Food Chemistry 248 (2018) 29–36

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Meat species identification using DNA-luminol interaction and their slow diffusion onto the biochip surface

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ARTICLE INFO

Keywords:
Luminol
Loop-mediated isothermal amplification
Electrochemiluminescence
Porcine DNA
DNA detection
Screen-printed electrode

ABSTRACT

Recently, there has been a growing concern of consumers on the authenticity of food ingredients including adulteration with porcine and/or its derivatives. Therefore, this work reports on the development of a novel, simple, sensitive and rapid luminol-based electrochemiluminescence (ECL) technique for *Sus scrofa* (Porcine) DNA detection. Porcine DNA was firstly amplified using loop-mediated isothermal amplification (LAMP) method and subsequently added to luminol solution for further ECL analysis for quantification. The DNA-luminol complexes were formed causing the diffusion of luminol towards the electrode surface to be slowed down and hindered that resulted in low luminol intensity. The LAMP-ECL sensor is shown to be highly specific, sensitive ($\geq$0.1 pg/μL) and rapid ($\sim$5 min for detection). Hence, by employing this principle with carbon screen-printed electrode (SPE), this technique has a potential to be developed further into a compact biosensor for the verifying food authenticity.

1. Introduction

In recent years, there is a growing concern of consumers on the authenticity of food ingredients including adulteration with porcine and/or its derivatives. Muslims and people who are practicing Judaism are prohibited to consume porcine meat and/or its derivatives since this is “haram” (not permissible) and “non-kosher”. Moreover, porcine is a source of pathogenic worm and its fats are incompatible with the systems in human body and therefore, can cause illness to those who consume it (Awan, 1988; Riaz & Chaudry, 2003). Although not widely reported, porcine serum albumin (PSA) is also acknowledged as a significant allergen found in porcine meats (Kim et al., 2011). Known as pork-cat syndrome, this pork allergy was first reported in 1994 by Drouet et al., that occurred in patients sensitized to cat epithelium, which was observed to trigger an IgE-mediated hypersensitivity upon ingestion of pork meat (Drouet & Sabbah, 1996; Drouet et al., 1994).

With a rapidly expanding global population, the development of low-cost point-of-care (POC) device has becoming a top priority to improve the quality of food and health. To this end, researchers around the globe have been focusing on new innovation to improve detection procedures and tools (Neethirajan et al., 2011). POC device allows measurements to be taken quickly, at the convenience of the users and without the needs of skilled operators, which could even make a life-or-death difference (Gubala, Harris, Ricco, Tan, & Williams, 2012).

Several DNA detection procedures have been developed to replace or complement conventional diagnostics methods. Prior to detection, DNA molecules are firstly amplified upon extraction from a sample in order to increase the sensitivity, reliability and accuracy of results obtained. There are various DNA amplification methods such as ligase chain reaction and strand displacement amplification (Tröger, Niemann, Gärtig, & Kuhlmeier, 2015). The most popular and conventional amplification technique is polymerase chain reaction (PCR), which requires several different temperatures, and takes approximately 2 h for a reaction to complete using a heavy thermal cycler. Furthermore, by employing qPCR (real-time polymerase chain reaction) or combining PCR with electrochemistry together for food analyses meat species can be identified quicker compared to the conventional methods (Munirah, Roy, Ying, Rahman, & Ahmed, 2016; Salihah, Hossain, Lubis, & Ahmed, 2016). In 2000, Notomi and co-workers introduced loop-mediated isothermal amplification (LAMP) that is able to replicate the target DNA sequence up to 10^9 copies and eliminates the lengthy steps of PCR amplification (Notomi et al., 2000). Another advantage of LAMP technique is that it only requires a single temperature with a simple and small heat block eradicating the need of a thermal cycler. In addition, the reaction takes about 45–60 min to complete and therefore less time-consuming compared to PCR technique or other post-amplification conventional processes such as gel electrophoresis (Mueller, Putz, & Hofler, 1997). Denaturation template is also not required for LAMP

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https://doi.org/10.1016/j.foodchem.2017.12.046
Received 10 April 2017; Received in revised form 26 November 2017; Accepted 13 December 2017
Available online 14 December 2017

method (Tröger et al., 2015). High specificity is provided as LAMP is able to distinguish six different regions with the help of four primers with another four distinct regions can be identified using two more primers (Notomi et al., 2000). This method is economically viable as no bulk and expensive instruments are needed to perform reactions and hence, highly suitable for POC detection (Roy, Wei, Ying, Safavieh, & Ahmed, 2016). The amplified DNA targets (amplicons) can then be detected using various methods such as by gel electrophoresis although this method is quite time-consuming as ~1 h is required to detect the amplicons (Le, Nguyen, Truong, & Van De, 2012; Mori, Nagamine, Tomita, & Notomi, 2001).

