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A multiplex DNA probe-based method for simultaneous identification of adulteration in meat samples

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ABSTRACT

Meat adulteration and admixing are prevalent malpractices observed in processed and raw meat samples, where the consumption of adulterated meat has been associated with food allergies, financial losses, and consumer distrust. Meat authentication is pivotal to address these concerns. The meat authenticity can be determined through genetic, protein, and immunological markers and advanced detection methods. However, these methods often target a single species and lack the specificity to distinguish closely related species. Here, in the present study, we have developed a multiplex detection method based on the species-specific primers and probes, that can target four meat species in one reaction. The developed method amplifies the mitochondrial genomic regions of chicken, pork, sheep and goat using TaqMan multiplex probe-based RT-qPCR assay. Unique pairs of species-specific primers and probes that target specific mitochondrial DNA (mtDNA) regions of each species were designed and screened for specificity and sensitivity. The detection limit for species identification using the designed primers in real-time qPCR assays was 0.1 picogram per microliter (pg/μL) DNA detected in singleplex reaction and facilitates the simultaneous detection of closely related species, such as goat and sheep. Further, DNA-based probes were utilized in a multiplex real-time qPCR assay to identify chicken, pork, sheep and goat DNA in a single tube reaction. The multiplex assay was validated for raw and processed meat products, demonstrating its applications in ensuring the quality of meat products and safeguarding consumer interests.

1. Introduction

Meat is a rich source of essential micro and macro-nutrients, such as protein, iron, zinc and vitamin B12 (Geiker et al., 2021; Du et al., 2023). The global meat production has significantly increased in recent years (OECD, Food, A.O.o.t.U. Nations, OECD-FAO Agricultural, 2020–2029, 2020.). The majority of this expansion is predicted to be in developing countries, with chicken meat accounting for over half of the total meat production, followed by pork, beef, sheep and goat meat (Charles et al., 2018; Whitton et al., 2021). The demand for meat products continues to rise due to their nutritional composition, desirable organoleptic characteristics such as flavour and palatability, the challenge of obtaining essential nutrients from vegetarian sources and growing consumer preference for high-protein products in the diet (Resurreccion, 2004; Arshad et al., 2018). The growing demand for meat products has increased the risk of deliberate adulteration in various meat-based foodstuffs (Adesogan et al., 2020; Di Pinto et al., 2015). Meat adulteration refers to the practice of admixing or substituting meat of inferior quality or low-priced meat with high-priced meat products to increase profit margins. Due to this, the meat industry faced many instances of false labelling, adulteration and unfair practices for financial gain (Di Pinto et al., 2015; Cao and Li, 2013). In addition to meat adulteration, false labelling also poses a significant risk to both consumer health and market standards (Sammut et al., 2021). Such incidents of false labelling violate religious sentiments, cause economic loss, compromising consumer trust and satisfaction (Li et al., 2020; Visciano and Schirone,

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2021). Numerous cases of adulteration in meat have been reported worldwide (Yamoah, 2017; M. Afifa khatun, A. Hossain, M.S. Hossain, M. Kamruzzaman Munshi, R. Huque, , 2021; Li et al., 7 (2023)). These malpractices and adulterations in raw and processed meat have highlighted the need for more rigorous testing and quality control measures in the meat industry. It has become imperative for regulatory agencies to effectively detect the mixing of meat species and enforce stricter regulations to ensure consumer safety and product authenticity (Sammut et al., 2021).

Based on their genetic, protein and immunological markers, conventional and molecular biology-based techniques have been developed to identify and authenticate meat and meat products. Among the molecular biology methods, PCR-based gene amplification techniques are being extensively used, such as PCR, real-time quantitative PCR (RT-qPCR), multiplex PCR, nested PCR, droplet digital PCR (ddPCR) and isothermal nucleic acid amplification (LAMP-Loop mediated isothermal amplification, SEA-Strand exchange amplification) (Yat-Tung and Shaw, 2018; Yang et al., 2014; Liu et al., 2019). The PCR-based techniques are precise, specific and rapid, thus applicable for high-throughput screening of large numbers of meat samples (Ren et al., 2017; Zahradnik et al., 2015). Other non-PCR-based techniques available for species identification, such as enzyme-linked immunosorbent assay (ELISA), proteomics analysis of proteins and peptides of meat samples using mass spectrometry (Sentandreu and Sentandreu, 2014), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and lipidomics-based identification (Jia et al., 2022; Mandli et al., 2018; Pu et al., 2023) have also been explored. The limitation of protein-based techniques is their dependability on sophisticated instrumentation and inability to correctly detect the processed/cooked samples due to denaturation/degradation of the protein markers (Balakrishna et al., 2019; Mane et al., 2009). In addition to the protein and DNA-based approaches, meat authenticity is analyzed using various infrared spectroscopy techniques such as Raman chemical imaging (RCI), spatially offset Raman spectroscopy (SORS) and surface-enhanced Raman spectroscopy (SERS) (Qu et al., 2022). These methods can be used in combination with PCR-based techniques for better accuracy and reliability in species identification (Mane et al., 2012; Parchami Nejad et al., 2014).

