

Preliminary communication

**LOOP-MEDIATED ISOTHERMAL AMPLIFICATION BASED
APPROACH AS AN ALTERNATIVE TO RECOMBINASE
POLYMERASE AMPLIFICATION BASED DETECTION
OF MANGALITZA COMPONENT IN FOOD PRODUCTS**

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(Received: 7 November 2016; accepted: 16 January 2017)

We used an alternative approach, loop-mediated isothermal amplification, to detect Mangalitza component in food products, and it has been compared to an established Recombinase Polymerase Amplification test. The correlation between the assays was significant ($P < 0.01$). Linear determination coefficient between the assays was 0.993 and level of diagnostic agreement was high ($\text{Kappa} = 0.971$).

Previously, a real-time PCR method based on TaqMan probe was developed (SZÁNTÓ-EGÉSZ et al., 2013) for detection of Mangalitza meat in food products, using a Mangalitza specific sequence. Other Mangalitza specific sequences suitable for the same purpose are also in use (V. STÉGER, personal communication).

Approaches like real-time monitoring of accumulation of the specific DNA product usually require specialised laboratory equipment. For Mangalitza detection, portable Recombinase Polymerase Amplification (RPA) approach has been developed (SZÁNTÓ-EGÉSZ et al., 2016), which requires a device capable of maintaining 39 °C and a lateral flow strip with easy yes/no indication of the successful amplification.

We wanted to develop another fast, non-PCR based test with minimal laboratory requirement to provide a third possibility to detect Mangalitza component in food.

Keywords: food, LAMP, RPA, Mangalitza

Nowadays, a wide range of non-PCR amplifications are available, such as Helicase Dependent Amplification (VINCENT et al., 2004), Transcription Mediated Amplification (GUATELLI et al., 1990), Self-Sustained Sequence Replication (3SR) (GUATELLI et al., 1990), Rolling Circle Amplification (FIRE and XU, 1995), Standard Displacement Amplification (SDA) (WALKER et al., 1992), and loop-mediated isothermal amplification (LAMP) (NOTOMI et al., 2000). They usually do not require expensive instrumentation and have comparable diagnostic power to

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PCR. LAMP applicability of amplification of DNA region in interest has been widely tested, and it has found its way mainly in pathogen detection (NIESSEN et al., 2013) and GMO detection in maize (HUANG et al., 2014) or soy (FUKUTA et al., 2004). There are examples for identification of ostrich meat (ABDULMAWJOOD et al., 2014) and detection of swine, chicken, and bovine species in food as well (AHMED et al., 2010).

To test LAMP capability to identify Mangalitza specific DNA, we designed primers and tested against our previously described RPA method (SZÁNTÓ-EGÉSZ et al., 2016).

1. Materials and methods

1.1. Samples

Pig, cattle, chicken, and turkey muscle samples were collected in abattoirs. We have obtained Mangalitza samples from our collection (ZSOLNAI et al., 2013). Wild boar samples were collected at different hunting events at different sites. Mangalitza sausage and liver paté were produced by a reliable manufacturer and also in the laboratory of NARIC-Food Science Research Institute (FSRI). Non-Mangalitza sausage and paté samples were purchased at the market and also prepared in the laboratory of FSRI. DNA sources like liver paté or sausage and muscle samples were processed as described by SZÁNTÓ-EGÉSZ and co-workers (2016) using Wizard® procedure (Promega, USA). Simple grinding and homogenisation were also applied parallel on liver paté and sausage (SZÁNTÓ-EGÉSZ et al., 2016) to test LAMP sensitivity to sample impurity.

1.2. Primers and detection of amplified products

RPA primers and probes were used as described in SZÁNTÓ-EGÉSZ and co-workers' (2016) work. Amplification conditions for 50 µl RPA reaction (TwistDx, UK) were 39 °C for 30 min. RPA amplified product was detected by Universal MileniaHybriDetect (MileniaBiotec, Germany) (KERSTING et al., 2014).

LAMP primers were designed by LAMP Designer 1.12 (<http://www.premierbiosoft.com/isothermal/lamp.html>).

The two outer primers are F3 (Forward outer primer) and B3 (Backward outer primer). They have a role in strand displacement. The internal primers are FIP (Forward Inner Primer) and BIP (Backward Inner Primer) having sense and antisense sequences helping in the formation of loops. Two additional forward and backwards primers (LoopF, LoopB) are optional. The LoopF and LoopB oligonucleotides are serving for acceleration of the reaction by binding to sites, which are not covered by the other four primers (PARIDA et al., 2008).

