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RECOMBINASE AMPLIFICATION METHOD APPLIED TO MEAT AND HALAL AUTHENTICATION: TRENDS AND POTENTIAL IMPLICATIONS

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ABSTRACT

The rapid evolution of technology and the food industry have raised concerns about the accurate labelling of Halal food and meat authentication. The Halal authentication system maintains a zero-tolerance policy regarding the halal status of food products from farm to fork. As a result, innovative technologies such as recombinase polymerase amplification are necessary to address the growing issues of food fraud and enhance the current species identification method. Recombinase amplification method is an isothermal amplification process that operates at 37-42°C without the need for specialized equipment or technicians. A systematic review was conducted to analyse research on the application of Recombinase Amplification method in meat and Halal authentication, covering literature published from 2018 to 2024. Relevant studies were identified through a comprehensive search of established databases in which 25 studies met the inclusion criteria, focusing specifically on meat and Halal authentication, while studies related to clinical diagnostics and microbiological testing were excluded. This paper aims to analyse the various detection technologies integrated with Recombinase Polymerase Amplification (RPA) and to provide a comprehensive review of the application of RPA in meat and Halal authentication. The review will summarise current trends and evaluate the potential future developments and implications of RPA-based methods for establishing a reliable and rapid on-site auditing system.

Keywords: *RPA, Halal, Meat Adulteration, Food Authentication, Recombinase-Aided*

1. INTRODUCTION

Maintaining and verifying the Halal status of food products presents

several challenges, including issues of food adulteration, non-Halal contamination, and the high demand for rapid verification tools to authenticate food innovations while upholding Halal certification standards. Meat is a frequent target of food fraud due to its high market value. Fraudulent practices include substituting different meat species, mislabelling Halal status and origin, and misrepresenting meat processing details (Ballin, 2010). According to the Food Fraud Database (2017), meat products ranked second in product fraud, with 264 reported cases in 2017 and 1,682 in 2021. Research conducted in Chinese markets by Li et al. (2023) revealed that the second highest category of food fraud involved "illegally imported meat," comprising 813 cases (40.9%). The most common issues were adulteration with other animal ingredients, use of meat from unknown sources, and inclusion of low-quality meat. It is important to note that these figures only account for reported incidents, excluding unreported food frauds. This issue highlights the urgent need for reliable detection methods like the Recombinase Amplification Method to ensure food authenticity and safety (Li et al, 2023).

In addition, with the rapid development of the food industry, there is an urgent need for a fast and reliable verification tool to address the limitations of current meat and Halal authentication methods, whilst maintaining the specificity, sensitivity and robustness of the authentication methods. While Polymerase Chain Reaction (PCR) and Enzyme-Linked Immunosorbent Assay (ELISA) are widely used in diagnostic laboratories due to their high specificity and sensitivity, they have significant drawbacks such as longer analysis times, the need for well-trained technicians, and unsuitability for on-site authentication. To meet the growing demands of the food industry, these limitations must be overcome. Consequently, there is increasing interest in recombinase amplification methods. Recombinase Amplification is an isothermal process that operates without multiple thermal cycles, eliminating the need for elaborate equipment and specialized technicians (Mao et al., 2022). Recombinase Polymerase Amplification (RPA), in particular, has been extensively studied for clinical diagnostics, including the detection of bacterial, fungal, and viral diseases (Euler et al., 2015; Li et al., 2019; Wang et al., 2022). Additionally, RPA is being explored for use in diagnosing plant pathogens (Zhao et al., 2025), demonstrating its versatility and potential for broader applications such as veterinary diagnostics, meat authentication, and Halal verification (Yusop et al., 2022; Sedykh et al., 2022; Zhang et al., 2022). The application of a rapid, reliable and sensitive RPA technology could ease on-site auditing in which supposedly ease producers, manufacturers or food preparers to follow the Halal certification.

Currently, there are several research experiments conducted for the application of authentication of meat or Halal status of meat and meat mixture. Henceforth,

the purpose of this review articles is to overview the current literature bodies and identify any potential implications. The objectives of this review article are to examine various detection assays integrated with Recombinase Polymerase Amplification (RPA) and provide a comprehensive overview of application of RPA in authenticating different animal or meat species particularly in food products. This article will also highlight current development and future implications of RPA-based methods as reliable and rapid on-site verification system.

Table 1. Summary of the Recombinase Amplification research experiments in Review

Target Species	*Sample source & Applicability*	*LOD*	*Relative LOD (Binary mixture)*	*References*
RPA- SYBR® Green I dye				
Duck, Chicken, Cow, Sheep, Porcine	Boiled, Microwave, High pressured & fried	-	1%	Cao et al, 2018
RPA-Lateral Flow				
Mutton	Shish (Skewer) and Kebab products	200 fg/ 50 μL reaction	-	Li et al, 2019
Duck, Beef	Raw meat samples & commercially processed beef	0.1ng	5%	Fu et al, 2020
Formosan Reeves' Muntjac	Boiled, Pan-fried, Roasted, Stir-fried, Stewed	-	-	Hsu et al, 2021
Cattle, Buffalo, Pig	Cytb, Cytb, D-loop	10 pg	1%	Kumar et al, 2021
Sheep, Beef, Porcine, Chicken, Duck	Boiled, Microwave, Pressure-cooked & Fried	10^1-10^2/μL	1%	Lin et al, 2021
Octopus	Frozen, Cooked, Canned	50×10^{-2} ng/ μL	-	Velasco et al, 2021

