



DIFFERENTIATION OF MEAT FROM DEAD AND SLAUGHTERED ANIMALS BASED ON HEMOGLOBIN CONTENT

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Abstract

Meat from dead animal is not good for human consumption. Slaughtering of animal results in 75-85% bleeding efficiency whereas dead animals do not bleed at all. Considering these views, this work aimed to develop a protocol for the differentiation of meat from dead and slaughtered animals based on hemoglobin content. Meat samples collected from dead and slaughtered layer, broiler and Sonali chickens and cattle were analyzed with two reagents viz. leucomalachite green (LMG) and tetramethyl benzidine (TMB). In brief, the muscle tissue was chopped, in triplicate, and placed in distilled water to liberate hemoglobin, which was allowed to react with hydrogen peroxide followed by reaction with LMG and TMB reagents for color production. The color output was graded by visual inspection as well as by measuring optical density on a spectrophotometer. The optical density ratios of meat from dead to slaughtered animals were 1.41±0.13, 1.89±0.43, 2.31±0.41, 2.01±0.34 with LMG and 4.76±1.13, 2.31±0.60, 6.55±0.25, 2.31±1.02 with TMB for white layer, brown layer, broiler and Sonali chickens, respectively. Meat from dead cattle produced blue color whereas that of slaughtered cattle produced green to light blue color. The intensity of color output was proportionate to the amount of hemoglobin content. The two chemicals, LMG and TMB, were equally effective in differentiating meat from dead and slaughtered animals.

Key words: Dead animal, Hemoglobin content, Leucomalachite green, Meat, Slaughter, Tetramethyl benzidine.

Introduction

Meat is an important source of nutrients essential for human health. Bleeding efficiency is an important consideration for consumption of meat from animals and birds in Muslim and Jews community, consumption of blood is forbidden in Islam and Jewish religions (Farouk 2013, Nakyinsige et al. 2013, Novita et al. 2023). Moreover, hemoglobin of erythrocytes is a potent promoter of lipid oxidation that deteriorates flavor and taste of meat (Everse and Hsia 1997, Alvarado et al. 2007). Bleeding efficiency depends greatly on slaughtering methods such as halal for Muslim, kosher for Jewish, stunning for industrial slaughter and Jhatka for Sheik employed to kill the animals. Additionally, bleeding efficiency is also affected by pre-slaughter stunning, the practice of electrically, mechanically or chemically inducing unconsciousness of an animal before slaughter (Addeen et al. 2014, Nakyinsige et al. 2014, Sabow et al. 2016). Slaughtering results in bleeding efficiency of 75-85% (Farouk 2013, Farouk et al. 2014). Bleeding efficiency also varies due to slaughtering with stunning and without stunning. By contrast, dead animals do not bleed at all and meat from dead animal is not suitable

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for human consumption irrespective of religious belief, cultural, aesthetic and public health point of view. Furthermore, blood acts as an important media for bacterial growth and degrades meat quality in terms of taste, flavor and nutrition. Therefore, bleeding efficiency is considered an important criterion to people with all cultural and religious faith for meat to be consumed. Halal slaughtering method results in the highest bleeding efficiency and is considered the most important criteria than any other factors for meat to be consumed by Muslim people (Ghovvati et al. 2009, Murugaiah et al. 2009). Bleeding efficiency may be determined by measuring the residual blood content in the carcass. Several methods may be employed to determine the residual amount of blood in meat such as measuring the hemoglobin content or enzymatic activity of the heme (Atamna et al. 2015, Hopp et al. 2020, Gupta et al. 2021). Several chemicals and reagents are used for presumptive detection of blood stains including leucomalachite green, tetramethyl benzidine and Kastle-Meyer (Matheson et al. 2022, Gautam et al. 2023, Jaremko et al. 2023). So far, there is no protocol for differentiation of meat from dead and slaughtered animals by chemical test. Considering these views, the research was planned to differentiate meat from dead and slaughtered animals based on its residual blood hemoglobin content.

