Development and comparison of recombinase polymerase amplification assays for the detection of chicken-derived ingredients in food products

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RESEARCH ARTICLE

Abstract

The recombinase polymerase amplification (RPA)-based assays, formulated with the *ND5* gene, were developed to meet the requirement of detecting different breeds of chicken-derived ingredients in deep-processed foods. The RPA assay demonstrated good inter-species specificity and intra-species conservation, exhibited high sensitivity (10 pg genomic DNA/reaction), high limit of detection, 0.1% (w/w). In all, 20 samples, including sausages and compound seasonings were used to compare the RPA assay developed for this study and other assays. RPA worked along with the polymerase chain reaction method described in SN/T 2978-2011 standard and a previously described protocol. Three compound seasonings containing small amounts of chicken juice or chicken meat showed discrepancies between GB/T 38164-2019 and the remaining methods because of sensitivity issues. Overall, the chicken-specific RPA assay was successfully developed, taking 20–25 min from sample processing to final output.

Keywords: chicken ingredients; real-time RPA; LFS RPA; authenticity identification

Introduction

The European Commission prioritizes food safety as a cornerstone of its policy on food and health, and that the food authenticity is one of the most important elements for ensuring this (Razzak *et al*., 2015). Food adulteration is the intentional substitution or addition of cheaper varieties to products for higher profits (Spink and Moyer, 2011; Wu *et al*., 2020). Meat products are one of the main types of foods that are adulterated for economic motives. A common form of adulteration in meat products is counterfeiting with cheap meat varieties, such as duck, chicken, pork, and horse meat, with expensive ones, such as beef, lamb, and donkey meat. Worse still is the counterfeiting of meat that has not been inspected and quarantined as an edible meat (Bittante *et al*., 2022; Mayer *et al*., 2012). A false ingredient lists could also conceal potential allergens, posing serious health risks to consumers and potentially including life-threatening allergic reactions (Bartuzi *et al*., 2017). Food adulteration not only undermines the fair business environment but also violates the legitimate rights and interests of consumers. Previous

studies conducted in European, South American, and Asia-Pacific nations have demonstrated that food adulteration is a global problem (Afifa Khatun *et al*., 2021; Pierina and Maria, 2021; Song *et al*., 2019). Enhanced surveillance and enforcement efforts are currently underway globally to reduce the incidence of food adulteration. Compared to other animal-derived ingredients, adulteration with chicken-derived ingredients is not only found in meat products but in compound seasonings produced using chicken as well. Chicken essence seasoning without chicken-derived ingredients is of concern and it infringes on the interests of consumers. The relevant Chinese standards for chicken essence seasoning (SB/T 10371-2003; National Developed and Reform Commission of the People's Republic of China, 2003) and chicken powder seasoning (SB/T 10415-2007; National Developed and Reform Commission of the People's Republic of China, 2007) rely on total nitrogen content to verify the presence of chicken-derived ingredients. However, the excessive addition of other nitrogen-containing substances, such as monosodium glutamate, and flavor-presenting nucleotides may confound the accuracy of the chicken-derived ingredients (Zhang *et al*., 2007). Consequently, there is an urgent need of specific and rapid detection methods that can accurately identify chicken-derived ingredients in meat products and seasonings.

The polymerase chain reaction (PCR)-based methods are the most well-established and widely used methods in detecting animal-derived ingredients (Zhao *et al*., 2020). China has also developed PCR-based assays for detecting chicken-derived ingredients, such as SN/T 2978-2011 (State General Administration of the People's Republic of China for Quality Supervision and Inspection and Quarantine, 2011), and GB/T 38164-2019 (State Market Regulatory Administration of the People's Republic of China, 2019) for detecting animal-derived ingredients in common poultry and livestock. However, the PCR-based techniques rely on the sophisticated thermal cycling instruments and thus cannot be used outside the laboratory settings. The alternatives, isothermal DNA amplification techniques, including the transcription-mediated amplification (TMA), strand-displacement amplification (SDA), rolling circle amplification (RCA), helicase-dependent amplification (HDA), cross-priming amplification (CPA), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), etc., have distinct requirements. Some of these, such as TMA and RCA, require higher annealing temperatures, while others need relatively high temperatures and/or a phase for complex primers design, such as LAMP requiring four to six primers to recognize discrete regions and reacting at 60–66°C, and CPA relying on five primers to detect target sequence at 63°C. Some such as HDA and SDA have long reaction periods of up to 1–2 h. In contrast, the RPA method works perfectly over a wide range temperatures (37–42°C), uses just one primer pair and a probe (Kumar, 2021). The lower reaction temperature makes it easier to operate, and a single primer pair and probe makes it simpler to design. Therefore, RPA can be applied in resource-limited field detection using hot water bags, metal baths, and other portable heating device to have a constant temperature. Furthermore, RPA amplification products can be coupled with different endpoint assays, such as flocculation assay, electrochemical detection, chemiluminescent detection, and so on for visualization of results (Li *et al.*, 2018).

Recombinase polymerase amplification is widely used in the detection of pathogenic microorganism, genetically modified foods, gene mutations, and so on (Du *et al*., 2018; Li *et al*., 2021; Zhang *et al*., 2020). The reported studies using RPA to detect chicken-derived ingredients mainly focused on the inter-species specificity validation while ignoring the intra-species conservation validation, with the scope of application being restricted to chicken products only and not the seasonings (Cao *et al*., 2018; Ivanov *et al*., 2021; Lin *et al*., 2021). In view of this, this study sought to improve the reliability of finding chicken-derived ingredients from different breeds, and to expand the scope of application to meet the demands of processed foods, such as compound seasonings. The chicken-specific real-time RPA assay and RPA combined with lateral flow strip (LFS RPA) assay were developed with the *ND5* gene as a target, and compare the performance with counterparts in SN/T 2978-2011, GB/T 38164-2019, and a previously described protocol in Liu *et al*. (2020). These methods were applied to actual samples to analyze the applicability of the developed RPA assays with the aim of providing a reference for selecting appropriate method for detecting chicken-derived ingredients.

