International Food Research Journal 24(4): 1357-1361 (August 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

A rapid and sensitive Loop-mediated isothermal amplification assay for detection of pork DNA based on porcine *tRNA lys* and *ATPase 8 genes*

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Article history

Received: 30 July 2016
Received in revised form: 22 August 2016
Accepted:24 August 2016

Keywords

LAMP
DNA
Food-Forensic
Amplification
Assay
Pork

Abstract

This study describes the development of a rapid and sensitive Loop-mediated isothermal amplification assay for detection of swine DNA in adulterated meat and meat products. The need to protect consumer's right to eat foods of their choices, has made it imperative for researchers to develop efficient means of screening and certification of food products. Six sets of LAMP primers designed based on porcine tRNA lysine gene and ATPase subunit 8 genes were used for the assay. Amplification was carried out under constant temperature (63^0C), using a simple laboratory water bath. Average time spent in amplification and detection of results was 25 min. All results were visually detected and confirmed by electrophoresis. Detection limit of the assay was 0.03 femtogram (fg) much high than the PCR assay, and detection probability of the assay was 100%. Detection of 0.5% of pork spiked with 99.5% of cattle beef is indicative of the sensitivity and robustness of the assay. This could serve as a prototype for development of a sensitive and inexpensive Swine DNA LAMP detection kit.

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Introduction

Screening and verification of meat and meat derivate is essential towards ensuring safety of products made available to consumers (Tanabe *et al.,* 2007a). Researchers in the area of food forensics are motivated to conduct research, to meet the rising demands for authenticity and credibility of food product labeling. Despite all efforts been made by food regulatory authorities towards stemming the challenge of food adulteration, the trend has regrettably continued.

One of the most widely publicize case of meat product fraud, is the beef burger scandal in the united kingdom commonly known as "Horse gate", where porcine and horse DNA were said to have been discovered in the affected products. This led to withdrawal of millions of the adulterated beef burger from various super markets across the United Kingdom (Stevens, 2013). Therefore, this call for a review of the methods currently employ in screening of meat and meat products. Swine tissue is said to be the most used adulterant in the meat industry (Syahariza *et al.,* 2010; Fadzillah *et al.,* 2012). Financial rewards benefited from partial or outright substitution of other animal meat and meat products with that of the swine, which is relatively cheap and readily available is the primary motivating factor behind this fraudulent practice (Al- Jowder *et al.,* 1997; Aida *et al.,* 2005; Grundy *et al.,* 2012).

Some categories of people exposed to consumptions of pork and its derivatives are at a high risk of health challenges, ranging from allergies, zoonotic infectious diseases and metabolic diseases (Rashood *et al.,* 1995; Rashood *et al.,* 1996). Ethical and religious rights of Muslim are seriously compromised by fraudulently exposing them to such food (Ali *et al.,* 2011). Hence, proper labeling of meat and meat products is nonnegotiable. Food regulatory authorities are saddled with the responsibility of ensuring compliance to global food safety standards. At present, the swine specie-specific PCR has been developed and adopted for screening and authentication of meat (Tanabe *et al.,* 2007a; Tanabe *et al.,* 2007b; Erwanto *et al.,* 2011). The PCR been a molecular technique is no doubt an effective forensic technique, capable of detecting few copies of DNA in adulterated sample. A number of gaps are however been identified from its application, these gaps are suggested to be responsible for the lingering cases of swine adulterated meat. High financial demands of setting up a PCR assay amongst other factors, has limit its application to highly resourced settings (Erwanto *et al.,* 2011; Parida *et al.,* 2008).

We therefore, found it imperative to develop

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cheap and cost effective Swine species specific Loop mediated isothermal amplification assay, based on conserved regions of the swine mitochondrial gene. LAMP has been proven to be a much more rapid and sensitive molecular diagnostic technique, when compare to PCR and molecular techniques (Erwanto *et al.,* 2011; Abdullahi *et al.,* 2015). It amplifies few copies of nucleic acid to a detectable level within an hour, under isothermal condition (Dhama *et al.,* 2014; Notomi *et al.,* 2000; Negamine *et al.,* 2002). Unlike the PCR, the LAMP technology can be adopted in low, middle and high income countries due to cheapness of the technique, requiring only simple water bath or heat block for amplification process (Boehme *et al.,* 2007; Tomita *et al.,* 2008).