Other detection techniques have also been reported that manipulate fluorescence and colorimetry (Denschlag, Vogel, & Niessen, 2012; Guan, Guo, Shen, Yang, & Zhang, 2010). Electrochemiluminescence (ECL) is a method that merges chemiluminescence and electrochemistry together and can be used to detect specific DNA. For ECL, a redox active species such as luminol or ruthenium complexes are employed to emit light as a signal resulting from oxidation or reduction processes (Ahmed, Nahar, Safavieh, & Zourob, 2013; Roy, Rahman, Santos, & Ahmed, 2016). ECL is a favourable technique as it provides reproducible data, sensitive (signal-to-noise ratio can be improved) and the luminophore used is not consumed during the reaction (Chu, Yan, & Tu, 2010; Miao, 2008). Additionally, by applying different potential, the production of different active species can be controlled selectively compared when using the chemiluminescence technique alone (Miao, 2008). Three-electrode system is typically used for ECL method where different types of electrode can be used as the working platform such as glassy carbon electrode, coiled platinum wire and also graphite electrode (Chu, Guo, Di, Wu, & Tu, 2009; Cui, Zou, & Lin, 2003; Yildiz, Tasdoven, & Menek, 2014). Screen-printed electrode (SPE) encompasses the three-electrode system being imprinted on a substrate made of plastic or ceramic (Saito, Kitsunai, Ahmed, Sugiyama, & Tamiya, 2008). For this research, SPE was opted due to its portability, low cost, simplicity and rapid detection platform for ECL technique (Ahmed et al., 2016). Additionally, by utilising SPE, only small volume of samples is required (of microliter range) and offers lower detection limit with high sensitivity (Ahmed, Saaem, Wu, & Brown, 2014; Ahmed et al., 2016).

5-Amino-2,3-dihydro-1,4-phthalazinedione, also known as luminol, was chosen as the luminophore dye for this study. Luminol is normally coupled with an oxidising agent, such as H_2O_2, which consists reactive oxygen species (ROS) (Chu et al., 2009; Yildiz et al., 2014). This is due to the ability of ROS to enhance the signal emitted by luminol in basic medium (Chu et al., 2009). Nevertheless, since luminol exhibited high intensity on its own, an oxidising agent was not used in this investigation.

In this work, LAMP amplification technique coupled with ECL using luminol as the redox intermediary for ECL analysis was developed to detect porcine DNA. ECL method that exploited the capability of luminol to emit light, which varies in the presence and absence of target DNA was chosen due to its straight-forward handling process whereas SPE was used as the working platform as it does not require any reagents with high health risks (Ligler & Taitt, 2002). ECL measurements were taken based on the fact that in the absence of DNA molecules, high intensity of luminol luminescence was detected since luminol could diffuse freely and swiftly in close proximity to the electrode surface. However, in the presence of DNA molecules, the luminol molecules were obstructed, causing the diffusion of luminol towards the electrode surface to be slowed down and hindered that resulted in lower luminol intensity (Fig. 1).

2. Materials and methods

2.1. Reagents and material

Luminol solution was prepared from 97% luminol (purchased from Sigma-Aldrich Co. LLC., Singapore) with 1 M of NaOH. The solution was kept in a tube shielded with a silver-mirror film as it was light sensitive. 5 mM Tris-EDTA (TE) was used as buffer for all of the ECL analysis. The buffer was prepared in our laboratory using 1 M Tris-HCl and 0.5 M EDTA. All DNA preparation and LAMP reactions used TE buffer (pH 7.5).

2.2. Instrumentation

SPE selected for the analysis was purchased from BioDevice Technology (Ishikawa, Japan). The working and reference electrodes were made up of carbon and the counter electrode was made of Ag. The three electrodes were imprinted on a plastic substrate with the dimension of 12.5 × 4 × 2 (LWH). MPI-A Capillary Electrophoresis Electrochemiluminescence Analyzer System was purchased from Xi'an Yima Opto-Electrical Technology Co., Ltd. (Xian, Shaanxi, China). The ECL analyser was connected to a computer and experimental parameters were adjusted using MPI-A Analysis System software. Autolab system PGSTAT 101 (Metrohm, the Netherlands) was used in conjunction with Nova 1.10 software for chronocoulometric study.

2.3. Experimental design

2.3.1. DNA extraction

Salmon double-stranded DNA (dsDNA) (Sigma-Aldrich, MO, USA) was prepared and diluted with TE buffer for optimisation studies. Forty-six food samples were originated from different sources and various animal and plant species including porcine meat (*Sus scrofa*) were bought from local markets in Brunei Darussalam. The samples included meat-based processed food such as chicken nuggets and beef loaf, seafood-based processed food (e.g. fish balls), complex food such as seasoning powder with beef extract and gelatine-based food products (e.g. marshmallow). DNA from nine different animal species was also extracted to investigate the specificity of this method. For binary mixture study, DNA was extracted by mixing chicken and porcine raw meat depending on the required percentage (w/w) of porcine DNA in the mixture. The DNA was extracted using a commercial kit, Biokits DNA Extraction Kit (Speciation) (Neogen Corporation, USA).