Materials and Methods

Sample preparation and DNA extraction

Pork, beef, chicken (white-feathered chicken), duck, and goose meat samples were purchased from local supermarkets. Different breeds of chicken, such as green bird chicken, crow chicken, apricot chicken, orangery chicken, and triple yellow chicken as well as turkey meats were obtained through online shopping. Donkey, horse, pigeon, goat and sheep meat samples were collected from a local farm, while Yak, buffalo and camel meat samples were collected from local markets of Lhasa, Kunming, and Xilingol in China, respectively.

All meat samples were churned separately, dried and crunched into a powder. DNA was extracted from 50 mg of powder using a Wizard® Genomic DNA purification kit (Promega Corp., Madison, WI, USA). The genomic

DNA concentration was adjusted to 10^5 pg/μL. The genomic DNA of each species was used as template in subsequent validation experiments. Chicken genomic DNA was further subjected to serial gradient dilutions of 1.0×10^5 pg μL to 1.0×10^{-1} pg/μL approximately.

The chicken and pork meat powder samples were mixed in different ratios to make binary mixtures with 25.0%, 10.0%, 5.0%, 1.0%, 0.5%, and 0.1% (w/w) of target species composition. A 50-mg sample of each binary mixture was taken for DNA extraction.

A total of 20 test samples, comprising nine sausages and eleven seasonings were purchased from local supermarkets. Each of the seasonings were powdered or homogenized with pestle and mortar. DNA extraction was carried out from 50 mg of each sample using the Wizard® Magnetic DNA Purification System for Food (Promega Corp.) according to manufacturer's protocol. A small piece of each of the nine sausages was excised at multiple points using sterile scissors and crunched into powder using liquid nitrogen. Then each sample was taken for DNA extraction carried out from 50 mg of each crunched sausage using Wizard® Genomic DNA Purification Kit (Promega Corp.) according to manufacturer's protocol.

All DNA extractions were examined for concentration and quality using a NanoDrop spectrophotometer (ND-2000c; Technologies Co. Ltd., Wilmington, DE, USA) and stored at -20°C to maintain their stability for downstream applications.

Primers and probes of RPA assays

The *ND5* gene was selected as the target gene. The published nucleic acid sequences of chicken (*Gallus gallus*, NC_053523.1, AB086102.1, AP003319.1, GU261687.1, KX987152.1, LC082227.1, LC082354.1, MN013407.1, MT471352.1, and OM634640.1), duck (*Anas platyrhynchos*, NC_009684.1), pig (*Sus scrofa*, NC_000845.1), goat (*Capra hircus*, NC_005044.2), donkey (*Equus asinus*, NC_001788.1), sheep (*Ovis aries*, NC_001941.1), and cattle (*Bos taurus*, NC_006853.1), horse (*Equus caballus*, EU939445.3), buffalo (*Bubalus bubalis*, NC_049568.1), bactrian camel (*Camelus bactrianus*, NC_009628.20), yak (*Bos grunniens*, NC_006380.3), cow (*Bos taurus*, NC_006853.1), rabbit (*Lepus capensis*, NC_015841.1), domestic goose (*Anser answer*, NC_011196.1), turkey (*Meleagris gallopavo*, NC_010195.2), dog (*Canis lupus familiaris*, NC_002008. 4), fox (*Vulpes vulpes*, NC_008434.1), American mink (*Neogale vison*, NC_020641.1), and raccoon dog (*Nyctereutes procyonoides*, NC_013700.1) were collected from GenBank. These sequences were analyzed using the MegAlign software (version 7.0; DNASTAR Inc., Madison, WI, USA). Regions with both intra-species conservation and inter-species specificity were chosen to design RPA primers and probes. The RPA assay design manual of TwistXD was referenced during the designing process for primers and probes in the subsequent phases. PCR primers and TaqMan probes from SN/T 2978-2011, GB/T 38164-2019-based detection protocols, and the protocol described by Liu *et al*. (2020) were synthesized by Generay Biotechnology (Shanghai, China). The primer and probe sequences are shown in Table 1.

Detection protocols reaction system

Polymerase chain reaction

A PCR assay from SN/T 2978-2011 was performed on thermal cycler (T100™ Thermal Cycler, Bio-Rad Corp., CA, USA). The reaction mix constituted was as follows: 12.5 μL of 2× GoTaq® green Master Mix (Promega Corp.), 1 μL of animal genomic DNA or 2 μL of test sample DNA as template, 2 μL of each primer (10 μmol/L) and deionized distilled H_2O (ddH_2O) was added up to 25 μL. The reaction conditions included were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min; and final extension at 72°C for 5 min, and storage at 4°C. The results were viewed on 2% agarose gel using the Fusion FX5gel Imaging System (Viber Lourmat Corp., Paris, France). The amplified products with expected target band were sent for sequencing.

Real-time polymerase chain reaction

The real-time PCR assays from GB/T 38164-2019 and Liu *et al*. (2020) were performed on ABI Quant Studio 5 real-time PCR system (Applied Biosystems Inc., Waltham, MA, USA). The reaction mix was constituted as follows: 12.5 μL of 2× PerfectStart® II Probe qPCR SuperMix (TransGen Biotech Inc., Beijing, China), 1 μL of animal genomic DNA or 2 μL of test sample DNA as template, 1 μL of each primer and probe (10 μmol/L), and ddH_2O was added up to 25 μL. The reaction condition were set as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles consisting of 95°C for 5 s and 60°C for 30 s, with the cycle threshold (Ct) values recorded.

