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Article *in* Fresenius Environmental Bulletin · April 2021

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MOLECULAR TYPING OF *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM DIFFICILE* FROM SALAD AND DRINKING WATER OF QUETTA

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ABSTRACT

Clostridium perfringens and *Clostridium difficile* are significantly important pathogens due to their ability of causing various diseases in animals and human beings. Samples (n=100) including water (n = 50) and salad (n = 50) were collected from households and retail shops of Quetta city. The samples were processed for pathogen isolation by culturing and preliminary conformed with the help of biochemical profiling. Molecular conformation was done through PCR. Out of the total samples, 22% (22/100) were found positive for the presence of *C. perfringens* whereas, *C. difficile* was found in 11% (11/100) samples. The overall incidence of *C. perfringens* was higher in water samples (34%) in comparison to salad samples (10%), the difference was statistically significant (p=0.004). *C. difficile* was detected in 6% salad and 16% water samples. However, the difference in the prevalence of *C. difficile* was non-significant between the water and salad samples (p=0.110). This study reveals that the microbiological quality of drinking water and vegetables (salads) in developing countries needs improvement in order to control the food borne pathogens and associated health risks.

KEYWORDS:
Food Safety, Food borne pathogens, Food analysis, Quality, Public Health

INTRODUCTION

The bacteria *Clostridium perfringens* and *C. difficile* are member of genus *Clostridium*, Gram-positive, spore forming, anaerobic with rod shaped morphology. *C. perfringens* is an important pathogen as it is the causative agent of many diseases, it is classified in 5 different types from A through E with four main toxins production, iota (ι), beta (β), alpha (α) and epsilon (ε) [1]. *C. perfringens* produce 16 toxins including enterotoxin called *Clostridium perfringens* enterotoxin (CPE) [2]. *C. perfringens* is associated food poisoning and it is a significant reason of outbreaks worldwide [3]. Its usual symptoms are diarrhea, abdominal cramps, and nausea [4]. *C. perfringens* food poisoning is amongst the major food borne disease in developed countries. Food borne illness triggered by plasmid CPE has been reported in Europe and Japan, gastroenteritis outbreaks (313) by *C. perfringens* has been reported in Wales and England from year 1992–2012 [5].

C. difficile is known as a hospital acquired pathogen mostly related with diarrheal disease in patients under antibiotics treatment [6]. Its infection is linked with two toxins, toxin A (tcdA) and B (tcdB): A (enterotoxin) and B (cytotoxin) these are the major virulence factors of this bacterium and are encoded by two genes [7]. Spores enter the body, reactivate in the colon and produce its toxins to cause health complications [8]. *C. difficile* is swallowed as resistant spores and passes through the acidic pH of stomach [9]. In small intestine, spores develop into the vegetative form and in the large bowel, *C. difficile* associated disease initiates if the indigenous flora has been disturbed by antibiotic therapy etc. It duplicates in the intestinal crypts, liberating A and B toxin, that cause severe inflammation [10]. *C. difficile* was reported to be associated with elevated death frequency in hospitalized patients. From 2007-11, in Canada mortality rate increased 33-38 each year due to *C. difficile* infection [11]. The ability of *Clostridium* spp. to survive under various environments has urged the researchers to explore the various possible routes of its transmission to humans. The temperature of water and ecosystem can affect the biotic and abiotic processing [12]. In current study the water and salad samples were analyzed for the presence of two pathogenic *Clostridium* spp. in Quetta region of Balochistan Pakistan.

