Incidence and toxigenic profile of Bacillus cereus in some fishes

Hassanien, F.S.[1]; Hassan, M.A.[1]; El-Hariri, M.D.[2], Eid Sayed*[3]

[1] *Food Hygiene Dep., Fac. Vet. Med., Benha Univ ,* [2] *Microbiology Dep., Fac. Vet. Med., Cairo Univ ,*
[3] *Directorate of Veterinary Medicine, Giza.*
Corresponding author E mail: agyad 2014@yahoo.com

A B S T R A C T

A total of 300 fish samples were collected from Giza and Cairo shops, supermarkets and restaurants. These samples were 150 raw fish samples of Tilapia, Mackerel and Sardine (50 for each) and 150 ready to eat fish samples of fried Tilapia, grilled Mackerel and grilled Sardine (50 for each). Each sample was subjected to bacteriological and molecular examination for detection of *Bacillus cereus* and its toxins. The obtained results using PCR indicated that the incidence of *B. cereus* in raw Tilapia, Mackerel and Sardine were 24% (12/50) , 26% (13/50) and 40% (20/50) respectively ; while for hemolytic toxin was 75% (9/12) , 62% (8/13) and 60% (12/20) , non-hemolytic toxin was 25% (3/12) , 38% (5/13) and 40% (8/20), cytotoxin K was 16.5% (2/12), 15% (2/13) and 0 and Cereulide was 16.5% (2/12) , 7.5% (1/13) and 5% (1/20) in positive samples of *B. cereus* in raw Tilapia, Mackerel and Sardine respectively. In ready to eat Tilapia, Mackerel and Sardine the incidence of *B. cereus* were 20% (10/50) , 18% (9/50) and 30% (15/50) respectively ; while for hemolytic toxin were ٧٠% (٧/١٠) ,67% (6/9) and 73% (11/15) , non-hemolytic toxins were 30% (3/10) ,33% (3/9) and 27 % (4/15) in in positive samples of *B. cereus* in ready to eat Tilapia , Mackerel and Sardine respectively. In this study we didn't detected any cytotoxin K or cereulide toxins genes in any of the ready to eat fish samples.

Keywords: *Bacillus cereus*, toxins, fish, ready to eat fish, PCR.

1. INTRODUCTION

Food and Agriculture Organization (1994) asserted that fish contributes about 60% of the world's supply of protein and that 60% of the developing world derives more than 30% of their annual protein from fish. Fish and ready to eat fish are susceptible to a wide variety of bacterial pathogens, most of which are capable of causing disease. Among these bacterial pathogens; Bacillus cereus, it is important duo to its ability to produce spores that survive in high temperature and grow at low temperatures (van Netten et al., 1990).

Bacillus cereus, a Gram-positive, rod shaped endospore-forming bacteria is an important cause of food-borne illness in humans and is frequently involved in food-borne outbreaks (Hall et al.,2001). The resistance of its spores to adverse environmental conditions has enabled it to

get distributed widely in the environment. When food is not adequately refrigerated and in the absence of competitive flora, B. cereus grows well after cooking (Kramer and Gilbert ,1989).

B. cereus is frequently associated with diarrheal and emetic types of food borne illness. Out of two, diarrheal type syndrome caused by enterotoxin (s), results in diarrhea and the emetic type induces nausea and vomiting. B. cereus produces emetic toxin (Agata et al., 1995) and four other enterotoxins: hemolysin BL or Hbl (Beecher and Wong ,1994), nonhemolytic enterotoxin or Nhe (Lindback et al., 2004), Cytotoxin K or cytK (Lund et al.,2000) and enterotoxin FM or entFM (Asano et al., 1997).

The aim of the present investigation was to study the incidence and level of contamination with B. cereus in fish and ready to eat fish using polymerase chain reaction (PCR) besides detection of toxins gene profile of isolates to determine their pathogenic nature.

2. MATERIAL AND METHODS

2.1. Collection of the samples:

A total of 300 fish samples were collected from Giza and Cairo shops, supermarkets and restaurants. These samples were 150 raw fish samples of Tilapia, Mackerel and Sardine (50 for each) and 150 ready to eat fish samples of fried Tilapia, grilled Mackerel and grilled Sardine (50 for each). Each sample was subjected to bacteriological and molecular examination for detection of Bacillus cereus and its toxins.

2.2. Isolation and identification of B. cereus:

It was carried out according to (Hwang and Park, 2015) and (Harrigan and McCane , 1976) .