Real-time recombinase polymerase amplification

The real-time RPA was performed using the ZC BioScience™ exo kit (ZC BioScience Inc., Hangzhou, China) following the manufacturer's protocol. The reaction mix was constituted as follows: 25 μL of A buffer, 2 μL of each primer (10 μmol/L), 0.6 μL of exo probe (10 μmol/L), 1 μL of animal genomic DNA or 2 μL of test sample DNA as template, and ddH_2O was added up to 47.5 μL. An additional 2.5 μL of B buffer (magnesium acetate, 280 mmol/L) was added to the tube cap, followed by capping. The tube was inverted several times, and

Table 1. Primers and probes used in this study.

Assays	Primers and probes	Sequence (5′-3′)	Genes	Sources
RPA	JF1	CAATCTTCATCCACTCAGGGGCAGAAAGCAT	*ND5*	The present study
	JR1	CGATGGTTAGTGTTAATATGGCGATGAGGAA		
	JF2	CATCCGAACCATTTATTACAAAATTCTTTACC		
	JR2	CGATTCGGTTGTAGATTATTGCCTGTAGTGC		
	JF3	TGCACTACAGGCAATAATCTACAACCGAATC		
	JR3	GGTGAAGGCCAAATTGAGCGGATTTTCCTG		
	JF4	AGGCCCAACCCCTGTCTCCGCCCTACTCCAT		
	JR4	TGTTGCGGCAAAGAGTGTTGATAGAGCACCT		
	exo-P1	GAATTTTTTTACTCATCCGCACCCACCCCTTCC[FAM][THF][BHQ1] CATCCAATAAAACAG-C3-spacer		
	nfo-R	Biotin-TGTTGCGGCAAAGAGTGTTGATAGAGCACCT		
	nfo-P	FAM-GCACCCACCCCTTCCTGTCATCCAATAAAA[THF] AGCCCTGACAACGTG-C3-spacer		
Real-time PCR	F	TGCTGCACCTATGAAAATGAATG	*TGFB3*	Liu *et al.*, 2020
	R	AGAATGCAGTCTCAGCACAACAC		
	P	FAM-TGCCCCGGTCTCCCTATGGTGC-BHQ1		
	F	CCCTCCTCCTTTCATCCTCAT	*ND1*	GB/T 38164-2019
	R	GTCATAGCGGAACCGTGGATA		
	P	FAM-CTATGAATCCGGGCCTC-BHQ1		
	F	CTATAATCGATAATCCACGATTCA	*12S rRNA*	SN/T 2978-2011
	R	CTTGACCTGTCTTATTAGCGAGG		

centrifuged briefly. Then the reaction tube was immediately placed in the Genie III scanner device (OptiGene Co. Ltd., West Sussex, UK). The real-time RPA reaction was carried out at 39 °C for 20 min, with fluorescence signals collected every 30 s, and the threshold time (TT, mm:ss) was recorded.

LFS RPA assay

The LFS RPA in this study was performed using GenDx ERA kit (GenDx Biotech Co. Ltd., Suzhou, China) following the manufacturer's protocol. The reaction mix was constituted as follows: 20 μL of rehydration buffer, 2.1 μL of each primer (10 μmol/L), 0.6 μL of nfo probe (10 μmol/L), 1 μL of animal genomic DNA or 2 μL of sample DNA as template, ddH_2O was added up to 48 μL, and the additional 2 μL of magnesium acetate (280 mmol/L) was added to tube cap. The reaction mix was inverted repeatedly, followed by incubation in a metal bath. Subsequently, 5 μL of the reaction product was diluted to 40-fold with ddH_2O, and the lateral flow strips (GenDx Biotech Co. Ltd.) were inserted into the diluent. The results were visualized after 5 min, with positive determinations made when both control and test line were present, and negative determinations were made when only the control line was present. The result was deemed as invalid in the absence of a control line.

The reaction conditions, including incubation temperature and time, were optimized to improve amplification efficiency. Incubation temperatures ranging from 35°C to 43°C were tested with 1 μL of 1×10^2 pg/μL chicken genomic DNA as template, with the initial incubation time set as 20 min. The brightness of test line was used to determine optimum incubation temperature. The incubation time was assessed at 5, 10, 15, 20, 25, and 30 min under optimum temperature. Each reaction was performed in triplicate.

Specificity analysis of five detection assays

To assess the performance of the aforementioned protocols for cross-reactivity to other common poultry, 1 μL of chicken genomic DNA was used as a positive control and 1 μL of ddH_2O as no template control (NTC). The genomic DNA of different breeds of chicken, such as green bird chicken, crow chicken, apricot chicken, orangery chicken, triple yellow chicken, were used as templates to validate intra-species conservation. The genomic DNA of turkey, pig, duck, horse, donkey, cow, sheep, goat, buffalo, yak, camel, goose, and pigeon were used as templates to validate inter-species specificity. All specificity tests were conducted in triplicate to confirm the results.

Sensitivity analysis of five detection assays

Chicken genomic DNA was serially diluted to 10-fold from 1×10^5 pg/μL to 1×10^{-1} pg/μL with 1 μL of each dilution used as template to validate different protocols. All sensitivity tests were repeated for five times. The lowest DNA concentration with a detection probability of at least 95% was the sensitivity of the method.

Limit of detection (LOD) analysis of five detection assays

To validate the LOD of each protocol, 1 μL of each proportion of prepared binary mixture DNA was used as a template. Every LOD test was conducted for five times to confirm the result. The LOD was defined as the lowest proportion with at least 95% probability of being detected as positive.

Evaluation of five detection assays for samples

The DNA extracted from nine sausages and 11 seasonings were used as templates for five different protocols. The results of five detection assays were compared to validate the practical efficacy.