The conventional DNA-based identification methods, such as DNA hybridization, restriction fragment length polymorphism (RFLP) and other protein-based markers pose constraints in distinguishing closely related targets in a mixture of multiple species due to inconsistent protein expression in different tissue types and high genetic similarity (Alikord et al., 2018). The PCR amplification method is suitable for the identification of single or two distantly related species in one reaction via inspection of distinct bands on agarose gel electrophoresis. Multiplexing in conventional PCR is laborious and not successful enough for identifying closely related species. The advanced TaqMan probe-based amplification method enables the detection of multiple species in a single reaction. The TaqMan probe-based amplification is based on the mechanism of a fluorescent-tagged probe complementary to an internal segment of the target DNA. During amplification, the probe is degraded by the polymerase, resulting in the release of reporter and quencher fluorescent moieties enhancing the fluorescence signal. The fluorescence increases with amplification cycles and can be monitored in real-time (Hossain et al., 2023). Various multiplex techniques utilizing the TaqMan probe exist; these include multiplex TaqMan, probe-based TaqMan allelic discrimination (Heissl et al., 2017), high-resolution melting multiplex real-time (Xiu et al., 2020) and digital droplet PCR using TaqMan (Edwards et al., 2023), these methods are typically used for gene screening on a large scale within a large population size. Here, the advantages of TaqMan probe multiplexing to detect meat adulteration in the food industry was utilized to achieve a lower limit of detection specifically tailored for meat authenticity applications (Hossain et al., 2023).

In the present study, a probe-based multiplex detection method utilizing species-specific primers and DNA probes has been developed. Unique pairs of species-specific primers and probes that target specific mitochondrial DNA (mtDNA) regions of each species were designed and screened for specificity and sensitivity. The selected set of primers employed in the assay facilitates the simultaneous detection of closely related species, such as goat and sheep in a single reaction mixture with a detection limit of 0.1 pg/μL concentration of DNA. Further, the DNA-based probes were utilized in a multiplex real-time qPCR assay to identify chicken, pork, sheep and goat DNA in a single tube reaction. The assay was validated using 19 diverse commercial meat products demonstrating its practical applicability and reliability in real-time matrices. The TaqMan multiplex assay was found to be more rapid and sensitive for detecting adulteration in meat products as compared to conventional PCR-based methods. The detection limit was found to be 0.1 pg/μL indicating high sensitivity, efficient for detecting small amounts of adulterants in meat products. The developed methodology has a promising approach for rapid and accurate species identification in meat products and can potentially be a valuable tool for quality control and fraud detection in the food industry. It will enable the regulators, exporters and other stakeholders in the meat industry to apply the advanced probe-based amplification method to check meat authenticity.

2. Materials and methods

2.1. Sample collection, preparation, and DNA purification

The raw meat samples of the target species: chicken (*Gallus gallus*), pork (*Sus scrofa*), sheep (*Ovis aries*) and goat (*Capra hircus*) were purchased from local merchants and stored at −20°C until further use. The ethical approval for the study was obtained from the Institutional Animal Ethics Committee (IITR/IAEC/69/21). Whole genomic DNA (gDNA) was isolated from 25 mg of tissue from raw chicken, pork, sheep, and goat using the DNeasy Blood & Tissue Kit (Qiagen, Germany cat no. #69506) according to the instructions of the manufacturer. Briefly, for DNA isolation, the tissues were homogenised in the buffer provided in the kit and Protinease K was added. Further, the homogenised tissues were incubated at 56-60°C for 2 hrs with periodic gentle tapping. Post incubation, the samples were washed using wash buffer and DNA was eluted using elution buffer. On completion of isolation, the purity and concentration of the isolated gDNA was assessed spectrophotometrically and quantified using the A260/280 absorbance ratio (1.8–2.0) using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). All the isolated DNA were stored at −20°C before use. Similarly, commercial frozen meat products were purchased from the local market outlets and the whole DNA was extracted with a DNeasy Mericon Food Kit (Qiagen, Germany cat no. #69514) using the manufacturer's protocol. The DNA isolation was done from 200 mg of tissue (meat products) with a yield of 100–200 ng/μl and an acceptable A260/280 ratio (1.8–2.0).

