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Rapid detection of *Clostridium perfringens* in seafood

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ABSTRACT

Background: *C perfringens* is one of the most widely spread pathogens in the environment and an important cause of food poisoning. Seafood is a poor vehicle of *C. perfringens* food transmission. The enterotoxigenic strains are a common cause of food poisoning outbreaks worldwide. The symptoms, predominantly diarrhea and abdominal pain, appear 6 to 24 h after ingestion of contaminated food. Vomiting and fever are unusual. **Objective:** The objective of this work to study the prevalence of *C.perfringens* in seafood samples [Finfish (Salmon and Tilapia), Crustaceans (Shrimp and Crab) and Molluscs (Calm)], through isolation, identification and rapid detection of their toxins and *cpe* genes in seafood samples by Conventional PCR and SYBR Green real time PCR. **Results:** one hundred and fifty seafood samples [Finfish (Salmon and Tilapia), Crustaceans (Shrimp and Crab) and Molluscs (Calm)] collected from different supermarkets in El Dakahlya Governorate, Egypt. Culturing, identification and serotyping of the isolates showed that *C.perfringens* isolates were detected with an incidence of 13%. The prevalence of *C.perfringens* in the exanimated samples in Tilapia, Shrimp, Crab and Calm were 10%, 36.6 %, 13.3% and 6.6%, respectively. While *C.perfringens* was not recorded in salmon at all. Twenty seafood samples were identified as *C.perfringens* type "A" (alpha toxin) by using Multiplex PCR and these were tested by uniplex PCR and SYBR Green real time PCR for *cpe* gene detection, results showed the positive results for *cpe* gene were 15% and 20% respectively. **Conclusion:** Good hygienic conditions, prevent the cross contamination of *C.perfringens* from environment to raw seafood. SYBR Green real time PCR more sensitive, qualitative and quantitative to rapidly detection *C.perfringens* and their toxins directly from seafood.

KEYWORDS: Seafood, *Clostridium perfringens*, toxins, enterotoxin, Conventional PCR, Syber Green Real time PCR.

INTRODUCTION

C.perfringens being responsible for food poisoning; it also causes a number of human diseases ranging from necrotic enteritis to wound infection and life threatening gas gangrene. Such pathogenicity is associated with the lethal extracellular toxin which has defined as enzyme activity as collagenas, hyalauronidase and deoxyribonuclease[1].

PCR-based techniques are used increasingly in food-microbiology research as they are well developed and when applied as culture confirmation tests, they are reliable, fast and sensitive. PCR methods offer a sensitive and specific detection of *C.perfringens* and its enterotoxins in food samples [2,3].Many authors have proposed the use of PCR for the detection of food-borne pathogens to replace the time consuming culture based classical techniques [4, 5].Both of which are time consuming and laborious. However, products of PCR can also be detected by using a DNA binding dye, such as SYBR Green Real-time PCR assays can be automated and are sensitive and rapid. They can also quantify PCR products with greater reproducibility while eliminating the need for post-PCR processing, thus preventing carryover contamination [6].This study was aimed to rapid diagnosis of *C.perfringens* from seafood samples using of multiplex PCR for typing *C.perfringens* through detection of their toxins gens and

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using uniplex PCR and real time SYBR Green to directly detect *cpe*gene in DNA extracted from seafood samples and comparison between them according results.

MATERIAL AND METHODS

Sampling:

One hundred and fifty seafood samples including Finfish (Salmon and Tilapia), Crustaceans (Shrimp and Crab) and Molluscs (Calm), 30 each, were collected randomly from supermarkets in El-dakahlya Governorates, Egypt. The collected samples were prepared according to previously published protocol [7, 8] under aseptic condition.