Results

Screening of optimal primers combination

Optimal primer design was done according to the RPA Assay Design Manual of TwistXD, resulting in four primer pairs from JF1R1 to JF4R4 (Table 1). All primer pairs were validated using the basic RPA with 1 μL of 1×10^5 pg/μL chicken genomic DNA as a template. All reactions produced the expected amplified band (Supplementary Figure S1A). JF4R4 was selected as an optimal pair because it produced a single band with expected amplified fragment size and was brighter than other primer pairs.

The specificity of JF4R4 was validated before the designing of exo and nfo probes. The genomic DNAs of fox, donkey, duck, pig, horse, goat, and cow were first chosen as templates for cross-reactivity analysis. The results showed that JF4R4 specifically produced the target band for chicken but not for any other species (Figure S1B). The real-time RPA and LFS RPA probes were designed on the basis of JF4R4 amplification fragment. The exo and nfo probes shared the same nucleic acid sequence, but with different group modification modes.

Optimization of incubation temperature and time of LFS RPA

The incubation temperature and reaction time were optimized to achieve the optimal performance of LFS RPA. The incubation temperature was optimized between 35°C and 43°C with 1 μL of 1×10^2 pg/μL chicken genomic DNA as template and an incubation period of 20 min. The optimal temperature determined was 37°C as it had the clearest test line (Figure 1A). Furthermore, the incubation time was optimized at 37°C. The results showed that the test line first emerged after 15 min, with the brightness of test line increasing with time (Figure 1B). The optimal incubation time was determined as 20 min because it was sufficient to determine the results.

Specificity analysis of real-time RPA and LFS RPA

The results of specificity analysis on real-time RPA and LFS RPA showed that both produced specific amplification curves and test lines for different breeds of chickens, indicating good intra-species conservation (Figure 2).

For non-target species, only turkey genomic DNA generated an amplification reaction. However, no specific amplification curves or test lines were observed, suggesting a certain degree of good inter-species specificity (Figure 2).

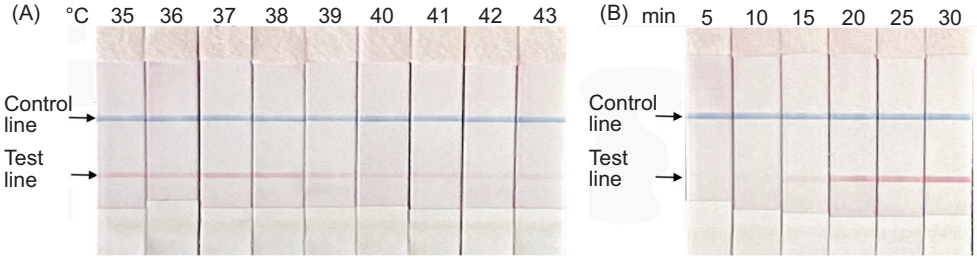

Figure 1. Optimization of reaction conditions for the LFS RPA assay. Optimization of (A) incubation temperature; and (B) incubation time.

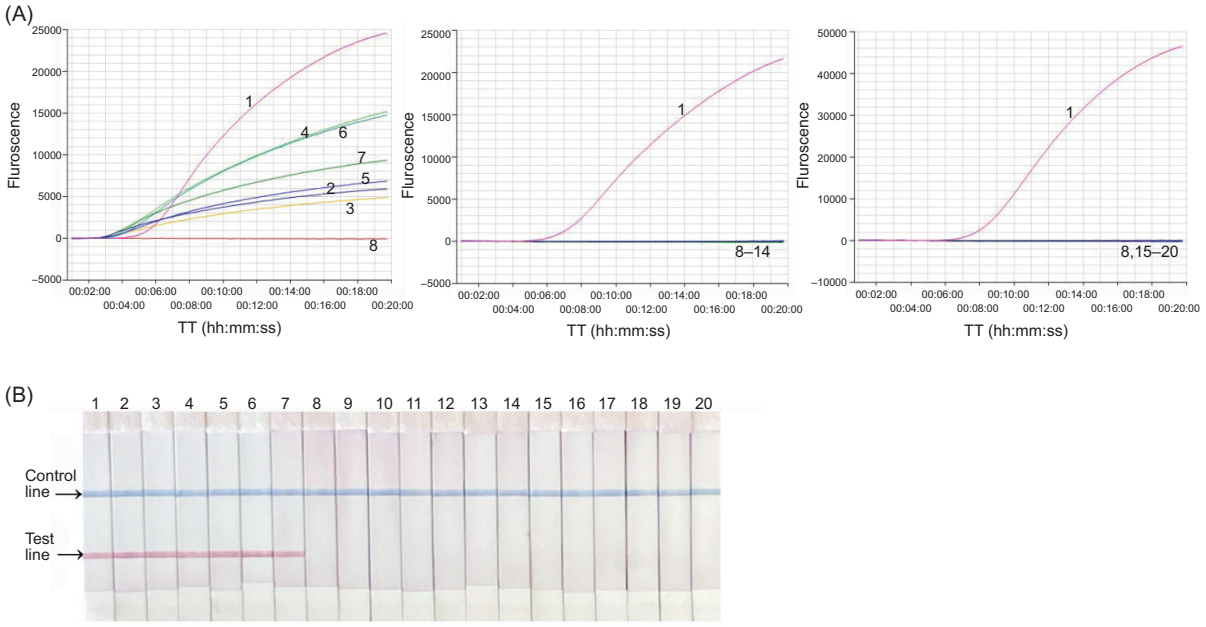

Figure 2. Specificity analysis of RPA assays. Line/lane 1, chicken; line/lane 2, green bird chicken; line/lane 3, crow chicken; line/lane 4, apricot chicken; line/lane 5, orangery chicken; line/lane 6, triple yellow chicken; line/lane 7, turkey; line/lane 8, ddH_2O; line/lane 9, pig; line/lane 10, duck; line/lane 11, horse; line/lane 12, donkey; line/lane 13, cow; line/lane 14, sheep; line/lane 15, goat; line/lane 16, buffalo; line/lane 17, yak; line/lane 18, camel; line/lane 19, goose; and line/lane 20, pigeon.