Materials and Methods

Sample collection and DNA extraction

Total DNA extracted from 48 raw meat samples, these includes; meats from cattle, sheep, goat, chicken, swine, flesh from tilapia and cat fish. The above mentioned meat samples were purchased from various local and supermarkets across 2 states of the Peninsular Malaysia, August 2014. Commercial Animal tissue DNA isolation and purification kit (Qiagen, Dneasy, Dermadst Germany) was employed in isolation and purification of DNA. Protocol prescribed in the kit manual was strictly adhered to. Purity and concentration of the extracted DNA was determined by spectrophotometric analysis, using Biophotometer plus (Eppendorf, Hilden, Germany), data not available.

LAMP primer design

Six LAMP primers were manually designed for this work. Segments of porcine mitochondrial tRNA lys gene and ATPase subunit 8 genes, with accession number: AF034253.1 were the primer target. The gene sequences were sourced from the gene bank nucleotide archive of the national center for biotechnology (NCBI). Details of the primers are highlighted in (Table 1) below.

LAMP reaction mixture and amplification conditions

Optimized porcine LAMP reaction was done with a final volume of 25 µl LAMP reaction mixture, an aliquot of 8 µl LAMP primers was used, 0.2 µM (1 µl) of each of the outer primers (F3 and B3), 1.6 µM (2 µl) of each of the inner primers (FIP and BIP), and 0.8 µM (1 µl) of loop primers each were the oligonucleotide concentrations. Other constituents of the mixture are 1.2 µM in 1 µl of each of the dNTP (New England Biolabs, Beverly USA), 0.5 µM of betain (Sigma), 8 mM of $MgSO_4$, 2.5 µl of 1 x Thermopol reaction buffer (New England Biolabs). Also contained in the reaction are 8 unit of Bst DNA polymerase large fragment (New England Biolabs, Beverly USA), 3 µl of specified amount of DNA and finally the mixture was adjusted to 25 µl with nuclease free water.

Table 1. LAMP Oligonucleotide Primers Based on Swine tRNA Lys and ATPase 8

Primer designation	Sequences (5'- 3')	Oligo base (bp)	Melting temp (Tm)
FOP (F3)	GTCAGCACTAACCTTTTAAG	20	48.6 °C
BOP (B3)	ATCGTAGGGGCAATGAAAGA	20	53.8 °C
FIP	AGAGATCGGGAGCCTAAATCCATGTAGATGTATCTAGTTGTGGC	44	65.4 °C
BIP	GGCAAATAGATTTTCGTTCATCCGAAATCAAAACTCAAAAACA	43	62.7 °C
FLP	ATACCATTGAGGGGA	15	45.0 °C
BLP	ATAGCACCCCTTGAGAAATA	20	50.6 °C

Results and Discussion

A total of 96 LAMP reactions were performed with 24 DNA samples of 8 different animal species. Twelve reactions tested positive to the established assay, while remaining 84 were negative. The positive LAMP results recorded were those carried out with pork DNA, positivity was determine through UV light aided visualization after amplifying for 25 min at 63 ^{0}C (Figure 1a). Bright greenish colour appearance of the positive reaction tubes after addition of SYBR green dye was a reflection of turbidity resulting from presence of magnesium pyrophosphate, a byproduct of a positive LAMP. Following suggestions by Mori *et al.,* (2004); Sen and Ashbolt (2011), we used (SYBR green x 10,000) as DNA binding dye to make turbidity visualization easier.

However, all the negative result recorded in this research were containing non porcine DNA templates, thus, indicating high assay precision. After addition of same quantities of SYBR green dye with the positive reactions, the colour remained unchanged (pinkish orange), thus, implies absence of amplicon. Furthermore, we subjected all result to confirmatory test, by running the LAMP products on 2% (w/v) agarose gel electrophoresis. The electrophoresis in figure 1b upheld the visually detected result (Figure 1a), hence, our newly established swine meat LAMP

assay is absolutely accurate, having achieved 100% positive and negative predictive values.

Analytical sensitivity of swine meat LAMP assay

Quality of a molecular diagnostic test is determined by its sensitivity and specificity, we therefore, performed an analytical sensitivity of the assay using series of serially diluted swine meat DNA samples. Ten-fold serial dilution of the pork DNA was done with sterile distilled water, starting and final concentration was calculated with biophometer plus. Serially diluted DNA (10-1 10-10) with final concentration of (3 ng- 3 ag), was used as template for the LAMP assay analytical sensitivity reactions. Nine out of the ten serially diluted templates were amplified, results were detected visually and confirmed by electrophoresis. Limit of detection of 0.03 fg was obtained, hence, the assay was highly sensitive compared with 1 pg and 10 fg limits of detection from previous studies done with conventional and real time quantitative PCR respectively (Tenabe *et al.,* 2007a; Ali *et al.,* 2011). High analytical sensitivity of this LAMP assay was also in concordance with LAMP assay developed for ostrich meat detection (Abdulmawjood *et al.,* 2014).