Chicken, Pig	Mixed meat samples and processed meat.	0.2pg/ μL	**0.001%**	Ivanov et al, 2021
Horse	Commercial donkey meat	-	0.01%	Chen et al, 2022a
Duck	Meat products from local market	23 copies/ μL	1%	Chen et al, 2022b
Chicken	Commercial beef samples	-	0.01% w/w	Liu et al, 2022
Porcine	Meatball & cooked products	0.01ng/μL	-	Mohd Yusop, M.H, et al, 2022
Duck	Various animal-derived products	10pg/ μL	0.1% w/w	Zhou et al, 2023a
Horse, Donkey, Porcine	Raw meat, Marinated minced meat	10 pg/ μL	0.1% w/w	Zhou et al, 2023b
Chicken, Duck	Commercial meat products	-	0.01%	Liu et al, 2023
Duck, Porcine	Raw meat	101 copies/ 50μL	-	Cao et al, 2023
Porcine, Chicken	Raw meat, Meat mixtures, Commercial products	-	0.1% (w/w)	Feng et al. 2024
Porcine. Horse	Donkey products	-	0.01%	Liu et al, 2024
RPA-CRISPR/Cas12a				
Porcine	Raw meat, Boiled, High-pressure	3 -10 ng (3000 pg/μL)	0.1% & **0.001% w/w**	Zhao et al, 2022
Porcine	Processed meat products	6.4 pg/μL	5%	Wang et al, 2023
Porcine	Commercial food products	5 pg/μL	-	Janudin, A.A.S et al, 2023
Beef, Mutton, Porcine, Chicken, Duck	Meat mixtures of low priced meat (pork,chicken,duck) in high price meat (beef, mutton)	10pg/μL	1%	Ding et al, 2023

2. METHODOLOGY

This review synthesises research experiments on the application of Recombinase Amplification method in meat and halal authentication from literature published since 2018 until 2024. Relevant studies were retrieved from established databases, including ScienceDirect, PubMed, Multidisciplinary Digital Publishing Institute (MDPI) and American Chemical Society Publications (ACS Publications). A systematic search of these databases identified 25 potentially relevant research papers. The inclusion criteria focused specifically on studies of recombinase amplification method in the application of meat and halal authentication, while studies employing recombinase amplification for clinical diagnosis and microbiological testing were excluded as ample review studies have been done comprehensively.

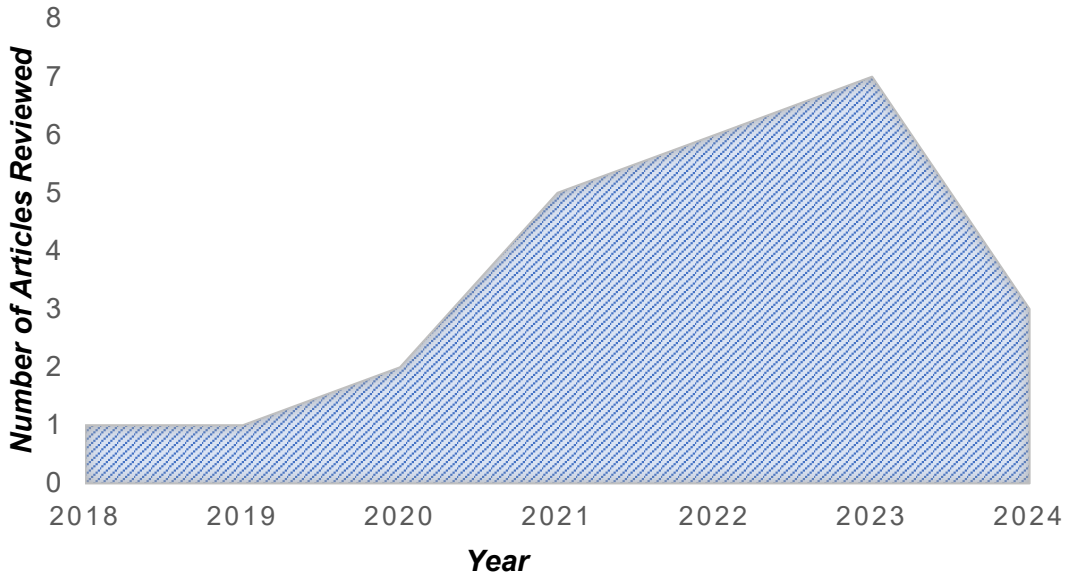

Figure 1. Frequency Of Articles of Recombinase Amplification Method For Meat and Halal Authentication Reviewed by Year