Conventional DNA Isolation: Apart from using a commercial DNA isolation kit, to avoid time consumption and to reduce the use of reagents, a one-step DNA isolation method was optimized using Guanidine hydrochloride (GuHCl) lysis buffer (4 M GuHCl cat no #45539, 1 mM DTT cat no. #D9779-50G, 20mMTris-HCl, pH 7.5 cat no. #T2319) (Batule et al., 2020). The lysis buffer was used to homogenize the tissues for 1 minute and the prepared lysate was centrifuged at 8500 rpm for 1 min. After centrifugation, the supernatant was collected in a fresh tube. The protocol was standardized for the detection of chicken, pork, sheep and goat meat samples. The obtained DNA was further quantified using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). The one-step DNA isolation protocol was suitable for the downstream processing of the TaqMan multiplex assay. In this method, the limit of detection (LOD) is, however high, possibly due to high salt concentration which can further be reduced by passing the samples through a desalting column (e.g., DNeasy Mini Spin Column).

Both conventional and commercial kit methods were used for DNA

isolation. However, owing to higher yield and better purity of DNA obtained through the commercial kit, DNA isolated by this method was used for all subsequent RT-qPCR reactions. After completion of each experimental step, excess tissues and generated waste were disposed of in leakproof, disposable bags according to waste disposal guidelines of the "Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEA) for Institutional Animal Ethics Committee (IAEC)".

2.2. Design of species-specific primers and probes

The complete genome sequences for chicken (*Gallus gallus*), pig (*Sus scrofa*), sheep (*Ovis aries*), and goat (*Capra hircus)*, were accessed from the NCBI Database. From these, conserved mitochondrial gene regions were selected for designing species-specific primer pairs: Cytb (Cytochrome *b*) and 12S rRNA for chicken; ND5 (NADH dehydrogenase) and 12S rRNA for goat; ND5 and 12S rRNA for pork; 12S rRNA, Cytb and COX1 (cyclooxygenase) for sheep (Uddin et al., 2021; Giglioti et al., 2022). Using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) multiple pairs of primers were designed for each species and used for further screening as listed in Table S1. To avoid any possible cross-reaction with other non-target species, the selected primer pairs were checked for species specificity *in silico* and those showing no cross-reactivity were selected for further experimentation.

Probes: For the simultaneous detection of four targets, 04 species-specific DNA probes were designed for the screened sets of primers. The probes were labelled with four different reporter molecules ABY (580 nm) for chicken, JUN (617 nm) for sheep, FAM (517 nm) for goat, and VIC (551 nm) for pork. Each probe contained a distinct reporter fluorescence molecule at the 5′ and a QSY or NFQ-MGB at the 3′ end to enable the detection of four target meat species at a time. The fluorescence reporters were selected having distinguishable wavelength differences to avoid any overlapping during the detection. Mustang purple was used as a passive reference dye in the TaqMan multiplex master mix (Thermo Fisher Scientific, USA cat no. #4461882) for signal normalization during detection. The primers and TaqMan probes were synthesized by Integrated DNA Technologies, USA and Thermo Fisher Scientific, USA respectively as listed in Table 2.

2.3. SYBR-based Real-Time Quantitative PCR (RT-qPCR)

Real-time qPCR was performed using ABI QuantStudio 6 Flex Real-time qPCR (Thermo Fisher Scientific, USA) instrument. The reaction mixture contained SYBR green master mix (Thermo Fisher Scientific, USA cat#A25742), forward primer (800 nM), reverse primers (800 nM), and gDNA (1000 pg/µl) as templates. The amplification was carried out with an initial denaturation step at 95°C for 1 minute, then at 95°C for 15 s and 60°C for 1 min followed by 40 cycles and a final melt curve stage (Li et al., 2019). In each reaction setup, a no template control (NTC) with nuclease-free water (Thermo Fisher Scientific, USA cat no. #AM9932) was run. The threshold cycle (Ct) obtained through automatic baseline values and melting curves were key determinant factors in this RT-qPCR assay.