Specificity analysis of real-time polymerase chain reaction assays

The results of the chicken-derived ingredient specificity analysis of the real-time PCR assay showed the GB/T 38164-2019 assay as having nonspecific amplifications for duck, cow, and donkey with Ct values < 35. Nonspecific amplifications with Ct values ≥ 35 were observed for rest of the species, with the exception of camel and yak (Figure 3A). A typical amplification curve was produced for chicken using the real-time PCR assay described by Liu *et al.* (2020). Nonspecific amplification was observed for turkey, duck, and cow DNA with atypical curves and Ct values ≥ 35 (Figure 3B).

Specificity analysis of polymerase chain reaction assay

The results of specificity analysis of the PCR assay for chicken-derived ingredient in SN/T 2978-2011 showed nonspecific amplifications with cow, duck, donkey, goose, pigeon, and turkey DNA (Figure 4). There was less than 98% homology between the sequences of nonspecific PCR amplicons and chicken. This indicated that the

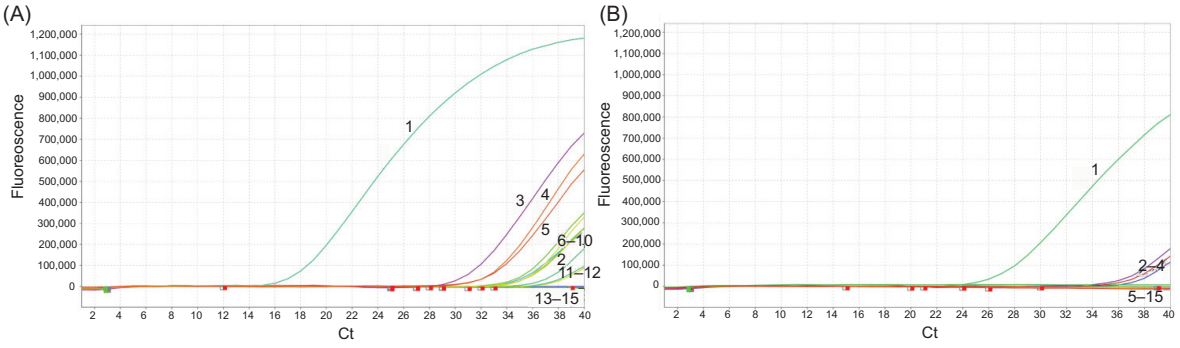

Figure 3. Specificity analysis of real-time PCR assays of chicken-derived ingredients in GB/T 38164-2019 and described by Liu *et al.* (2020). Line 1, chicken; line 2, turkey; line 3, duck; line 4, cow; line 5, donkey; line 6, goat; line 7, sheep; line 8, buffalo; line 9, goose; line 10, pigeon; line 11, horse; line 12, pig; line 13, camel; line 14, yak; and line 15, ddH_2O.

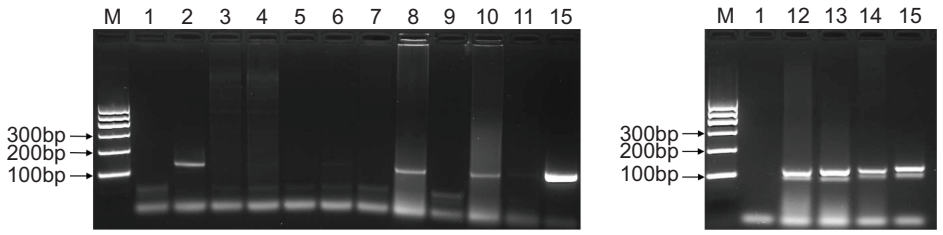

Figure 4. Specificity analysis of the PCR assay for chicken-derived ingredients in SN/T 2978-2011. Lane 1, ddH_2O; lane 2, cow; lane 3, goat; lane 4, sheep; lane 5, pig; lane 6, buffalo; lane 7, yak; lane 8, duck; lane 9, camel; lane 10, donkey; lane 11, horse; lane 12, goose; lane 13, pigeon; lane 14, turkey; and lane 15, chicken.

specific detection of chicken-derived ingredient using SN/T 2978-2011 PCR protocol could be realized by combining it with sequence analysis.

Sensitivity analysis of five detection assays

Sensitivity analysis of five assays with chicken genomic DNA ranging from 1.0×10^5 pg/μL to 1.0×10^{-1} pg/μL as template indicated that the PCR assay in SN/T 2978-2011 consistently produced target bands at 100 pg/μL (Figure 5C), while the real-time RPA, LFS RPA and the real-time PCR from Liu *et al.* (2020) achieved stable amplification at 10 pg/μL (Figures 5A, 5B, and 5E). The GB/T 38164-2019 real-time PCR achieved stable amplification curves at concentrations as low as 1 pg/μL (Figure 5D).

LOD analysis of five detection assays

The LOD analysis of five detection assays showed that all achieved stable amplification at all ratios, that is, 25%–0.1% (w/w) (Figure 6).