3. PRINCIPLES OF RECOMBINASE AMPLIFICATION METHOD

3.1 *Recombinase Polymerase Amplification*

Recombinase Polymerase Amplification (RPA) is an isothermal nucleic acid amplification technique designed to address the limitations of conventional Polymerase Chain Reaction (PCR). Unlike PCR, which relies on thermal cycling and necessitates specialized equipment, extended analysis times, laboratory environment and highly trained personnel, RPA operates at a constant temperature which makes it more accessible and efficient for on-site applications (Lobato & O'Sullivan, 2018). The Recombinase Polymerase

Amplification (RPA) system comprises three key enzymes: recombinase, single-stranded DNA binding protein (SSB), and strand-displacement DNA polymerase. In this system, recombinase enzymes first bind to the primers and facilitate the search for homologous sequences within the double-stranded DNA template. Upon locating the homologous sequence, the primers are integrated into the template, initiating strand displacement. The single-stranded DNA binding protein (SSB) then binds to the displaced DNA strand, thereby stabilizing the primer-template complex. Following this stabilization, the recombinase dissociates, exposing the 3' end of the primer. This exposed primer end is subsequently recognized and extended by the strand-displacement DNA polymerase, enabling the amplification process (Feng et al, 2023 & Lobato & O'Sullivan, 2018).

Compared to conventional PCR, real-time PCR and other isothermal amplification, RPA has the advantages of amplification operating in 37- 42°C, able to amplify as low as 1-10 DNA target copies and in duration time of less than 20 minutes. In addition, RPA requires only two primers (forward and reverse), does not require initial heating, allows multiplexing and therefore could be conducted on-field as incubation temperature could be achieved by using body heat, portable incubators, heat blocks and chemical heaters. Although the optimum temperature of RPA is between 37° and 42°C, amplification can still occur between 22°C and 45°C. RPA technology offers the advantage of enabling the development of multiplex assays and demonstrates higher amplification efficiency, and without instruments, the results are equivalent to conventional PCR (Fu et al, 2020). Rapid detection methods are particularly essential for samples with high decay rates, which permit only a limited window for testing. Additionally, RPA surpasses other isothermal amplification methods due to its simpler primer design process, compatibility with various nucleic acid templates, and cost-effectiveness, given the relatively low expense of the enzymes involved. An initial finding by Hsu et al (2021) demonstrated that RPA could successfully amplify DNA with purified DNA extracts such as Qiagen DNeasy which produce higher DNA and pure DNA yield, and crude DNA extracts such as UniversAll™ Tissue Extraction Buffer which only require a single-step method and could be incubated in room temperature.

3.2 *Recombinase Aided Amplification & Enzymatic Recombinase Amplification*

There have been several studies which use recombinase-aided amplification (RAA) and enzymatic-recombinase amplification (ERA) instead of recombinase polymerase amplification. Both RAA and ERA have the same basic principles of RPA, the differences are in the sources of enzymes obtained.

Recombinase enzymes used in RAA are obtained from bacteria or fungi, and exploits four enzymes: UvsX, UvsY, SSB and polymerase. According to Zheng et al (2017) in Xiong et al (2020), in RAA the UvsX enzyme, in conjunction with the UvsY accessory protein, facilitates the binding of oligonucleotide primers to form a complex which actively searches for homologous sequences within double-stranded DNA. Upon locating these homologous regions, UvsX mediates strand displacement, resulting in the displacement of single-stranded DNA, and following that, the processes are similar to RPA (Xiong et al, 2020). Apart from that, RAA is specifically employed as amplification tools for diagnosis of infectious disease (Xiong et al, 2020 & Wang et al, 2021), parasites (Li et al, 2022) and food contaminations in detecting various types of bacteria and fungi, and genetically modified crops (Wang et al, 2021). For meat authentication, Cao and Song (2023) developed dual-RAA assay for species identification of duck, bovine, and porcine with sensitivity of 10^1 copies/50 μL reaction system (0.001%) within 20 minutes.

Recent studies by Zhou et al. (2023) and Wang et al. (2023) have highlighted the effectiveness of ERA when combined with advanced detection methods such as real-time ERA, lateral flow assays and CRISPR/Cas12a-based systems. While both methods share similarities in their underlying principles and use of recombinase enzymes, they are distinct techniques. The primary difference lies in the specific recombinase enzymes used in the amplification process. Munawwar (2022) clarified that the enzymes utilized in ERA and RAA are sourced from various biological origins, including bacteria, viruses, and bacteriophages, and are often modified for optimal performance. In contrast, the enzymes used in Recombinase Polymerase Amplification (RPA) are exclusively derived from T4 bacteriophages. This distinction in enzyme sources and modifications leads to variations in the amplification mechanisms and efficiencies of these methods.

The two relevant studies which use the ERA/RAA in combination with real-time quantification as the authentication technology tools which targeted horse, donkey and pig as target species have reported that real-time ERA and lateral flow ERA have good specificity with no cross-reactions with sensitivity of 10pg DNA/reaction and 0.1% w/w for raw and marinated meat burgers. As for research study by Wang et al (2023), the assay named CAMERA has high specificity for porcine detection with high sensitivity between 20- 0.032 ng/μL in which the assay uses both fluorescent signal, I,e, using BioRad Real-Time PCR detection system with similar operating temperature as ERA of 37°C and also visual detection using LED blue light illuminator in which uses smartphone to capture the imaging, make it suitable for a rapid on-site auditing. However, there are problems in detection of the green fluorescent signal using the LED blue light illuminator as the signal was too low to be

recognised by smartphone when the concentration of DNA was at 0.032 ng/μL (Wang et al., 2023).