Materials and Methods

Sample collection: Fat free back muscle tissue (sirloin) from cattle and breast muscle from white layer (Isa White), brown layer (Hi-Lyne), broiler (Hubbard Classic) and Sonali (Rhode Island Red × Fayoumi) chickens were collected from dead and slaughtered animals/birds and brought to the Laboratory of Veterinary Pathology, University of Rajshahi. The muscle tissues were divided into an aliquot of 6 g and tested immediately or placed in falcon tubes and stored at -20°C until use.

Preparation of reagents: Leucomalachite green (LMG) reagent was prepared by mixing 0.25 g of LMG crystal (CAS # 129-73-7, Sigma-Aldrich, Germany) to a solution containing 100 mL glacial acetic acid and 150 mL distilled water. Finally, 5 g of zinc dust was added and kept the reagent at 2-8°C. For preparation of tetramethylbenzidine (TMB) reagent, 0.2 g of TMB (CAS # 54827-17-7, Sigma-Aldrich, Germany) was added to 10 mL of glacial acetic acid, mixed well and stored refrigerated (2-8°C). Thirty percent of hydrogen peroxide was diluted 10 times with distilled water to get 3% hydrogen peroxide and stored refrigerated (2-8°C).

Leucomalachite green (LMG) test: Muscle tissue (6 g) from dead and slaughtered white layer, brown layer, broiler and Sonali chickens and cattle, fresh or frozen and in triplicate, were chopped and placed in 14 mL of distilled water and allowed to stand at room temperature for 20 minutes. Fifty microliter of leucomalachite green reagent was added to the 700 µL of supernatant followed by addition of 50 µL of 3% hydrogen peroxide in an Eppendorf tube and allowed to react for 15 minutes at room temperature. The color output developed was graded by visual inspection and quantified on a spectrophotometer (LisaScan EM, Erba Mannheim, Germany) at a wavelength of 630 nm. The optical density (OD) ratio of meat obtained from dead to slaughter (D/S) were calculated by a formula as stated below:

$$D/S \text{ ratio} = \frac{\text{OD of dead meat} - \text{OD of vehicle}}{\text{OD of slaughtered meat} - \text{OD of vehicle}}$$

Tetramethyl benzidine (TMB) test: Muscle tissue (6 g) from dead and slaughtered white layer, brown layer, broiler and Sonali chickens and cattle were chopped and placed in 14 mL of distilled water as mentioned above. Seven hundred microliter of clear supernatant aspirated was mixed with 20 µL of TMB reagent

followed by addition of 50 μL of 3% hydrogen peroxide in an Eppendorf tube and allowed to react for 15 minutes at room temperature. The color output developed was graded by visual inspection and quantified on a spectrophotometer at a wavelength of 630 nm. The D/S ratio was calculated by a formula as stated above.

Statistical analysis: The result data are expressed as mean OD \pm standard deviation (SD) and statistical analysis was performed using student's t test. A $p < 0.05$ was considered significant.

Results

Residual blood content in meat from slaughtered and dead animals

Leucomalachite green (LMG) test: Muscle tissues of dead and slaughtered white layer, brown layer, broiler and Sonali chickens and cattle collected were analyzed immediately or after storage at -20°C . The reaction product, the color change of leukomalachite green reagent from colorless to blue color, was observed by visual inspection. The meat from slaughtered white layer, brown layer, broiler and Sonali chickens and cattle developed light blue color compared to dark blue color in meat from dead counterparts; the vehicle control did not develop any color change (Fig. 1). The optical density of meat from slaughtered and dead chickens and their ratios (D/S) are presented in Fig. 2. The optical density ratios of meat from dead to slaughter (D/S) were 1.41 ± 0.13 , 1.89 ± 0.43 , 2.31 ± 0.41 , 2.01 ± 0.34 for white layer, brown layer, broiler and Sonali chickens, respectively (Fig. 2). It was worthy to note that the meat stored at -20°C for two weeks showed the color output similar to the color developed in the case of fresh meat.

