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Microbiological Quality Assessment of Frozen Beef in Bangladesh

Md. Al-Amin*, Md. Mizanur Rahman, Md. Mostofa Kamal

Microbial Food Safety Section, Quality Control Laboratory, Department of Livestock Services, Savar, Bangladesh

Email address:
alamin_magura@yahoo.com (Md. Al-Amin), mostofa.kamal.phd@gmail.com (Md. M. Kamal)
*Corresponding author

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Abstract: The demand for both homegrown and imported frozen beef sold through super shops and online sales channels in Bangladesh has increased in recent years. These frozen beef may harbor potential microbiological health hazards which are unexplored yet. Hence, the present investigative research was envisaged to assess the potential microbiological public health hazards lying in these frozen beef. A statistically valid 72 frozen beef samples from the 204 sales points were collected during June 2021 to April 2022. The beef samples were subjected to aerobic plate count (APC) to assess the total viable bacterial load. Prevalence of major frozen meat-borne pathogens *Salmonella*, *Escherichia coli*, *Campylobacter*, and *Listeria monocytogenes* were investigated. The pathogens were isolated following US Food and Drug Administration Bacteriological Analytical Manual, henceforth, confirmatory identification was made by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology. *Real-time* Polymerase Chain Reaction was used to detect pathogenic *Escherichia coli*. Out of 72 samples, 33 samples (46.0%) were found satisfactory, 32 samples (44.0%) were found marginal, and 7 samples (10.0%) were found unsatisfactory based on total viable bacterial load in frozen beef. *Escherichia coli* was identified in 63 samples (88%), but none of them was found pathogenic for humans. Based on the most probable number (MPN) of *Escherichia coli*, 49 samples (68%) were found satisfactory (< 50 MPN/g), 18 samples (25%) were found marginal (50 to 500 MPN/g), and 5 samples (7%) were found unsatisfactory (> 500 MPN/g). *Salmonella* was identified in two (2.7%), and *Listeria* was identified only in one (1.3%) sample. All the samples were found free from *Campylobacter*. Overall 93 ± 3% of frozen beef sold through different super shops in different areas in Bangladesh has been found safe for human consumption.

Keywords: Beef, Quality, Salmonella, *Escherichia coli*, Campylobacter, Listeria

1. Introduction

Meat constitutes the most important items of human food, because of its palatability and nutritional value. Muscles of healthy animals usually do not contain microorganisms, but meat tissues get microbial contamination during the various stages of its value chain [1]. Frozen meat is often more heavily contaminated due to the presence of spoilage microorganisms responsible for objectionable changes or pathogens lead to either food infection or intoxication [2]. Microbiological quality assessment of meat is an important concern in the context of public health.

Microbiological quality assessment of meat includes identification of both indicator and foodborne pathogens in meat. Total viable bacterial count (TVC) is used as an indicator of level of bacterial population in meat and sanitary condition at abattoirs and other processing stages [3]. Other indicator bacteria are coliforms, *Escherichia coli* and *Listeria* spp. The presence of these indicator bacteria in excess of satisfactory level in meat indicate inferior sanitary practices throughout the value chain [4].

The major foodborne pathogens are *Salmonella*, *Escherichia coli* (*E. coli*), *Campylobacter*, *Listeria monocytogenes*, Coagulase positive Staphylococci (CPS), *Clostridium perfringens*, *Bacillus cereus* and *Vibrio parahaemolyticus*. Among these, mainly *Salmonella*, *E. coli*, *Campylobacter* and *Listeria monocytogenes* can be found in frozen meat. *Salmonella* causes gastroenteritis, which can be severe in the young and elderly people, and in patients with compromised

immunity. Pathogenic *E. coli* are associated with diarrhea, abdominal pain, fever, kidney failure and even death in humans. Both *Listeria* and *Campylobacter* causes fever, chills, stomach cramp, nausea and vomiting along with diarrhea [5].