Isolation and identification of C.perfringens:

Each sample was inoculated onto a tube of sterile freshly prepared cooked meat medium (CMM) then the tube was incubated anaerobically at 37°C for 24-48 hours after that a loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 µg /ml) and the plate was incubated anaerobically at 37°C for 24-48 hrs. [9].Bacterial colonies were purified individually based on the size, shape, color, hemolysis pattern. The suspected colonies of *C. perfringens* were picked up and examined for their morphological and culture characters microscopical examination of stained films with Gram's stain and biochemical tests such as gelatinase, fermentation of sugars, gelatin liquefaction, litmus milk, catalase, indole tests were identified [10].

C.perfringens enumeration [9]:

Appropriate 1 ml of dilution was spread over the surface of duplicate TSC agar plates. Plates were overlaid with TSC agar and incubated at 37°C for 24hours in an anaerobic jar. Black CFU was counted as presumptive *C.perfringens*/g of sample. Black colony confirmed by lactose sulphid.

Nagler's Test by Half Antitoxin Plate [11]:

Detection of lecithinase activity of *C.perfringens* alpha toxin on lecithin of an enriched egg yolk agar medium.

Typing of C. perfringens toxins by dermonecrotic test in albino guinea pigs:

It was applied by preparation of the toxins and their treatment [11], application of dermonecrotic test I/D of an albino guinea pig [12, 13] and interpretation of the results according to colour degree of the dermonecrotic reaction and its neutralization [14].

Toxin antitoxin neutralization test:

It was performed by injection of the toxin antitoxin mixture intraperitoneally in mice or intradermally in an albino guinea pig [15].

Molecular Assay for identification of C.perfringens:

Extraction of DNA according to QIAamp DNA mini kit instructions using 200 µl of seafood sample for using in Conventional and Syber green real timePCR.

Conventional PCR:

Preparation of PCR Master Mix for preparation of four Clostridium toxins and preparation of uniplex PCR Master Mix for *cpe* gene and Cycling conditions of the primers during cPCR temperature and time conditions of the primers during PCR according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit: multiplex and uniplex PCR-based protocol is described with 5 primer sets to simultaneously identify the toxins together with all primers used in the study,

The oligonucleotide primers used in this study and their amplicon sizes are listed in Table 1 (16, 17). Uniplex PCR was performed in 25 µl of reaction volume consisting of 12.5 µl of Emerald Amp GT PCR mastermix (2X premix) (Takara) Code No. RR310A kit, 1 µl of 20 pmole of each primer (Sigma, USA), 6µl of template DNA and water nuclease free up to 25 µl. PCR cycling program was performed in PTC-100 TM programmable thermal cycler (Peltier-Effect cycling, MJ, Research, INC., UK) for detection of Alpha, Beta, Iota and Epsilon genes as following: initial denaturing step at 95°C for 10 min; followed by 35 cycles of 94°C for 5 min, 94 for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min [16] and for detection of *cpe* gene as following: initial denaturing step at 95°C for 10 min; followed by 35 cycles of 94°C for 30 sec., 94 for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final extension step at 72°C for 7min[17].Aliquot of each amplicon, along with a 100-600 bp molecular weight DNA ladder (QIAGEN, USA) were subsequently separated by electrophoresis on 1.5% molecular biology grade agarose gel (Sigma, USA) stained with 0.5µg/ml ethidium bromide (Sigma, USA) on a mini slab horizontal electrophoresis unit (Bio-Rad,

USA) at 100 V for 30 min. DNA bands were visualized under UV transilluminator (Spectroline, USA) and photographed[18].

Table 1: Oligonucleotide primers sequences Source: Midland Certified Reagent Company_ oilgos (USA).