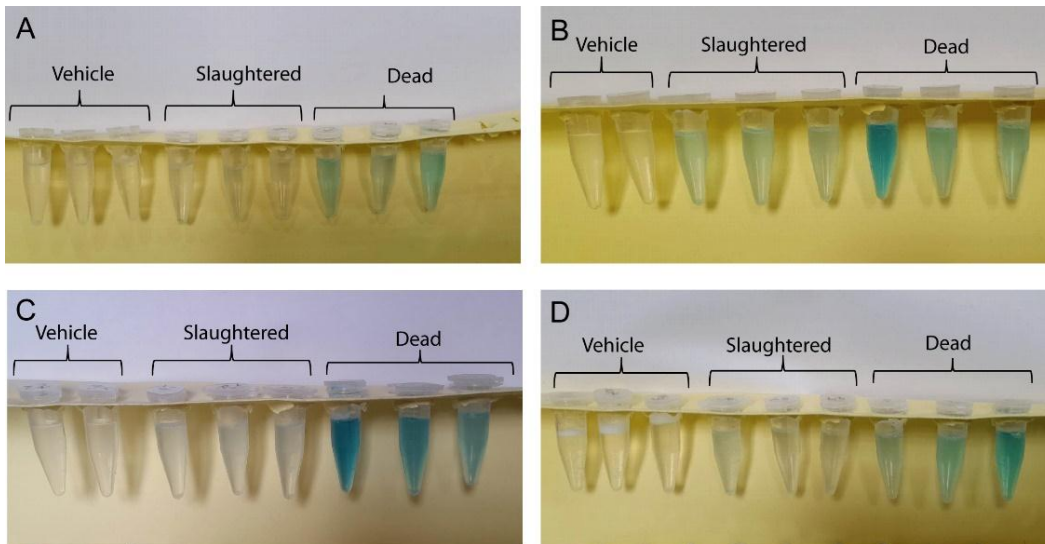


Fig. 1 (A-D): Color output of meat from dead and slaughtered chickens with leucomalachite green (LMG) reagent. Meat from slaughtered white layer (A), brown layer (B), broiler (C) and Sonali (D) chickens produced light blue color with LMG compared to dark blue color in meat from dead counterparts. Control (vehicle) did not produce any color change.

