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ELISA-based detection of fresh and frozen–thawed lamb meat: a promising analytical approach for meat authentication†

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Fresh/chilled meat differs in quality from frozen–thawed meat, and consumers prefer fresh meat over frozen–thawed meat. Differentiating between the two types of meat is an important part of the meat quality control system. This study aimed to develop an ELISA based on cytoplasmic antioxidant enzyme superoxide dismutase (SOD) as a biomarker. IgGs were raised in experimental animals and purified using immunoaffinity chromatography. The assay was optimized with guinea pig anti-SOD pAb as the capture antibody and rabbit anti-SOD pAb as the detection antibody. The assay showed excellent performance for differentiation, as the ROC area under the curve values were >0.9. A sensitivity of 95.8% and specificity of 95.0% were observed at a positive percentage (PP%) criterion value of 52.752. Sandwich ELISA results showed a significant difference between the chilled and repeatedly frozen–thawed lamb samples. Lamb meat stored for five days in a chiller showed a PP% value below the threshold PP% value, indicating that the assay can differentiate meat until 5 days of chiller storage. In addition, a thawing time of more than 18 h at 4 ± 1 °C is required to differentiate between both types of lamb meat with a size of 500 g. The developed ELISA can be applied at various points in the meat value chain to differentiate fresh meat from frozen–thawed meat.

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1 Introduction

Food authentication in the context of meat and meat products is essential in addressing the growing problem of food fraud in the meat food sector. The meat and meat processing industry has witnessed numerous types of fraudulent practices so far.[1] One common practice in the meat industry involves falsely labeling or misrepresenting the origin, quality, or content of meat products.[2] This can include passing off lower-quality meats as premium cuts misleading consumers about where the meat comes from, labeling frozen–thawed meat as fresh/chilled meat, *etc.* Delivering the right information about the product such as its type, nutritional composition, and preservatives added if any through mandatory labeling of food items is the right of consumers.[3] Presence of any kind of false labelling or misguiding information on the food label is a critical public health concern and illegal.[4] Many countries have legal restrictions against labeling meat that has been frozen and thawed as 'fresh'.[5,6] However, loopholes in these laws are exploited by some to label frozen–thawed meat as 'fresh/chilled' meat. Studies have reported that 8–15% of labeled fresh meat was frozen–thawed meat.[5] Since food fraud including false labelling decreases the confidence of the consumers, it is important to address the issue to protect the credibility of the sector as well as to protect the confidence of consumers.

The global market for frozen lamb, as measured by its monetary value, was assessed at $5.9 billion in the year 2022.[7] Forecasts indicate that this market is anticipated to expand to $10.7 billion by the year 2032, demonstrating a Compound Annual Growth Rate (CAGR) of 6.3% from 2023 to 2032.[8] Lamb or mutton is one of the favourite meat options in many countries. Globally, Australia and New Zealand act as the major exporters of sheep meat (either mutton or lamb) with a share of 38 and 33%, respectively.[9] Of this, around 62% is exported in frozen form, and hence freezing plays an important role in the global lamb/mutton supply chain.[9] From years ago, freezing technology was an integral part of the global meat industry, and has been employed for the preservation/transport of meat across the globe. Freezing and subsequent thawing affect the quality of meat significantly.[10] Repeated freezing and thawing can cause the loss of nutrients including cellular enzymes through the drip, which originates through the cell damage caused by the formation of ice crystals during the freezing

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process. In addition to nutrient loss, protein and lipid oxidation under refrigeration has a negative impact on the texture and colour stability of meat products.[11] Protein oxidation results in a loss of amino acids, protein-polymer formation, loss of solubility, and increased carbonyl groups in proteins, while lipid oxidation leads to the development of malondialdehyde (MDA) and cholesterol oxidation products, resulting in the creation of oxy-free and lipid-free radicals.[12,13] Furthermore, freezing and subsequent thawing of meat accelerate microbial deterioration and lipid peroxidation processes that release toxic compounds such as MDA, 4-hydroxynonenal (4-HNE), and propanol, of which 4-NHE is the most mutagenic and toxic.[14] So, fresh meat possesses superior quality attributes compared to frozen–thawed lamb meat, so it should be labeled accordingly. However, frozen meat is an integral component of the global lamb supply chain, as production and consumption of lamb are concentrated in different parts of the globe, and transportation of frozen lamb is unavoidable.[2,15] During frozen transportation, one should ensure the uninterrupted cold chain and avoid repeated freezing and thawing due to power failure. To ensure the meat transported has not undergone repeated freezing–thawing and subsequent quality loss, there are limited analytical techniques available currently.[16] There is a scope of developing analytical methods to resolve the issue. Rahman *et al.* (2024) reported that cytoplasmic SOD can act as an excellent biomarker for differentiation of fresh and frozen–thawed goat meat.[17] Jangir and co-workers developed a colour-based assay for the differentiation of fresh and frozen–thawed buffalo meat based on the superoxide dismutase enzyme biomarker.[18] Cu–Zn superoxide dismutase, a cytoplasmic antioxidant enzyme, was proven to be a reliable biomarker for this purpose as the activity of the enzyme was higher in samples obtained from frozen thawed meat as compared to chilled meat.[18] During the freezing of meat, ice crystals are formed.[19] These ice crystals possess sharp edges capable of inflicting damage to the cell membranes.[20] Consequently, this damage can lead to the leakage of cytoplasmic contents from the cell in the form of drip. Therefore, the drip contained biomolecules present in the cytoplasm at a higher level. The enzyme activity of the sample prepared from frozen thawed meat will be higher than that of chilled meat, since there is limited scope for cellular injury and subsequent escape of enzymes from the cytoplasm. There is potential to develop enzyme biomarker-based immunoassays, as enzyme biomarkers represent an accurate and underexplored area for this purpose. Immunoassays offer distinct advantages over alternative techniques, including high throughput screening capabilities, specificity, selectivity, cost effectiveness, and simplicity. These advantages underscore the importance of employing immunoassays for meat authentication purposes.[21]