2.3. DNA template preparation:

One typical colony from each of the isolates was picked up and inoculated in 5 ml of Brain Heart Infusion (BHI) broth and incubated overnight at 35 °C. The broth culture was subjected for DNA extraction using QIA amp DNA mini kit as per the instructions of manufacturer.

2.4. Molecular characterization of B. cereus and its toxins:

The primers used for the detection of toxin genes (*hbl*, *nhe*, cytK and *ces*) in the current study are described by (Ehling-Schulz *et al.,*2006) (Table 1).

The PCR amplification mixture (20 μl) which was used for the detection included 2x master mix, 5 μl of template DNA, 1 μl for forward and reverse of each oligonucleotide primers. The volume was completed by sterile free water to 20 μl, and the vortex was used to mix them well.

The cycling conditions for PCR for detection of *B.cereus* toxins genes were : initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation step at 94°C for 15 min, annealing step at 60°C for 45 min, extension step at 72°C for 2 min, and final extension step at 72°C for 5 min. PCR products were detected in 1.5 % agarose gel stained with ethidium bromide, viewed by U.V. light.

Table (1): Oligonucleotide primers sequences of toxins genes in *Bacillus cereus* strains: Source (Ehling-Schulz *et al.,* 2006) Metabion (Germany):

Gene	Primer Sequence (5'-3')	Amplified product
hbl	HD2 F (GTA AAT TAI GAT GAI CAA TTTC) HA4 R (AGA ATA GGC ATT CAT AGA TT)	1091 bp
Nhe	NA2 F (AAG CIG CTC TTC GIA TTC) NB1 R (ITI GTT GAA ATA AGC TGT GG)	766 bp
cytK	CK F (ACA GAT ATC GGI CAA AAT GC) CK R (CAA GTI ACT TGA CCI GTT GC)	421 bp
Ces	Ces F (GGTGACACATTATCATATAAGGTG) Ces R (GTAAGCGAACCTGTCTGTAACAACA)	1271 bp

3. RESULTS

Table (2) revealed that by using PCR for detection of *Bacillus cereus* and its toxins genes in raw samples of Tilapia, Mackerel and Sardine. The percentage of positive samples for *B. cereus* were 24% (12/50), 26% (13/50) and 40% (20/50) respectively while for hemolytic, non-hemolytic toxins, cytotoxin K and Cereulide toxins in positive samples of *B. cereus* were 75% (9/12) , 62% (8/13) and 60% (12/20) - 25% (3/12) , 38% (5/13) and 40% (8/20)- 16.5% (2/12), 15% (2/13) and 0 – 16.5% (2/12) , 7.5% (1/13) and 5% (1/20) in Tilapia- Mackerel - Sardine respectively.

Table (3) showed that detection of *Bacillus cereus* and its toxins genes in ready to eat samples of fried Tilapia, grilled Mackerel and grilled Sardine. The percentage of positive samples for *B. cereus* were 20% (10/50), 18% (9/50) and 30% (15/50) respectively while for hemolytic, non-hemolytic toxins in positive samples of *B. cereus* were 70% (7/10) ,67% (6/9) and 73% (11/15) - 30% (3/10) ,33% (3/9) and 27 % (4/15) in fried Tilapia - grilled Mackerel - grilled Sardine respectively. In this study we didn't detected any cytotoxin K or cereulide toxins genes in any of the ready to eat fish samples.

Table (2) incidence of *Bacillus Cereus* and its toxins genes in raw fish by using PCR:

	Tilapia	Mackerel	Sardine	p-value
No. of Samples	50	50	50	
Bacillus cereus	12 (24%)	13 (26 %)	20 (40%)	0.203
*hbl	9 (75%)	8 (62%)	12(60%)	0.658
*nhe	3 (25%)	5 (38 %)	8 (40 %)	0.312
*Cyt K	2 (16.5%)	2 (15%)	0	0.547
*Ces	2 (16.5%)	1 (7.5 %)	1 (5%)	1.000

hbl hemolysin BL, *nhe* non-hemolytic enterotoxin, *Cyt K* cytotoxin K ,*Ces* cereulide,