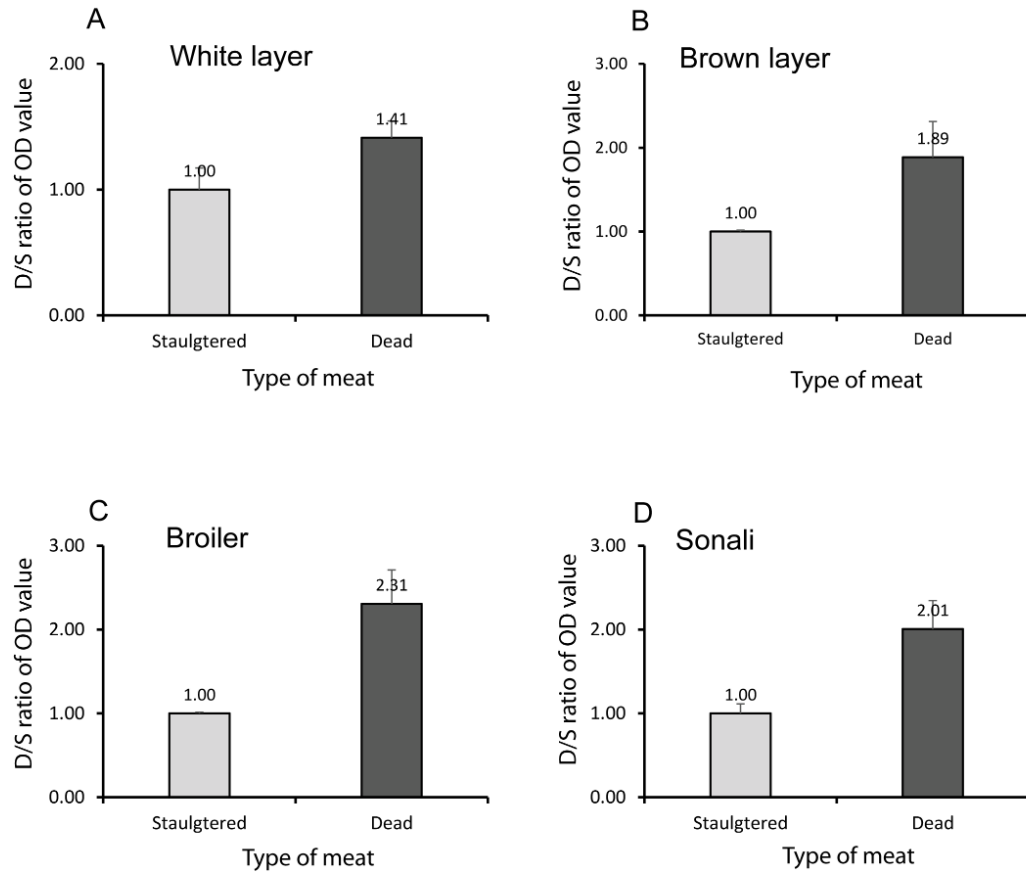


Fig. 2 (A-D): Quantification of color output of meat from slaughtered and dead chickens with leucomalachite green (LMG) reagent. Quantification of color output (ODs) showed D/S of 1.41 times in white layer (A), 1.89 times in brown layer (B), 2.31 times in broiler (C) and 2.01 times in Sonali (D) chickens.

Tetramethyl benzidine (TMB) test: Muscle tissues of dead and slaughtered white layer, brown layer, broiler and Sonali chickens and cattle were also tested immediately or two weeks after storage at -20°C . The reaction product, color change of TMB reagent from light green to blue was observed by visual inspection. The meat from slaughtered white layer, brown layer, broiler and Sonali chickens developed light green color compared to dark blue color in meat from dead counterparts (Fig. 3).

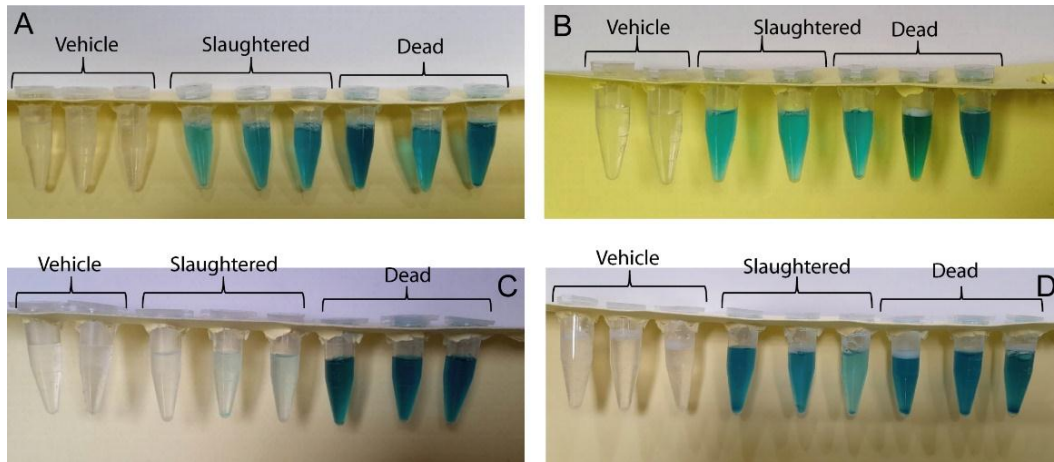


Fig. 3 (A-D): Color output of meat from slaughtered and dead chickens with tetramethyl benzidine (TMB) reagent. Meat from slaughtered white layer (A), brown layer (B), broiler (C) and Sonali (D) chickens produced light blue color with TMB compared to dark blue color in meat from dead counterparts. Control (vehicle) did not produce any color change.

The optical density ratios of meat from dead to slaughter (D/S) chickens were 4.76 ± 1.13 , 2.31 ± 0.60 , 6.55 ± 0.25 , 2.31 ± 1.02 (Fig. 4). Meat from slaughtered cattle produced green to light blue color whereas that of dead animal produced blue color. The fresh meat and meat stored at -20°C for two weeks also showed the same results.

