow; however, it was far more expensive than the commercial ELISA and real-time PCR with designed primers. Furthermore, it showed only 75% concordance among duplicate samples for the beef products tested in this study (Figure 4B, Table 2). Another shortcoming of the mericon ready-to-use assay is its level of detection for pork matter, which was above 1% (w/w).

## CONCLUSION

Here, for the first time, we report that on a level of incorrect information on labelling of the commercial products sold at retail in Kosovo, beef products had a higher incidence (32%) of adulteration compared to chicken products that show relatively low incidence (8%). ELISA, mericon-real-time PCR and real-time PCR are useful for detecting pork content in commercial meat products, being the real-time PCR with designed primers the cheapest and more reproducible one. When ingredients of vegetable sources are present in the samples being tested, the mericon-real-time PCR ready to use kit is suggested, as it offers the possibility to monitor PCR inhibition and excludes thus false negative results. Real-time PCR with pork specific primers in combination with ELISA commercial kits provides suitable and affordable testing and monitoring strategy in retail markets.

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