The current study aimed at the development, optimization, and evaluation of sandwich ELISA for differentiation of frozen–thawed and chilled/fresh lamb meat, based on a cytoplasmic antioxidant enzyme, superoxide dismutase, biomarker. To the best of our knowledge, this study represents the inaugural application of the ELISA technique for discriminating between frozen–thawed meat and non-frozen (chilled/fresh) meat.

2 Experimental

2.1. Materials and apparatus

The reagents used in the current study were of analytical reagent grade and procured from standard firms. MaxiSorp ELISA plates were obtained from Nunc (Nunc, Roskilde, Denmark), and conical centrifuge tubes, microcentrifuge tubes, and micropipette tips were procured from Tarsons (Kolkata, India). Whatman No. 1 filter paper was obtained from Whatman Cytiva (Little Chalfont, Buckinghamshire, UK). Reagents employed in this study include polyvinylpyrrolidone K-30 (HiMedia, Maharashtra, India), bovine serum albumin (SRL, Mumbai, India), Tween 20 (Amresco, OH, USA), sodium citrate, citric acid, sodium bicarbonate (Loba Cheme, Mumbai, India), potassium chloride, sodium chloride, potassium dihydrogen phosphate, disodium phosphate, disodium hydrogen phosphate (SRL, Mumbai, India), H_2SO_4, H_2O_2 (Merck, Bengaluru, India), and *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Antibodies used for assay were the goat anti-guinea pig IgG–HRP conjugate and goat anti-rabbit IgG–HRP conjugate (Santa Cruz Biotechnology, Dallas, TX, USA). The standard antigen was superoxide dismutase from bovine erythrocytes (Sigma-Aldrich, St. Louis, MO, USA). Adjuvants were procured from Sigma-Aldrich (St. Louis, MO, USA). The protein concentration was estimated using a BCA assay kit (RealGene, Baden-Wűrttemberg, Germany).

Lamb meat was kept frozen at -20 ± 2 °C in a deep freezer (Elanpro, Gurugram, India). The chiller storage and thawing procedures were done at 4 °C in a refrigerator (Whirlpool, MI, USA). Immunoglobulins (IgG) were separated by immunoaffinity chromatography using a protein-A agarose column (RealGene, Baden-Wűrttemberg, Germany) for purification of IgG antibodies. A 135 kDa dialysis membrane was procured from HiMedia (Mumbai, India). Immunoglobulins were lyophilized using a bench top lyophilizer (IlshinBiobase, Dongducheon, South Korea). ELISA results were measured at 492 nm using a microplate reader (Biotek, WA, USA), and results were obtained using Gen5 3.09 software version 3.09.07.

2.2. Methods

2.2.1. Raising of antisera for polyclonal antibodies. Two guinea pigs (Dunkin Hartley strain) and two albino rabbits (New Zealand White) were immunized with bovine Cu–Zn superoxide dismutase. A protein dose of 200 μg per animal was emulsified in Complete Freund's Adjuvant and administered intramuscularly and subcutaneously at distinct sites. For booster doses, 100 μg of protein was emulsified in Incomplete Freund's Adjuvant and was administered after 21, 35, and 42 days. Seven days after administration of the final booster dose, from 12 h fasted animals, blood was collected under 20% (200 mg ml^{-1}) urethane anaesthesia. Blood was drawn from the ear vein (for rabbits) and retro-orbital sinus (from guinea pigs). The collected blood was kept at an angle of 45° and left undisturbed at 4 °C overnight. The next day, the serum was carefully pipetted out and collected in 2 ml microfuge tubes. Furthermore, it was centrifuged at 2000*g* at 4 °C to obtain clear serum. The serum

obtained contained anti-SOD polyclonal antibodies, which were confirmed using a dot blot assay. All experiments were approved by the Institute Animal Ethics Committee (IAEC/20.06.2024/S1) as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

Immunoglobulins (IgG) were separated by immunoaffinity chromatography. In brief, the column was preequilibrated with 0.15 M NaCl (pH 7) and 2 ml of antisera diluted with 2 ml Tris–HCl (pH 8) was added. Unbound proteins were removed using Na_2HPO_4 (pH 7) and 0.15 M NaCl. Elution was done with 0.1 M glycine (pH 3) elution buffer. The eluate was further purified by dialysis by using a molecular cut-off (135 kDa) dialysis membrane. The obtained immunoglobulins were lyophilized at −55 °C and 100 mTorr pressure and reconstituted to the desired volume with 100 mM PBS, pH 7.4. The protein concentration was estimated by BCA assay.