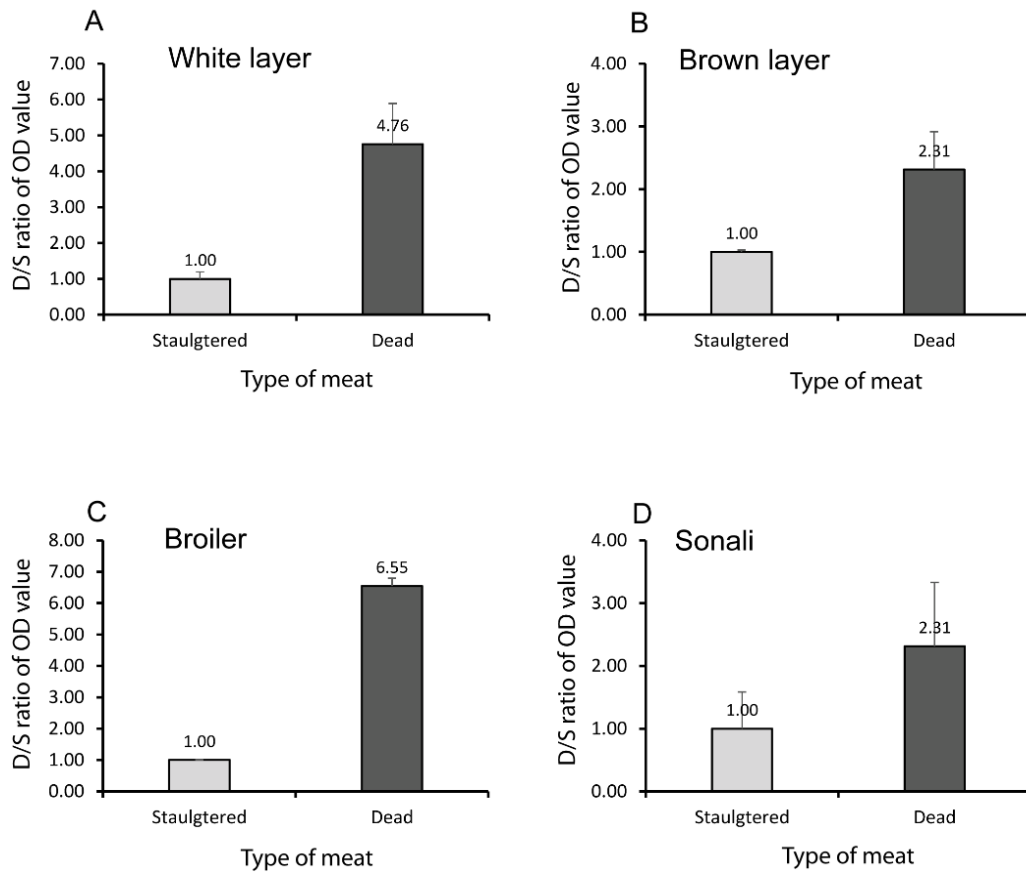


Fig. 4(A-D): Quantification of color output of meat from slaughtered and dead chickens with tetramethyl benzidine (TMB) reagent. Quantification of color output (ODs) showed D/S of 4.76 times in white layer (A), 2.31 times in brown layer (B), 6.55 times in broiler (C), and 2.31 times in Sonali (D) chickens.

Discussion

Meat is an important source of animal protein and consumed by people of all religious and cultural belief. However, meat from dead animal is not suitable for human consumption. An important consideration of differentiating meat from dead and slaughtered animal is its bleeding efficiency, the percent of blood expelled from the body by the process of slaughtering. Although greatly affected by pre-slaughter stunning methods, slaughtering usually results in bleeding efficiency of 75-85% (Farouk 2013, Farouk et al. 2014). In this study the authors attempted to differentiate meat from dead and slaughtered animal based on bleeding efficiency. As the dead animals do not bleed at all, the residual blood content in meat from dead animal is higher compared to the meat from slaughtered animal. For qualitative detection of blood by calorimetric test the authors used inherent property of hemoglobin of erythrocyte. Hemoglobin has peroxidase-like activity i. e. hemoglobin cleaves oxygen from hydrogen peroxide with the production of water and oxygen radicals (Slocombe and Colditz 2011). The LMG and TMB are oxidized with the oxygen cleaved from the hydrogen

peroxide and develop color output and the intensity of color output is proportionate to the extent of oxidation (James et al. 2005). Although both LMG and TMB were equally effective in producing darker color and D/S ratio > 1 to help differentiating meat from dead and slaughtered chickens/cattle, however, Cox (1991) described LMG as the most specific (Gomes et al. 2017, Gautam et al. 2023) and TMB as the most sensitive in detecting hemoglobin of blood. In agreement with these findings, TMB resulted higher D/S ratio (2.31 ± 1.02 to 4.76 ± 1.13) compared to D/S ratio with LMG (1.41 ± 0.13 to 2.31 ± 0.41) in the current study.