Table (3) incidence of *Bacillus Cereus* and its toxins genes in ready to eat fish by using PCR:

|	Tilapia (fried)	Mackerel (grilled)	Sardine (grilled)	P. value
No. of Samples	50	50	50	
Bacillus Cereus	10 (20%)	9 (18%)	15 (30 %)	0.330
hbl	7 (70%)	6 (67%)	11 (73%)	0.461
nhe	3 (30%)	3 (33%)	4 (27 %)	1.000
Cyt K	0	0	0	
Ces	0	0	0	
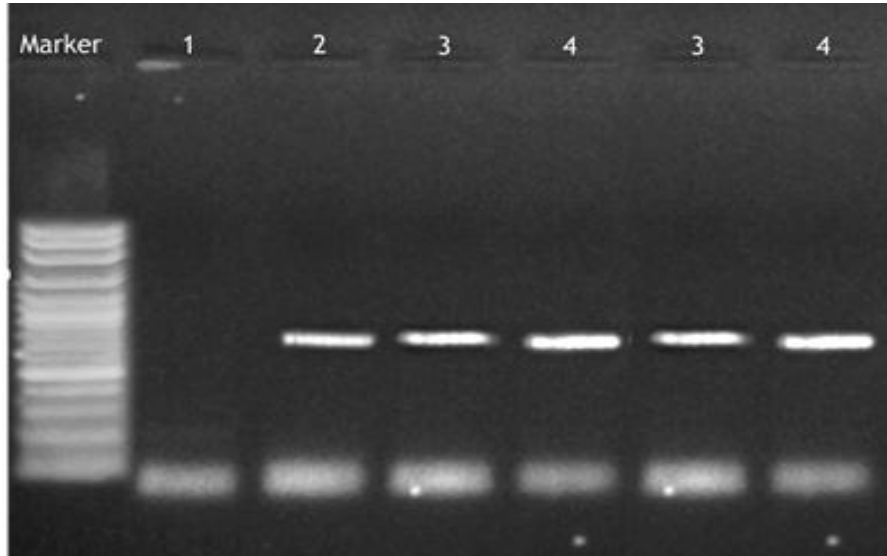
Photo (1) Agarose gel electrophoresis of PCR for detection of *hbl* gene of *B. cereus.* (1091-bp fragment) in examined samples. Marker (Gel Pilot 100 bp. ladder). Lane1, negative control for *Bacillus cereus.* (Master Mix without any DNA). Lane 2 to 6: positive results for *hbl* toxin gene of *B. cerus.*
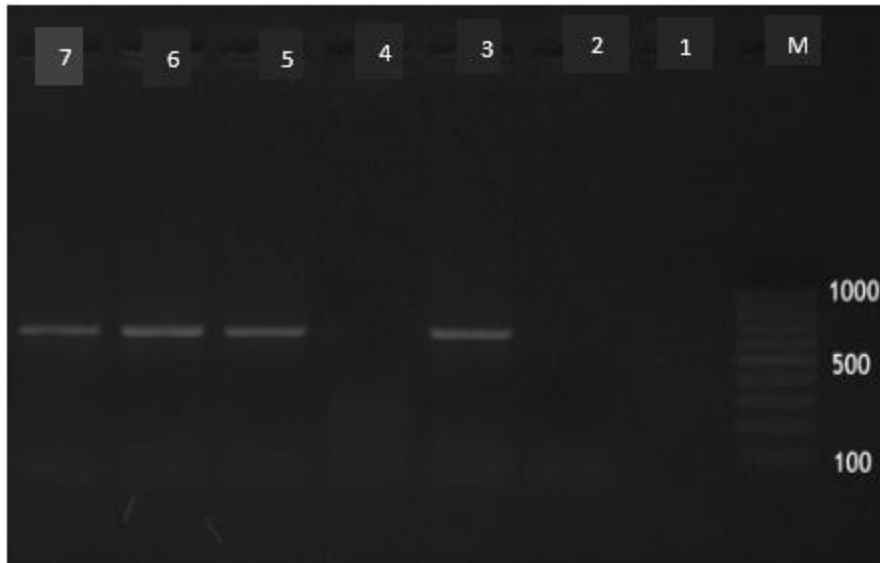
Photo (2) Agarose gel electrophoresis of PCR for detection of n*he* gene of *B. cereus.* (766-bp fragment) in examined samples.Marker (Gel Pilot 100 bp. ladder).Lane1, negative control for *Bacillus cereus.* (Master Mix without any DNA). Lane 2 and 4: negative results for *B. cerus* toxins. Lane3, 5 ,6 7: positive results for *nhe* toxin gene of *B. cerus*.
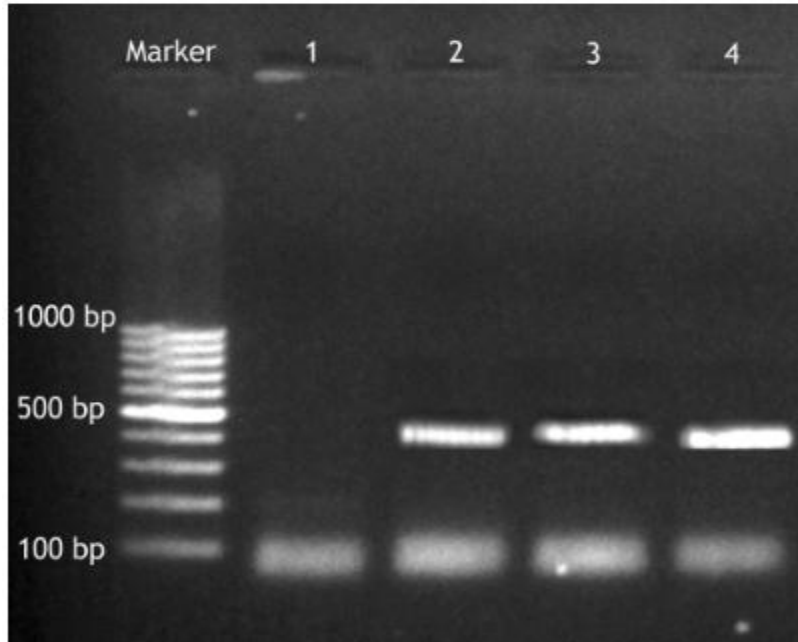