2.4. Determination of limit of detection

The sensitivity of the assay was determined using a 10-fold serial dilution from 10000 pg/µL to 0.01 pg/µL of the DNA. To determine the LOD for each primer pair, singleplex RT-qPCR was carried out using SYBR green chemistry for each species. RT-qPCR reactions for the four species were carried out as follows: initial denaturation at 95°C for 1 min, at 95°C for 15 s and 60°C for 1 min followed by 40 cycles and with melt curve stage. To determine product size, the amplified RT-qPCR products were validated on a 3% agarose gel electrophoresis similar to conventional PCR. The gel containing ethidium bromide was run in TBE buffer (pH 7.4) at 55 V for 60 minutes and visualized using a Biorad ChemiDOC system. The respective bands were compared with the results obtained from *in silico* analysis (Fig. S3. a-d).

2.5. Standard curve plot

For each species, standard curves were plotted (using GraphPad Prism 8.4.2) to determine the linearity of the reaction using Ct scores generated from DNA isolates of chicken, pork, sheep and goat. The DNA samples were 10-fold serially diluted from 10000 pg/µL − 0.01 pg/µL and the Ct scores were plotted against the logarithmic DNA concentrations (pg/µl) (GraphPad Prism 8.4.2) for each screened primer pair and the efficiency of the method was determined from the standard curve (Fig. S2. and Table S2(a–d). The reaction efficiency (E) of the assay was computed based on the slopes of the standard curves according to (Druml et al., 2015) and the efficiency values were found to be within the acceptable range of 80–110%.

2.6. Singleplex RT-qPCR assays optimization

Singleplex RT-qPCR assays were developed to precisely target the DNA of chicken, pork, sheep and goat before being applied to the multiplex RT-qPCR assay. Reaction conditions were optimized according to the manufacturer's manual to standardize the concentration of primers and probes. The reaction mixture contained 900 nM of each primer, 250 nM of the probe, nuclease-free water (Thermo Fisher Scientific, USA cat no. #AM9932), TaqMan multiplex master mix (Thermo Fisher Scientific, USA cat no. #4461882), and DNA (1000 pg/µl) in a final reaction volume of 5 µL. For determining of Multiplexing RT-qPCR assay was performed using conditions as provided by manufacturer with a hold stage at 95°C for the 20 s, followed by 40 cycles at 95°C for 1 s and 60°C for 20 s. The isolated DNA from raw chicken, pork, sheep and goat were used as positive and the no template control (NTC) as negative controls for every reaction. All RT-qPCR reactions were performed in triplicate (n = 3) using QuantStudio 6 Flex Real-time qPCR instrument (Thermo Fisher Scientific, USA), all singleplex RT-qPCR assays were carried out with automatic baseline and threshold parameters.

2.7. TaqMan multiplex assay conditions

Multiplex RT-qPCR using TaqMan probes was carried out using Quant Studio 6 Flex real-time PCR system (Applied Biosystems, USA) in a 10 µL total volume reaction mixture. According to the manufacturer's protocol, the reaction contained TaqMan multiplex master mix (Thermo Fisher Scientific, USA cat no. #4461882), forward primers (900 nM), reverse primers (900 nM), probes (250 nM), and DNA templates (1000 pg/µl) of all the 04 species. Multiplex RT-qPCR assay was performed with the reaction conditions as mentioned in section 2.6. DNA isolated from non-adulterated samples was used as positive and no template control (NTC) was used as the negative control for each reaction. All the reactions were repeated in triplicate (n = 3).

Similarly, to validate the applicability of the developed TaqMan multiplex assay in real food matrices, meat products (19 samples) such as nuggets, meatballs, sausages, and hams, etc. were procured from the local markets and e-commerce portals. The market samples were subjected to multiplex RT-qPCR using the protocol as discussed in section 2.6.

2.8. Data acquisition and statistical analysis

All the datasets, and amplification curves were acquired using ABI QuantStudio 6 Flex Real-time qPCR software (Thermo Fisher Scientific, USA) and analysed using MS Excel. Standard curves were generated using GraphPad Prism 8.4.2. All the statistical calculations (mean, standard deviation) were carried out using GraphPad Prism 8.4.2. The mean value obtained refers to the quantity indicating the central tendency of the data sets. Standard deviation (SD) was calculated to

Table 1
Sensitivity and linearity results for the selected primers with 10-fold serially diluted DNA (10000 pg/µl − 0.01 pg/µl) along with Standard error mean for determination of LOD.