Myoglobin of muscle also has the peroxidase-like activity similar to hemoglobin (Giulivi and Cadenas 1994, Mannino et al. 2019). In this study chopped meat was placed in distilled water which acted as hypotonic solution resulting in the bursting out of the erythrocytes liberating hemoglobin. As the meat is insoluble in water, the participation of myoglobin in the reaction process was ruled out. Another key consideration was liberation of hemoglobin of frozen meat upon defrosting. The meat sample was kept in an aliquot of desired amount (6 g) to overcome the loss of hemoglobin through water generated at defrost of frozen meat. Therefore, freezing of meat did not hamper the output of the test results.

The developed protocol estimated residual blood of meat in terms of hemoglobin content irrespective of species of origin and, so far, this was the first attempt of this kind in differentiation of meat from dead and slaughtered animals/birds. However, blood content of meat varies with respect to difference in location of muscles as well as within the same muscle, light vs. dark, in dead animal carcass due to hypostatic congestion after death. However, variation in residual blood content due to difference in muscle or within the same muscle was not evident in cattle tissues examined. This experiment also estimated almost the same color output in meat from slaughtered broiler chicken and light muscle of dead broiler chicken. This means that care should be taken in sampling muscle tissues from dead chickens, and sampling of light color muscle tissues need to be avoided. On the other hand, quantification of the color output and calculation of D/S value may mitigate the discrimination.

To set a cut-off value to differentiate meat from dead and slaughtered animals, the OD or hemoglobin content in meat from dead and slaughtered animals need to be quantified by spectrophotometric analysis. The cut-off value is generally set at average OD or hemoglobin content of meat from slaughtered animal + $2/3$ SD (standard deviation) values. We attempted to calculate D/S (Dead to Slaughter ratio) based on OD values obtained from meat of dead and slaughtered chickens by a formula as stated in materials and methods section. Our results showed that OD values are higher in meat from dead chickens with respect to that of slaughtered chickens (D/S ratio >1). These results also indicated that D/S ratio is a bit higher with TMB compared to that with LMG. The higher D/S ratio (>1) indicates meat from dead animal and lower D/S (<1) ratio indicates meat from slaughtered animals. However, the protocol needs to be validated by examining significant number of meat samples from different species with respect to age, sex and nutritional status of the animals/birds.

Color output due to reaction of tetramethyl benzidine and leucomalachite green reagents with hemoglobin of erythrocytes in meat were clearly different with meat from dead and slaughtered animal. Therefore, the protocol could be used for differentiation of meat from dead and slaughtered animal qualitatively and quantitatively. For determination of cut-off value, the hemoglobin content in meat from dead and slaughtered animals need to be quantified by modified protocol. Because the protocol is based on detection of

hemoglobin of erythrocytes of blood, the protocol may also be used in detection of crime scene (detection of blood) in forensic medicine to aid court in medico-legal practices.

Conclusion

Meat from slaughtered animal origin is an important consideration for consumption. Herein, we describe the development of a new protocol for differentiation of meat from dead and slaughter animals by biochemical test. Therefore, this protocol could potentially be used for resolving conflict in distinguishing meat from dead and slaughtered animals scientifically in suspected or disputed cases.

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Author's contribution: Conceptualization- SMAR and HMG; Formal analysis- MIAB, SMAR and HMG; Methodology- MQA, FA, MIAB, MGH; Investigation- MQA, FA; Writing (original draft preparation)- HMG and SMAR; Writing (review and editing)- MQA, FA, MIAB, MGH, SMAR and HMG; Supervision- SMAR and HMG; Acquisition of fund- SMAR and HMG. All authors have read and agreed to the published version of the manuscript.

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