TABLE 1
Specie-specific primers of *C. perfringens* and *C. difficile* based on 16S rDNA sequence

Primers	Specie	Primer sequence (5'-3')	Amplicon Size	References
ClPER-F	*C. perfringens*			[12]
ClPER-R		AGA TGG CAT CAT CAT TCA AC	793-bp	
		GCA AGG GAT GTC AAG TGT		
ClDIF-F	*C. difficile*	CTT GAA TAT CAA AGG TGA GCC A		[12]
ClDIF-R		CTA CAA TCC GAA CTG AGA GTA	1,085- bp	

TABLE 2
Detection of *C. perfringens* and *C. difficile* from water and salad samples by culture

Sample type	*Clostridium perfringens*		*Clostridium difficile*	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
Water (n= 50)	15 (30%)	35 (70%)	05 (10%)	45 (90%)
Salads (n= 50)	05 (10%)	45 (90%)	02 (04%)	48 (96%)
Total (n= 100)	20 (20%)	80 (80%)	07 (07%)	93 (93%)
Chi-square	6.250		1.382	
P value	0.012*		0.240^{ns}	

*$p \leq 0.05$ (Significant); $p^{ns} > 0.05$ (Non-significant)

TABLE 3
Detection of *C. perfringens* and *C. difficile* from water and salad samples through PCR

Sample type	*Clostridium perfringens*		*Clostridium difficile*	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
Water (n= 50)	17 (34%)	33 (66%)	8 (16%)	42 (84%)
Salads (n= 50)	05 (10%)	45 (90%)	3 (6%)	47 (94%)
Total (n= 100)	22 (22%)	78 (78%)	11 (11%)	89 (89%)
Chi-square	8.392		2.554	
P value	0.004*		0.110^{ns}	

*$p \leq 0.05$ (Significant); Pns>0.05 (Non-significant)

MATERIALS AND METHODS

Sampling. Sampling was done during February to July 2018, which was comprised of 50 salad and 50 water samples. Sterilized stomacher bags were used for salad collection from hawkers and shops whereas, sterilized bottles were used for water samples collection from households in Quetta city. The samples were processed in the Center for Advanced Studies in Vaccinology and Bacteriology, University of Balochistan within 6h of its collection and not later than 24 h in any case.

Sample processing. For the isolation of *C. perfringens* and *C. difficile* each salad sample was processed by homogenizing 10 g and mixing it into 100 ml of peptone saline water. While the water samples were processed by mixing 10 ml into 90 ml of peptone saline water. The sample were normally incubated at 37 °C for 16-24 h before selective culturing and isolation of the target pathogenic bacteria.

Isolation and identification. The homogenized mixture of samples was then transferred to Thioglycolate Broth (Oxoid, UK) for anaerobic growth and incubated at 37 °C for 16-24 h. Growth on Thioglycolate media were streaked on Sheep Blood Agar dishes and incubated at 37 °C for 16-24 h anaerobically. Suspected colonies from Sheep Blood Agar were finally streaked over Reinforced Clostridial Agar (RCA) (Oxoid, UK), for 16-24 h, at 37 °C, anaerobically.

Suspected colonies from RCA were processed for Gram's staining and microscopy. Which were further analyzed for biochemical profiling catalase, oxidase, indole test, nitrate reduction, VP, motility test, lecithinase test and dulcitol, glucose, mannitol, ribose, xylose, sucrose, lactose, fructose, arabinose, sorbitol sugar fermentation tests for preliminary identification and conformation.

Molecular detection of the isolates by PCR. Primers specific for Clostridium species based on 16S rDNA gene, previously used by Kikuchi et al. [13] were used in this study which gave amplification product of 793-bp for *Clostridium perfringens* and 1,085bp for *Clostridium difficile* (Table 1). PCR reaction comprising of 20 µl was prepared for PCR amplification by using 10 µl master mix, 3 µl DNA template, 2 µl (1+1) forward and reverse primers and 5 µl molecular grade water.

The PCR program comprised of initial denaturation for 3-4 min at 94 °C, 35 cycles of DNA denaturation for 30 sec at 94 °C, annealing was done for 30 sec at 48-51°C for *Clostridium perfringens* and 50-53 °C for *Clostridium difficile*, and extension for 2 min at 72 °C. The final extension was carried out for 7 min at 72 °C. Annealing temperature was set to taper down of -0.5 °C, 7 cycles with 30 sec intervals repeated of 5 times with total 35 cycles.