M.O.	Toxin	Primer	Amplified product(bp)	Annealing temp. (°C)	Ref
C.perfringens	Alpha	GTTGATAGCGCAGGACATGTTAAG	402	55 1 min.	16
		CATGTAGTCATCTGTTCCAGCATC			
	Beta	ACTATACAGACAGATCATTCAACC	236		
		TTAGGAGCAGTTAGAACTACAGAC			
	Epsilon	ACTGCAACTACTACTCATACTGTG	541		
		CTGGTGCCTTAATAGAAAGACTCC			
	Iota	GCGATGAAAAGCCTACACCACTAC	317		
		GGTATATCCTCCACGCATATAGTC			
	Enterotoxin (*cpe* gene)	GGAGATGGTTGGATATTAGG	233	50 30 sec.	17
		GGACCAGCAGTTGTAGATA			

Real time PCR Amplification and cycling protocol (MX3005P QPCR system) it was applied in the following steps:

QPCR reaction setup:

DNA samples were amplified in a total of 25 µl of the following reaction mixture: 12.5µlQuantiTect SYPR Green (2X), 0.5µl of each primer (50 pmol), 7 µl template DNA and 4.5 µl water, nuclease-free. The samples were transferred to each well of a PCR plate.

Running of the QPCR:

It was applied in 40 cycles according to the following program: enzyme activation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds then annealing 50°C for 30 seconds, extension at 72°C for 30 seconds. PCR results were given as the increase in the fluorescence signal of the reporter dye detected and visualized by The MX3005P QPCR system. Ct values (threshold cycle) represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred, for each amplification plot.

Analysis of the results using the standard curve method [19]:

The standard curve method is based on using a DNA sample of known concentration to construct a standard curve. Once the standard curve has been generated, it can then be used as a reference standard for the extrapolation of quantitative information regarding the unknown concentration.

RESULTS AND DISCUSSION

In this study *C.perfringens* was detected in seafood samples 20 (13%) out of 150 collected samples. The prevalence of *C.perfringens* in Tilapia and (Shrimp, Crab and calm) was 10% and 18% (36.6, 13.3 and 6.6) %, respectively. 100% of Salmon fish samples were free from *C.perfringens* (Table 2).

Salmon fish samples were free from *C.perfringens*[20, 21, 22]and *C.perfringens* was highly detected with a percentage of 84% [23].*C.perfringens* was detected from tilapia with a percentage of 1%, 4%, 6%, 16%, 16.92%,18.35%, 18.36%, 27.24%, 84 %, 30% and 62.5 % [20, 24, 25, 26, 27, 28, 29, 30, 23, 31, 21].*C.perfringens* was isolated from seafood (shrimp, crab and clam) with percentage of 17%, 9%, 7%, 6% and 4.7%, 0%[31, 24, 22, 26, 32, 20]

The obtained results revealed that the average *C.perfringens* counts of seafood samples (tilapia, shrimp, crab and clam) were 6.5×10^2, 1.7×10^3, 1.3×10^3 and 2.2×10^3 CFU/g (Table 2). The average count value of *C.perfringens* results were 1.82 - 4.26 ×10 and 3.5 ×10 CFU/g obtained by [23, 27].

Seafood might have come from contaminated water bodies, which in turn get infected due to excretion of the organism in feces of various carrier animals or man or the water used for washing seafood might be contaminated [25].

Poor hygiene from fishing to marketing is main purpose of prevalence of and multiplication of *C.perfringens* and poor conditions of storage such as the environments attracted flies and insects and prolonged preservation facilitate the reproduction of *C.perfringens* spores which might also have contributed to the higher bacterial counts and hence poor quality of fish are presented to costumers[33].

Table 2: Prevalenceof *C.perfringens* in seafood samples

Seafood Samples (No.)		*C.perfringens*type A NO. (%)	Count of *C.perfringens*		
			Minimum count (CFU)	Maximum count (CFU)	Average (CFU)
Finfish	Salmon (30)	0(0)*	0	0	0
	Tilapia (30)	3(10)*	10	1.3×10^3	6.5×10^2
Crustaceans	Shrimp (30)	11 (36.6)*	2×10	3.4×10^3	1.7×10^3
	Crab (30)	4(13.3)*	7×10	2.5×10^3	1.3×10^3
Molluscs	Calm (30)	2(6.6)*	4×10	4.3×10^3	2.2×10^3
Total	150	20(13)**	----------		

* % of positive samples of *C.perfringens* according to total No. of each type of samples.
**% of positive samples of *C.perfringens* according to total No. of samples.

