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Detection of *Vibrio* spp., *Salmonella* spp., and *Shigella* spp. among the frozen food samples employing enrichment culture technique

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Freezing has long been an established method for food preservation. Freezing temperature may act as a stress factor for microbial cells, transforming the cells into injured or dormant state. Upon inoculation, these debilitated cells cannot grow on solid media and hence produce false negative results. Foods contaminated with injured cells of pathogenic bacterial strains are of potential health risk. Employing enrichment cultivation technique, present study attempted to detect such injured, dormant or viable but non culturable (VBNC) cells in different frozen food samples, collected from local markets and super-shops of Dhaka metropolis. Compared to the conventional cultivation means, the enrichment procedure revealed a significant increase in bacterial burden as well as increase in the pathogenic load. A maximum of 3 log increase in case of total bacterial load while 4 log, 5 log and 2 log increase in case of *Vibrio* spp., *Salmonella* spp. and *Shigella* spp., consecutively were observed. These findings clearly demonstrated the presence of injured cells in frozen foods which could be lethal under normal condition thereby posing public health risk.

Key words: Frozen food; Injured cells; Viable but non-culturable (VBNC) cells; Pathogenic microorganisms; Enrichment cultivation procedure; Public health

Foods are very much likely to be microbiologically contaminated during its production, packaging, transportation and storage (1-9). An array of microorganisms including enteric bacteria, *Bacillus* spp., *Listeria monocytogenes*, *Yesinia enterocolitica*, *Pseudomonas* spp., *Clostridium perfringenes*, *Staphylococcus aureus*, *Campylobacter jejuni* and *vibrio* spp. have been reported to be the common food spoilage bacteria (1, 3, 4, 8-11). An ever-enlarging world population has increased demands on frozen foods worldwide. Freezing has long been established as an excellent method of preserving high quality of foods (7, 10). However, due to high demand and popularity of frozen food, quality analysis of frozen food is receiving increased attention (7). The major consideration for recovery and isolation of microorganisms from frozen food could be the high proportion of sub-lethally injured cells; i.e., reversibly damaged or debilitated cells (1, 2, 4, 7, 10). Injured microorganisms present a potential threat in food safety since they may repair themselves under suitable conditions (2, 3, 6, 7, 12).

Storage at freezing temperatures is known to exert effects on microbiological loads of a variety of food products; due to its ability to lower metabolic activities (6, 7, 13-17). Distribution and preparation prior to consumption of such products lead to the onset of several disease outbreaks (15-18). Bacterial pathogens such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Vibrio* species are well known for their etiological dominance in triggering enteric diseases in humans (18-24). Therefore, the aptitude of progression as well as the continued existence of bacteria must be measured not only to detect the microbiological quality but also to assess the consumer safety of such stored frozen food products (22-24).

To ensure a safe state of public health, there is a need for readily available methods to detect and enumerate bacterial pathogens in the commonly consumed foods (8, 24). A complication arises in this regard that most enteric pathogens appear intermittently and often in the viable but not non-culturable (VBNC) in the stored frozen foods. Thus, potentially pathogenic organisms present in a frozen food sample, may go undetected, largely due to their low numbers. The procedure of enrichment thus claims significance in order to avoid the possible false negative results (8). Along these lines, current study focused on recovery of the VBNC or injured cells (i.e., cells which can be enumerated after enrichment but not before that) among the common frozen food samples by employing the enrichment culture technique.

MATERIALS AND METHODS

Sampling, sample processing and enrichment of samples. Collection of samples was performed aseptically in the month of September 2014 from different places of New market, Malibag, Siddeswari and Mogbazar area in the Dhaka city of Bangladesh. Eight Samples of different categories including meat, fish, shrimp, squid, poultry and processed frozen foods (one chicken ball and one mutton roll) were collected and quickly transported to laboratory keeping in an ice box maintaining the temperature at 4 °C (25, 26). Ten gm of each sample was weighed

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and added in a sterile homogenizing beaker containing 90 ml of sterile normal saline, and then homogenized to prepare 100 ml sample suspension for microbiological examinations.