Statistical Analysis. Statistical analysis was done using statistical package software SPSS 20.0, Cross tabulation and Chi square tests were used to determine the significant difference ($p < 0.05$) among variables.

RESULTS

Out of the total 100 samples (50 water and 50 salads) analyzed, *C. perfringens* were found positive in 20% (20/100) and *C. difficile* in 07% (7/100) by culture. *C. perfringens* was detected in 30% (15/50) water and 10% (05/50) salad samples by culture. a significant difference has been revealed in the detection rate of *C. perfringens* between water and salad samples (p=0.012) by Chi-square test. *C. difficile* was detected in 10% (05/50) water and 4% (02/50) salad samples. However, the difference in the prevalence of *C. difficile* was non-significant between the two sample types (p=0.240) (Table 2).

The samples analyzed for the target pathogens by culture were processed with PCR conformation and it was found that out of 100 (50 water and 50 salads) samples analyzed, the *C. perfringens* presence were conformed in 22% (22/100) and *C. difficile* in 11% (11/100) by PCR. The prevalence of *C. perfringens* was found higher 34% (17/50) in water samples as compared to salad sample 10% (5/50). This difference was statistically significant (p=0.004). *C. difficile* was detected in 16% (08/50) water and 6% (03/50) salad samples. However, the difference in the prevalence of *C. difficile* was non-significant between the water and salad samples (p=0.110) (Table 3). The PCR conformed higher number of *C. perfringens* and *C. difficile* compared to culture and biochemical conformation. Figures 1 and 2 show the molecular conformation of the target pathogens through PCR.

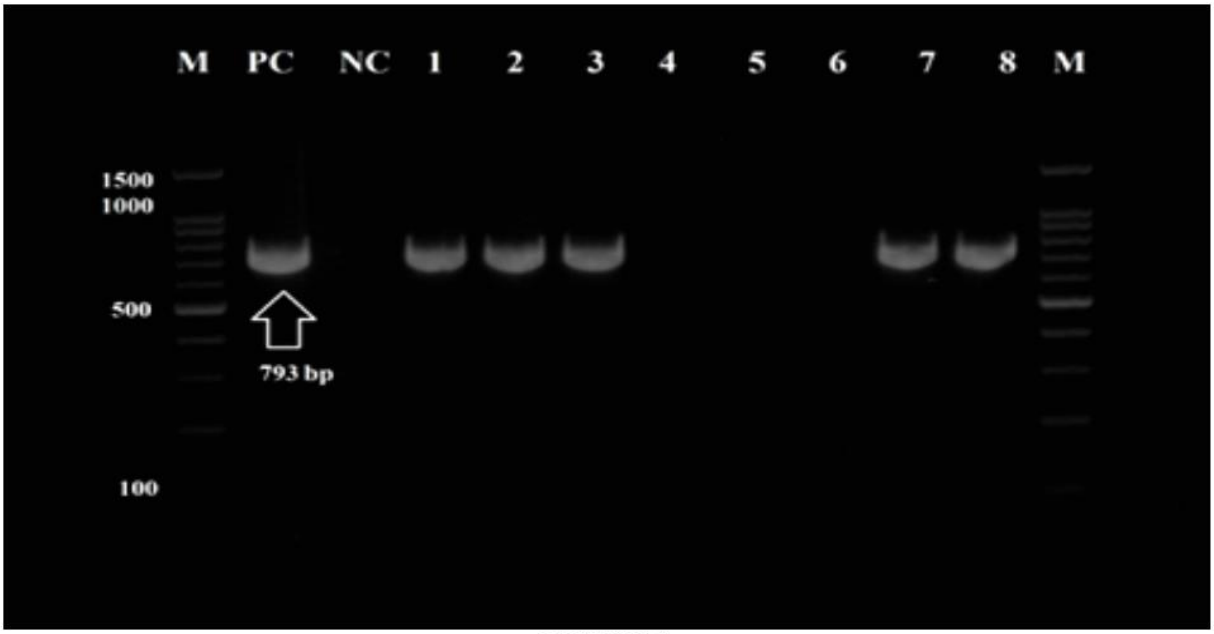

FIGURE 1
Electrophoresis showing amplification of 16S rDNA (793-bp) of *C. perfringens* by PCR. Lane 1,2,3,7, and 8= positive amplification., M= DNA Marker, PC= Positive Control, NC= Negative Control