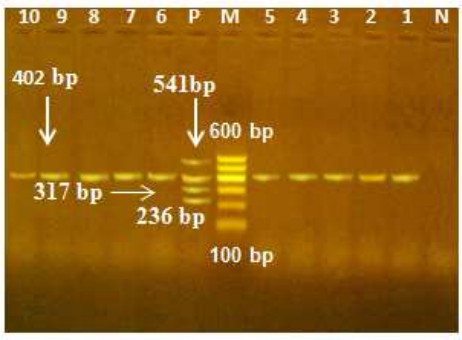
Regarding to conventional methods for identification of *C.perfringens* recovered from seafood the present results revealed that *C.perfringens* was Gram positive short plumb rarely sporulated and non-motile bacilli. It was apparent that sheep blood agar with neomycin sulphate (200 µg/ml) was a perfect medium for isolation of *C.perfringens* rather than other *Clostridium*spp and gave double zones of haemolysis. All the recovered strains in this work were fermentative to different sugars as glucose, maltose, lactose, sucrose and mannitol with production of acid and gases, gelatin liquefiers, litmus milk positive, catalase, oxidase and indole tests negative[34,35, 36].

Nagler`s test represented the action of *C.perfringens* alpha toxin (lecithinase) on lecithin of egg yolk onto enriched egg yolk agar medium which appeared as pearly opalescence zone surround the colonies while this reaction was inhibited by *C.perfringens* alpha toxin antiserum[15].

Biotyping of *C.perfringens* isolates was applied by dermonecrotic reaction in albino gunia pigs[14]. All*C.perfringens* isolates recovered from seafood (Tilapia, Shrimp, Crab and Calm) were identified into toxigenic strains type A100%[28, 30,31,37].

Rapid identification of pathogens may prevent foodborne diseases through better control of foods. Pathogenic bacteria that were previously isolated and identified by conventional testing procedures can be easily detected quickly and reliably by rapid testing methodologies, including molecular biological assays. However, DNA based techniques can be adversely affected by interfering substances in the sample or lack the sensitivity needed to detect bacteria in very low levels[37]. Therefore, in this study, we first developed sensitive and specific representative molecular assays. However, even though molecular methods are often touted as being highly sensitive (detection limit of 1 to 10 gene targets), they are generally not of value if employed directly for the detection of organisms in food or environmental samples [38].

In this study, Conventional PCR for Detection of the presence of *C.perfringens* toxins in seafood samples by using Multiplex PCR results. All the examined seafood samples were identified as *C.perfringens* type "A" (alphatoxin) and gavecharacteristic bands at 402 bp.Figures (1,2).



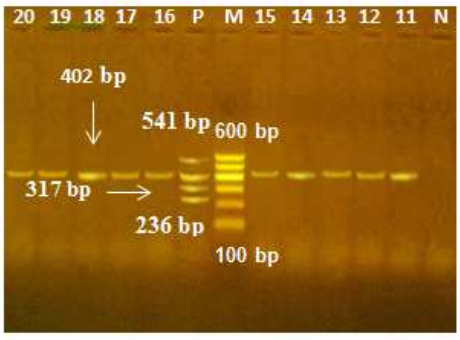


Fig. (1)Fig. (2)

Fig.1,2: Muliplex PCR analysisby Agarose gel electrophoresis of representative *C.perfringens* type A, B, C and D "alpha, beta, epthilon, iolta toxins gene" amplicons. Lane M: Marker 100 bp DNA ladder (Pharmacia). Lane P:positive control forharbouring*C.perfringens* alpha, beta, epthilon and iolta toxins gene obtained from reference laboratory for veterinary in animal health research institute, Dokki, Giza

Lane N:Negativecontrol for *C.perfringens*. Lane 1-20 seafood samples positive for alpha toxin gene of 402bp fragment only.