3.3 *Applications in Meat and Halal Authentication*

Most meat authentication uses DNA and protein-based molecular assays for species identification, as it does not only accurately analysed it, but also capable of providing detailed information about the origin and processing history of the meat products such as the geographic source of the meat and any treatments or modifications that has undergone, ensuring both authenticity and quality control in the meat supply chain. This is particularly important as to detect meat and non-Halal adulteration which most often substitute a higher-quality meat with lower-quality meat or non-meat ingredients. Zhou et al (2023a) and Fu et al (2020) highlighted that 0.1% adulteration or below 100pg/μL reaction will not bring economic benefit in meat adulteration products.

Thus far, a number of studies have explored the recombinase polymerase amplification technology for the purpose of meat and Halal authentication due to its reliability from other detection system such as short detection or amplification time, simple operational procedure, low cost, possible visual detection when combined with colorimetric analysis, multiple detection, high detection efficiency and high sensitivity, and low sample size. A rapid authentication system is necessary, as prolonged analysis can result in economic losses for restaurants if the seized meat products are ultimately deemed legal (Hsu et al, 2021). This situation necessitates the development of a fast, simple, and cost-effective identification technique for meat and Halal authentication officers to accurately identify meat sources on-site. However, until this point, there has not been any reported application of recombinase polymerase amplification in Halal Certification bodies either for laboratory scientific analysis or on-site auditing, which has been presumed that this technology is in the exploration stage.

In the development of assays for meat and Halal authentication, a key requirement is the design of species-specific primers that do not cross-react with commonly adulterated species. To achieve this specificity, many studies focus on targeting the *cytochrome b* gene, as it exhibits greater variability compared to other genetic markers (Hsu et al. 2021). In regard to design primers and probes, the amplification products need to be shorter in size as the purpose of amplifying a short DNA fragment was to maximize performance and ensure successful amplification, particularly in highly processed tissues (e.g., canned products) where DNA may be degraded or fragmented (Velasco et al, 2021).

4. TARGET SPECIES STUDIED IN RESEARCH AMPLIFICATION METHOD

4.1 *Porcine (Sus scrofa)*

Contamination and adulteration with porcine substances are significant concerns within the Halal industry, primarily due to the strict prohibition against the consumption of pork and pork-derived products, where even the slightest presence is unacceptable. Henceforth, the development of RPA-based assays for the detection of porcine DNA is a rapidly advancing field, particularly for meat and halal authentication. As illustrated in Table 1, the study by Velasco et al. (2021) demonstrated high sensitivity, with a detection limit of 0.2 pg/μL and the ability to detect porcine DNA at a concentration of 0.001% in a binary mixture. While the applicability of these assays in commercial meat products was not assessed in this study, subsequent research by Liu et al. (2022) has confirmed the effectiveness of RPA-LFA for detecting porcine DNA in commercial products, with a relative limit of detection of 0.01%. Various porcine target genes were tested, and those located within mitochondrial DNA, such as cytochrome b, ND2, and CO1, exhibited strong specificity without cross-reactivity with DNA from other species. The development of CRISPR/Cas12a-based assays has also shown significant promise, as demonstrated by Zhao et al. (2022), with further validation provided by subsequent studies by Wang et al. (2023) and Janudin et al. (2023). These studies explored the efficacy of CRISPR/Cas12a in detecting porcine DNA in commercial and processed meat products, highlighting its potential as a reliable tool for meat authentication.

4.2 *Poultry (Duck-Anas platyrhynchos, Chicken-Gallus gallus)*

Although chicken and duck are generally considered halal, concerns have emerged regarding their slaughtering methods, particularly the use of stunning, which raises questions about permissibility under Islamic law in countries such as Australia, New Zealand, and Malaysia. Additionally, products labelled as vegetarian or vegan may undergo testing to ensure they are free from animal-derived ingredients. In meat authentication studies, chicken and duck have been used to assess whether products are genuinely composed of duck meat or have been adulterated, altered, or substituted with cheaper alternatives like chicken. Duck meat is often adulterated with beef in Chinese barbecue stalls and hot pot restaurants due to their similar texture and colour and the addition of additives like butter and beef essence could be used to mask the odor and enhance flavour (Zhou et al, 2023a). Hence, there have been several research experiments which examined the presence of DNA of poultry species, i.e. chicken and duck (Table 1). Recent advancements in RPA

research have concentrated on developing rapid, on-site species identification assays for chicken by refining extraction methods, shortening analysis times, and reducing overall assay costs. Notably, Feng et al. (2024) have introduced a novel assay capable of being completed in 18.5 minutes, addressing previous research gaps related to on-field extraction methods for commercial meat products. This assay maintains high specificity and sensitivity (0.1%) while offering a quantitative analysis solution through RPA-LFA by integrating imaging software such as ImageJ.