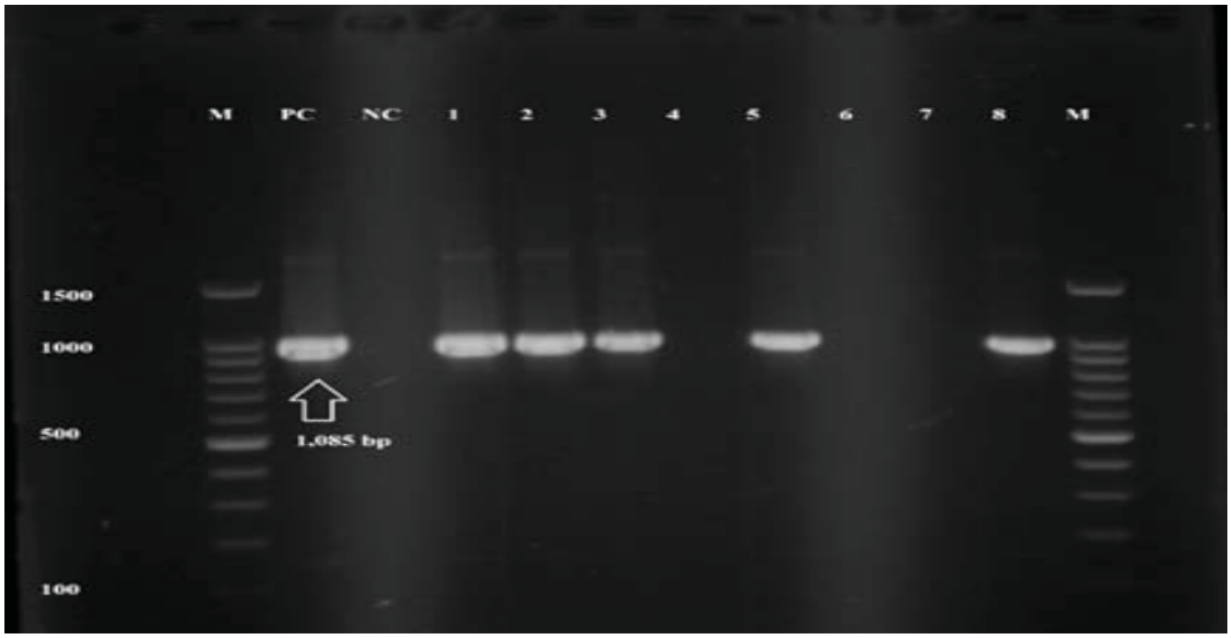

FIGURE 2
Electrophoresis showing amplification of 16S rDNA (1,085 bp) of *C. difficile* by PCR. Lane 1,2,3,5, and 8= positive amplification, M= DNA Marker, PC= Positive Control, NC= Negative Control

DISCUSSION

In current study the occurrence of *C. perfringens* in water was found 34% (17/50). Due to lack of proper filtration facilities in Quetta city and old corroded pipes for transportation of water are reason for water contamination. The findings of this study are in compliance to Hörman et al. [14] similar study where he reported 31.7% prevalence of *C. perfringens* in water, whereas some studies with lower finding have also been reported by Pitkänen et al. [15] from Finland where he found 10% (8/80) prevalence of *C. perfringens* in water indicating that water supply plants in developed countries face limitation of funding as well [16].