Swine meat LAMP assay on spiked meat samples

DNA collected from spiked meat samples containing variable amounts of pork and cattle beef was used in performing LAMP reactions in line with the establish assay, this was done to test the ability of our assay to detect swine adulterated meat. A typical scenario of beef adulterated with pork was mimicked, using 25%, 20%, 10%, 5%, 1%, and 0.5% (w/w) of pork equivalent to 10 mg, 8 mg, 4 mg, 2 mg, 0.4 mg, and 0.2 mg respectively. The result indicated that DNA from 0.5% (0.2 mg) pork spiked with 99.5% (39.8 mg) of beef was isolated and amplified. However, this remarkably result also reflects on the sensitivity and specificity of the LAMP primers. Furthermore, accuracy of the assay is thought to be attributed to the choice of highly conserved swine tRNA Lys and ATPase 8 gene, on the basis of which the primers were designed, this is in accordance to Tartaglia *et al.,* (1998). This study produced similar result as our previous work, where same primers were used to detect porcine DNA in blood samples, hence, reflects high analytical specificity of the LAMP primers.

Conclusion

All results recorded in this study were visually detected under UV light, although, the resultant

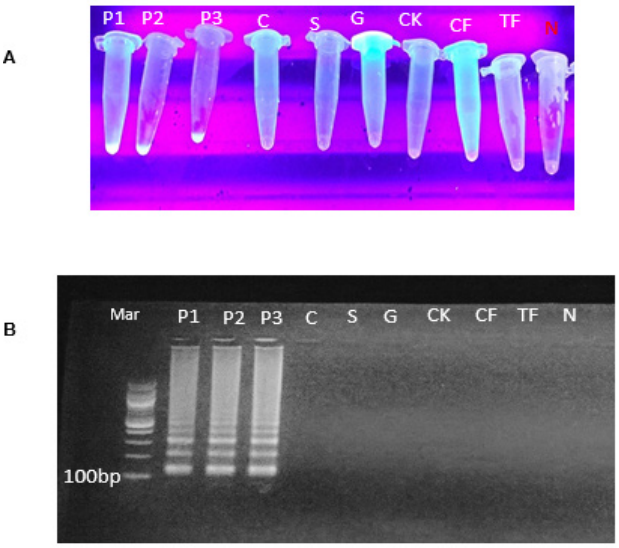
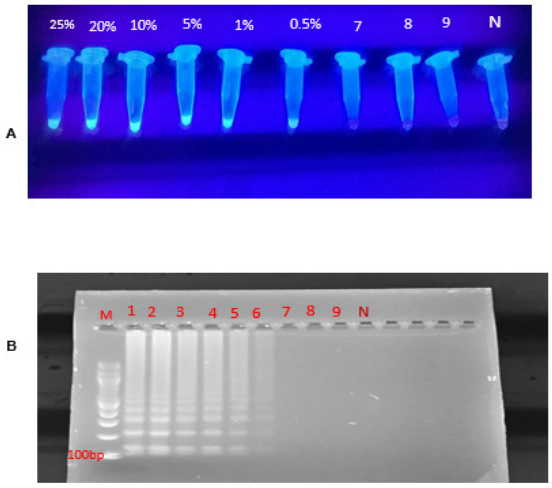


Figure 1. Lane Mar in (1B) represents 100bp DNA marker. Tubes 1-3 labeled P1, P2 and P3 are positive LAMP product with pork DNA, the 3 products corresponds with P1, P2 and P3 lane in the agrose gel electrophoresis image (B), showing typical ladder like LAMP amplicon bands. By contrast, tube C, S, G, CK, CF, TF in (1A), containing cattle, sheep, goat, chicken, cat fish and tilapia flesh DNA, all remained pinkish orange in color when viewed under same UV light wavelength (306 nm). Absence of bands in lanes C, S, G, CK, CF and TF in (B), confirms the negativity of the reactions. The negative control reaction tube N containing no DNA also remained unchanged and presented no band when electrophoresed.