As it is normally assumed that *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. remain in viable but non-culturable (VBNC) state or in stressed condition in the environment (24, 27-29), therefore, enrichment technique was employed for the isolation and identification of these bacteria. One ml sample was added to 9 ml selenite cysteine broth (SCB) (Difco Laboratories, Detroit, Mich.) and alkaline peptone water (APW) (Difco Laboratories, Detroit, Mich.), respectively. Culture suspensions were incubated for 4 hours at 37 ºC with shaking at 100 rpm (24). A series of 10 fold serial dilutions were made up to 10^{-6} for both non-enriched and enriched samples. Then 0.1 mL from each of the enriched broth was spread over Salmonella-Shigella agar (SSA) and Thioglycollate citrate bile salt (TCBS) agar respectively. Moreover, nutrient agar plates were also used for the determination of total viable bacteria and for the detection of psychrophiles by incubating the respective agar plates at 37 **°C** for 12-24 hours and at 2-7 ºC for 14 days (25).

Detection of *Vibrio* spp., *Salmonella* spp., and *Shigella* spp. Thiosulfate Citrate Bile Salt Sucrose (TCBS) (Difco Laboratories, Detroit, Mich.) agar plates were used to quantify the contaminating *Vibrio* spp. within the samples. While *Salmonella-Shigella* (SS) agar (Difco Laboratories, Detroit, Mich.) was used both for the isolation and quantification of *Salmonella* spp. and *Shigella* spp. After incubation at 37 ºC for 24 hours, characteristic colonies were detected and enumerated (9, 11, 24). Yellow colored colony on TCBS is characteristic of *Vibrio* spp. while the black centered colony on *Salmonella*/*Shigella* agar was considered as *Salmonella* spp. and the colorless colony on the same agar plates was noted as *Shigella* spp. A deduction of the pre-enriched bacterial load from the enriched ones detected the amount of injured cells of *Vibrio* spp., *Salmonella* spp., and *Shigella* spp. in all the samples examined. Finally, a series of biochemical tests were performed following the standard method to confirm the presence of these isolate

RESULTS AND DISCUSSION

Freezing is considered as one of the important tools for preserving materials of biological origins (30). Gram negative pathogens such as *Salmonella* and other *Enterobacteriaceae* are sensitive to freezing injury and there is also some mortality of mesophilic *Vibrios* (30). Various stress factors may lead to the generation of a dormant or viable but non-culturable state in the bacterial cells which direct bacteria to be unable to produce observable colonies when cultured on selective and non-selective agar media (31). Despite this condition, these bacteria possess the ability to cause disease in suitable conditions (32, 33, 34). Previously a range of microorganisms have been isolated from various food items; however, investigation on the frozen foods by our group remained bared. Thus the present study attempted to examine the effect of enrichment technique in the recovery or the enhancement of the growth of microorganisms in different frozen food items in terms of total viable count as well as in the detection of various pathogenic organisms such as *Vibrio* spp., *Salmonella* spp. and *Shigella* spp.

In the present study, the total aerobic viable bacterial load ranged from 2.6×10^5 to 3.7×10^7 cfu/g (Table 1), observed in shrimp and chicken respectively. After enrichment an elevated count was found ranging from 2.9×10^7 to 5.6×10^9cfu/g (Table 1). A maximum of 3 log increase was noticed in beef and shrimp samples. Other samples also augmented at least 1 or 2 log. Hasan et al. (7) perceived TVC to be 2.6×10^5 cfu/g in frozen fish samples. Another study estimated TVC to be around 3.7×10^6 cfu/g without enrichment which is lower than this experiment. In the current study, we also attempted to observe the presence of psychrophilic bacteria in frozen food. Before enrichment 2 samples, chicken and shrimp showed presence of psychrophiles and after enrichment squid along with chicken and shrimp samples found to be positive. Maximum 3 log increase was observed in squid, 1 log increase in chicken and shrimp remained unchanged after enrichment (Table 2). Psychrophiles were absent in other 5 samples. Psychrophilc organisms are not usually pathogenic but can cause spoilage of food products. Thus Psychrophilc load in frozen food has economic importance. Frozen fish can even be infected with this organism after coming in contact with ice made from *Vibrio* contaminated water (33, 35). In the present study, presence of *Vibrio* was found in all samples except rui fish and mutton roll. For the rest 6 samples, load of *Vibrio* spp ranged from 6.0×10^2 to 2.8×10^4 cfu/g (Table 1 & 3). After 4 hours of enrichment all the samples except the mutton roll showed an elevated count, with a maximum of 4 log increase in growth was observed in rui fish (Table 1). (According to Massachusetts Department of Public Health, 1959 (36) Total viable bacteria should be 5.0×10^4 cfu/g, coliform should be 10 cfu/g and *Staphylococcus* spp., *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. should be absent per gram of frozen pre-cooked food**.**