Vierheilig et al. [17] claimed 100% incidence rate of *C. perfringens* from a water samples in from Japan and Austria respectively. This high prevalence rate in two different studies was because samples were collected from river mouth and wastewater treatment plants which were vulnerable to contamination with pathogens.

In this study water samples were evaluated, and 16 % samples were found positive for *C. difficile*. Janezic et al. [18] reported 14.4% water samples contamination with *C. difficile* in a study from Chile, this percentage is almost similar to the percentage of our study. The prevalence rate may be due to the lack of filtration and water treatment facility for the community water plants in Quetta city [19]. Low prevalence has been reported in Canada house tap water samples which were checked for the presence of *C. difficile* and 2/20 (10%) samples were found positive. In developed countries like Canada where water is properly treated the presence of *C. difficile* in water indicates that *C. difficile* survive the process of filtration to some extent and enter the municipal system [20]. From drinking and tap water the prevalence of *C. difficile* was reported to be 6% in Zimbabwe [21]. Detection of *C. difficile* in stored and well water demonstrates the capacity of water as a source of infection.

The highest rate of *C. difficile* detection was reported from Slovenia where 25 river water samples were evaluated and 68% were found contaminated. High percentage of positive samples were related with the increased population in those sites [22]. Salads provides important nutrients but at the same time if not properly handled it can act as a medium for the spread of gastrointestinal infections [23, 24].

Salad samples were evaluated in this study to check the incidence rate of *C. perfringens* and 05 (10%) of the samples were found contaminated. The findings of this study agree with some previously reported studies. Lin and Labbe [25] and El-Tawab et al. [26] reported the incidence 10 and 10.5% in vegetables and herbs from USA and Egypt, respectively. The presence of *C. perfringens* in salads is because the vegetables and salads grown locally in Quetta city are irrigated with sewage water.

Tango et al. [27] studied microbiological quality of fruits and vegetables in Korea and found *C. perfringens* as the predominant organism in vegetables 13.3% (48/360). While the packing of vegetables, the rate of respiration of packed and crowded fresh produce rise, thus producing an anaerobic environment serving in growth of bacteria such as

C. perfringens [28]. Percentage of *C. difficile* that was detected from salads in this study was (6%) 3/50 this is in compliance with many other similar studies conducted. The 5.66% incidence of *C. difficile* from Iran was reported by Yamoudy et al. [23] in salads. In another study salad samples bought from seven superstores, 3 (7.5%) were found positive for *C. difficile* in Scotland [29]. Due to shortage of water for agriculture purpose, the farmers in Quetta use waste and polluted water for farming of vegetables which could possibly be the cause for incidence of *C. difficile* in samples processed.

Prevalence of *C. difficile* in salads and vegetables in some other studies ranged from 0-4.5% lower than the findings of this study the lower detection rate in these studies could possibly be due to collection of samples over a short period of time and small sample size [30, 31].

CONCLUSION

The microbiological quality was found to be unsatisfactory thus the presence of *C. difficile* and *C. perfringens* in salads and drinking water collected in the city of Quetta is a main risk to the health of the community, so it is important to take measures to improve the quality of drinking water and salads. Policy makers must develop strategies and device them to ensure the supply of pure and clean drinking water and safe and healthy vegetables / salads for the consumer. In Pakistan the burden of these pathogens in salads and water is less reported, so further research is suggested so the health risks due to these pathogens could be highlighted and preventive measure could be considered as soon as possible.

ACKNOWLEDGEMENT

Hafsa Jamil and Hamida Ali contributed to sample collection and experiment. Dr. Abdul Samad and Ali Akbar designed the study and supervised for different task throughout the research and writing process. Muhammad Naeem, Nosheena, Shabir Ahmad Khan and Zarghoona Farooq Shah helped in experimentation and data analysis. Dr. Muhammad Bilal Sadiq co-write the article and conduct brief revisions.

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Received: **30.10.2020**
Accepted: **10.01.2021**

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