4.3 *Bovine (Cow-Bos Taurus, Buffalo- Bubalus bubalis)*

Although bovine meat is not considered particularly expensive, its high demand among the general population serves as a significant driver for adulteration by food producers and preparers. Additionally, economically motivated adulteration often stems from the intent to substitute high-value meats with undeclared low-cost alternatives, such as lower-quality meats or vegetable proteins, through unethical practices (Lin et al, 2021). Fu et al (2020) have stated that beef products are frequently reported as being among the most adulterated foods, often substituted with lower-cost meats such as pork, chicken, or duck. Several horse meats scandals have also heightened the interest of relevant stakeholders in enhancing meat control measures and food fraud detection. Notable cases include Malaysia's 'meat cartel' scandal and the 2013 horse meat scandal, where horse DNA was detected in over one-third of beef burger samples, and porcine DNA was identified in 85% of the products (Guardian News and Media, 2013). The Malaysian meat cartel scandal involved meats with falsified documentation being sold for over 40 years, originating from non-Halal sources such as kangaroo, horse, and pork, smuggled from countries like Argentina, Brazil, China, and Ukraine. These meats were then repackaged and falsely labelled with Halal certification (Adam, A., 2021).

4.4 *Ovine and Caprine (Sheep – Ovis aries, Goat – Capra hircus)*

According to Li et al (2019), in China as mutton shish and kebabs are one of the most frequently eaten food which are frequently adulterated with cheaper poultry or porcine meat, hence research experiments by Li et al (2019), Lin et al (2021), Ding et al (2023) conducted were to authenticate the lamb or mutton meat products. However, investigations done by Zhou et al (2024) revealed that milk is also one of the most adulterated food products as reported by 2013 report from the European Parliament, henceforth claimed high value milk such as camel milk, goat, and donkey milk were often adulterated, whether substituted or added with low-value milk such as cow's milk (Bittante et al, 2022 in Zhou et al, 2024). The researchers have developed RPA assay with real-time and lateral flow method within 30 minutes and experimented with

crude extraction method in which RPA could still successfully amplify despite the presence of potential amplification inhibitors. In a research study by Li et al (2019), the authors have demonstrated the high reliability and sensitivity of RPA with lateral flow over RPA-Gel electrophoresis as RPA-LFA showed a positive clear line at 200fg/reaction while RPA-gel electrophoresis showed a clear line at 2 pg/reaction.

4.5 *Equine (Horse-Equus caballus, Donkey- Equus asinus)*

Meat adulteration of horse meat in beef-based products had become a concern due to ethical, social, health, and religious issues, as the origin of horse may be from retired racehorses injected with veterinary drugs which could be harmful for human consumption. In addition, although horse is considered a halal meat for muslims, however undeclared horses most likely not slaughtered according to the Syariah principles. Meat authentication tests were conducted involving donkey meat, which has gained popularity among Chinese consumers due to its high nutritional value and tender taste. However, the scarcity of donkey meat has led to its increased market value, making it a target for adulteration with porcine and horse meat. The study demonstrated significant occurrences of such adulteration, with detection rates reaching 53.33% for porcine and horse meat used as adulterants (Zhou et al, 2023).

Numerous experiments have been conducted to investigate meat adulteration, including a study by Kissenkötter et al. (2020) that focused on recombinase polymerase amplification (RPA) with real-time detection. This research demonstrated that the limit of detection (LOD) for horse meat, at 5.4 pg, was lower than that for porcine meat at 5 pg, with sensitivity extending to as low as 0.1 pg, and 0.1% in meat mixture (Kissenkötter et al, 2020). Although studies by Chen et al (2022) have demonstrated a higher sensitivity of 0.01% in meat mixture of 0.01%. The study also successfully designed primers with no cross-reactivity to donkey DNA, despite the close genetic relationship between horse and donkey, with the minimal mismatches between the primers and donkey's ATP 6-8 gene sequence being eight. Furthermore, the research confirmed that myoglobin, haemoglobin, heparin, and ethanol did not adversely affect RPA performance, thereby eliminating the need for purification steps in the assay.

Previous investigation of Zhou et al (2023) have demonstrated the applicability of recombinase polymerase amplification in marinated donkey meat burgers using real-time ERA and lateral flow ERA, high specificity of donkey-meat with no cross-reactivity as donkey, horse and pig usually have high homology with each other. Zhou et al (2023) have identified the donkey-specific and horse-specific target genes ATPase 6 and ND2 which exhibit significant intraspecies conservation and substantial interspecies variability, however the target genes

could not overcome the issues of crossbreeding between horse and donkey, i.e. Mules/hinnies which may also be used as adulterants. Research by Liu et al (2024) have begun the use of Surface-Enhanced Raman Scattering (SERS) to RPA which enhances the sensitivity of detection of amplified DNA on lateral flow, and also integrate magenta spherical and cyan urchin for two-colour lateral flow assay allowing multiple species detection (Liu et al,2024). The research has shown no cross-reactivity and has high sensitivity of 0.01% (wt%) with cytochrome b as the target genes, although the overall duration took 26 minutes to conduct and requires a Raman spectroscopy and later flow strips reader to quantify the DNA samples.