Multiplex PCR was used as a sensitive technique and time saver than traditional methods to detect *C.perfringens* in seafood. All seafood samples were examined were identified as *C.perfringens* type "A" (alpha toxin)[30, 31].

The genotyping by PCR suggested that alpha toxin is linked to *C.perfringens* type A in fish and seafood. Type A was the most predominant one which was responsible for potential food poisoning and gastroenteritis.[28]

UniplexPCRresults showed that 3 out of 20 samples (15%) positive for *C.perfringens* enterotoxin gene and gavecharacteristic bands at 233 bp[31] Table (3) andFigures(3, 4).

Real Time PCR technique based SYBR Green dye for detection of *cpe*were show 4/20seafood samples (20%) gave a positive result by amplification of *cpe*gene Table (3) and Figures (5, 6)[28].

SYBR Green real time PCR, with its combination of speed, sensitivity, specificity ,qualitative and quantitative technique detection of *C.perfringens*strains (1-10) gene targets in a wide range of food homogenate samplesharbouring the enterotoxin gene cluster in seafood [37, 38, 39]positive results was showed as curve.

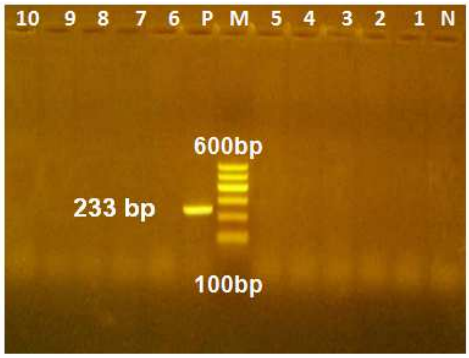
The presence of *cpe*gene in *C.perfringens* type A is very uncommon, and <5% of global *C.perfringens* type A isolates are *cpe*positive [28].

Isolates carrying the *C.perfringens* enterotoxin gene (*cpe*) are the most important food borne pathogens. Previous surveys indicated that *cpe* positive *C.perfringens* isolates are present in only 5% of nonoutbreak food samples and then only at low numbers, usually less than 3 cells/g.[38]

Table 3: Detection of *C.perfringens* enterotoxin genes in seafood samples using uniplex PCR and SYBR Green real time PCR:

Samples (NO.)	Code number	*C.perfringens* enterotoxin genes		
		Uniplex PCR	SYBR Green RT PCR	
			Result	Ct*
Shrimp (8)	1, 4,7, 9, 11, 15, 17, 18	-	-	-
Tilapia (3)	2,6,8	-	-	-
Clam (2)	3,10	-	-	-
Crab (3)	5,19,20	-	-	-
Shrimp (1)	12	+	+	24.34
Shrimp (1)	13	-	+	26.17
Shrimp (1)	14	+	+	24.57
Crab (1)	16	+	+	23.56

*Ct:Cycle thresholder



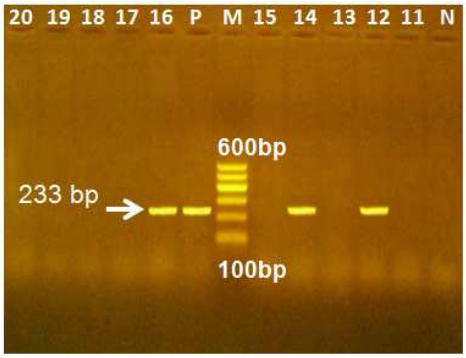


Fig. (3) **Fig. (4)**

Fig. 3,4: Agarose gel electrophoresis of *cpe* gene amplicons.
Lane M: Marker 100 bp DNA ladder (Pharmacia). Lane P:Positive control obtained from reference laboratory for veterinary in animal health research institute, Dokki, Giza. Lane N:Negativecontrol.Lane 12 and 14 shrimp samples and Lane 16 crab sample were positivefor *cpe* gene of 233bp.