Processed frozen and ready-to-cook foods are important part of daily foods of the people of the developed and developing countries [6]. In Bangladesh, a rapid rise in the demand for all forms of frozen food products with a compound annual growth rate of 6.2% is being witnessed. The drivers behind this increased demand of frozen foods are fast-paced urban life, rising disposable incomes, increasing number of women in the workforce and resultant shortage of time, minimal processing for cooking, along with the preference for nuclear families. The frozen food market in Bangladesh is segmented by food types into frozen fruits and vegetables, frozen meat and fish, frozen-cooked ready meals, frozen desserts, frozen snacks, and other applications. Among the frozen meats, frozen beef is the highest consumed meat in Bangladesh [7].

Though Bangladesh is self-sufficient in producing meat to full fill its demand, frozen beef is also being imported from abroad, mainly from India and surprisingly imported cheaper frozen meat is aggressively displacing homegrown beef in Bangladeshi eateries [8-10]. Both locally produced and imported frozen meats are mainly sold through super shops and online sales channels [7, 11]. The contamination of frozen meats by pathogenic bacteria may pose a potential public health hazard due to its close contact with handlers at various stages. Thus, the microbiological safety assessment of the frozen meat is an important concern in the context of public health. Though the microbiological quality of the frozen chicken sold in Bangladesh has been investigated by several previous studies, the same of the frozen beef is still unexplored [11-14]. Therefore, this research was envisaged to the microbiological quality assessment of the frozen beef sold through different super shops and online sales channels in Bangladesh. Thus, the specific objectives of the research were-

a) determination of microbial load in frozen beef;
b) detection of *Salmonella*, *E. coli, Campylobacter* and *Listeria monocytogenes* in frozen beef; and
c) microbiological risk assessment of frozen beef to public health.

2. Materials and Methods

In this research, the microbiological quality of the frozen beef sold in different super shops and online sale channels in Bangladesh were assessed through the determination of microbial load by aerobic plate count (APC) as well as through the detection of major frozen meat borne bacterial pathogens *Salmonella*, *Escherichia coli*, *Campylobacter* and *Listeria monocytogenes*.

2.1. Study Area and Sample Collection

The study area of this investigative research was entire country. All the identified outlets of the super shops located at different cities in Bangladesh were included in this study. Only the frozen beef samples were collected following the procedure described in US Food and Drug Administration Bacteriological Analytical Manual (FDA/BAM) chapter 1 [15]. Super shops along with their outlets situated at different cities of Bangladesh and online meat sale channels were identified using *Google* search and henceforth, a database listing 204 number of meat sales points were prepared. A statistically valid 72 number of the frozen meat samples were collected at 95% confidence and 0.09 precision level using the following formula [6]:

$$n = \frac{N}{1 + N\,(e)^2}$$

Here, *n* is the sample size, *N* is the population size, and *e* is the level of precision. Frozen meat sales points were randomly selected from the database using IBM SPSS Version 20.0 software. Upon collection, the samples were transported to the QC Lab, DLS, Savar, Dhaka maintaining cool chain and stored at -20°C until analysis. A pre-structured form was used during sample collection for proper record keeping.

2.2. Aerobic Plate Count

Aerobic plate count (APC) was used for the determination of total viable bacterial load in beef samples and the test was carried out following FDA/BAM chapter 3 [16]. Briefly, a 25 g sample portion was homogenized in 225 ml of Butterfield's phosphate-buffered water diluent to prepare 10^{-1} dilution. Henceforth, serial 10-fold dilutions up to 10^{-7} or higher were prepared in the same diluent. One ml of appropriate dilutions were inoculated in duplicate plate count agar plates following pour-plate method. Plates were incubated at 35°C, and bacterial colonies were counted after 48-72 hours of incubation. Results were recorded as $\log_{10}$ values and meat samples were categorized into three groups based on the bacterial load-Satisfactory (<5.0 log/g), Marginal (5.0-7.0 log/g), and Unsatisfactory (>7.0 log/g) [4].