Figures 2. LAMP assay results conducted with DNA obtained from spiked pork and beef samples: Visual detection of LAMP reaction tubes (A), labeled as 25%-0.5% containing DNA from (30 mg- 39.8 mg) of beef spiked with (10 mg-0.2 mg) of pork. The 6 tubes with greenish coloration indicates positive LAMP reaction which corresponds to lanes labeled 1-6 in (B), however, colors of tubes 7-9 as well as N containing only beef DNA and negative control (no DNA) respectively. They correspond with lanes 7-9 and N of (B) which shows no LAMP amplicon bands. The tubes remained pinkish orange in color following addition of 1ml (1/10) SYBR green x 10,000 dye and illuminated under UV light indicating negative reaction.

LAMP reaction products were electrophoresed on 2% agarose gel as a means of confirmation. Hence, the established assay provides a platform for detection of swine meat DNA, via direct visualization of the product, thereby eliminating risk of exposure to potential mutagenic DNA staining dyes, used in electrophoresis of nucleic acid. The use of six primers which includes 2 loop primers may have potentiated the amplification time as well as the sensitivity of the assay. This is in comfort with the study conducted on the accelerative effect of loop primers in LAMP reaction (Negamine et al. 2002; Tomita *et al.,* 2008). Other studies where four regular primers were used for LAMP reaction were shown to take longer amplification time (Ravan *et al.,* 2012). The most crucial step in any successful molecular diagnostic test is the quality of primer design. It is therefore, logical to attribute the success of this work to the specially designed LAMP primers, which passed series of tests conducted in this study. This includes amplification of 0.03 fg of target DNA as well as specifically amplifying only 12 DNA templates representing 100% of the pork DNA, in a total of 96 LAMP reactions.

Our aim in this study is to develop a rapid, cheap and cost effective LAMP assay for detection of swine DNA in meat and meat products adulterated with pork and its derivatives. Identification of such adulterated products requires a highly sensitive and robust food forensic approach. In reality, considering the degree of degradation of meat products resulting from hydrolysis and other rendering activities which occur in meat processing. It became imperative to take into account the possibility of having denatured DNA samples. Hence, our LAMP primers were designed in line with LAMP primer design principles targeting gene fragment of (200- 300) bp (Erwanto *et al.,* 2011; Dhama *et al.,* 2014). Thus, in agreement with Buttler (2005) and Periera *et al.* (2010). The choice of a short nucleotide gene fragment as primer target, as well as the highly stable mitochondrial genes present in numerous amount in eukaryotic cells, allowed for better chance of amplification.

To the best of our knowledge, there is currently no commercial LAMP assay kit developed for this purpose. Hence, our rapid and highly sensitive porcine LAMP assay can serve as headway towards developing a cost effective LAMP kit, for the screening and detection of swine meat in food products. The swift and seamless application of LAMP technique has made it the most user-friendly molecular diagnostic technology. This assay has proven to have several advantages over the PCR, especially in the aspect of rapidity and affordability, requiring no sophisticated and expensive thermocycling device. Thus, it is expected to serve as an alternative to PCR and perhaps, most beneficial to low-resource settings.

References

Abdullahi, U.F., Naim, R., Aliyu, S., Mu'azu, A., Wan-Taib, W.R., Saleh, A. and Baig, A.A. 2015. Loop-Mediated Isothermal amplification (LAMP), an innovation in gene amplification: Bridging the gap in Molecular diagnostics; A Review. Indian. Journal of Science and Technology 8(17): 55-67

Abdulmawjood, A., Nils, G., Svenya, F., Sophie, K., Helga, N. and Guenter, K. 2014. Development of Loop mediated isothermal amplification (LAMP) assay for Rapid and sensitive identification of Ostrich meat. PLoS ONE 9(6): e100717.

Aida, A.A., Che Man, Y.B., Wong, C.M.V.L., Raha, A.R. and Son, R. 2005. Analysis of raw meats and fats of pigs using polymerase chain reaction for halal authentication. Meat Science 69(1): 47-52.

Al- Jowder, O., Kemsley, E.K. and Wilson, R.H. 1997. Mid- Infrared Spectroscopy and authenticity problems in selected Meats, a feasibility study. Journal of Food chemistry 59(2): 195-201.

Ali, M.E., Hashim, U., Mustafa, S. and Che Man, Y.B. 2011. Swine-specific PCR-RFLP Assay Targeting Mitochondrial Cytochrome b Gene for Semiquantitative Detection of Pork in Commercial Meat Products. Food Analytical Methods 5: 613-623.

Boehme, CC., Nabeta, P., Henostroza, G., Raqib, R., Rahim Z., Gerhardt M., Sanga E., Hoelscher, M., Notomi, T., Hase, T. and Perkins, MD. 2007. Operational feasibility of using loop-mediated isothermal amplification for detection of pulmonary tuberculosis in microscopy centers of developing countries. Journal of Clinical Microbiology 45(6): 1936-1940.