2.2.2. ELISA protocol development

2.2.2.1. Buffer preparation. The buffers used in the sandwich ELISA protocol were prepared according to standard procedures. 50 mM carbonate buffer of pH 9.6 was prepared as a coating buffer. 2% polyvinylpyrrolidone (PVP) in 10 mM PBS at pH 7.4 was used for blocking, and one half dilution of the blocking buffer (1% PVP) was used as the dilution buffer throughout. Washing was done with PBST, prepared by the addition of 0.05% (v/v) Tween 20 to 10 mM PBS (pH 7.4). Sodium citrate buffer at pH 3.6 was used as a substrate buffer, and OPD was used for developing the substrate solution. 2 M H_2SO_4 was used to stop the reaction.

2.2.2.2. Optimization of antigen concentration. Indirect ELISA was performed for optimization of the concentration of SOD (antigen of interest). MaxiSorp 96-well ELISA plates were coated with the serially diluted SOD protein, and 1× PBS (100 mM) was used as a negative control. The coating was done overnight at 4 °C. Washing was done with PBS-T, followed by blocking with 300 µl of blocking buffer (2% PVP) for 60 min at 37 °C. After washing, the primary antibodies (rabbit anti-SOD pAb and guinea pig anti-SOD pAb) were added by serial dilution (from 1 : 250 to 1 : 36 000), and plates were incubated at 37 °C for 60 min. After washing, the respective anti-antibody conjugates (anti-rabbit IgG–HRP conjugate and anti-guinea pig IgG–HRP conjugate) were added to each well at 1 : 1000 dilution and incubated at 37 °C for 60 min. Plates were washed, and a developing solution was added, followed by incubation at 37 °C for 15 min. The reaction was terminated by the addition of 100 µl of stop solution, and the absorbance was measured at 492 nm using a microplate reader (Biotek, WA, USA).

2.2.2.3. Optimization of the sandwich ELISA protocol. The optimization of sandwich ELISA was conducted in two distinct configurations. In the first configuration, the capture antibody employed was rabbit anti-SOD pAb, complemented by the detector antibody, guinea pig anti-SOD pAb. Conversely, in the second configuration, guinea pig anti-SOD pAb was utilized as the capture antibody, juxtaposed with rabbit anti-SOD pAb serving as the detector antibody. ELISA plates were coated with 100 µl of guinea pig or rabbit anti-SOD pAb, serially diluted two-fold (ranging from 1 : 200 to 1 : 409 600 in coating buffer) and incubated overnight at 4 °C. Washing with a wash buffer (PBS-T) was performed three times to remove unbound antibodies, and each well was blocked with 300 µl of blocking buffer (2% PVP) and incubated at 37 °C for 60 min as before. After washing three times, 100 µl of 10 µg ml^{-1} diluted SOD protein in a diluent (positive control; optimised by indirect ELISA) and 1× PBS (as a negative control) were added to each plate well. The plates were then incubated for sixty minutes at 37 °C. Following three rounds of washing with wash buffer (PBS-T), 100 µl of either guinea pig anti-SOD pAb or rabbit anti-SOD pAb that had been serially diluted two times (1 : 500–1 : 64 000) in the diluent was added to each well of the corresponding plates. The plates were then incubated at 37 °C for 60 minutes. After three washes with PBS-T wash buffer, 100 µl (1 : 1000 dilution) of the goat anti-rabbit IgG–HRP or goat anti-guinea pig IgG–HRP conjugate was applied to each well of the corresponding plates. The plates were then incubated for sixty minutes at 37 °C. After four rounds of washing with PBS-T wash buffer, 100 µl of OPD solution was added to each well, and the plates were incubated for fifteen minutes at 37 °C. After adding 100 µl of stop solution to end the reaction, the absorbance was measured at 492 nm, as previously described.

Interaction analysis was done to determine which combination of capture and detector antibodies would produce the highest sensitivity with SOD antigen. Based on the best results of a previous experiment, pairs of varying concentrations of capture antibodies (guinea pig anti-SOD pAb) and detector antibodies (rabbit anti-SOD pAb) were selected. Similar procedures to those outlined in the previous section were adopted for carrying out the sandwich ELISA. Each reaction was carried out in triplicate.