2.3. Isolation, Identification and Enumeration of E. coli

E. coli in frozen beef samples were detected and enumerated as the most probable number (MPN) following the procedure described in FDA/BAM chapter 4 [17]. In brief, a 25 g sample portion was homogenized in 225 ml of Butterfield's phosphate-buffered water to prepare 10^{-1} dilution. Thereafter, serial decimal dilutions of 10^{-2}, 10^{-3}, and others as appropriate, of food homogenate were prepared. One ml aliquot from each of 10^{-1}, 10^{-2}, and 10^{-3} dilutions was inoculated into 3 Lauryl tryptose (LST) broth tubes for a 3 tube MPN analysis. LST tubes were incubated at 35°C± 0.5°C for 24 to 48 h and examined for gas formation. A loopful of suspension from each gassing LST broth was transferred to EC broth for 24 to 48 h at 44.5°C and examined for gas production. Finally, a loopful of broth from each gassing EC tube was streaked on Levine-EMB agar plates and incubated for 18-24 h at 35°C ± 0.5°C. The colonies on Levine-EMB agar plates were examined by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) for

confirmatory identification of *E. coli* [18]. MPN of *E. coli* was calculated based on the proportion of EC tubes in 3 successive dilutions that contained *E. coli* [17]. Based on *E. coli* load, beef samples were categorized into Satisfactory (<50 MPN/g), Marginal (50 to 500 MPN/g), and Unsatisfactory (>500 MPN/g) groups [19].

2.4. Isolation and Identification of Salmonella

Salmonella sp. in frozen beef samples were detected by combined use of FDA/BAM Chapter 5 reference method and Association of Analytical Communities (AOAC) Official Method 2017.09 [18, 20]. A 25 g sample portion was pre-enriched in 225 ml of Lactose broth at 35°C for 24 ± 2 h. Pre-enriched suspension was inoculated for selective enrichment in both Rappaport-Vassiliadis (RV) and Tetrathionate (TT) broth, and incubated for 24 ± 2 hours at 42 ± 0.2°C and 35 ± 2.0°C respectively. Suspensions from both RV and TT broths were streaked on BS, XLD, and HE agar plates for isolation of *Salmonella*. Thereafter, the isolated organisms were tested by MALDI TOF MS for confirmatory identification of *Salmonella*.

2.5. Isolation and Identification of Campylobacter

Campylobacter spp. in frozen beef samples were detected by combined use of FDA/BAM Chapter 7 reference method and AOAC Official Method 2017.09 [18, 21]. Briefly, 25 g sample was rinsed in 100 ml of Bolton broth and incubated micro-aerobically 48 h at 42°C for enrichment. Enriched suspension was streaked on Modified Campy blood-free agar (mCCDA) plates and incubated anaerobically at 42°C for 24-48 hours. Finally, the isolates were identified by MALDI TOF MS.

2.6. Isolation and Identification of Listeria Monocytogenes

FDA/BAM Chapter 10 reference method and AOAC Official Method 2017.09 were used jointly to detect *Listeria monocytogenes* in frozen beef samples [18, 22]. A 25 g representative sample was weighed into a sterile net-lined stomacher bag and 225 ml of buffered *Listeria* enrichment broth (BLEB) was added to it and homogenized. The homogenate was enriched for 24-48 h at 30°C. Thereafter, the enriched suspension was streaked on PALCAM agar, incubated 24-48 h at 35°C for isolation of *Listeria*. Confirmatory identification of *Listeria* was done by MALDI TOF MS.