2.3.2. Preparation of LAMP reaction

A set of six different primers which has been previously optimised was chosen for the porcine DNA amplification (Roy et al., 2016). The sequence of these six primers are: 5′-TCGCCTACGCTATTCTAC-3′ for F3 (Forward Outer Primer); 5′-GGAAGTATAAGATGGAGGCTA-3′ for B3 (Backward Outer Primer); 5′-ATTAGGATTAGGATGGAGGCTA-3′ for LF (Loop Forward Primer); 5′-CTAGTAGCAGACCTCATTACAC-3′ for LB (Loop Backward Primer); 5′-GGATGTGTGTAGTATGGGCATTAACTAG GTGGAGTGTTGG-3′ for FIP (Forward Inner Primer); and 5′-TTCGACC ACTAAGTCAATGCCGGTTGTCCTCCAATTCATG-3′ for BIP (Backward Inner Primer). A total of 25 μL of LAMP reaction mixture was prepared consisting of 10× ThermoPol Reaction buffer (New England Biolabs, MA, USA), 3 mM of MgSO4 (New England Biolabs), 0.4 mM dNTP (New England Biolabs), 0.64 mM Betaine (purchased from Sigma-Aldrich Co.), 0.2 μM of F3 and B3 primers, 0.8 μM LF and LB loop primers and 1.6 μM FIP and BIP primers, 8000 U/mL *Bst* (*Bacillus stearothermophilus*) DNA polymerase large fragment (New England Biolabs) and 5 μL of target DNA. 10 μL of mineral oil was also added in order to prevent evaporation. The LAMP reaction was carried out at 65 °C for different time ranging from 5 to 60 min. The amplicons were then visualised via gel electrophoresis using 2% agarose gel (1st Base, Singapore) and 1X TBE (Tris-Borate EDTA) buffer. FloroSafe DNA stain (1st Base, Singapore) used as the staining agent to visualise the positively-amplified DNA via the UV-light irradiation. The gel was run for 80 min at 80 V and photographed.

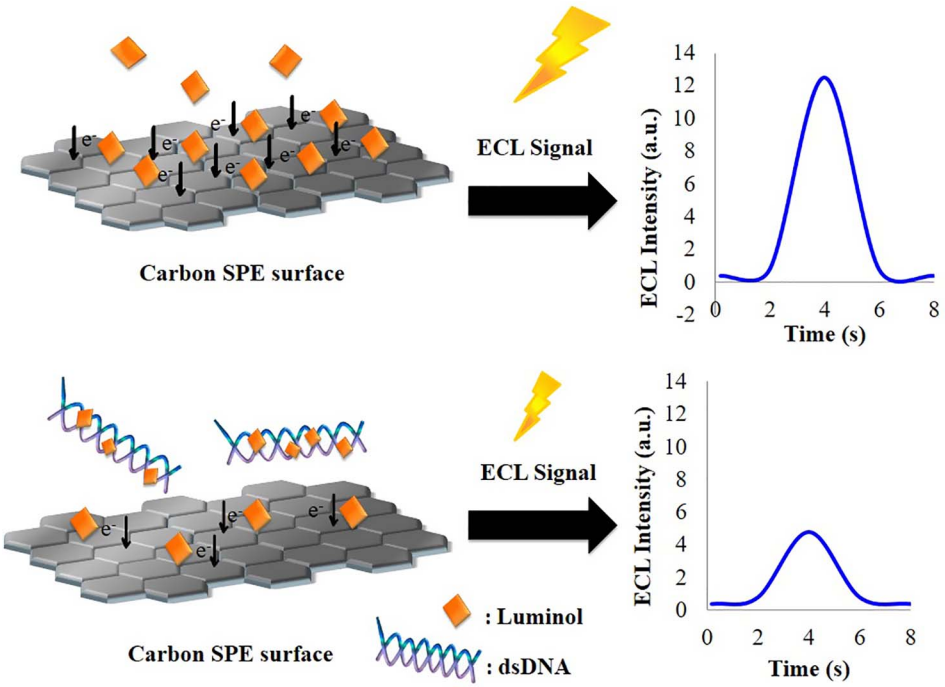

Fig. 1. Schematic diagram of the principle of detection using Luminol-ECL based technique.

2.3.3. ECL measurement of signal

A 2 mL tubular vial was used for ECL analysis. The vial was completely covered with a silver-mirror film except for the bottom of the vial (with the diameter of 0.8 cm) that served as a 'window' allowing the transmission of the light. The vial (ECL cell) was secured at the centre of a photomultiplier tube (PMT). The electrode was immersed in the ECL cell and analysed using cyclic voltammetry (CV) technique was chosen to study the oxidation/reduction, which occurred when a potential is applied. The measurement parameters set-up was 800 V for PMT High Voltage, $1.e^{-005}$ A/V for the sensitivity, amplifying series of 3 and scan rate of 0.13 V/s. The sample solution was analysed at the potential of 0.2 V to 1.25 V. Otherwise stated, all measurements were taken for at least three times for each analysis.

2.3.4. Chronocoulometric study

The same carbon SPE was used for the investigation of chronocoulometry of luminol in the absence and presence of dsDNA. The SPE was connected to a potentiostat by a connector. The potentiostat utilised was the Autolab system PGSTAT 101 (Metrohm, the Netherlands) which was also connected to a computer for viewing the results. The sample (30 μL) was spiked on top of the working electrode of SPE and then, the analysis was run in triplicate. The current used was 10 mA.

3. Results and discussion

Currently, conventional processes for the identification of species are unsophisticated requiring many complicated steps that need individual biochemical confirmation of the species being studied. Furthermore, these conventional techniques are not that sensitive, costly, labour-intensive, time-consuming and not reliable at the end. As a result, efforts being put in towards performing experiments may be in vain. In today's point of care research, time and miniaturization of instruments are important factors to consider when designing and developing a sensitive sensor. Herein, this work designed and developed a process towards robust, faster and simple electrochemiluminescence detection of meat species.