2.2.2.4. Specificity & sensitivity of the developed sandwich ELISA. Using 100 random known samples (samples were either fresh/chilled or frozen–thawed; prepared as per the protocol described below), a receiver operating characteristic (ROC) analysis was performed. The results included the computation of the Youden index (J), specificity, sensitivity, and area under the curve (AUC) as per standard procedures.[22,23] Interpretation of results was done by calculation of percent positivity (PP%) as in eqn (1). A test sample was considered positive (frozen–thawed) when the percent positivity (PP) of the sample was equal to or was more than the threshold PP% value.

$$\text{Percentage positivity} = \frac{\text{mean OD 492 of test sample}}{\text{mean OD 492 of positive control}} \times 100 \quad (1)$$

Chilled and frozen–thawed lamb samples were prepared to evaluate the specificity of the assay. Muzaffarnagari breed sheep under one year of age raised at the Indian Veterinary Research Institute's sheep and goat farm were humanely slaughtered, and their meat was collected following strict hygienic measures. Fresh lamb meat samples comprising *M. supraspinatus* (from the square-cut shoulder) and tenderloin/*M. psoas major* (from the hindquarter/loin) were used for making samples for the experiment. After passing the rigor state, the chilled sample was prepared, and another set of samples were kept in a deep

freezer at −20 ± 2 °C. After 7 days of freezing, the meat samples were thawed at 4 ± 1 °C for 24 h, and samples were prepared. For both chilled and frozen–thawed meat, 10 g of meat was cut into 1 cm^3 size, and cubes were completely immersed in 10 ml of 75 mM Tris buffer (pH 7.4) for 15 min. After completion of the dipping time, meat express juice (MEJ) was filtered through Whatman No. 1 filter paper and used as a sample for ELISA. The drip was collected from frozen–thawed meat and stored separately for analysis. Drip at a 1 : 10 dilution with sample buffer was used as the sample for ELISA. The dipping/immersion sample preparation method was adopted throughout the experiment over the clamp/press method as the press method can produce false results, as described by Biswas and co-workers (2023).[2]

To evaluate the effect of different chilling times on assay specificity, which simulated the market conditions, 100 g of each sample of lamb meat was kept at a chiller temperature (4 ± 1 °C) for different periods (12, 24, 48, 72, 96, and 120 h) and evaluated by the developed assay. The experiment aimed to investigate variations in enzyme released over a period of up to five days of chiller storage. To evaluate the impact of different durations of temperature abuse on assay specificity, 500 g samples of lamb meat were initially frozen at −20 ± 2 °C and subsequently stored at the chiller temperature (4 ± 1 °C) to simulate temperature abuse conditions. Meat samples were collected at 2, 4, 6, 8, 12, and 24 hour intervals, and meat extract juice (MEJ) was prepared according to a previously described protocol. The samples were then analysed using a sandwich ELISA, and the results were evaluated.

2.2.2.5. Statistical analysis. The sandwich ELISA absorbance data were obtained by measuring each sample in triplicate and performing multiple analyses on separate days. The findings are presented in the form of mean ± standard deviation (SD). Data analysis involved applying one-way analysis of variance (ANOVA) with Dunnett's post-hoc test conducted using SPSS version 25 (IBM Corp., Armonk, NY, USA). ROC curve analysis was performed using the MedCalc® statistical software version 22.020 (Ostend, Belgium). Statistical significance was set at a *P*-value of <0.05.

3 Results and discussion

3.1. Optimization of antigen concentration by indirect ELISA

For the development of the assay, antigen concentration was optimized by indirect ELISA. A minimum antigen concentration with a higher OD_{492} was selected for developing the assay (maximum measurable OD by the equipment was avoided). The optimal optical density with a minimal concentration of SOD was found at 10 μg ml^{-1}, and for further optimisation of ELISA, this concentration was used. Rabbit anti-SOD pAb was found to be a suitable detector antibody (data not given). Fig. 1(a) shows the results of antigen optimization for assay development.

3.2. Optimization of sandwich ELISA

The sandwich ELISA format using guinea pig anti-SOD IgG as the capture antibody and rabbit anti-SOD IgG as the detector antibody demonstrated a superior diagnostic window compared to the format using rabbit anti-SOD IgG as the capture antibody and guinea pig anti-SOD IgG as the detector antibody. Further optimization was performed using different antibody dilutions to determine the minimum dilution that still provided optimal results. For this, the capture antibody (guinea pig anti-SOD IgG) was tested at dilutions of 1 : 1000, 1 : 1500,