2.7. Confirmatory Identification of Bacteria by Matrix Assisted Laser Desorption ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS)

MALDI TOF MS using MALDI Biotyper (Daltronic Microflex LT-MS System, Bruker, Germany) was employed for confirmatory identification of the bacterial isolates in the study. Samples were prepared from isolated colonies on reusable steel target plates following extended direct transfer (eDT) procedure [18]. Briefly, a smear of an isolated colony of bacteria was prepared as a thin film directly onto an appropriate sample position of target plate. The smear was overlaid with 1 µl 70% aqueous formic acid and dried at room temperature. A1 position on the target plate was selected for bacterial test standard (BTS) control in each run and 1 µl of BTS solution was added there. After samples and BTS dried, 1 µl HCCA matrix was added to each position, and dried at room temperature. Thereby, the target plate was ready for examination. Target plates were read by *flexControl* and *MBT Compass* softwares following manufacturer's protocol. The spectrum patterns acquired from bacterial ribosomal proteins were used to reliably and accurately identify the bacteria [23]. The spectrum values generated log scores ≥2.0 were considered to be acceptable with high confidence identification. Those presented in yellow with log scores between 1.70 and 1.99 were considered to be acceptable with low confidence identification. Results presented in red with log scores ≤1.70 were considered not acceptable for identification [18].

2.8. Polymerase Chain Reaction (PCR)

The already identified *E. coli* isolates were subjected to sybr green multiplex *real-time* PCR for the confirmatory identification of shiga toxin-producing *E. coli* (STEC) which are harmful to humans [24, 25]. Four primer sets (*O157, eae, stx1, stx2*) were used for the detection of pathogenic *E. coli* (Table 1) [26]. *E. coli* isolates positive to either *O157*, or *eae* and *stx1* and/or *stx2* were considered 'pathogenic'. In addition to pathogenic genes, *yaiO* gene was also targeted for the specific identification of *E. coli [27]*. In each PCR run, a 'Melt curve' step starting from 65°C to 95°C was added to observe the specificity of amplification by the primers used here.

Table 1. *List of primers used for the detection of pathogenic E. coli.*

Primer pair no.	Primer name	Sequence (5′-3′)	Product size (bp)	PCR condition
1	O157-F	TCG TGA CAA CCA TTC CAC CTT	123	Multiplex sybr green *real-time* PCR: Initial denaturation step of 94°C for 3 min, then 35 cycles of 94°C for 20s, 58°C for 30s, and 72°C for 30s [26, 27]
	O157-F	GCG CTG AAG CCT TTG GTT CT		
2	eae-F	CAT TGA TCA GGA TTT TTC TGG TGA TA	102	
	eae-R	CTC ATG CGG AAA TAG CCG TTA		
3	stx1-F	GTG GCA AGA GCG ATG TTA CGG TTT G	182	
	stx1-R	ATG ATA GTC AGG CAG GAC GCT ACT C		
4	stx2-F	ACG AGG GCT TGA TGT CTA TCA GGC G	200	
	stx2-R	GCG ACA CGT TGC AGA GTG GTA TAA C		
5	yaiO-F	TGATTTCCGTGCGTCTGAATG	115	
	yaiO-R	ATGCTGCCGTAGCGTGTTTC		

2.9. Data Analysis

Raw data of all experiments were subjected to statistical analyses. Graphs were built in Microsoft Excel 2013, and mean values were compared in IBM SPSS Version 20.0 software. Analyses were carried out at 95% confidence level and *p*-values less than 0.05 were considered significant.