3.1. Optimisation of experimental parameters

In this paper, luminol-ECL based DNA detection with carbon SPE was developed for the first time. To maintain stability of our developed method, numerous optimisation studies have been undertaken. Firstly, optimisation of the luminol-ECL method was performed by studying the trend of the luminol intensity in the presence and absence of salmon dsDNA. Experimental parameters that was also optimised included concentration of luminol and the incubation time with DNA sample to determine the positivity of result. The optimisation of luminol was initially carried out by analysing luminol that was pre-mixed with different kinds of buffer. This is an important factor since polarity of a solvent contributes charge to the ECL intensity of luminol and has a significant role in the fluorescence quenching mechanism (Rothschild, 1971). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), TE buffer, Tris-HCl and phosphate buffered saline (PBS) were the four varieties of studied buffer solution. It was observed that luminol dissolved in TE buffer produced the most stable peak in comparison to other buffers such as PBS (Supplementary Table 1 and Fig. 1). A range of pH from acidic to basic was also investigated to obtain the optimal working buffer and pH for luminol. As depicted in Fig. 2A, TE buffer exhibited a wide working range, ranging from pH 3.5 to pH 10.5 and this further illustrated its high suitability as buffer for luminol. It was observed from the trend that luminol intensity was lower in the presence of dsDNA when compared in the absence of dsDNA as depicted in Fig. 1.

At acidic pH of 3.5–5.5, luminol intensity was indistinguishable in the absence and presence of DNA. This could be due to the low reactivity of luminol in acidic medium and additionally, as its pKa is 6, luminol did not release its protons towards DNA molecules at low pH (acidic) (White, Zafiriou, Kagi, & Hill, 1964). Therefore, no or low interactions between the luminol and DNA molecules occurred and subsequently, causing the difference of the intensity in the absence and presence of DNA to be imperceptible. As the pH increased beyond the pKa of luminol (pH 6.5–pH 9.5), the ECL intensity also gradually increased as luminol was becoming more soluble from neutral to basic medium (Yildiz et al., 2014). However, when pH 10.5 was used, the intensity suddenly increased greatly, implying that luminol was at its most stable state. Khan et al. also demonstrated luminol molecules are

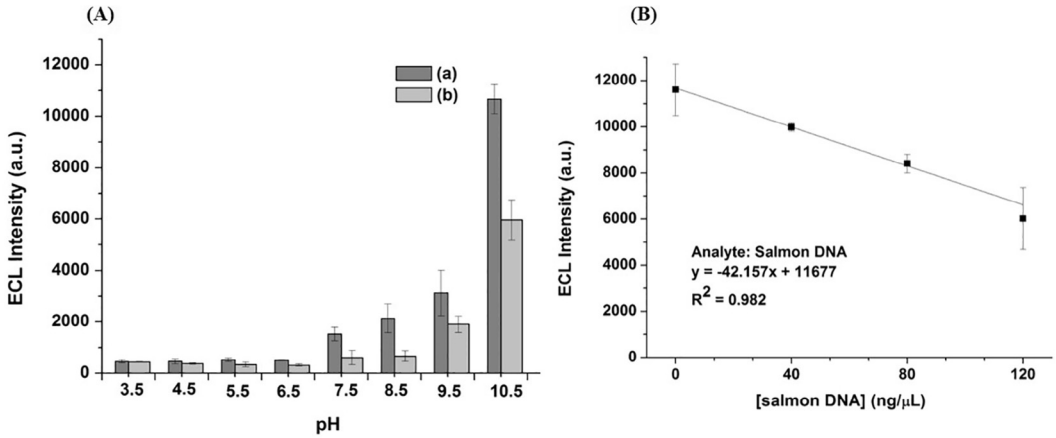

Fig. 2. (A) Working range of TE buffer showing different ECL intensity in (a) absence of salmon DNA and (b) presence of salmon DNA. (B) The curve showing the ECL intensity of luminol at different salmon DNA concentration ranging from 0 to 120 ng/μL. For all of the analyses, 100 μM luminol and TE buffer (pH 10.5) were used. The error bars denote the standard deviation of the three measurements recorded.

more stable at higher pH in their investigation (Khan et al., 2014). Moreover, the difference of the intensity in the absence and presence of DNA was outstandingly discernible. This is due to the donation of protons by the amine groups of luminol molecules towards the phosphate groups of DNA molecules and therefore, leading to the formation of hydrogen bonding (Will et al., 1999). The interactions caused the diffusion of luminol molecules towards electrode's surface to be obstructed and hence, lower intensity was exhibited than in the absence of DNA. At pH 10.5, the error bars did not coincide with each other and able to produce the highest intensity amongst the other pH. Henceforth, pH 10.5 was selected to be the optimum working pH for luminol.