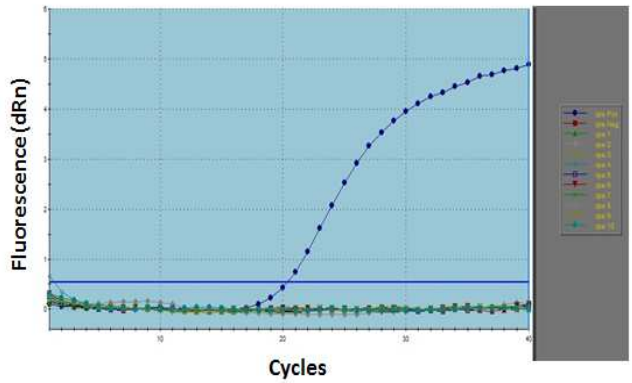


Fig. 5: Data sheet of RT-PCR showing amplification curves of DNA of standard samples and seafood samples number 1-10 for detection of *cpe* gene (the colour of each curve is corresponding to well content in data sheet). Amplification plot was generated by Stratagene MX3005P software. The fluorescence emission intensity is plotted on the Y axis versus the cycle number on the X axis.

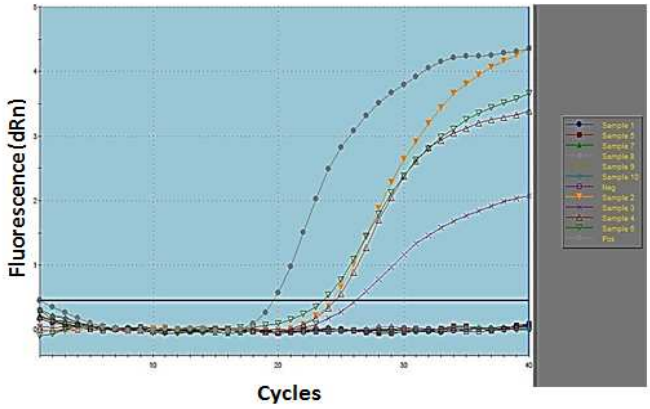


Fig. 6: Data sheet of RT-PCR showing amplification curves of DNA of standard samples and seafood samples number 11-20for detection of *cpe* gene.Display Real-Time PCR amplification plots that appeared from the (23.56 to 26.17 cycles). Samples were negative which appeared under threshold line.

Conclusion:

This work revealed the presence of *C.perfringens*in seafood. Seafooda probable health risk for consumers of raw seafood. Improvement of the effective sanitary conditions inhandling and processing operations from fishing to marketing is needed to minimize therisk of infections associated with consumption of these products. SYBR Green real time PCR more sensitive, qualitative and quantitative than Conventional PCR to detect food microorganism and their toxins directly from seafood for rapidly diagnosis of food poisoning outbreak.

REFERENCES

[1] Ammar, A.M., S. Basma, A. Ahlam and S.A. Walaa, 2011. Study on the effect of some spices and organic salts on *Clostridium perfringens* during cooling of raw and cooked ground beef.Zagazig VeterinaryJournal, 39(5): 138-151.

[2] Anbudhasan., P., A. Uma and G. Rebecca, 2012. Assessment of bacteriological quality in selected commercially important processed seafood (PCR). International Journal of Food, Agriculture and Veterinary Sciences, 2(3): 20-25.

[3] EI Jakee, J., S.A. Marouf, S.A. Nagwa, H. Eman, I. Sherein, A.A. Samy and E.E. Walaa, 2013. Rapid Method for Detection of *Staphylococcus aureus* Enterotoxins in Food. Global Vet., 11(3): 335-341.

[4] Gravet, A., M. Rondeau, C.H.F. Monteil, H. Grunenberger, Monteil, J.M. Scheftel and G. Prevost, 1999. Predominant *Staphylococcus aureus* isolated from antibiotic-associated diarrhea is clinically relevant and produces enterotoxin Aand the bicomponent toxin LukE-LukD. J. Clin. Microbiol., 37: 4012-4019.