4.6 *Other Species*

In other cases, meat authentication was conducted to ensure that there are no illegal hunting of wildlife and no consumption of bushmeat as both activities are recognized as significant threats to biodiversity and potential sources of zoonotic disease transmission. As in the case of research study by Hsu et al (2021) which uses RPA-lateral flow to identify Formosan Reeves' muntjac species, a deer species which were hunt for cultural or ritual purposes by Taiwan indigenous people in raw and cooked meat (boiled, pan-fried, roasted, stir-fried, or stewed) (Hsu et al, 2021). In the areas of fisheries, meat authentication was particularly important in the authentication of the common octopus (*Octopus vulgaris*) as the market could reach tens of thousands of tons per year and have higher susceptibility to food mislabelling in some countries (Velasco et al, 2021).

5. INTEGRATION WITH OTHER ANALYTICAL TECHNIQUES'

5.1 *SYBR® Green I*

One of the initial developments of recombinase polymerase amplification coupled with SYBR® Green I as it is one of the colorimetric analyses that does not require any sophisticated equipment and procedures to provide an instant visualization (Chang et al, 2023) (Table 2). Chang et al (2023) also highlighted that use of SYBR® Green I is cost-effective and a robust detection method that works well in challenging samples like serum, meat, and milk and the rapidity, visually intuitive, and user-friendly detection method make it ideal for resource-limited settings. Study by Cao et al (2018) utilized this assay for the purpose of meat authentication of duck, chicken, cow, pig and sheep in boiled, microwaved, fried and high-pressured food samples, and the results have shown that relative limit of detection of 1% (in binary mixture) which are a bit higher in comparison to real-PCR standard (0.01%). The RPA-SYBR® Green I assay achieved visual detection down to 200 fg of genomic

DNA across all species tested, showing significantly higher fluorescence intensities compared to negative controls ($p < 0.01$), demonstrating superior sensitivity outperforming conventional PCR in detecting most species tested (Cao et al., 2018). However, after this experiment, there appears to be a paucity of research investigating the application of SYBR® Green I in conjunction with Recombinase Polymerase Amplification (RPA) for meat authentication. Several factors may account for this gap in the literature: non-specific binding, sensitivity to contaminants in food samples, complexity of interpretation and better visualisation alternative. For the quantification of SYBR® Green I dye, the use of fluorescence intensity readers, such as the QuantStudio 6 Flex Real-Time PCR system, may be necessary to ensure accurate and reliable analysis (Cao et al., 2018).

Table 2. Comparison Of Integration of Recombinase Amplification With Different Analytical Technique

Assay	*Key Features*	*Advantages*	*Limitations/ Concerns*	*References*
SYBR® Green I	Fluorescent dye used for instant visualisation	-No sophisticated equipment required -Effective in challenging samples	-Influences background signal in visual analysis -Potential false positives	Chang et al (2023), Cao & Song (2023)
Real-Time Detection	Monitors fluorescence during amplification without opening tube	-Eliminates need to open reaction tube -Overcome issue of false positive	-Requires fluorescence reader -Difficulty detecting low levels of adulteration due to background DNA	Zhou et al (2023a), Kissenkötter et al (2022)
Lateral Flow	Field-based test, visual detection via strips	-Simple, convenient, instant -Multiple detection with fluorescent-labelled primers -Two types: immunoassay & nucleic acid-hybridised	-Potential false positives -Requires thorough examination to avoid inaccuracies	Zhou et al (2023a), Kumar et al (2020), Liu et al (2024)

CRISPR/Cas12a	Gene-editing based detection method	-Addresses nonspecific recombinase amplification -High sensitivity	-Limited multiplex capability	Li et al (2022), Wang et al (2023)

5.2 *Real-Time Detection*

Kissenkötter et al. (2020) highlighted that employing real-time detection, as opposed to methods like lateral flow or SYBR® Green I, offers the significant advantage of eliminating the need to open the reaction tube from the amplification stage through to the detection stage (Table 2). This is achieved by analyzing the DNA using a fluorescence reader with a fluorophore, streamlining the process. Additionally, their developed RPA assay, integrated with real-time detection, reduced the authentication time to as little as 15 minutes. The limit of detection of the assay for porcine is 16 DNA molecules/μL and 1 DNA molecules/μL for horse DNA. One of the earliest studies conducted using real-time RPA was by Zhou et al (2023a) which examined the application or real-time RPA in various cooked and commercial products and the author raised the concerns of the impact of background DNA on detection sensitivity across varying levels of adulteration ratios. The results indicated that background DNA reduced the detection ability of the assay, leading to failure in identifying low levels of adulteration. Consequently, artificial samples with varying proportions of the target ingredients were employed to assess the adulteration sensitivity of the developed RPA assays.