Photo (3) Agarose gel electrophoresis of PCR for detection of *cytK* gene for *B. cereus.* (421-bp fragment) in examined samples. Marker (Gel Pilot 100 bp. ladder). Lane1, negative control for *Bacillus cereus.* (Master Mix without any DNA). Lane 2 to 4: positive results for *cytK* toxin gene of *B. cerus.*
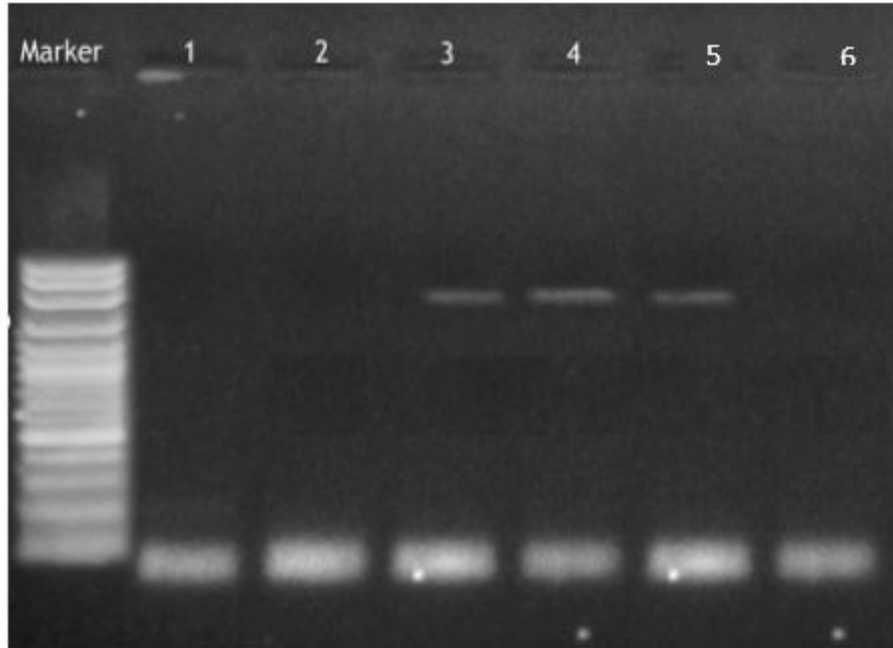
Photo (4) Agarose gel electrophoresis of PCR for detection of *ces* gene for *B. cereus.* (1271 fragment) in examined samples.
Marker (Gel Pilot 100 bp. ladder). Lane1, negative control for *Bacillus cereus.* (Master Mix without any DNA). Lane 2 and 6: negative results for *B. cerus* toxines. Lane 3 to 5: positive results for *ces* toxin gene of *B. cereus*

4. DISCUSSION

Fish is very important for human nutrition because it is rich in animal protein and other elements for the maintenance of healthy body (Andrew, 2001). Raw or cooked fish may be harbored *B. cereus* which can cause human illness duo to it characterized by spore formation and can produce two types of toxin; diarrheic and emetic toxins.