Next, a range of luminol concentration was studied from 100 to 1000 μM. The ECL intensity increased as the concentration of luminol increased. Furthermore, the error bars were observed to be wider as the concentration increased. This might indicate that luminol molecules were becoming unstable at increasing concentration. The intensity of 100 μM luminol showed better error bar compared to other concentrations, which implied luminol molecules were stable at that particular concentration and hence, 100 μM was chosen as the optimal concentration for luminol. In this research, only luminol produced significant signal with DNA interaction, which means that it is not necessary for hydrogen peroxide to be added. In previous studies, all luminol-based detections require hydrogen peroxide to be used. According to Leca and Blum, in the presence of hydrogen peroxide, luminol can be catalysed by peroxidase to emit light (Leca & Blum, 2000). Nevertheless, in this study we observed that without adding any hydrogen peroxide, interaction with targeted DNA amplicons emits successful amount of light emission for analysis. This work has been performed on carbon screen-printed electrodes coupled with LAMP amplicons. We can hypothesise from this incident that the phosphate backbone of DNA interacts strongly with luminol (Chu et al., 2010). In this case since the concentration and the charge of LAMP amplicon are much higher than any other isothermal or polymerase reactions, it vigorously reacted with luminol to obtain significant signals for further analysis.

To obtain maximum signal, the effect of incubation time is crucial. DNA molecule with luminol solution was tested for the ECL intensity together with stability of luminol. The mixture of DNA and luminol solution was incubated for 0, 2.5, 5 and 7.5 min respectively, inside PMT before analysed using the ECL analyser. This was to allow the DNA and luminol molecules to interact homogeneously.

The ECL intensity decreased as the incubation time increased from 0 to 7.5 min (Supplementary Fig. 2). This is because of the excited states of luminol, which emits the light signal, starts to undergo a decaying process, producing lower signal as the incubation time used is longer (Menezes, 2010). All of the error bars were quite similar to each other showing that luminol was stable regardless of the incubation time used. The ideal incubation time was 0 min as it displayed the highest ECL intensity.

Successively, chronocoulometric study (CC) was conducted to further corroborate the expected trend of luminol intensity and analyse any dissimilarity between the charges in the absence and presence of salmon DNA based on the different resulting charge of luminol molecules (Supplementary Fig. 3). Two concentrations of salmon DNA (20 ng/μL and 100 ng/μL) investigated along with blank (only luminol and buffer) solution to compare the effect of different DNA concentration on the overall charge of luminol molecules. As can be observed in Supplementary Fig. 3A and B, solution consisting of only luminol acquired the highest charge of ~250 μC whereas in the presence of salmon DNA, the charge decreased to ~100 μC and ~80 μC for 20 ng/μL and 100 ng/μL DNA, respectively. Hence, it was evident that as the concentration of DNA added increased, the intensity of luminol decreased due to the charge compensation (Ahmed et al., 2013).

The trend of luminol intensity upon addition of increasing salmon DNA concentration was also examined. The analysis was performed with the addition 0 ng/μL, 40 ng/μL, 80 ng/μL and 120 ng/μL salmon DNA into individual sample vial. The resulting intensities were plotted as shown in Fig. 2B. The correlation coefficient (R^2) value was 0.982 as indicated in Fig. 2B and this signified the reliability of data (since R^2 value is more than 0.95). It was apparent that as the concentration of DNA increased from 0 ng/μL to 120 ng/μL, the luminol intensity decreased. This demonstrated that as more amount of DNA molecules present in the luminol solution, luminol molecules were not able to diffuse quickly and close towards the electrode surface. This is due to the hampered movement of luminol molecules as they interact with DNA molecules. Consequently, limiting the luminol intensity detectable by the electrode.

However, when the concentration of DNA reached up to a certain point, the trend discontinued where luminol intensity became unobservable because the solution had reached its saturation point. This is due to the absence of free-diffusing luminol molecules as the number of DNA molecules present in the same solution is equivalent, if not more, than the number of luminol molecules. Inevitably, all of the luminol molecules in the solution interacted with the DNA molecules, disrupting the movement of luminol molecules towards the electrode's surface. Concurrently, the electrode's surface was completely covered by the DNA molecules and thus, could not detect the light emitted by luminol molecules.