Primers used for LAMP reaction:

F3: CCACAGAAGGAGTAAGAGTTG,

B3: CAACGCTGAACACAGTGT,

LoopF: TGCTTCTCTAACGTTTTGCTC,

LoopB: TTTCATGTTTGAAACAAGCAT,

FIP: ACTGGGTCTTAAGGTAAGTCACTGTACAATAACAAAGGTCAA,

BIP: AGTGTCCTATGCTATGAATCACACAATGTAGCCACCTACTAA.

Primer concentrations were 0.2 μM for F3 and B3, 0.4 μM for loopF and loopB, and 0.8 μM for FIP and BIP. Isothermal Master Mix (OptiGene, UK) was applied in 25 μl LAMP reaction according to the manufacturer's instruction. The reaction was carried out at 65 °C for 30 minutes. LAMP products were visualised on 2% MetaPhore agarose (Fig. 1., 5 V cm^{-1} , 15 min; Fig. 2., 3 V cm^{-1} ; 60 min).

1.3. Statistical analysis

RPA and LAMP assays were compared with correlation and regression functions of SPSS software. Kappa value was also determined to inquire agreement level of assays.

2. Results and discussion

Altogether 711 samples have been tested by RPA previously (SZÁNTÓ-EGÉSZ et al., 2016). A smaller set, 70 samples has been selected to test LAMP reaction in triplicates (Table 1.).

Table 1. Results of LAMP assay on purified (Wizard[®] procedure) DNA samples.

Species or food product	Breed or species	Number of samples	Number of positive LAMP reactions		
			Replicate1	Replicate2	Replicate3
Pig	Mangalitza Blond	5	5	5	5
	Mangalitza Swallow-Belly	5	5	5	5
	Mangalitza Red	5	5	4	5
	Mangalitza \times Duroc	5	5	5	5
	Duroc	5	0	0	0
	Large White	5	0	0	0
	Wild Boar	5	0	0	0
Other animals	Chicken	5	0	0	0
	Turkey	5	0	0	0
	Cattle	5	0	0	0
Sausage	Mangalitza	5	5	5	5
	Non-Mangalitza	5	0	0	0
Liver paté	Mangalitza	5	5	5	5
	Non-Mangalitza	5	0	0	0

Each sample has been measured in triplicates.

LAMP reaction detected 30 positive samples out of 30 Mangalitza samples (including 20 Mangalitza or its cross and 10 Mangalitza food products) in accordance to RPA assay.

There was only one muscle sample, where one reaction among the LAMP-triplicates has failed. Repeating the LAMP assay again on this particular sample several times, the results were positive. The false negative reaction could have been a consequence of unidentified human error. Neither cross sensitivity of LAMP or false positives among the 40 non-Mangalitza samples were observed. Differentiation of positive and negative samples was based on the electrophoretic pattern of the LAMP reactions (Fig. 1A). In order to resolve the pattern of positive LAMP reaction better, lower field strength and longer run might be applied

if required (Fig. 2). The Pearson correlation coefficient was 0.997 at $P < 0.01$ level. Linear determination coefficient (r^2) between the assays was 0.993. The kappa value between the tests was 97.1%, displaying a perfect diagnostic agreement.

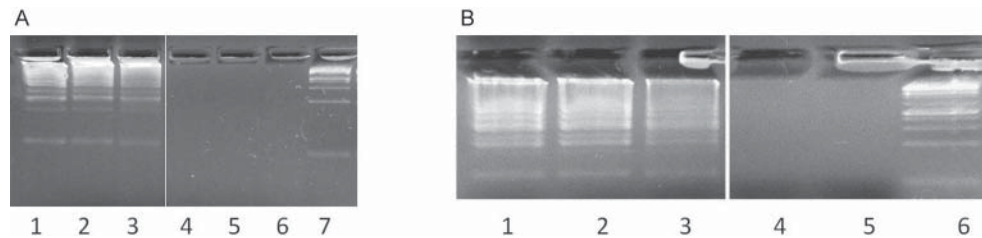


Fig. 1. Electrophoretic patterns of Mangalitzta specific LAMP reactions.

(Section A) Mangalitzta (lanes 1, 2 & 3), Duroc and Wild Boar (lanes 4 & 5), a non-template control (lane 6), Gene Ruler 1 kb Plus DNA ladder, lane 7).