Evaluation of five assays on actual samples

The chicken-derived ingredients were detected in 14 samples (70%) by the real-time RPA, LFS RPA, the PCR assay in SN/T 2978-2011, and the real-time PCR described in Liu *et al.* (2020). The real-time PCR in GB/T 38164-2019 was detected in 17 samples (85%, 17/20). Notably, chicken-derived ingredients were detected by all assays in one sausage with no chicken in the ingredient

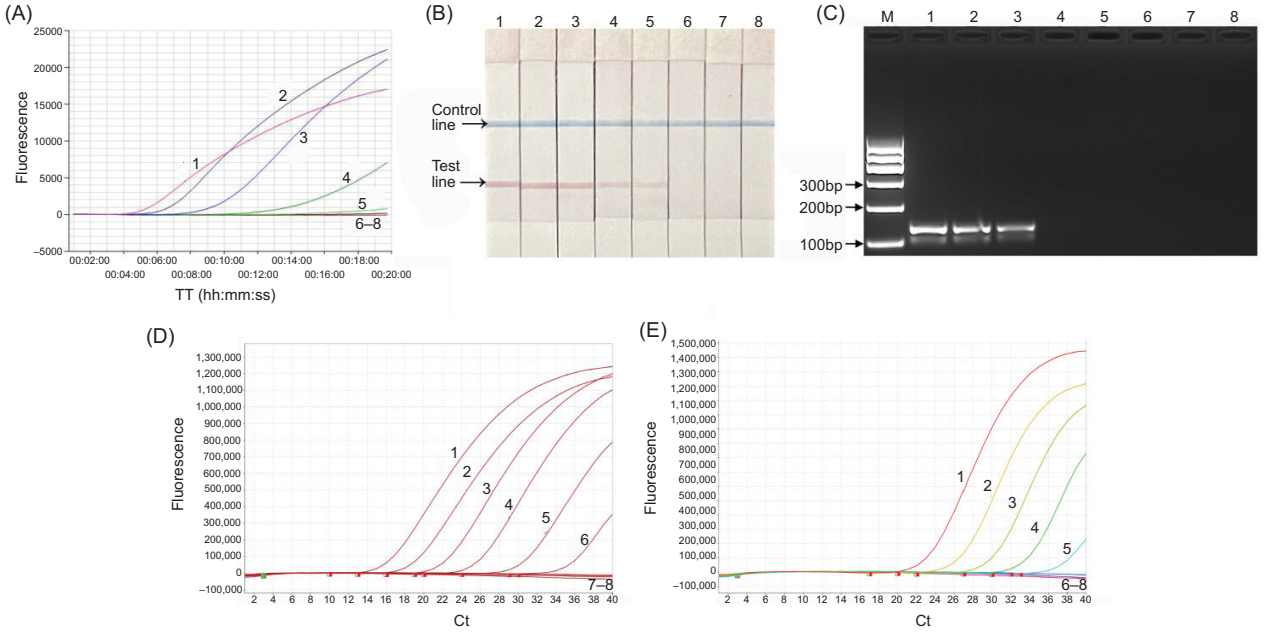

Figure 5. Sensitivity analysis of five detection assays. Line/lane 1, 1.0×10^5 pg/μL; line/lane 2, 1.0×10^4 pg/μL; line/lane 3, 1.0×10^3 pg/μL; line/lane 4, 1.0×10^2 pg/μL; line/lane 5, 1.0×10^1 pg/μL; line/lane 6, 1.0×10^0 pg/μL; line/lane 7, 1.0×10^{-1} pg/μL; and line/lane 8, ddH_2O.

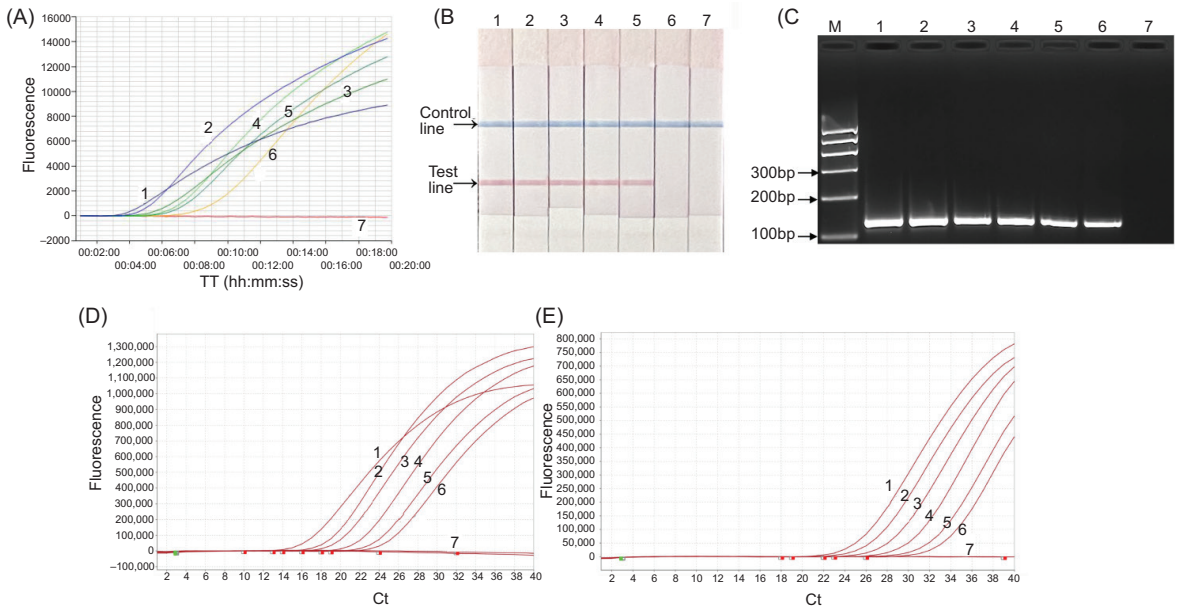

Figure 6. LOD analysis of five detection assays. Line/lane 1, 25.0% chicken+75.0% pork; line/lane 2, 10.0% chicken+90.0% pork; line/lane 3, 5.0% chicken+95.0% pork; line/lane 4, 1.0% chicken+99.0% pork; line/lane 5, 0.5% chicken+99.5% pork; line/lane 6, 0.1% chicken+99.9% pork; and line/lane 7, ddH_2O.

list, while only real-time PCR in GB/T 38164-2019 with Ct values of 31.81, 31.9, and 34 detected chicken in ingredient list in three seasoning samples with chicken-derived ingredients.