DNA Concentration	Ct mean ± Standard Error Mean (SEM) for varying DNA concentrations			
	Chicken_CYTB	Goat_ND5	Sheep_COX1	Pork_ND5
10000 pg/µl	13.91 ± 0.70	11.72 ± 0.39	13.94 ± 0.15	13.21 ± 0.30
1000 pg/µl	17.62 ± 0.55	14.84 ± 0.59	17.408 ± 0.177	17.18 ± 0.45
100 pg/µl	21.39 ± 0.23	19.19 ± 1.99	20.600 ± 0.399	23.11 ± 3.05
10 pg/µl	25.23 ± 0.45	22.99 ± 1.97	24.17 ± 0.274	26.78 ± 3.30
1 pg/µl	28.14 ± 0.25	26.05 ± 1.87	28.028 ± 0.171	29.99 ± 1.36
0.1 pg/µl	30.96 ± 0.36	29.49 ± 1.23	31.196 ± 0.231	30.94 ± 1.41
0.01 pg/µl	32.59 ± 0.63	32.33 ± 1.65	32.89 ± 0.446	ND
NTC	ND	ND	ND	ND

determine the degree of dispersion of data distribution and the degree of its deviation from the arithmetic mean. All the experiments using different selected sets of primers were performed in triplicate (n = 3).

3. Results & Discussion

3.1. Real-time detection of meat species

Based on the market share of the meat products and commonly used for consumption, four species, chicken, pork, sheep and goat were selected as target meat species for the present study. The mitochondrial barcoding gene regions specifically, Cytochrome *b* (Cytb) and 12S rRNA for chicken; NADH dehydrogenase (ND5) and 12S rRNA for goat; ND5 and 12S rRNA for pork; 12S rRNA, Cytb, cyclooxygenase (COX1) for sheep were selected as the target regions for amplification due to their stability under food processing treatments. The species-specific primer pairs were designed using the NCBI Primer Blast tool and NCBI GenBank Database selected for amplification (Table S1) (Uddin et al., 2021; Giglioti et al., 2022). The mitochondrial region has higher thermal stability over the genomic DNA and is present in thousands of copies per DNA (Toews & Brelsford, 2012). As meat products are exposed to extreme temperatures during cooking/processing, there is a high risk of DNA being broken into small fragments (Musto, 2011). Such primer pairs designed using the mitochondrial region increase the chances of successful amplification while studying the species. Thus, making it an ideal barcoding region for species identification in processed as well as non-processed meats. The primers were selected based on the general criteria of qPCR primer designing steps including high GC content (50–60%), T_m value difference not more than 3°C, and no 3′ end complementarity. For chicken, 03 primer pairs from the Cytb region and 02 sets from the 12S rRNA region; for goat species 05 primer pairs from the ND5 gene region and 02 sets from the 12S rRNA region; for pork 03 primer sets from the ND5 gene region, and 02 sets from the 12S rRNA region; for sheep 04 primer pairs from Cytb region and 02 sets each from Cox1 and 12Ss rRNA regions were selected Table S1). To select the best performing primer pairs based on LOD and sensitivity, each primer pair was used for amplification of its target region of the related meat species. The LOD of each species was determined using 10-fold serially diluted DNA (10000 pg/µl − 0.01 pg/µl) and amplified using RT-qPCR assay with SYBR green chemistry. At the highest concentration of DNA (10000 pg/µl), the Ct scores were 14.61 ± 0.27 for chicken, 11.53 ± 0.03 for goat, 13.39 ± 0.12 for pork, and 13.94 ± 0.15 for sheep. Similarly, at the lowest concentration of the DNA (0.01 pg/µl), the Ct scores were in the range of 30–32 for chicken, goat and sheep. However, for pork, the Ct was 31.57 ± 0.11 at 0.1 pg/µl concentration (Table 1). All the designed primer sets for each meat species were screened for the efficiency of the method and based on linearity of the standard curve of amplification one set of primer was selected for further studies (Fig. 1a–d). For species chicken, goat and sheep, the LOD obtained was 0.01 pg/µl whereas for pork, it was 0.1 pg/µl. The Ct scores of the selected primer pairs have been summarised in Table 1.