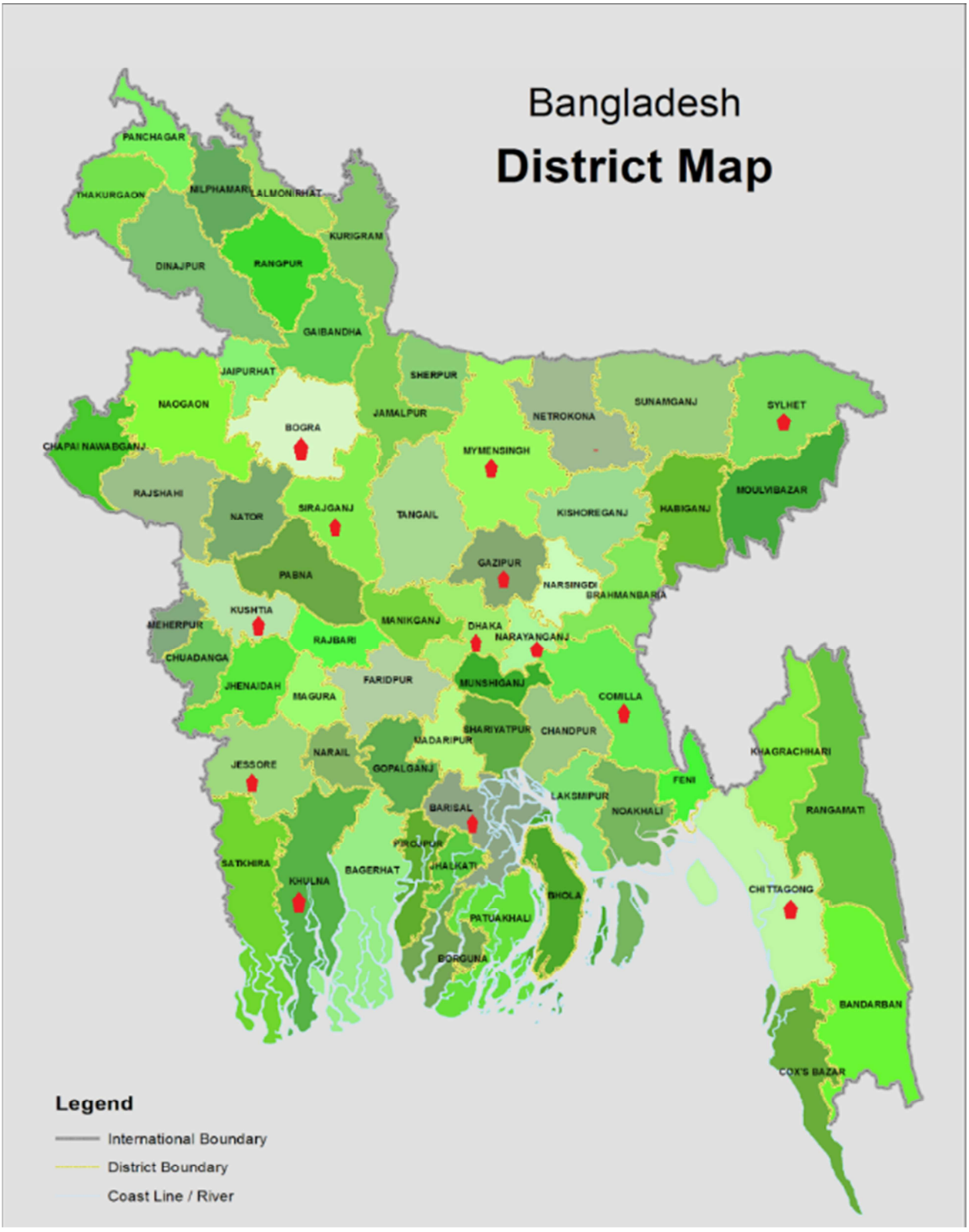

Figure 1. *Red marked symbols in the map of Bangladesh are showing the sample collection areas.*

3. Results and Discussion

3.1. Sample Collection

A total of 72 frozen beef samples were collected during June 2021 to April 2022 from 6 different super shops in 13 districts of Bangladesh (Figure 1). Forty eight samples were collected from Dhaka district, eight from Sylhet district, four from Gazipur, two from each of Chattogram and Khulna, and one from each of Narayanganj, Jashore, Kushtia, Cumilla, Bogura, Mymensingh, Barishal, and Sirajganj district. All samples were found suitable for testing. Most of the samples (93%) were collected from three branded chain super shops (Bengal meat, Agora, and Shwapno) located at different cities in selected 13 districts of Bangladesh.