Results in Table (2) agree with (Abd-Elaziz,2016) who reported that the Positive *B. cereus* isolates in raw meat, minced meat and meat luncheon were 15% (3 / 20) ,20% (4 / 20) and 20% (4 / 20) respectively while hemolytic and non-hemolytic toxins among

the positive isolate were 33% (1/3) and 100% (3/3), 44% (1/4) and 100% (4/4) and 50% (2/4) and100% (4/4) in raw meat, minced meat and meat luncheon respectively.

Also, the result agrees with (Abbas *et al.,* 2014) who detect the presence of hemolytic (*hblA*, *hblC* and *hblD*) and non-hemolytic enterotoxin (*nheA*, *nheB* and *nheC*) genes in *Bacillus cereus* strains isolated from milk and milk product collected from local markets. they revealed that the percentage of these genes in *B. cereus* isolates were 22.58, 51.61, 9.67, 90.32, 58.06 and 54.83%, respectively. The result agreed with (Tewari *et al.,* 2015) who isolated *B. cereus* from 30.9 % (29) of the 94 raw meat and meat products samples and

its toxin genes *nhe*, *hbl* and cyt K were isolated from 89.7 % (26), 55.2 % (16) and 41.4 % (12), respectively. And also agree with (Ehling-Schulz *et al.*, 2006) who detected *nhe, hbl, cytK and ces* toxins genes from *B.cereus* food isolates and clinical isolates.

The result is low than that of (Rather, 2011) who detected the incidences of various enteroxigenic genes of *Bacillus cereus* in meat and meat products by PCR and the result of hblD, hblA, hblC, nheA, nheB, nheC, cytK and entFM were 66.10%, 66.10%,67.78%, 96.61%, 96.61%, 93.22%, 67.78% and 100%, respectively. Also, the result is low than that detected by (Khudor *et al.,* 2012) who make Screening of *B. cereus* isolates from milk, soft cheese, curls cheese and yogurt by polymerase chain reaction (PCR) and revealed the presence of diarrheal toxin *cyt K* gene in 87.09 % of isolates.

The low result of the emetic toxin (cereulide) in our study agree with the result of (Abbas *et al*., 2012) who detected the emetic gene in a percentage of 0.00 %, 10.00 %, and 20.00 % in milk, yogurt and curls cheese, respectively.

In relation of presence of *B. cereus* in raw fish the result agrees with result obtained by (Prasad, 2014) who recovered *B. cereus* from shark, anchovy, ribbion fish, sole, mackerel, seer, tuna, snapper, sardine, and pomfret commercially available in Bangalore region- India by using PCR and with (Das *et al.,* 2009) who detect *B. cereus* and enterotoxigenic *B. cereus* in fish samples by PCR in a percentage of 36.7% and 29.41%

respectively also they interpreted that all the diarrheal enterotoxin producing isolates showed the presence of *hbla* gene, but *hbla* gene was not present in any of the non-enterotoxigenic isolates.i.e; *hbla* gene specific PCR can be employed for differentiation of enterotoxigenic *B. cereus* isolates from non-enterotoxigenic isolates. And agree with (Rahmati And. Labbe, 2008) who reported that in 347 fresh and processed retail seafood samples (including Mackerel. Tilapia, Salmon, Trout, Carp and fish past) Sixty-two samples contained *B. cereus* and Thirty (48%) of 62 isolates produced both the hemolysin BL (HBL) and nonhemolytic (NHE) enterotoxins, and 58 (94%) and 31 (50%) produced NHE or HBL toxins, respectively.

Counts ranging from 200 to 10^9 gm^{-1} (or ml^{-1}) *B. cereus* have been reported in the incriminated foods after food poisoning, giving total infective doses ranging from about 5×10^4 to 10^{11} partly due to the large differences in the amount of enterotoxins produced by different strains, the total infective dose vary between 10^5 and 10^8 viable cells or spores. So, any food containing more than 10^3 B. *cereus*/g^{-1} cannot considered completely safe for consumption (Granum and Lund, 1997).