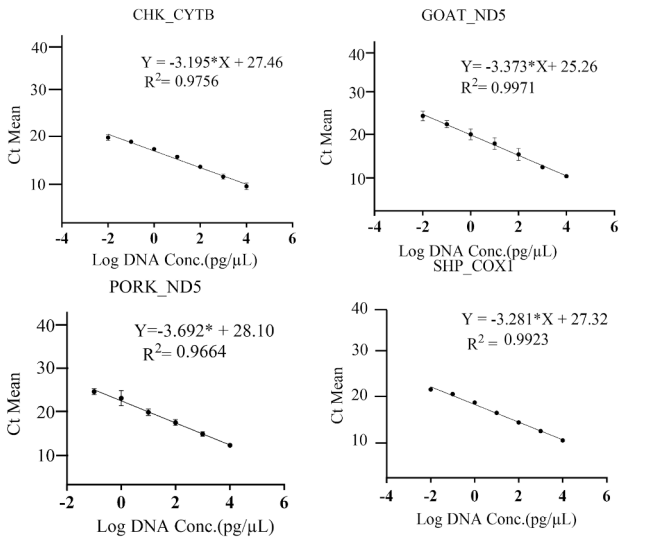

Fig. 1. Species-specific efficiency of selected primers (a-d): standard curves for the selected sets of primers CHK_CYTB, GOAT_ND5, PORK_ND5, SHP_COX1 were plotted against the obtained Ct mean and 10-fold serially diluted DNA (10000 pg/µl − 0.01 pg/µl) extracted from (a) chicken, (b) goat, (c) pork, (d) sheep. The standard curves were plotted using GraphPad Prism 8.4.2.

3.2. Specificity of the designed primers

To employ these primers for multiplexed detection, species-specificity was checked, as this is an essential characteristic to avoid cross-interaction between the primer, probes, and non-target species. Therefore, the amplification of selected primer pairs with non-target

Table 2
The table summarizes the species-specific probes of screened primers selected for TaqMan Multiplexing Assay using QSY and NFQ-MGB quenchers. The table indicates that ABY and JUN were paired with the QSY quencher and FAM and VIC were paired with the NFQ-MGB quencher.

Species	Primers (5′-3′)	Amplicon Size (bp)	Target gene	Probe
Chicken	Forward: AGCAGACACATCCCTAGCCT Reverse: TAGGAGCCGTAGTATAGGCCTC	153 bp	Cytochrome *b*	5′ABY-CCGGAATCTCCACGCAAACGGC-QSY
Sheep	Forward: CTAGCAACGCTTCATGGG Reverse: GCCTCCGACTGTGAAAAGA	87 bp	COX1	5′JUN-CCTAGGGCTCATATTATGGCAGGAGATCA-QSY
Goat	Forward: TTCTTCTCTTGCACTAACCAC Reverse: GAAGCTGAGCGATAATTTAAGG	229 bp	ND5	5′ FAM- AAGGCACATGAAACGAC-NFG-MGB
Pork	Forward: AACCCATTCGCCTCACTCAC Reverse: GGCGTAGGATACGGTGGTTT	126 bp	ND5	5′ VIC- TAGATGTTTGAGTTGGATATTAT-NFQ-MGB

Table 3
Comparison of Ct values for singleplex and multiplex reaction.

Target Genes	Singleplex Reaction	Multiplex Reaction
	Ct mean ± Standard Error Mean (SEM)	
CHK_CYTB	21.697 ± 0.95	23.435 ± 0.315
GOAT_ND5	19.34 ± 0.132	24.374 ± 0.376
SHP_COX1	20.862 ± 1.80	23.731 ± 0.335
PORK_ND5	20.230 ± 0.413	20.030 ± 0.904

DNA samples was performed with four DNA samples in one reaction mixture. As shown in Fig. S1(a–d), the specificity of species-specific primers was cross-tested with 1000 pg/µl DNA concentration for each meat species chicken, pork, sheep and goat. The single tube reaction was performed with selected sets of primer pairs using the conventional PCR method which resulted in four separate DNA bands as observed on 3% agarose gel (Fig. S1(a–d). Primer pairs along with their target species were used as the positive control (PC). The molecular weight of amplified products matched with their product size as per the *in silico* analysis. From these results, the primers were confirmed to be species-specific showing no cross-reactivity with the non-target species thus acceptable for multiplexed detection with TaqMan probes.