5.3 *Lateral Flow*

Lateral flow assay is a point of care testing which is performed over a strip which made of different components: sample application pad, conjugate pad, nitrocellulose membrane and adsorption pad (Kumar et al, 2020). Lateral flow assays can take the form of conventional immunoassays, which utilize antigen-antibody interactions to capture tagged nucleic acid amplicons, or nucleic acid hybridization assays, where two pairs of nucleic acid-tagged RPA primers generate double-tailed RPA amplicons (Table 2). These amplicons are initially recognized by probes on gold nanoparticles and subsequently captured by nucleic acid probes pre-labelled on the two test lines of the lateral flow strip (LFS), resulting in the formation of two visible coloured lines detectable to the naked eye. The use of conventional immunoassay techniques in rapid on-site authentication tests raises concerns due to the high cost of antibodies and their susceptibility to environmental interference. Additionally, labelling antibodies with gold nanoparticles can be time-intensive, particularly

in self-assembled lateral flow assays, limiting the efficiency of these methods (Liu et al, 2023). Nevertheless, the cost of nucleic acid-based lateral flow assays (NABLFA) is not generally lower than that of immunoassay lateral flow assays, as the former remains a relatively novel technology requiring complex reagent preparation and intricate design for consistent performance (Dryan et al, 2022). In contrast, traditional immunoassay lateral flow tests are significantly more cost-effective, especially when purchased in bulk.

The optimisation of RPA-LFA assay was investigated by Hsu et al (2021) on effect of incubation temperature and time, as well as extraction methods using purified and crude DNA extracts on RPA-LF assay. The study has shown that 5 minutes incubation are sufficient time for detection of amplified products with incubation temperature between 30 to 45°C, although 10 minutes incubation was required for 30°C incubation temperature due to low enzyme kinetics. Whilst if the amplification temperature is at 50°C, Velasco et al (2021) stated that the assay produced a weaker signal. Another foundational exploration of optimisation of lateral flow strips in RPA-LFA for meat and halal authentication is by Kumar et al (2021) which have optimised the pore size of nitrocellulose membrane, concentration of anti-fluorescein on test line, concentration of anti-biotin on control line, size of streptavidin gold conjugate on conjugate pad. The study revealed that the optimal specifications for the nitrocellulose membrane were identified as a pore size of 5.0 microns and a width of 5.0 mm. For the test line, 1 μL of anti-FITC at a concentration of 1 mg/mL was determined to be ideal, while 1 μL of BSA-biotin at 2 mg/mL was optimal for the control line. The distance between the test and control lines was set at 5.0 cm, and streptavidin gold nanoparticles with a diameter of 20 nm were utilized (Kumar et al, 2021). However, the author provided limited justification for the selection of the optimal specifications in the lateral flow assay. A critical examination on the optimisation of RPA-LFA conditions conducted by Lin et al (2021) demonstrated that optimum magnesium acetate concentrations were different for each target species ranging from 1 μL, 1.5 μL, 2 μL, and 2.8 μL. Whilst the optimum amplification duration was found to be 20 minutes, although no significant difference on test lines were found between incubation temperature of 37, 39, and 45°C.

One notable drawback of using RPA-LFA is the potential for false positives, an issue observed in several studies on this technique. Velasco et al. (2021) highlighted that in some cases, betaine was added to mitigate this problem, although this adjustment may also affect the assay's sensitivity. Zhou et al. (2020) further validated this approach, emphasizing that the addition of 0.8M betaine in the amplification tube could slightly reduce non-specific amplification by approximately 8%.

Nucleic acid-hybridised lateral flow utilised complementary DNA sequences as recognition elements, instead of conventional antibody-antigen binding mechanism traditionally relied upon in lateral flow assay. Liu et al (2022) demonstrated that nucleic acid hybridisation could be performed by the utilisation of probes of different types which will be coated on the conjugate strip, test and control strip as replacement to anti-fluorescein or anti-biotin, forward and reverse primers with spacer9 (isp9) and additional extended sequences as tags or labels to each primer. Henceforth, although nucleic acid hybridised lateral flow strips may potentially be cheaper than conventional lateral flow, however it will only happen if it is designed and produced in bulk. Experiments conducted by Liu et al. (2023) and Liu et al (2024) successfully uses nucl**e**ic acid hybridisation lateral flow strip assay coupled with RPA for the purpose of meat and halal authentication for chicken and duck, pork and horse respectively. Experiment by Liu et al (2023) have demonstrated multi-coloured nucleic acid hybridization lateral flow strip for authentication of multiple species reducing the number of tests conducted with sensitivity on par with the real-time PCR of 0.01%, better sensitivity than conventional PCR with no cross-reactivity. Quantitative analysis can be performed using image processing software such as ImageJ (NIH) which assess the colour intensity of the pressed test line, by converting to a grey-scale value and measure the average pixel intensity along the entire length of the strips. Analysis could also be done as demonstrated by Liu et al. (2024), using a Raman detection reader for relative quantification.