Buttler, J M. 2005. Forensic DNA typing. 2nd Edition Elsvier Academic press London. Retrieved on 13th of April 2014. *http://www.cstl.nist.gov/biotech/strbase/fdt2e.htm.*

Dhama, k., Karthik, K., Chakraborty, S., Tiwari, R., Kapoor, S., Kumar, A. and Thomas, P. 2014. Loop-mediated Isothermal Amplification of DNA (LAMP): A New Diagnostic Tool Lights the World of Diagnosis of Animal and Human Pathogens: A Review. Pakistan Journal of Biological Sciences 17(2): 151-166

Erwanto, Y., Abidin, M.Z., Rohman, A. and Sismindari, M. 2011. PCR-RFLP using BseDI enzyme for Pork authentication in Sausage and Nugget Products. Journal of Animal Science and Technology 34(1): 14-18.

Fadzillah, N.A., Che Man, Y.B., Jamaludin, MA. and Abdullrahman, S. 2012. The Contribution of Science and Technology in Determining the Permissibility (Halalness) of Food Product. Revelation and Science 2(1): 1-9.

Grundy, HH., Kelly, SD., Charlton, AJ., Donarski,

JA., Hird, HJ. and Collins, MJ. 2012. Food authenticity and food fraud research, Achievement and emerging issues. Journal of Association of Public analysis 40: 65-68.

Mori, Y., Kitao, M., Tomita, N. and Notomi, T. 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. Journal of Biochemistry and Biophysics Methods 59(2): 145-157.

Nagamine, K., Hase, T. and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular Cells and Probes 16(3): 223-229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28(12): e63.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L. and Morita, K. 2008. Loop mediated isothermal amplification (LAMP): A new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Review in Medical Virology 18(6): 407-421.

Periera, F., Carneiro, J. and Van-Asch, B. 2010. A guide for Mitochondrial DNA analysis in None human foriensic investigations. The journal of open forensic science 3(1): 33-44.

Rashood, K.A., Shaaban, R.R.A., Moety, E.M.A. and Rauf, A. 1995. Triacylglycerols-profiling by high performance liquid chromatography: a tool for detection of pork fat in processed foods. Journal of Liquid Chromatography 18(13): 2661-2673.

Rashood, K.A., Shaaban, R.R.A., Moety, E.M.A. and Rauf, A. 1996. Compositional and thermal characterization of genuine and randomized lard: A comparative study. Journal of the American Oil Chemistry Society 73(3): 303-309.

Ravan, H. and Yazdanparast, R. 2012. Development of a new Loop mediated Isothermal amplification assay for (rfbs) gene to improve the identification of Salmonella sero group D. World Journal of Microbiology Biotechnology 28(5): 2101-2106.

Sen, K. and Ashbolt, NJ. 2011. Environmental microbiology: current technology and water application. Nortfolk UK. Casier Academic press ISBN, 978-1-9044.

Stevens, R. 2013. Burger scandal reveals food adulteration in Britain. Retreved on 6th of May 2014. I*CFI.www.wsws.org/er/article/2013/01/31/meat-j31.htm.*

Syahariza, Z.A., Che Man, Y.B. and Rohman, A. 2010. Discrimination analysis of selected edible fats and oils and those in biscuit formulation using FTIR spectroscopy. Journal of Food Analytical Method 4(8): 404-409.

Tanabe, S., Hase, M., Yano, T., Sato, M., Fujimura, T. and Akiyama, H. 2007a. A Real-time Quantitative PCR detection method for Pork, Chicken, Beef, Mutton and Horseflesh in foods. Japanese journal of Bioscience Biotechnology and Biochemistry 71(12): 3131-3135.

Tanabe, S., Miyauchi, E., Muneshige, A., Mio, K., Sato, C. and Sato, M. 2007b. PCR method of detecting pork in foods for verifying allergen labeling and for identifying hidden pork ingredients in processed foods. Japanese Journal of Bioscience Biotechnology and Biochemistry 71(7): 1663–1667.

Tartaglia, M.E., Saulle, E., Pestalozza S., Morelli, L., Antonucci, G. and Battaglia, P.A. 1998. Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for presence of bovine derived materials. Journal of Food Protection 61(5): 513-518.

Tomita, N., Mori, Y., Kanda, H. and Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nature Protocols Journal 3(5): 877-882.