3.2. Bacterial Load in Frozen Beef

Distinct bacterial colonies on plate count agar plates were found after 48 h of incubation in most of the cases. Bacterial load in meat samples varied from 4.0 to 7.8 log/g with mean 5.3±1.2 SD log/g. Out of 72 samples, 33 samples (46.0%) were found satisfactory (<5.0 log/g), 32 samples (44.0%) were found marginal (5.0 to 7.0 log/g), and 7 samples (10.0%) were found unsatisfactory (>7.0 log/g) (Figure 2). Considering satisfactory and marginal categories as consumable, 90% frozen beef found safe for human consumption.

No significant differences ($p=0.115$) were found in bacterial loads in frozen beef sold in Dhaka city and outside Dhaka city. Likewise, differences in bacterial loads in frozen beef sold through major three chain super shops were insignificant ($p=0.191$).

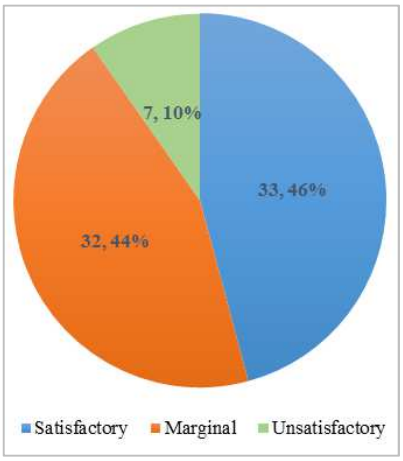

Figure 2. *Pie chart showing the number and percentage of meat samples in each category based on total viable bacterial load.*

3.3. E. coli in Frozen Beef

E. coli produced black colonies with metallic sheen on the Levine-EMB agar plates (Figure 3). Confirmatory identification by MALDI FOF MS revealed that 63 samples (88%) were positive to *E. coli*, and 9 samples (12%) were found negative to the bacteria (Figure 4).

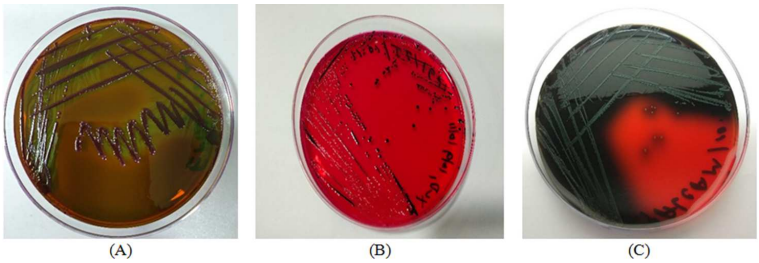

Figure 3. *Growth of different bacteria on different isolation agar plates. Brown colonies by E. coli on Levine-EMB agar plates with metallic sheen (A); black colonies by Salmonella on XLD agar plates (B); and black colonies with black halos on PALCAM agar by Listeria monocytogenes (C).*

MPN of *E. coli* in beef samples ranged from <3 to 1100/g. Based on MPN of *E. coli*, 49 samples (68%) were found satisfactory (<50 MPN/g), 18 samples (25%) were found marginal (50 to 500 MPN/g), and 5 samples (7%) were found unsatisfactory (>500 MPN/g). Considering satisfactory and marginal categories as consumable, 93% frozen beefs sold through super shops in Bangladesh were found safe for human consumption. In PCR tests, all *E. coli* isolates were found positive to *yaiO*, but negative to pathogenic genes *O157, eae, stx1,* and *stx2*.