There is numerous research that have been conducted in integrating RPA with lateral flow, and further developments have been analysed and examined such as the integration of Surface-Enhance Ramen Spectroscopy and the use of natural dyes to create a multiple colour allowing multiple species detection and quantitative analysis. This integration has been done by Liu et al (2024) in detecting donkey and horse species for meat authentication. The use of SERS and two-coloured lateral flow test strips could be employed for detection of potentially non-halal species for Halal Authentication. However, the downside of using the combinations of SERS and two-coloured lateral flow is the unsuitability of performing the assay on-site as equipment are required for Raman spectroscopy and in quantitatively analyse the multi-coloured test strips, although undeniably the fast duration of analysis and high specificity makes the test reliable for meat and halal authentication (Liu et al, 2024).

5.4 *CRISPR/Cas12a*

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas) constitute a specialized adaptive immune system that has evolved in many bacteria and archaea to protect

against foreign genetic elements. In this system, guide RNA (gRNA) and CRISPR-associated (Cas) genes are transcribed from the short DNA repeats within CRISPR arrays, leading to the subsequent translation of these transcripts into Cas endonucleases (Janudin et al., 2023). In regard to the integration of RPA with CRISPR/Cas12a detection technology, there are a number of successful applications as clinical diagnostic tools for detection of viruses, fungi and bacteria (Li et al., 2022), and a number of research studies have shown promising results on the application of RPA-CRIPSR/Cas12a in meat and Halal authentication (Zhao et al., 2022; Wang et al., 2023; Janudin et al., 2023). The technology showed its high limit of detection of 0.0064 ng/μL as investigated by Wang et al (2023) and have shown its validity by the test on cooked meat and processed meat products. The research has also investigated the specificity of the C-type lectin domain-containing 20A gene within nuclear DNA, which is rarely used as a target gene due to the higher variability typically observed in mitochondrial DNA. In contrast, Janudin et al. (2023) conducted experiments utilizing the COX1 gene, demonstrating superior sensitivity at a concentration of 5 pg/μL when tested with commercial food products. The extraction method studied by Zhao et al. (2022) revealed that the application of the CTAB method exhibited higher sensitivity in detecting binary mixtures at 0.001% w/w compared to the alkaline-lysis method.

6. FUTURE DIRECTIONS AND POTENTIAL IMPLICATIONS

To date, there are still not much known Halal certification bodies or meat authentication laboratories which uses this assay despite the recognition as an effective method for species identification, suitability in detecting within shorter amount of time, capability in working with crude DNA extracts and could be conducted on-site (Kua et al., 2022; Usman et al., 2023). This lack of practice could be researched further as to understand whether there are any other issues that need to be foreseen and tackled further for the assay to be feasible. In addition, the application of these authentication methods could be tested by meat and Halal authentication laboratories as part of interlaboratory validation. A key investigation by Velasco et al (2021) on interlaboratory validation has been conducted for common Octopus authentication to validate the authentication method. A standard operating procedure of RPA-LFA was elaborated and tested on several laboratories using 8 blind samples to measure the number of false positives and false negatives to calculate interlaboratory trial's specificity and sensitivity tests that were calculated using formula (1) and (2):

$$Specificity = \left[\frac{True\ Negatives}{True\ Negatives + False\ positives}\right] \times 100 \quad (1)$$

$$Sensitivity = \left[\frac{True\ Positives}{True\ Positives + False\ negatives}\right] \times 100 \quad (2)$$

Other experimental research that could be developed further by examining the implications of meat and halal authentications using recombinase polymerase amplification and lateral flow varied, such as the use of cartridge-based lateral flow strip for automatic connection to electronic system and the application of multiplex detection using molecular colorimetric 7-segement display. Land et al. (2018) highlighted the potential of cartridge-based microfluidics, integrated with a readout system and lateral flow devices, to enable real-time connectivity. This approach allows the technology to interpret lateral flow results and convert them into digital outputs, facilitating streamlined and automated data acquisition. Li et al (2019) studies the use of molecular colorimetric 7-segment display which combines lateral flow membrane with binary and molecular encoding as different forward primer labels are being used namely, Biotin, Digoxigenin, TAMRA, TexasRed-X, Cascade Blue C6-NH, DNP-X-C6-NH, Dansyl C6-NH with reverse primer and probe. This novel technology utilizes the integration of molecular labels to spatially differentiate nucleic acid species on a lateral flow membrane which results in the generation of seven-segment output display without increasing the size of the device which overcomes the issues of slow flow rate as distance increases from sample pad (Li et al., 2019).

7. CONCLUSION

In conclusion, Recombinase Polymerase Amplification (RPA) presents significant potential for further development, particularly in areas that remain underexplored in comparison to its advancements in clinical diagnostics. While some research has focused on integrating CRISPR/Cas12a with RPA, other detection methodologies, such as SERS, flocculation assays and electrochemical detection, have yet to be fully investigated. The extensive application of lateral flow assays in conjunction with RPA underscores the demand for rapid, user-friendly, and robust authentication tools. However, it is crucial that research in this field extends beyond academic inquiry and fosters collaboration with relevant stakeholders, such as Halal diagnostic laboratories and Halal certification agencies. Establishing laboratory validation processes will be essential to ensure the reliability and practical application of RPA in Halal and meat authentication.

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