Discussion

The authentication of animal-derived ingredients is included in the routine food risk monitoring programs in China (Wang *et al.*, 2020). Many studies on common animal-derived ingredients' detection and authentication are based on PCR technology (Sreenivasan Tantuan and Viljoen, 2021; Uddin *et al.*, 2021; Wang *et al.*, 2021; Yu *et al.*, 2021). In China, PCR or real-time PCR assay is often used as a standard method for the detection of animal-derived ingredients. The specificity of PCR in SN/T 2978-2011, real-time PCR assays in GB/T 38164-2019 and Liu *et al.* (2020) were verified in this study. The real-time PCR assay in GB/T 38164-2019 produced nonspecific amplification curves for several non-target species at 10^5 pg/μL of genomic DNA, suggesting that the template concentration should be restricted within a range of 5–50 ng/μL as referenced in GB/T 38164-2019. PCR in SN/T 2978-2011 also had nonspecific amplification for several non-target species. Although the species-specific differentiation could be realized through sequence analysis, the whole detection process was long and complicated. Although the real-time PCR in Liu *et al.* (2020) had a better performance, it also produced nonspecific amplifications in three different species and could not be applied independently from sophisticated thermal cycling instruments. In contrast to the above assays, the real-time RPA and LFS RPA assays demonstrated satisfactory specificity results with high intra-species conservation and a good inter-species specificity.

The advantages of its simple operation and availability of easy-to-carry equipment establish RPA a promising tool for rapid detection. However, most RPA primers are long (28–35 bp) and are tolerant to mismatches (5–9) bases, and strict primer and probe design principles make it more difficult to identify specific primers for developing RPA assays (Kissenkotter *et al.*, 2020; Munawar, 2022). Furthermore, the natural function of the enzyme involved in the homology-directed repair during the reaction limits the ability of RPA to identify major species with high sequence similarity (Li *et al.*, 2018). During the designing of primers, the presence of differential bases between species close to the 3'-end decreases the nonspecific amplification of non-target species and even inhibit cross-reactions, favoring the specificity of developed RPA assays (Daher *et al.*, 2015). In this study, real-time RPA and LFS RPA were developed to facilitate the detection of chicken-derived ingredients in food. The optimal reaction temperature for LFS RPA was 37°C, aligning with Zhao *et al.*'s (2020) findings but differing from 39°C as reported by Chen *et al.* (2022) and Ivanov *et al.* (2021) and 40°C as reported by Chen *et al.* (2022); Ivanov *et al.* (2021); Kumar *et al.* (2021);

Table 2. Detection results of chicken-derived ingredients in food for sale.

No.	Sample name	Major animal-derived ingredients	GB/T 38164-2019 (Ct)	Liu *et al*., 2020 (Ct)	SN/T 2978-2011	Real-time RPA (TT, mm:ss)	LFS RPA
1.	Beef soup stock	Dehydrated beef and edible beef oil	–	–	–	–	–
2.	Matsutake seasoning	Corn sauce and mushroom powder	–	–	–	–	–
3.	Chicken and corn soup ingredients	Ground chicken	20.51	31.14	+	8:30	+
4.	Compound seasoning with chicken-derived ingredients	Chicken and whole eggs	23.42	28.84	+	10:00	+
5.	Compound seasoning with chicken-derived ingredients	Ground chicken (chicken rack, chicken), edible chicken	23.36	32.23	+	8:30	+
6.	Stewed pork sausage	Pork and chicken	15.30	25.15	+	6:30	+
7.	Chicken sausage	Chicken	12.31	21.51	+	5:30	+
8.	Sausage	Chicken	13.00	22.38	+	6:00	+
9.	Sausage	Pork	13.51	23.43	+	6:00	+
10.	Halal beef-flavored sausage	Chicken and beef	13.12	24.48	+	6:30	+
11.	Pork bone bouillon-flavored soup mix	Pork bone bouillon powder and cooking lard	–	–	–	–	–
12.	Hen soup flavor gumbo	Cooking chicken oil and crunched chicken	21.22	33.15	+	8:30	+
13.	Compound seasoning with chicken-derived ingredients	Crunched chicken and egg yolk powder	31.81	–	–	–	–
14.	Compound seasoning with chicken-derived ingredients	Ground chicken, egg yolk powder, and chicken oil	34.01	–	–	–	–
15.	Compound seasoning with chicken-derived ingredients	Egg yolk powder and crunched chicken	31.90	–	–	–	–
16.	Compound seasoning with chicken-derived ingredients	Chicken, whole egg, and chicken bone extracts	22.91	30.19	+	9:30	+
17.	Sausages	Pork and chicken	15.46	22.14	+	6:20	+
18.	Sausages	Pork and chicken	14.02	20.43	+	6:00	+
19.	Sausages	Pork and chicken	16.00	23.29	+	6:30	+
20.	Sausages	Chicken	13.33	24.00	+	6:30	+

Ct: cycle threshold; TT: threshold time; "-" indicated a negative result; "+" indicated a positive result.

and Zhao *et al*., (2022). Interestingly, Lin *et al*. (2021) revealed no differences in test lines between 37°C, 39°C, and 42°C. The above-mentioned optimal temperatures were in the range of 35–42°C, which were commonly required for RPA assays (Kumar, 2021). Considering that the enzymes used in the RPA assay show activity in a wide range of temperatures, the optimal reaction temperature is largely influenced by the primers and probes used. Next, the specificity of the assays was classified as good intra-species conservation and good inter-species specificity. Amplification reactions occurred in different breeds of chickens, and no amplifications were recorded among other common poultry and livestock. Although the amplification reaction was detected in turkeys, the turkey was not the primary target for adulteration of chicken-derived ingredients.