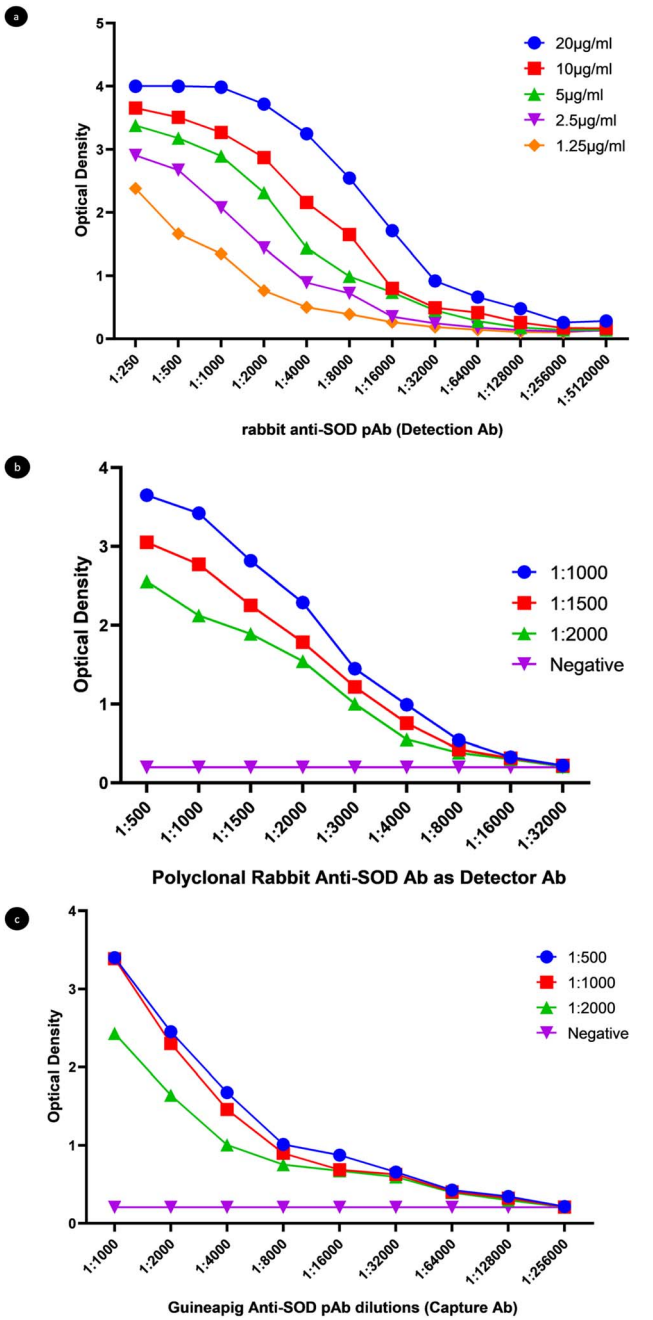

Fig. 1 (a) Optimization of antigen (SOD) concentration by indirect ELISA; 10 μg ml^{-1} was found to be the optimum concentration. The detector Ab used is rabbit anti-SOD pAb. (b) Optimization of capture antibody (guinea pig anti-SOD IgG) concentration. (c) Optimization of detector antibody (rabbit anti-SOD IgG) concentration (negative control – 100 mM PBS).

and 1 : 2000, while the detector antibody (rabbit anti-SOD IgG) was tested at dilutions of 1 : 500, 1 : 1000, and 1 : 2000. From the selected dilutions of guinea pig anti-SOD IgG (capture antibody) and rabbit anti-SOD IgG (detector antibody), six combinations were tested. A sandwich ELISA was performed using the previously optimized concentration of SOD at 10 μg ml^{-1}. The highest sensitivity was achieved using guinea pig anti-SOD polyclonal antibody at a 1 : 1000 dilution as the capture antibody and rabbit anti-SOD polyclonal antibody at a 1 : 1000 dilution as the detector antibody, as shown in Fig. 1(b) and (c). Both dilutions were optimized for the assay procedure.

3.3. Specificity and sensitivity of the developed ELISA

ROC analysis revealed 95.8% sensitivity and 95.0% specificity at 52.752% percentage positivity criterion value [Fig. 2(a) and (b); ESI†]. A threshold value of 52.752% positive percentage (PP%) was established to differentiate between chilled and frozen–thawed lamb meat. Samples with a PP% equal to or greater than this threshold were classified as frozen–thawed, while those with a PP% below the threshold were classified as fresh/chilled.