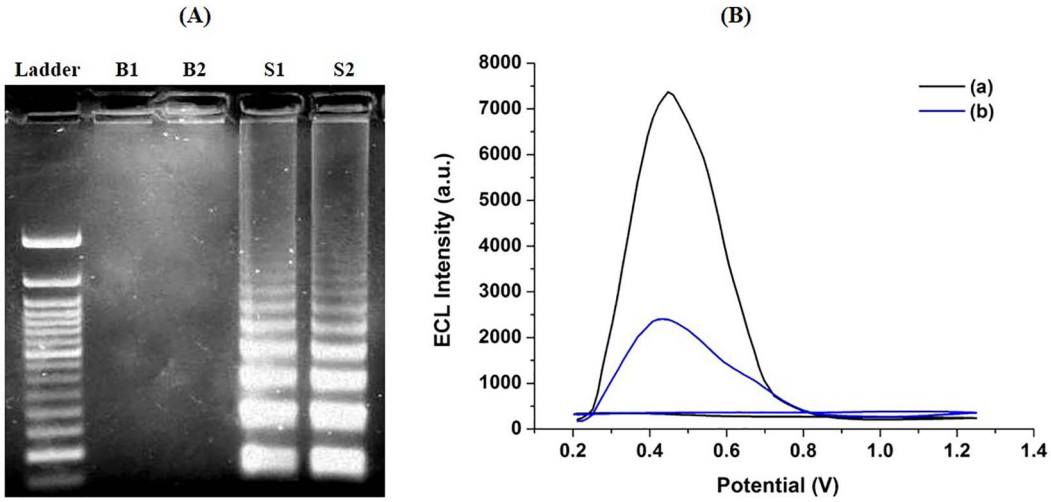

Fig. 3. (A) Gel Electrophoresis result showing the ladder and positive result for the DNA amplification. Ladder is used as a reference. B1 and B2 indicate the LAMP negative acting as the negative control whereas S1 and S2 signify LAMP positive consisting of the amplified target porcine DNA. TE buffer is used for the analysis. (B) Overlay CV graph of potential (V) against ECL intensity of (a) luminol solution in the presence of LAMP negative and (b) luminol solution in the presence of LAMP positive. The analyses were carried out at the potential of 0.2 V–1.25 V. The scan rate used is 0.13 V/s, sensitivity of $1.e^{-005}$ and amplifying series of 3. 800 V was used for the PMT high voltage.

3.2. Analytical performance of electrochemiluminescence

3.2.1. Detection of LAMP amplicons

Porcine DNA, being the target genomic DNA, was initially amplified using LAMP method to improve the sensitivity of the detection (Tlili et al., 2013). Since porcine DNA was not detected directly, amplification was required in order to make the detection specific towards porcine DNA (Ahmed, Hasan, Hossain, Saito, & Tamiya, 2010). Hence, primers that specifically targeted and amplified only porcine DNA were used (Roy et al., 2016). The porcine DNA was extracted using the Biokits DNA Extraction Kit from meat. After the amplification process was completed, the LAMP amplicons were detected using gel electrophoresis for corroboration of the amplification and luminol-ECL technique. 2% agarose gel was used for the gel electrophoresis method. The result from gel electrophoresis detection is as shown in Fig. 3A.

It was confirmed from Fig. 3A that the chosen primers only amplified the target DNA as there are only bands displayed on the S1 and S2 lane where both comprised of 1 ng/μL porcine DNA. Meanwhile, there were no bands displayed on the B1 and B2 lanes as they contained no porcine DNA.

Succeeding the detection with gel electrophoresis to verify the success of the amplification, the amplicons were then detected using the luminol-ECL method. The graphs of the individual samples were superimposed on each other in order to clearly perceive the differences in the luminol intensity between the two different samples.

As exhibited in Fig. 3B, the ECL intensity in the presence of LAMP-negative was significantly higher compared to the solution comprising of LAMP-positive. This is because the LAMP-negative solution (consisted of porcine primer and lamp reagents only) did not obstruct the diffusion of luminol molecules onto the electrode's surface as much as LAMP-positive solution (consisted of amplified porcine DNA, porcine primer and lamp reagents). The ECL intensity diminished in solution containing LAMP-positive since LAMP DNA formed complexes with luminol and this subsequently impeded effective diffusion onto or near the electrode's surface. The difference in the diffusion rate has been reflected on the calculated diffusion coefficient of luminol molecules in the presence of LAMP-negative and LAMP-positive which are 0.006248 cm^2/s and 0.001305 cm^2/s, correspondingly. Importantly, the result from the luminol-ECL method is consistent with the result shown in Fig. 3A.

3.2.2. Specificity and binary mixture study

Following the verification of the effective amplification with the chosen primers, the DNA from nine different species were then amplified using the porcine primers and LAMP method. This is to further validate the specificity of the porcine primers where the primers were added into individual sample tubes containing different species. After 60 min of amplification process, all of the samples were then analysed *via* ECL method.

It can be seen in Fig. 4A that the non-specific genomic DNA was evidently not amplified because the luminol intensity in the presence of the non-target DNA is high. The species involved were sheep, ostrich, turkey, goat, buffalo, duck and horse, respectively, which are not the species targeted by the primers. Simultaneously, the intensities in the presence of the target DNA from the same genus were found to be more than twice lower in contrast to in the presence of non-specific DNA. Both amplicons were wild boar and porcine DNA correspondingly. Both of the species are derived from the same genus of *Sus*, which is the target of the porcine primes used. Thus, the primers efficiently amplified these two species, leading to the low luminol intensity which subsequently, confirmed the specificity of this method.

In order to further justify the specificity and sensitivity of this detection method, binary-mixture DNA consisting of porcine and chicken DNA was extracted from raw meat. Five different binary mixture (w/w) was prepared, ranging from 10% to 0.001% of porcine DNA in total concentration of 10 ng/μL extracted DNA. Each of the samples including the negative control was first amplified at 65 °C for 60 min.

All of the samples consisting porcine DNA were successfully detected as shown from the Fig. 4B signifying that this method is specific towards the porcine DNA. The ECL intensities for the porcine DNA-containing samples were lower than the ECL intensity NC (LAMP negative). Moreover, all of the intensities are almost threefold lower than the intensity of NC. It was also observed that this ECL method was able to detect trace amounts (down to 100 fg/μL) of porcine DNA in the imitation binary mixture of porcine-chicken DNA.

3.2.3. Real-time quantitative detection of LAMP amplicons

One of the objectives of this research is to develop a fast detection method. In order to achieve our objective, the minimum time taken for primers to start amplifying specific target DNA was investigated.