[5] Miethke, T.C., H.K. Wahl, B. Echtenacher, P.H. Krammer and H. Wagner, 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. J. Exp. Med., 175: 91-98.

[6] Jothikumard, N. and M.W. Griffiths, 2002. Rapid detection of *E.coli* O157:H7 with multiplex real-time PCR assays. Appl. Environ. Microbiol., 68: 3169-3171.
[7] ISO/DIS 6887-3:2013:Microbiology of food and animal feeding stuffs Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products. International standard, ISO 6887-3, 2E, 1-11.
[8] Wallace, H.A. and S.H. Thomas,2003. Bacteriological Analytical Manual 8th Edition, Revision A, Food Sampling and Preparation of Sample Homogenate, Chapter 1.
[9] Rhodehamel, E.J. and M.H. Stanley, 2001. Bacteriological Analytical Manual 8th Edition, Revision A,*Clostridium perfringens*, Chapter 16.
[10] Koneman, E.W., S.D. Allen, V.R. Dowell and H.W. Summers, 1992. Colour atlas and text book of diagnostic microbiology. 4th Ed., Philadelphia, J.B. LippinCott. Co., New York, London.
[11] Bullen, J.J., 1952. *C.perfringens* in the alimentary tract of normal sheep. J. Path. Bact., 64: 201-210.
[12] Oakley, C.L. and G.H. Warrack, 1953. Routine typing of *C.welchii*. J. Hyg. Gamb., 51: 102-107.
[13] Quinn, P.J., B.K. Markey, M.E Carter, W.J. Donnelly, F.C. Leonard and D. Maguire, 2002. Veterinary microbiology and microbial disease. 2nd Ed., Blackwell Sci., pp: 84-96.
[14] Stern, M. and I. Batty, 1975. Pathogenic Clostridia. Butter Worth, London, Boston.
[15] Smith, L.D.S. and Holdeman, 1968. The pathoigenic anaerobic bacteria. 1st Ed.; Charles Thomas Publisher, USA., pp: 201-255.
[16] Yoo, H.s., S.U. Lee, K.Y. Park and Y.H. Park, 1997. Molecular Typing and Epidemiological Survey of Prevalence of *Clostridium perfringens* Types by Multiplex PCR. J. Clin. Microbiol., 35(1): 228-232.
[17] Heikinheimo, A. and H, Korkeala, 2005. Multiplex PCR assay for toxinotyping*Clostridium perfringens* isolates obtained from Finnish broiler chickens. Lett. Appl. Microbiol., 40: 407-411.
[18] Sambrook, J., E.F. Fritscgh and Mentiates, 1989. Molecular coloning.A laboratory manual. Cold spring Harbor Laboratory press, New York.
[19] Adams, P.S., 2007. Data analysis and reporting, In, Dorak, M. T. (Ed.): Real-time PCR, Taylor & Francis Group, New York: pp: 39-62.
[20] Papadopoulou, C., E. Economou, G. Zakas, C. Salamoura, C. Dontorou and J. Apostolou, 2007. Microbiologicaland pathogenic contaminants of seafood in Greece. J. Food Quality, 30(1): 28-42.
[21] ElShorbagy, M.M., M.R. Lamyaaand, H. Mona, 2012. Prevalence of *Clostridium perfringens* Alpha toxin in processed and unprocessed fish.Int. J. of Microbiol. Res., 3(3): 195-199.
[22] Rokibul, M.D.H.,A. Mrityunjoy, D. Eshita,K.D.Kamal, A. Tasnia,A.A.Muhammad, K.F. Kazi and N. Rashed, 2013. Microbiological study of sea fish samples collected from local markets in Dhaka city. Int. Food Res. J. 20(3): 1491-1495.
[23] Herrera, F.C.,J.A.Santos, A. Otero and M.L.Garc, 2006. Occurrence of foodborne pathogenic bacteria in retail prepackaged portions of marine fish in Spain. J. Appl. Microbiol., 100: 527-536.
[24] Martha, I.,A .Tracy,E.M. Barbara and L.S.David,2010. American Society for Microbiology. All Rights Reserved. Epidemiology of Seafood-Associated Infections in the United States. Clin. Microbiol. reviews 23(2): 399-411.
[25] Endale, B.G., A.H. Razzbuddin, B. Probodh and B. Acinto, 2013. Presence of enterotoxigenic *C. perfringens* in foods of animal origin, Guwahati, India J. Environ.Occup. Sci., 2(1): 45-50.
[26] Dalton, C.B.,J. Gregory,M.D. Kirk, R.J.Stafford, R. Givney, E. Kraa and D. Gould, 2004.Foodborne disease outbreaks in Australia, 1995 to 2000.Commun. Dis. Intell. Q Rep., 28(2): 211-224.
[27] Gamal, E.D. and M.R. El-Shamery,2010.Studies on contamination and quality of fresh fish meats during storage Egypt.Acad. J. biolog. Sci., 2(2): 65-74.
[28] Arunava, D. and J. Adarsh,2012a.*Clostridium Perfringens* type A from fresh water fishes. Int. J. Adv. Biotech.Res., 3(3): 680-687.
[29] Cai, Y.,J. Gao,X. Wang, T. Chai,X. Zhang, H. Duan,S. Jiang, B.A.Zucker and G. Schlenker, 2008.*Clostridium perfringens* toxin types from freshwater fishes in one water reservoir of Shandong Province of China, determined by PCR. DtschTierarzt Wochenschr. 115(8): 292-297.
[30] Arunava, D. and J. Adarsh, 2012b. Genotyping of *C. perfringens* from fresh water fish and fish pickles. J. Microbiol., Biotech. Food Sci., 2(1):162-174.
[31] Qiyi, W. and A.M.Bruce, 2004. Detection of Enterotoxigenic*Clostridium perfringens* Type A Isolates in American Retail Foods. Appl. Environ.Microbiol., 70(5): 2685-2691.
[32] Rahmati, T. and R. Labbe, 2008. Levels and Toxigenicity of Bacillus cereus and *Clostridium perfringens* from Retail Seafood, J. Food Protect., 71(6): 1178-1185.
[33] Maysoon, S.A.,2014. Isolation of bacteria from fish, Int. J. Adv. Res., 2(3): 274-279.
[34] Peter, H.A., S. Nicholas, S. Elisabeth and G. John, 1986. Bergey`s Manual Systemic Bacteriology. 2, Williams and Wilkins.