3.3. Multiplex RT-qPCR assay and its application in processed meat products

For multiplex detection of the target species, probes were designed based on the unique primers identified as previous results (Table 2). The primary step for reaching the goal of multiplex identification is to achieve precise target discrimination by probe-tagged fluorescence colours. We verified the performance of the designed multiplex probes in a singleplex reaction first to determine the efficiency of probes for their specific primer pair. Similarly, along with the amplification curves, Ct values obtained were also clearly distinguishable by different fluorescent colours of probes for four meat species (Table 3, Fig. 2a-e). The Ct scores for multiplex reaction 23.25 ± 0.06 for chicken, 24.16 ± 0.12 for goat, 23.53 ± 0.30 for sheep and 20.52 ± 0.005 for pork were consistent with the singleplex amplification assay as tabulated in Table. 3. This indicated the suitability of the assay for determining adulteration in processed meat samples. Further, to validate the applicability of the developed TaqMan multiplex RT-qPCR assay in food matrices, 19 samples consisting of minced, preserved, processed meat products, including salami, pepperoni, nuggets, sausages, meatballs, seekh kebab of chicken, pork, sheep and goat were collected from the local market. The isolated DNA from raw samples was used as positive controls to determine adulteration in the samples by multiplex assay.

Adulteration of chicken meat was observed in 02 goat-based products (goat seekh kebab and goat meatball), 02 pork-based products (pork momos and pork classic salami); goat meat adulteration in chicken pepperoni salami, Sheep meat adulteration in chicken pepperoni salami. Major adulteration of pork was also observed in 04 meat products chicken pepperoni salami, goat seekh kebab, goat meatball, and sheep pepperoni (Table 4). In these admixed samples, the verification of the presence of other meat species was done by comparing the Ct scores obtained previously and found significant to raw samples. With the developed multiplex method, detection of all admixed species was

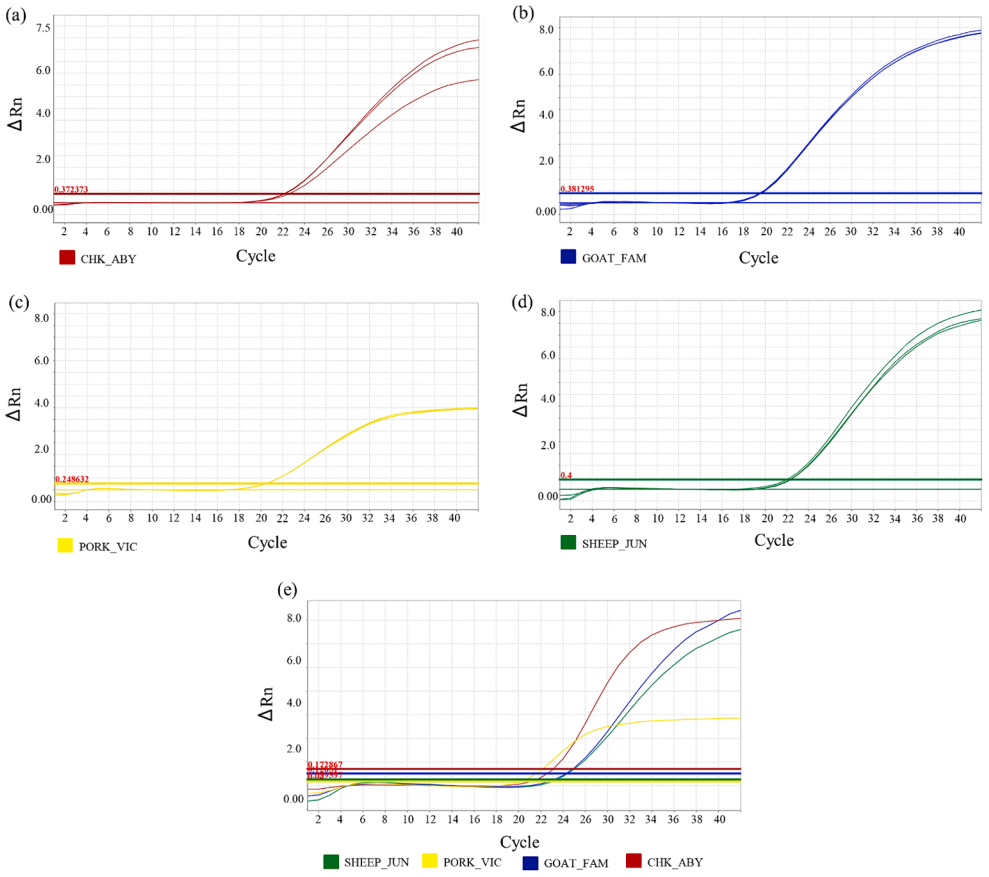

Fig. 2. Amplification of four meat species using TaqMan singleplex and multiplexed assay. (a-e). Amplification curves for singleplex was generated during TaqMan singleplex RT-qPCR assay: (a) chicken, (b) goat, (c) pork, (d) sheep (e) multiplex amplification curves for detection of four different target species obtained where each curve represents the amplification profile of a specific DNA probe of the target gene in the multiplex RT-qPCR assay. The difference is distinguishable in the form of different colour curves as well as Ct. The x-axis represents the number of cycles of PCR amplification and the y-axis represents the fluorescence intensity of each probe. The assay was able to detect all the targeted species.