Result in Table (3) agree with that recorded by (Jawad, 2016) who detected 35 *B. cereus* isolates from 70 ready to eat cooked rice samples and it was found that in the 35 *B. cereus* isolates 4 (11 %) isolates positive towards *hblB* and 12 (34%) isolates positive towards *nheA* by using PCR. The result disagree with (El- Sayed ,2015) who isolate *B.cereus* from samples of meat sandwiches of kofta, fried liver (kibda) and

shawerma in a percentage of 94.29 , 88.57 and 97.14, respectively. In addition that he didn't record *hbl* gene of *B. cereus* in any of examined samples but record (*nhe,cytk* and *ces*) genes in all examined samples.

Generally, the infection of raw fish with *Bacillus cereus* and its toxins is higher than the infection in ready to eat fish. This my duo to the fact that the cooking process may effect on the count of aerobic spore forming bacteria, its ability to form active spore or toxins. (Parry and Gilbert, 1980), (Houška *et al.,* 2007) and (Desai and Varadaraj, 2010).

The low percentage of cereulide detected in raw fish and its absence in ready to eat fish may be due to the fact that The emetic toxin only seems to be produced when *Bacillus cereus* is grown on particular substrates particularly rice and other farinaceous materials. Fried or cooked rice has been implicated in nearly 95% of all emetic toxin food poisoning outbreaks (Jenson and Moir, 1997). Also, these results may be attributed to that the emetic toxin titers were much highest when *B. cereus* was grown in skim milk, cooked rice suspension and minimum amino acid-defined medium (MADM) than growth in brain heart infusion (BHI) broth, trypto-soya broth, nutrient broth, trypto-soya agar and cooked rice agar (Agata *et al*., 1999) and (Shinagawa *et al.,* 1992).

Finally, it can be concluded that, it is important to make strict hygienic measures in handling and preparation of fish from fishing to consumption to avoid contamination with *Bacillus cereus* and its toxins.

5. REFERENCES

Abbas, B.A; Khudor, M.H.; Saeed, B.M.S. 2012. Molecular detection of Bacillus cereus emetic toxin gene by PCR and determine its susceptibility against punica granatum extracts. Basrah journal of veterinary research, vol. 11, 4, (79-94).

Abbas, B.A.; Khudor, M.H.; Saeed, B.M.S 2014. Detection of *hbl, nhe* and *bceT* Toxin Genes in *Bacillus cereus* Isolates by Multiplex PCR. Int. J. Curr. Microbiol. App. Sci., 3(11) 1009-1016.

Abd-Elaziz, M.H.G. 2016. Bacteriological and molecular studies on *Bacillus cereus* isolated from meat and meat products. Ph.D. Thesis. microbiology dep. Veterinary medicine, Cairo university.

Agata, N.; Ohta, M.; Arakawa, Y.; Mori, M. 1995. The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. Microbiology 141:983–988.

Agata, N., Ohta, M., Mori, M.; Shibayama, K. 1999. Growth conditions of and emetic toxin production by *Bacillus cereus* in a defined medium with amino acids. *Microbiology and Immunology,* 43: 15-18

Andrew, A.E. 2001. Fish processing Technology. University of Ilorin press, Nigeria, pp: 7-8.

Asano, S.I.; Nukumizu, Y.; Bo, H.; Iizuka, T.; Yamamoto, T. 1997. Cloning of novel enterotoxin genes from Bacillus cereu Bacillus thuringiensis. Appl. Environ. Microbiol., 63:1054–1057.

Beecher, D.J; Wong, A.C.L. 1994. Improved purification and characterization of hemolysin BL, a haemolytic dermonecrotic vascular permeability factor from Bacillus cereus. Infect. Immun., 62:980–986.

Das *et al.* (2009) found that B. *cereus* and enterotoxigenic *B. cereus* were found to be in 36.7 and 29.41 per cent of examined fish samples, respectively. All the diarrheal enterotoxin producing isolates showed the presence of *hbla* gene, but *hbla* gene was not present in any of the non-enterotoxigenic isolates.

Desai, S.V.; Varadaraj, M.C. 2010. Behavioural pattern of vegetative cells and spores of Bacillus cereus as affected by time-temperature combinations used in processing of Indian traditional foods. J Food Sci Technol., 47(5):549-556.

Ehling-Schulz, M.; Gmnebretiere, M.I.I.; Montlian, A.; Berge, O.; Fricker, M.; Svensson, B. 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol.. Lett., 260:.232-240.

El-Sayed, A.A. 2015. Probable dangers from take away meat products meals. Ph. D. thesis. Food Control Dep. Vet. Medicine, Zagazig University.