Several outbreaks of human disease both in developed and developing countries have occurred by *Salmonella* spp. (37). In our study, samples were of animal origin. *Salmonella* spp. were found to be absent before enrichment. But interestingly, after enrichment 5 samples showed high load of *Salmonella* spp. ranging from 2.3×10^2 to 1.6×10^5 cfu/g where squid sample harbored the highest load (Table 1 & 3). Hasan et al. (7), also observed *Salmonella* spp. in different frozen fish sample following enrichment technique within the margin of 1.6×10^5 to 1.3×10^6 cfu/g. High load of *Salmonella* spp. has also been detected in several milk and milk products (38). In the current study, *Shigella* spp. was found to be present in all samples before and after enrichment within the range of 1.8×10^2 to 3.6×10^4 cfu/g and 4.0×10^4 to 1.0×10^5 cfu/g, respectively (Table 1 & 3). A maximum two log increase was found in chicken ball and mutton roll while the other samples showed one log increase (Table 1).

CONCLUSION

An array of frozen foods contributes largely to the economy of Bangladesh, and it also serves as popular food items; therefore studies relating to disease onset, respective of storage, are required for the maintenance of national health and safety. Frozen meat, poultry especially shrimp which is a prime export product of Bangladesh have been studied previously. In our study, we attempted

TABLE 1. Enumeration of total viable bacteria and pathogenic bacteria (*Vibrio* spp., *Salmonella* spp., and *Shigella* spp.) in frozen food samples

Sample	TAVB (cfu/g)		*Vibrio* spp. (cfu/g)		*Salmonella* spp. (cfu/g)		*Shigella* spp. (cfu/g)	
	Non enriched	Enriched	Non enriched	Enriched	Non enriched	Enriched	Non enriched	Enriched
Chicken	3.7×10^{7}	1.11×0^{9}	8.01×0^{2}	1.61×0^{4}	0	2.0×10^{4}	2.5×10^{4}	6.4×10^{4}
Beef	2.3×10^{6}	5.61×0^{9}	6.01×0^{2}	3.61×0^{3}	0	0	1.4×10^{3}	6.0×10^{4}
Rui fish	1.3×10^{6}	2.91×0^{7}	0	1.21×0^{4}	0	4.0×10^{4}	3.0×10^{4}	1.01×10^{5}
Bele fish	6.9×10^{6}	3.2×10^{8}	4.01×0^{3}	3.0×10^{4}	0	0	2.0×10^{2}	2.0×10^{4}
Shrimp	2.6×10^{5}	$3.6x\times10^{8}$	6.61×0^{3}	1.5×10^{4}	0	2.3×10^{3}	3.6×10^{4}	1.0×10^{5}
Squid	1.9×10^{7}	2.51×0^{9}	2.81×0^{4}	1.2×10^{5}	0	1.6×10^{5}	5.0×10^{3}	4.0×10^{4}
Chicken ball	3.8×10^{6}	6.01×0^{8}	6.01×0^{2}	6.8×10^{3}	0	0	1.8×10^{2}	5.0×10^{4}
Mutton roll	2.81×0^{6}	3.11×0^{7}	0	0	0	2.3×10^{2}	2.0×10^{2}	6.3×10^{4}

TAVB = Total aerobic viable bacteria

TABLE 2. Load of bacteria at refrigeration condition

sample	TAVB (cfu/g)	
	Non enriched	Enriched
Chicken	1.0×10^{2}	2.0×10^{3}
Beef	0	0
Rui fish	0	0
Bele fish	0	0
Shrimp	2.0×10^{3}	2.0×10^{3}
Squid	0	1.8×10^{3}
Chicken ball	0	0
Mutton roll	0	0

TAVB = Total aerobic viable bacteria

TABLE 3. Summery of biochemical identification of *Vibrio* spp., *Salmonella* spp., *Shigella* spp.

Assumed Organism	TSI			H_2S creation	Indole test	MR test	VP test	Citrate test	Motility	Oxidase Test
	slant	Butt	gas							
Vibrio spp	Y	Y	-	-	+	+	-	+	+	+
Salmonella	R	Y	-	+	-	+	-	-	+	
Shigella	R	Y	+	+	+/-	+	-	-	-	

TSI = Triple Sugar Iron, Y = Yellow (Acid), R = Red (Alkaline), MR = Methyl red, VP =Voges-Proskauer

to detect microbiological quality as well as pathogenic load of different frozen foods, meat, poultry, shrimp with some processed frozen food and frozen squid which has recently been introduced to the urban consumer's of Bangladesh, Our study focused on enrichment is not suitable for enumeration rather a presence absence test, our future study may include establishment of quantitative method for injured cell analysis and risk assessment of different pathogens commonly observed in frozen food products.

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