Previous studies have shown that as target species DNA is being amplified with their respective primers, the intensity emitted by the luminol molecules becomes lower as the amplification time progresses

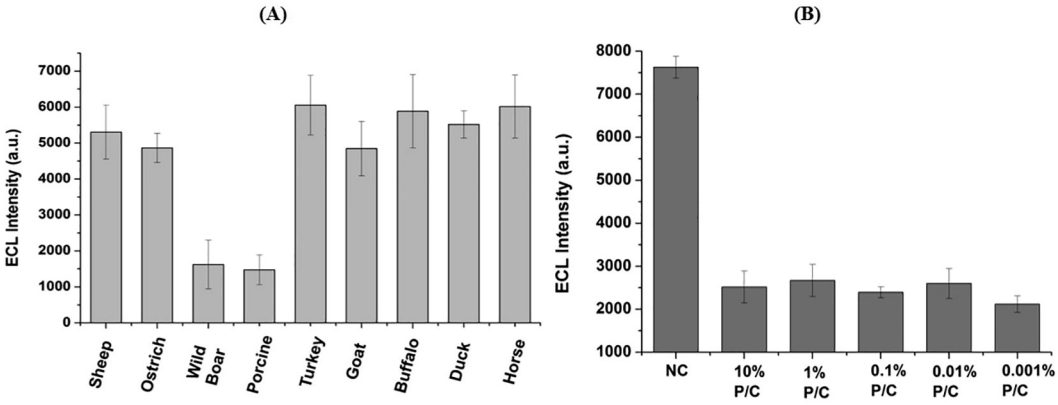

Fig. 4. (A) Specificity study of porcine primers and the species involved are sheep, ostrich, wild boar, porcine, turkey, goat, buffalo, duck and horse. The analyses were performed using CV method. TE buffer (pH 10.5), 100 μM luminol and 1 ng/μL of DNA were used for all of the analysis. (B) Bar graph showing the average ECL intensity for the negative control labelled 'NC' and the binary mixture samples consisting 10%, 1%, 0.1%, 0.01% and 0.001% of porcine DNA in 10 ng/μL of porcine-chicken extracted DNA.

(Ahmed et al., 2013; Safavieh, Ahmed, Ng, & Zourob, 2014). This is because of the increased amount of amplicons formed during LAMP reaction that interacts with the luminol molecules. Therefore, by employing this fact, quantitative detection of porcine DNA was studied at real-time to examine the minimum amplifying time required by the primers. For this evaluation, different concentrations of porcine DNA were selected, ranging from 1 pg/μL to 2000 pg/μL to examine the trend of amplification time. Negative control comprising the LAMP reagents and primers was also amplified acting as the reference to obtain peak height ratio. All samples were amplified for independent period of time varying from 5 to 60 min, with 5-min intervals.

Fig. 5 represents the resulting peak height ratio for all of the studied porcine DNA concentration with varying amplification times. From the early start of the amplification until about 5 min, the peak height ratio rapidly decreased. This could be attributed to complementary primers that were promptly annealed to the target DNA sequence and subsequent production of amplicons quickly. From 5 to 15 min, the amplification rate started to decrease. As the amplifying time transcended 15 min, the peak height ratio of all of the porcine DNA concentrations did not differ considerably until the end of the amplification process of 60 min. This is due to the increasing copies of target DNA exist in the solution and as the amplification reaching to completion, the solution became very saturated and hence, the luminol molecules could not diffuse freely to the electrode's surface (Ahmed et al., 2013).

Two threshold values were then selected from Fig. 5 to further study the rate of amplification at different concentration of porcine DNA and also, to clearly display the approximated minimum time required for the target to be amplified (Ahmed et al., 2016; Roy et al., 2016). Based on the linearity of the results obtained from the real-time detection, the 0.70 and 0.64 were chosen as the threshold ratio value. It was clear that the rates of amplification at the two thresholds are distinct from each other. At the threshold ratio of 0.70, the amplification rate was slower compared to the amplification rate at threshold ratio of 0.64. Porcine DNA with the concentration of 1 pg/μL, 100 pg/μL and 1000 pg/μL crossed over the threshold of 0.70 at 3.7, 2.9 and 2.5 min, respectively. Concurrently, at the threshold ratio of 0.64, the DNA templates of the same concentrations passed over the threshold at 4.8, 3.7 and 3.2 min into the amplification process, correspondingly. It was apparent by interpreting from Supplementary Fig. 4 that the concentration of DNA template used and the time required for the amplification acquired a linear relationship. The higher concentration of the DNA template used for the LAMP reaction, the shorter time required for starting the amplification process as more target DNA templates are present. Thus, the production of the amplicons were faster compared to when lower concentration of DNA template was used (Safavieh et al., 2014). The squares of correlation coefficient of the 0.7 and 0.64 threshold ratios were and 0.91045 and 0.92857 respectively. However, the threshold of 0.7 was preferable for the quantitative analysis as it represented a higher square of correlation value between the two threshold values.