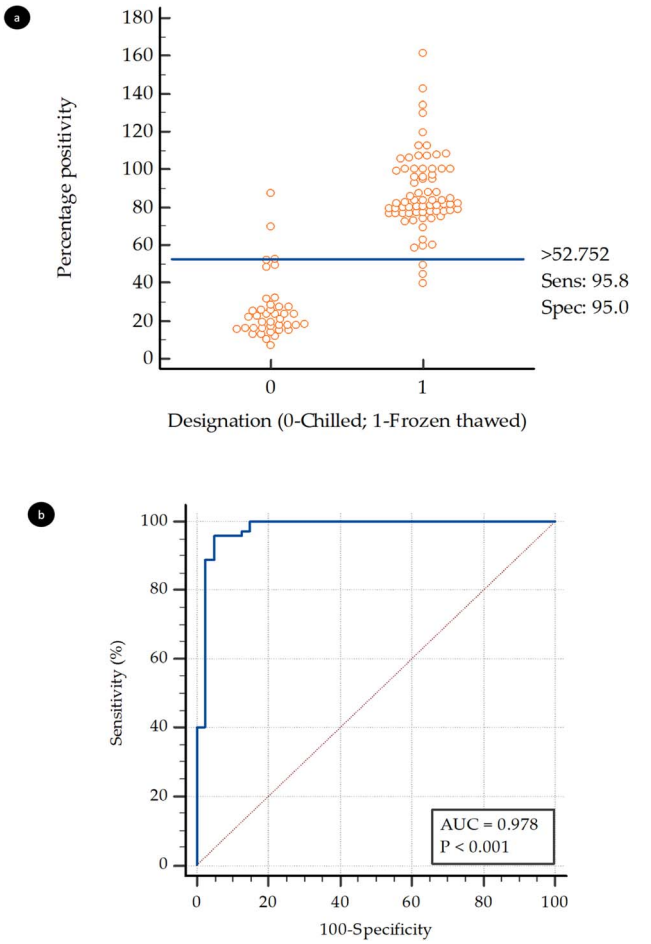

Fig. 2 (a) Receiver operating characteristic analysis showing the distribution of known samples. (b) Receiver operating characteristic area under the curve showing excellent performance (AUC > 0.9) for assay.

An area under the ROC curve (AUC) of 0.978 was achieved, indicating excellent performance for the assay development. The Youden index (J), which assesses the maximum potential effectiveness of the biomarker, was 0.908 for the developed assay. Based on these results, the assay was extended to include MEJ and drip samples.

The results of the sandwich ELISA revealed a significant difference in the percentage positivity values between repeated frozen–thawed (RFT-1 for one freeze–thaw cycle and RFT-2 for two freeze–thaw cycles) lamb MEJ samples and chilled samples. The results were significant for both shoulder and loin origin meat. A significant difference was observed in the shoulder origin MEJ between RFT-1 and RFT-2 samples. The loin-origin MEJ showed a significantly higher value of percentage positivity as compared to the shoulder-origin MEJ. Consistent with the findings of Jangir and co-workers, who reported higher SOD activity in loin-origin buffalo meat compared to shoulder-origin buffalo meat, similar patterns were observed in this study.[18] Biswas and co-workers observed a similar trend for other biomarker enzymes, including LDH, AST, and aconitase, reinforcing the observed differences in enzyme activity by meat origin.[2] These findings point out that loin-origin meat is more susceptible to freezing–thawing damage than shoulder-origin meat. When drip samples were employed in the sandwich ELISA, a minor variation in the results was observed. Frozen–thawed (RFT-1 and RFT-2) samples demonstrated a significant difference in percentage positivity compared to chilled samples. However, no significant difference was observed between RFT-1 and RFT-2 samples for either loin or shoulder origin samples. Thus, drip samples can also be utilized for ELISA if available, potentially bypassing the need for extensive sample preparation procedures. The results of sandwich ELISA using MEJ and drip samples are depicted in Fig. 3.

3.4. Evaluation of different chilling times on assay specificity

To check the effect of chiller storage on the SOD detection level using the developed assay, lamb samples were stored for 120 h, and samples were analysed by the optimised ELISA protocol. This may simulate the conditions of retailer chiller storage, where retailers do not store fresh meat for more than 120 hours (especially in countries like India, where meat was sold fresh without ageing). The results of the experiment revealed that upon chiller storage up to 120 h, there was no increase in the SOD level beyond the threshold level (52.75 PP%). So, the results of the experiment on evaluation of different chilling times on assay specificity showed that up to 120 h of chiller storage does not affect the results of the developed sandwich ELISA results. The results are shown in Fig. 4(a).

3.5. The assay can differentiate fresh and completely thawed meat

Thermal abuse, which may occur during transportation or storage when there is a power outage and meat thaws for a certain period, was simulated in this study. It was found that thawing for up to 12 hours (at 4 ± 1 °C) could not be detected by the developed assay. The meat must be thawed at 4 ± 1 °C for