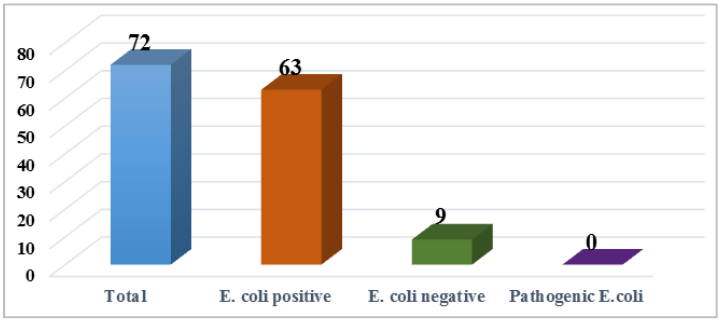

Figure 4. *Column chart showing the numbers of E. coli positive and negative samples, and pathogenic E. coli among the total.*

Melt Curve data in each PCR run delineates that Melt temperatures for both *E. coli* positive control and meat isolates were always very unique (86°C) [Figure 5].

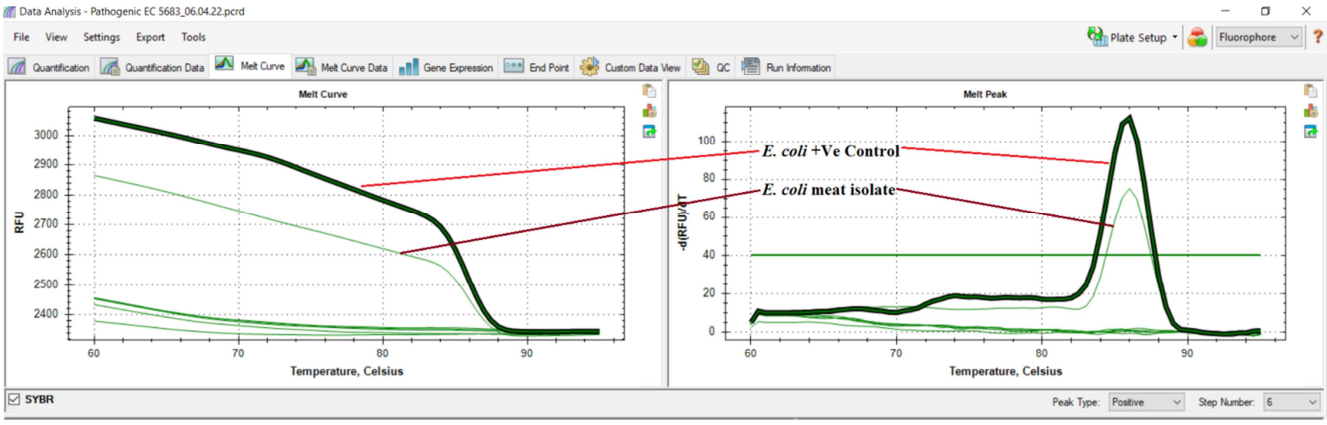

Figure 5. *Representative Melt Curve shows the very unique Melt temperatures (86°C) for both E. coli positive control and meat isolates.*

3.4. Salmonella and Listeria Were Identified in Very Few Number of Samples

Salmonella was identified in two (2.7%) frozen meat samples and *Listeria monocytogenes* was identified only in one (1.3%) samples out of 72. *Salmonella* formed black colonies with metallic sheen on BS agar, black colonies on XLD agar, and black centered blue colonies on HE agars. *Listeria monocytogenes* formed black colonies with black halos on PALCAM agar plates (Figure 3). The findings indicate that the majority of the frozen meat samples are free from these two pathogenic bacteria, and are safe for human consumption.

Campylobacter could not be identified in any of the 72 frozen meat samples, thus the frozen beefs sold through super shops in Bangladesh assumed to be safer for consumption.

4. Discussion

In Bangladesh, the demand of frozen beef is gradually increasing with resultant expansion of marketing channels. Heavy microbial load or contamination of frozen meats by pathogenic bacteria creates a potential public health hazard due to its close contact with handlers at various stages of value chain [28]. Thus, the microbiological quality assessment of the frozen meats is an important concern in the context of public health. Hence, the present study aimed to evaluate the frozen meat processing environment through the determination of microbial load and the detection of indicator bacteria in frozen meat. Moreover, this research also aimed to the detection of pathogenic bacteria harbored in frozen beef with a view to microbiological public health risk assessment.