Table 4
TaqMan probe-based multiplex assay for commercial meat products.

Sample	Detected species				Ct scores			
	Chicken	Goat	Sheep	Pork	Chicken	Goat	Sheep	Pork
Chicken raw meat (PC)	+	–	–	–	21.01 ± 0.83	–	–	–
Chicken pepperoni salami	+	–	+	+	18.67 ± 0.23	–	28.29 ± 2.67	22.70 ± 0.31
Chicken nuggets	+	–	–	+	19.85 ± 0.27	–	–	27.24 ± 0.63
Chicken momos	+	–	–	–	18.54 ± 0.11	–	–	–
Chicken seekh kebab	+	–	–	–	18.34 ± 0.63	–	–	–
Chicken hariyali kebab	+	–	–	–	17.55 ± 0.58	–	–	–
Chicken sausage	+	–	–	–	18.50 ± 0.95	–	–	–
Chicken keema	+	–	–	–	17.44 ± 1.57	–	–	–
Goat raw meat (PC)	–	+	–	–	–	19.74 ± 2.01	–	–
Goat seekh kebab	+	–	–	+	20.30 ± 0.89	–	–	18.12 ± 0.89
Goat meatball	+	–	–	+	20.23 ± 0.53	–	–	18.36 ± 0.64
Sheep raw meat (PC)	–	–	+	–	–	–	19.23 ± 0.233	–
Sheep pepperoni	–	–	+	+	–	–	19.06 ± 0.31	26.10 ± 4.76
Pork raw meat (PC)	–	–	–	+	–	–	–	21.57 ± 0.28
Pork sausage	–	–	–	+	–	–	29.29 ± 0.05	21.21 ± 0.67
Pork ham	–	–	–	+	30.92 ± 0.74	–	–	19.42 ± 0.35
Pork momos	+	–	–	+	24.44 ± 1.12	–	–	19.82 ± 0.78
Pork classic salami	+	–	–	+	20.55 ± 0.84	–	–	19.40 ± 0.45
Pork bacon	–	–	–	+	30.4 ± 0.68	–	–	18.44 ± 0.82

Note: + Presence, - Absence, PC; positive control.

possible in 45 min run-time compared to the SYBR green chemistry or conventional PCR followed by agarose gel analysis. Thus, the developed detection method to identify pork, chicken, sheep and goat in a single reaction can help in the fast identification of meat adulteration, admixing and identification in raw as well as processed meat samples.

4. Conclusion

Adulteration and admixing of meat products are significant concerns for the food industry and consumers due to the associated potential health risks and ethical concerns. To addess these concerns, a TaqMan multiplex probe-based RT-qPCR assay has been developed for the simultaneous detection of multiple meat species, such as chicken, pork, sheep and goat in a single reaction. This method can be used for the authentication of both raw and processed meat samples. The method is based on unique primers and TaqMan probes specific for each meat species to detect and quantify DNA from respective species in a single reaction. The method exhibited high specificity and sensitivity with a detection limit of 0.1 pg/μL DNA for each species in a multiplex reaction. Overall, the multiplex TaqMan-based method will enable the regulators, exporters and other stakeholders in the meat industry to apply the advanced probe-based amplification method to check the authenticity and identify the admixing in meat products.

CRediT authorship contribution statement

Smriti Singh Yadav: Investigation, Methodology, Visualization, Formal analysis, Writing - original draft. **Ramsha Tariq:** Validation, Methodology, Investigation. **Prabeen Kumar Padhy:** Writing – original draft, Visualization, Methodology, Investigation. **Apoorva Saxena:** Writing – review & editing, Methodology. **Pawankumar Rai:** Writing – review & editing, Visualization. **Vikas Srivastava:** Writing – review & editing, Conceptualization. **Navjot Kumar:** Writing – review & editing, Conceptualization. **Sandeep Kumar Sharma:** Writing – review & editing, Conceptualization. **Smriti Priya:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2024.100200.

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