[35] Vaikosen, E.S. and W. Muller, 2001. Evaluating biochemical tests for isolation and identification of*Clostridium perfringens* in faecal samples of small ruminants in Nigeria. Bull. Anim. Health and Prod. in Africa, 49 (4): 244-248.
[36] Liang, L., H. Li, E. Xian and B. Li, 2004. Isolation and identification of *Clostridium perfringens* from bovine.Chinese J. of Vet.Sci. Technol., 34(5): 58-60.
[37] Ismail, H.T. and Ö. Haydar, 2015.Evaluation of a Real-Time PCR Multiplex Assay For Detecting Foodborne Pathogenic Bacteria.Inter. J. Food Engin.Res., 1(1):7-16.
[38] Ikuko, K., M. Kazuaki, M. Kanako, Y. Natsuko, U. Hirotoshi, A. Shigeru and A.M. Bruce, 2011. Detection of Enterotoxigenic*Clostridiumperfringens*in Meat Samples by Using Molecular Methods. American Society for Microbiol. Appl. Environ. microbial., 21(77): 7526-7532.
[39] Xihong, Z., W.L. Chii, W. Jun and H.O. Deog, 2014. Advances in Rapid Detection Methods for Foodborne Pathogens, J.Microbiol. Biotechnol., 24(3): 297-312.