Food and Agriculture Organization of the United Nations, 1994. Review of the State of the World Fishery Resources; Marine Fisheries. FAO Fishery Circular No 920. Rome.

Granum, P.E.; Lund, T. 1997. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Lett., 157: 223-228.

Hall, J.A.; Goulding, J.S.; Bean, N.H.; Tauxe, R.V.; Hedberg, C.W. 2001. Epidemiologic profiling: evaluating foodborne outbreaks for which no pathogen was isolated by routine laboratory testing: United States,1982–89. Epidemiol Infect 127:381–387.

Harrigan, W.F.; Mccance, M.C. 1976. Laboratory methods in food and Dairy Microbiology. Academic press, New York, San Francisco.

Houška, M., Kýhos, K., Landfeld, A., Průchová, J., Schlemmerová, L., Šmuhařová, H., Špelina, V., Novotná, P. 2007. Dry heat inactivation of Bacillus cereus in rice. Czech J. Food Sci., 25: 208–213.

Hwang, J.Y.; Park, J.H. 2015. Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods. Department of Food Science and Biotechnology, Gachon University, Sungnam 461-701, republic of Korea. J. Dairy Sci., 98:1-9.

Jawad, N. 2016. Determination of Haemolytic and Nonhaemolytic Genes Profiles of *Bacillus cereus* Strains Isolated from fried Rice samples by Polymerase Chain Reaction (PCR) technique. International Journal of ChemTech Research, 9(11), pp. 151-156.

Jenson, I; Moir, C.J. 1997. *Bacillus cereus* and other *Bacillus* species, In A. D. Hocking, G. Arnold, I. Jenson, K. Newton, and P. Sutherland (ed.), Foodborne Microorganisms of Public Health Significance, 5th ed. Australian Institute of Food Science and Technology Inc., NSW Branch, Food Microbiology Group, Sydney, New South Wales, Australia. pp. 379–406.

Khudor, M.H.; Abbas, B.A.; Saeed, B.M.S. 2012. Molecular detection of enterotoxin (cytk) gene and antimicrobial susceptibility of bacillus cereus isolates from milk and milk products. Bas. J. Vet. Res., Vol.11, No.1. p.: 164-173

Kramer, J.M.; Gilbert, R.J. 1989. *Bacillus cereus* and other *Bacillus* species, P. 21-70. In Dayle, M.P. (ed) Food borne Bacterial pathogens, Marcel Dekkar, New York.

Lindback, T.; Fagerlund, A.; Rodland, M.S.; Granum, P.E. 2004. Characterization of the *Bacillus cereus Nhe* enterotoxin. Microbiology, 150: 3959-3967.

Lund,T.; Debuyser, M.L.; Granum, P.E. 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol. Microbiol., 38:254–261.

Parry, J.M.; Gilbert, R.J. 1980. Studies on the heat resistance of Bacillus cereus spores and growth of the organism in boiled rice. J. Hyg., Camb. 84, 77.

Prasad, M.P. 2014. Molecular characterization of Enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers. Int. J. Pure App. Biosci. 2 (4): 189-195.

Rahmati, T.; Labbe, R. 2008. Levels and toxigenicity of *Bacillus cereus* and *Clostridium perfringens* from retail seafood. J. Food Prot. 71, 1178-1185.

Rather, M.A.; Aulakh, R.S.; Gill, J.P.S.; Rao, T.S.; Hassan, M.N. 2011. Direct Detection of Bacillus cereus and its Enterotoxigenic Genes in Meat and Meat Products by Polymerase Chain Reaction. Journal of Advanced Veterinary Research Volume 1, 99-104.

Shinagawa, K., Otake, S., Matsusaka, N.; Sugii, S. 1992. Production of the vacuolation factor of Bacillus cereus isolated from vomiting-type food poisoning. *J. Vet Med Sci* 54: 443-6

Tewari, A.; Singh, S.P; Singh, R. 2015. Incidence and enterotoxigenic profile of *Bacillus cereus* in meat and meat products of Uttarakhand, India. J Food Sci Technol., 52(3):1796–1801.

Van Netten, P.; Van De Noosdijik, A.; Van Hoensel, P.; Mossel, D.A.A.; Perales, J. 1990. Psyehrotrophic strains of *Bacillus cereus* producing enterotoxin. J. Appl. Bacteriol., 69:73- 79.