Simple random selection of 72 frozen beef samples out of 204 sales points at different cities in the country made the sampling regimen representative [29]. Sampling covered almost all parts of the country (Figure 1). Moreover, popular meat brands in Bangladesh were included in the study. All the samples were transported to the laboratory timely, preserved properly, and found suitable for testing. The bacterial loads in frozen beef were found satisfactory (<5.0 log/g) in majority of the samples indicating good hygiene and precise storage practices in most of the meat processing plants and sales points. Though marginal bacterial loads (5.0 to 7.0 log/g) result in some unacceptable spoilage and texture, still these types of meats are consumable [30]. Hence, 90% frozen beef tested in the study found safe for human consumption. Unsatisfactory bacterial loads (>7.0 log/g) in frozen beef result in spoilage, slimy raw texture, and bad odors in meat [3]. Only 10% beef samples were found under this category and suggested to be condemned. Insignificant differences ($p=0.115$) in bacterial loads in frozen beef sold in Dhaka city and outside Dhaka city indicates precise storage in all sales points. Likewise, insignificant differences ($p=0.191$) in bacterial loads in beefs of major three branded super shops indicated that frozen beefs of all three brands are equally good.

Interestingly, majority of the beef samples (88%) were found positive to *E. coli,* but loads of the bacteria were under satisfactory level in 68% samples, and at marginal level in 25% samples. The findings explicit that, though majority of the samples got *E. coli* contamination, which was very minimal. *E. coli* is ubiquitously distributed in fecal materials from humans and warm-blooded animals [31]. Thus, *E. coli* contamination of frozen beef could be from gut of slaughtered animals. However, this type of contaminations indicates the poor hygiene practices during evisceration of carcasses.

Among the pathogenic *E. coli*, we targeted O157 strains responsible for gastrointestinal illness and prevalent in raw beef [32]. We also targeted to determine the prevalence of the STEC virulence genes *stx1* (encoding Shiga toxin 1), *stx2* (encoding Shiga toxin 2), and *eae* (encoding intimin). Shiga-toxin producing *E. coli* (STEC) is an important group of zoonotic human pathogens, with *E. coli* O157 being the best known and most studied serotype. STEC strains are generally carried asymptomatically by cattle and shed in their feces [33]. In PCR tests, all the MALDI TOF MS confirmed *E. coli* isolates were found positive to species specific to *yaiO* gene

and the findings further confirmed the isolates as *E. coli* [27]. Among the 63 *E. coli* isolates, none of them was identified pathogenic for humans in PCR tests. The findings indicate that, most of the frozen meats are safe for consumption, though they are contaminated with *E. coli.* Very unique Melt temperature (86°C) of the amplicons by the *E. coli* species specific *yaiO-F* and *yaiO-R* primers in each sybr green *real-time* PCR run for both positive control and meat isolates confirmed that there was no non-specific amplification by the primers (Figure 5) [34].

Detection of two important food-borne pathogens *Salmonella* and *Listeria monocytogenes* in very few number of frozen beef samples points out that the meats are coming from healthy animals, and processed and preserved in good hygienic environment as well. Our findings corroborated with the findings of Dong *et al*., 2014 [35]. Absence of *Campylobacter* spp. in meat samples further confirmed the good hygiene practices along the meat value chain.

5. Conclusion

Through this investigative research, the microbiological health hazards lying in frozen beef sold through super shops in Bangladesh have been assessed. Most of the frozen beef sold through different super shops of different areas in Bangladesh have been found safe for human consumption. The current research provides baseline information of microbiological quality of frozen beef in Bangladesh, which could be used as a reference for the future study. However, the current research is limited by inclusion of small sample size due to resource constraints. Therefore, an elaborate research with inclusion of large sample size on microbiological quality of frozen meats sold in Bangladesh is suggested.

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