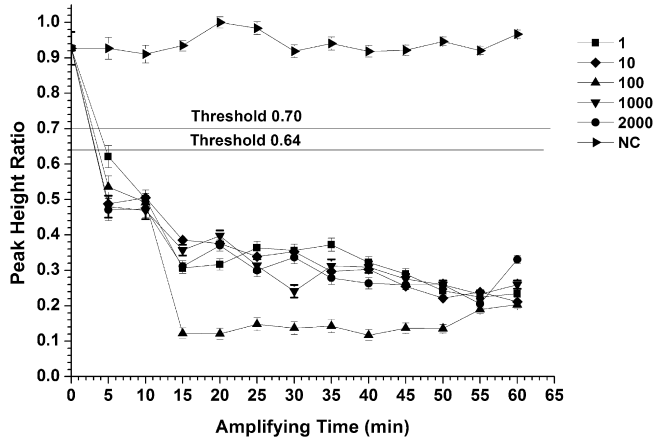

Fig. 5. Quantitative detection of amplified porcine DNA using different concentrations at 65 °C with amplification time ranging from 5 to 60 min.

Table 1 summarises and compares DNA-based detection techniques for porcine DNA as performed by other researchers. As seen from Table 1, our proposed assay can detect amplified porcine DNA within 30–40 s, which is faster than other methods such as gel electrophoresis and lateral flow dipstick detection approaches (Guan et al., 2010; Mori et al., 2001; Najian, Syafirah, Ismail, Mohamed, & Yean, 2016; Tsai et al., 2012). Our ECL-based detection method is also label-free that requires no customisation on the DNA for molecules to be detected. There is no modification made on the electrode surface that further demonstrates the simplicity of this technique in contrast to other DNA-based assays (Najian et al., 2016; Tsai et al., 2012). In addition, as can be observed from Table 1, this current work requires the least amount of amplifying time (5 min) and, ultimately, less detection time of LAMP amplicons than by other methods (Guan et al., 2010; Mori et al., 2001). A comparison of different techniques used to detect LAMP amplicons is also included in Supplementary Table 2. From this table, our LAMP-ECL method has been verified to exhibit high specificity with lowest limit of detection amongst the alternative published DNA-based methods shown in Supplementary Table 2.

3.3. Real food samples analysis

The feasibility of our proposed method for practical application was tested using various food samples acquired from several origins such as Brunei Darussalam, China, Denmark, Indonesia and Malaysia. These

Table 1
Comparison of different techniques used for detecting LAMP amplicons.

Detection techniques	Type of detection	Type of analysis	Show specificity	Minimum amplifying time required for detection	Detection time	References
ECL based detection using luminol	Label-free	Qualitative and quantitative analysis	Yes	5 min	30–40 s	This research
Gel Electrophoresis	Label-based	Qualitative analysis only	Yes	30 min	~1 h	Guan et al. (2010) and Mori et al. (2001)
Lateral Flow Dipstick based detection	Label-based	Qualitative analysis only	Yes	30 min	5–10 min	Najian et al. (2016) and Tsai et al. (2012)
Turbidity based detection	Label-free	Qualitative and quantitative analysis	No	~11–15 min	5–20 min	Mori et al. (2001)
Colorimetric-based detection	Label-free	Qualitative analysis	No	45 min	Immediate Response	Guan et al. (2010) and Safavieh et al. (2014)
Fluorescence Dye- based detection	Label-free	Qualitative analysis	No	35 min	30 min	Denschlag et al. (2012)

were extracted, diluted (if too concentrated), amplified using LAMP technique followed by luminol-ECL analysis. In addition, negative control and positive control were amplified and used as standard references for further analysis. All analyses were done in triplicate and comparison was made with the said negative and positive controls. Supplementary Table 3 demonstrates that samples containing the specific target DNA (porcine) produced average ECL intensity response either lower or within the range as exhibited by the positive control. Results also showed that the samples that do not contain porcine DNA produced ECL output higher than that of positive control and within the range of negative control. All of the food samples tested produced results as expected; porcine-containing food gave positive results for porcine DNA whereas food samples free from porcine showed negative results. These data clearly indicated an excellent practicality for qualitative and quantitative determination of porcine in real food samples.

4. Conclusion

In this work, we have first demonstrated a sensitive, label-free and fast detection technique using luminol-ECL-based method to determine porcine DNA qualitatively and quantitatively. It was shown that by combining the ECL method with LAMP technique for the DNA amplification, sensitivity of ≥ 0.1 pg/μL and 0.001% in pure meat and binary mixture respectively, were accomplished. The reported sensor was demonstrated to be highly specific towards its target as could be observed from specificity tests, rapid (detection can be made within 5 min from the start of amplification) and practical for food analysis. Our cross-reaction tests showed that the primers used for LAMP amplification were very specific to the targeted DNA samples. Furthermore, this technique only required small amount of sample (within microliter range) and the carbon SPE was utilised without any modification on the electrode's surface. In this research, neither a co-reactant nor a catalyst has been used to generate oxidation making this process simpler and straightforward. The overall approach of this paper was based on the interaction of amplified DNA sample with luminol in solution and the emitted light was delicately detected by PMT whilst the LAMP amplicons helps to produce more reliable outcome. It was also proven that this technique has successfully detected porcine in various types of food samples. Albeit the excellent sensitivity and specificity, necessary development still need to be done to further enhance the overall performance. Correspondingly, this method has an excellent potential to be developed further as a compact biosensor for on-site food authenticity verification.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

We would like to acknowledge the financial support for the project given by the Brunei Research Council [Grant# BRC-10] of Negara Brunei Darussalam. Sharmili Roy and Syazana Abdullah Lim wishes to thank the UBD's Graduate Research Scholarship (GRS) for their Ph.D. fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.12.046.

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