The developed real-time RPA and LFS RPA as well as SN/T 2978-2011, GB/T 38164-2019, and a previously described protocol (Liu *et al*. 2020) were applied to the

collected real samples to test their applicability. The above-mentioned five assays generated different results only in three samples. Notably, the real-time RPA and LFS RPA, the PCR assay in SN/T 2978-2011, and the real-time PCR described in Liu *et al*. (2020) did not detect chicken-derived ingredients in three seasoning samples, which were labeled with chicken in ingredient lists. In comparison, real-time PCR in GB/T 38164-2019 successfully detected chicken-derived ingredients. These differences could be related to the different sensitivity levels of each assay. Advancements in processing technologies have introduced more aggressive methods that can cause significant DNA fragmentation, potentially leading to undetectable or less sensitive detection of target-derived ingredients (Liu *et al*., 2021). To identify animal-derived ingredients in extensively processed foods, the target amplified fragments should be short enough to accommodate highly degraded DNA. For the developed chicken-specific RPA assays, the amplified fragment is 156 bp, which is suitable for small DNA fragments of approximately 350 bp after processing (López-Andreo *et al*., 2012). Sensitivity analyses showed that the sensitivity of RPA assays reached 10 pg genomic DNA/reaction, which was consistent with that of the real-time PCR method described in Liu *et al.* (2020) and was better than the PCR assay in SN/T 2978-2011. Therefore, it is ideal for detecting chicken-derived ingredients in meat products. Even in complex samples, such as soup stock and chicken essence, the method successfully detected chicken-derived ingredients in just 20–25 min. However, for extensively processed samples (with chicken powder and egg yolk powder as raw materials) or lower content of chicken-derived ingredients, the proposed RPA assays did not detect chicken-derived ingredients. In contrast, the real-time PCR in GB/T 38164-2019 effectively detected the same due to its higher sensitivity but in a time-consuming manner, taking 1 h. The appropriate assay should be able to meet different testing requirements under daily testing.

None of the commercially available chicken products listed their chicken content. A comprehensive identification of the species and content is advocated to provide a more accurate evaluation of food adulteration. The RPA assays developed in this study aimed to provide qualitative results, and future studies are needed to develop quantitative detection methods for chicken-derived ingredients.

Conclusions

In this study, the chicken-specific real time RPA and LFS RPA assays were developed exhibiting high intra-species conservation and good inter-species specificity. The sensitivity and LOD of the two methods were 10 pg genomic DNA/reaction and 0.1% (w/w), respectively. When applied for detecting chicken-derived ingredients in actual samples, such as sausages and compound seasonings, the developed RPA assays showed good performance, compared to that of SN/T 2978-2011 and previously described protocol, suggesting that the developed RPA assays were applicable to extensively processed foods obtained from different breeds of chicken. Compared to the current standards in China, the developed RPA assays showed simple operation and rapid detection characteristics, and took only 20–25 min to produce results. However, it is necessary to explore the quantitative detection of chicken-derived ingredients in the future to provide more comprehensive assessment of food adulteration.

Data Availability Statement

All data generated or analyzed in this study are included in this published article.

Author Contributions

Conceptualization: Jianchang Wang; Methodology: Cang Zhou and Jinfeng Wang; Investigation: Cang Zhou and Jinfeng Wang; Data curation: Minna Chen and Zhenguo Dong; Writing – original draft preparation: Cang Zhou; Writing – reviewing and editing: Jianchang Wang; Funding acquisition: Jianchang Wang; Validation and resources: Qi Fu, Zhenguo Dong, and Xiaoxia Sun; Supervision and project administration: Libing Liu and Xiangdong Xu. All authors reviewed and read the manuscript, and agreed upon to be published version of the manuscript.

Conflicts of Interest

The authors declared no conflict of interest.

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Supplementary

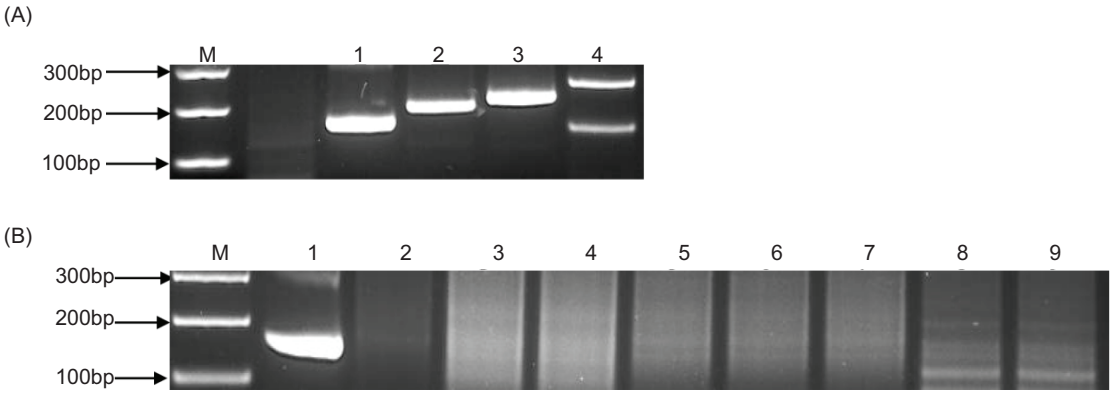

Figure S1. RPA primers screening for chicken. (A) Primers validation. Lane M: DNA marker; lane 1, JF4/JR4; lane 2, JF3/JR3; lane 3, JF2/JR2; lane 4, JF1/JR1. (B) Preliminary specificity validation of JF4/JR4. Lane M: DNA marker; lane 1, chicken; lane 2, fox; lane 3, donkey; lane 4, duck; lane 5, pig; lane 6, horse; lane 7, goat; lane 8, cow; and lane 9, ddH_2O as no template control (NTC).