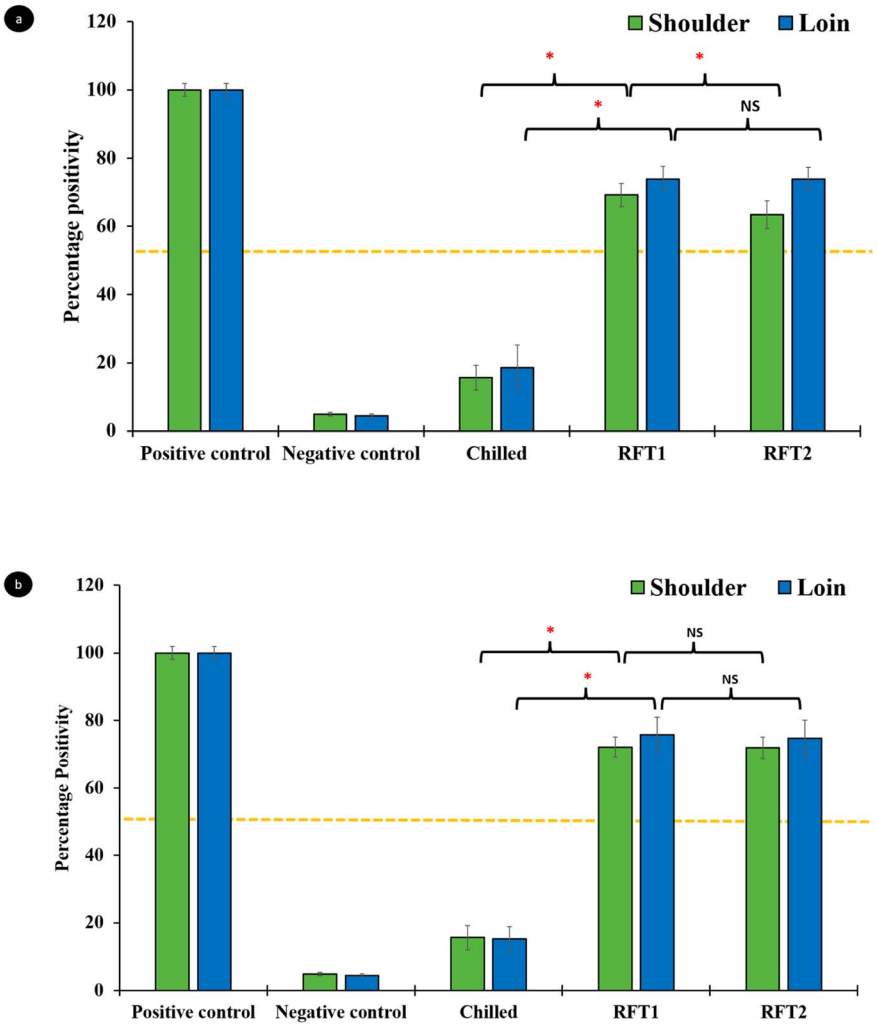

Fig. 3 Sandwich ELISA results showing a significant difference between chilled and repeated frozen–thawed lamb meat. (a) Meat express juice is used as a sample. (b) Meat drip diluted at 1 : 10 dilution is used as a sample. The MEJ sample revealed a significant difference between chilled and RFT samples, RFT-1 and RFT-2 of shoulder origin meat. The drip sample showed a significant difference between the chilled and RFT-1 samples for both shoulder and loin origin meat. * – significantly different; NS – non-significant; the dotted line depicts the threshold limit for differentiating chilled and defrosted meat samples (52.75 percentage positivity); positive control – standard SOD (10 μg ml^{-1}); negative control – 75 mM Tris buffer (sample extraction buffer).

more than 18 hours to achieve a percentage positivity value exceeding the threshold [Fig. 4(b)]. Unpublished data from the author's laboratory indicated that in a thermal abuse study involving 1500 g of buffalo meat chunks, the percentage positivity value exceeded the threshold level after more than 24 hours of thawing. In the current study, the lamb samples investigated had smaller meat chunk weights, which could affect both the rate of thawing and the time required to completely thaw the meat. Therefore, it can be concluded that the developed sandwich ELISA is capable of distinguishing between completely frozen–thawed and fresh/chilled lamb meat. In this context, the assay can be utilized to protect the integrity of meat sold as fresh/chilled in markets and can serve as a quality control tool to prevent food fraud.

The current study targeted one of the proven biomarker enzymes for developing the immunoassay. Besides the superoxide dismutase enzyme, there are multiple other biomarker enzymes discovered for differentiating chilled and defrosted red and white meat such as citrate synthase,[24,25] aconitase,[26] β-hydroxyacyl coenzyme A dehydrogenase (HADH),[27–29] and *N*-acetyl-β-glucosaminidase.[30] Further studies in this line can be extended to develop more immuno-diagnostic techniques including user-friendly rapid tests.

Table 1 compares some important analytical parameters as well as sample preparation methods employed in various studies as compared to the current study. The table depicts that a majority of studies followed the press method for sample preparation, which we avoided here because of the possible operator induced false positive results. Some studies adopted exudate as the sample, as we did in the case of drip samples. But, the drip/exudate may not be available always, as the presence of drip indicates that the meat is frozen–thawed. So, during fraudulence practice, some may use meat diapers or remove the exudate manually to avoid the presence of drip in