(Section B) Mangalitzta, DNA prepared from muscle (lane 1), Mangalitzta, DNA prepared from sausage (lane 2), homogenised liver paté, no additional DNA preparation (lane 3), non-Mangalitzta, DNA is prepared from sausage (lane 4), non-template control (lane 5), Gene Ruler 1 kb Plus DNA ladder, lane 6).

Field strength is 5 V cm^{-1} , separation time is 15 min.

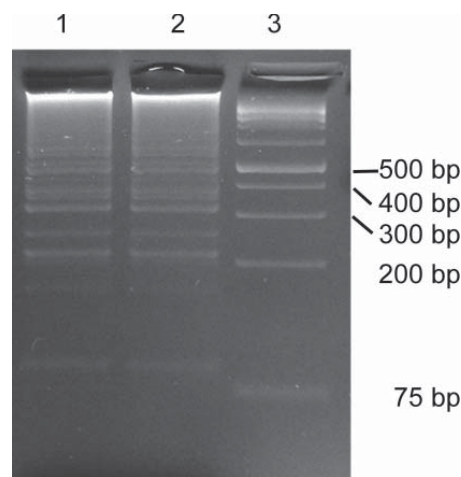


Fig. 2. Elongated electrophoretic separation of LAMP products and GeneRuler 1 kb Plus DNA ladder. Lanes 1 and 2 are fragments of positive LAMP reactions, lane 3 is a DNA molecule ladder. Field strength is 3 V cm^{-1} , separation time is 60 min.

Each technique has its pros and cons.

As for sample preparation, RPA is extremely insensitive to the quality of the DNA (KERSTING et al., 2014), even homogenised, unprocessed sausage sample can serve as a template for RPA reaction (SZÁNTÓ-EGÉSZ et al., 2016). Although LAMP produced successful amplification from raw Mangalitzta liver paté (Fig. 1B), it did not give a LAMP-characteristic pattern of amplification products from grinded, unprocessed Mangalitzta sausage. Sometimes LAMP has given false positive signal (data not shown) from grinded, non-Mangalica sausage.

In order to compare the performance of LAMP and RPA reactions, the dilution series of clean DNA were used described by SZÁNTÓ-EGÉSZ and co-workers (2016). LAMP reaction

was successful at 1.8 but it has failed at 1.44 copy number/ μ l reaction. It did not reach the sensitivity of RPA test; Mangalitza specific RPA reaction was successful at 1 copy number/ μ l.

Each method has special requirements for the primer sets. RPA involves incorporation of modified nucleotides. FAM label must be at the 5' end of the probe, tetrahydrofuran residue is to be in the probe sequence and a protective group at the 3' end. Additionally, biotin label is attached to the 5' nucleotide of a primer (PIEPENBURG et al., 2006). These modifications elevate the price of the primer-probe set. On the contrary, LAMP requires simple unlabelled oligos, which makes LAMP more attractive against RPA approach, especially at the design phase, where multiple primers and/or probes have to be tested to achieve sequence specific amplification. However, all six LAMP primers are unlabelled, special care must be taken to avoid primer-dimers of oligonucleotides, which can be a source of false positive reactions. Such false positive amplification occurred only in case of unpurified, grinded, non-Mangalitza sausages.

The temperature profile of isothermal reactions is not like in PCR (Polymerase Chain Reaction), where high- and low-temperature stages are alternating. LAMP and RPA reactions require one but different temperature levels. RPA reaction could be useful where incubators are not available. Maintaining 37–39 °C anywhere out of the lab is much easier than to maintain 65 °C necessary for LAMP reaction.

Visualisation of a properly labelled RPA product is a fast, 5-minute procedure by a strip used by SZÁNTÓ-EGÉSZ and co-workers (2016). The gel electrophoretic detection of LAMP product is more time consuming, but this phase can be spared when dsDNA binding SybrGreen or pH sensitive dyes are used in the reaction. The naked eye can distinguish between positive and negative LAMP reactions based on colour alteration (TANNER et al., 2015).

3. Conclusions

Both methods are appropriate to determine the presence of Mangalitza DNA, diagnostic results were in agreement between the RPA and LAMP when sample DNA was purified. When DNA is not extracted from the sample matrix, using LAMP directly on grinded sausage is not recommended.

The range of selectable methods has been widened to fight against food adulteration, so the choice for a method can be done depending on the investigator's aim and the available resources.

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