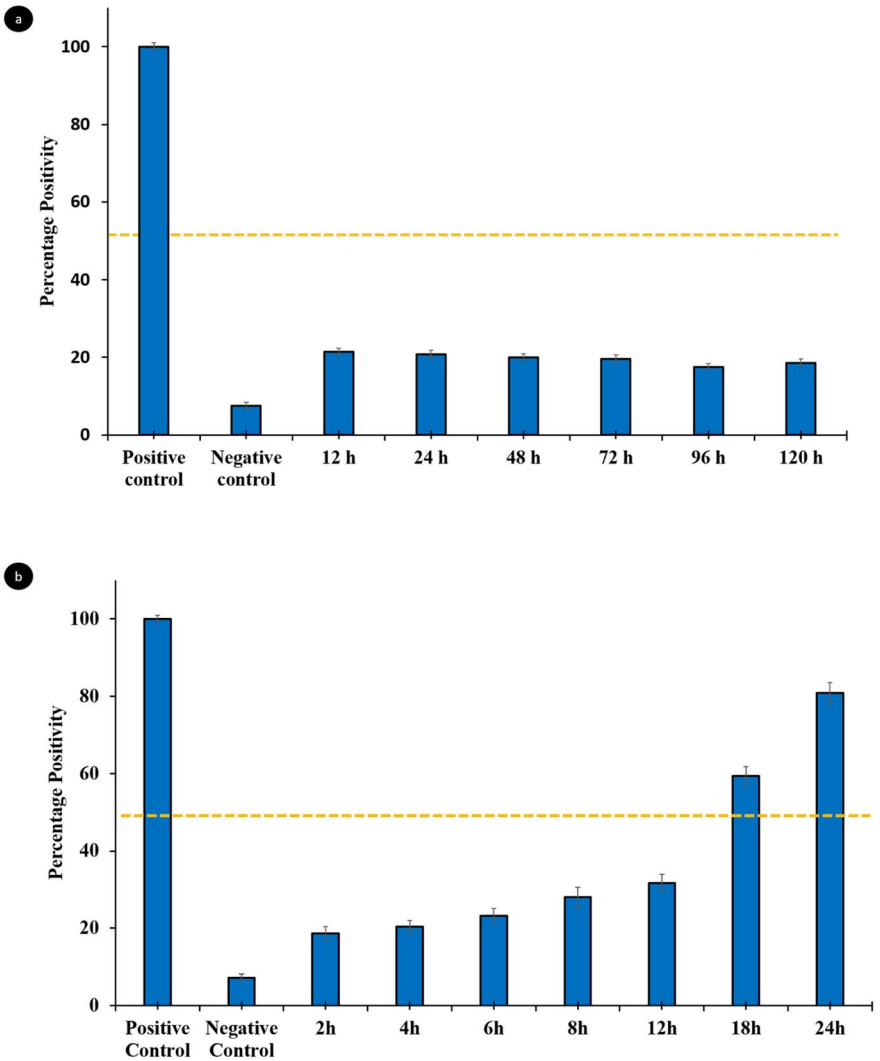

Fig. 4 (a) Chilling temperature storage up to 120 h showing a percentage positive value below the threshold limit for assay. (b) Thermal abuse up to 12 h did not produce results higher than the threshold limit for the assay. The dotted line depicts the threshold limit for differentiating chilled and defrosted meat samples (52.75 percentage positivity).

Table 1 Comparison of different enzymatic methods for differentiation of fresh and frozen–thawed meat with the currently developed ELISA protocol

Study	Sample preparation method	Differentiation method	Cut-off value for chilled meat ($R1$)	Specificity	Sensitivity
Šimoniová *et al.* (2013)[24]	Meat exudate collected directly	Enzyme activity assay; citrate synthase	—	—	—
Bomminayuni *et al.* (2020)[25]	Press method	Enzyme activity assay; citrate synthase	—	—	—
Jangir *et al.* (2024)[10]	Dip method	Enzyme activity assay; superoxide dismutase	0.9	—	—
Pipek *et al.* (2014)[26]	Meat exudate collected directly	Enzyme activity assay; citrate synthase and aconitase	—	—	—
Boerrigter-Eenling *et al.* (2017)[27]	Press method	Enzyme activity assay; HADH	0.9	—	—
Cheung *et al.* (2015)[28]	Press method	Enzyme activity assay; HADH	0.6	—	—
Fernández *et al.* (1999)[29]	—	Enzyme activity assay; HADH	—	—	—
Ellerbroek *et al.* (1995)[30]	Meat exudate collected directly	Enzyme activity assay	—	—	—
Sandwich ELISA developed in the current study	Dip/immersion method	Immunoassay	52.75 (PP%)	95%	95.8%

the package. But, even in that case, the currently developed methodology can be adopted as MEJ can be prepared by dipping meat cubes in sample buffer. Moreover, the developed study having high sensitivity and specificity supports the credibility of the meat quality control system as well as consumer rights.

4 Conclusions

This study presents the development of an ELISA-based immunological method for distinguishing fresh/chilled lamb meat from frozen–thawed lamb meat. A comprehensive review of the literature revealed that no previous studies have employed immunological assays to address this particular food authenticity challenge. Colorimetric, spectrophotometric, and mass spectrometric methods previously explored for this purpose often suffer from drawbacks such as high operational costs, complex procedures, and potential accuracy issues. As a sensitive (>90%) assay with high throughput, ELISA can resolve some of the issues of the analytical methods currently employed. The developed assay holds promise as a valuable tool for authenticating meat freshness within the industry, safeguarding consumers, and bolstering the integrity of the meat supply chain.

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

Conceptualization, A. K. B., C. K. F., and R. K. A.; methodology, C. K. F., B. B., and T. S. A.; investigation, C. K. F., R. K. A., and S. K. B.; data curation, C. K. F., R. K. A., A. A., and T. S. A.; writing, C. K. F.; writing-review and editing, A. K. B. and R. K. A.; project administration, A. K. B. and A. R. S.; funding acquisition, A. K. B.

Conflicts of interest

There are no conflicts to declare.

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