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PREVALENCE AND RISK FACTORS ANALYSIS OF SOIL - BORNE *CLOSTRIDIUM PERFRINGENS* IN SELECTED DISTRICTS OF PUNJAB PROVINCE, PAKISTAN

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ABSTRACT

Clostridium perfringens is a Gram positive anaerobe which makes spores that help it to persist under different environmental conditions. Spores of *C. perfringens* have the potential to remain in the fecal matter and soil for many years due to which control of transmission of pathogen becomes difficult. The pathogen is responsible for various diseases in both humans and animals. We determined the prevalence of *C. perfringens* in the soil of selected districts of Punjab province of Pakistan along with potential association of different categorical variables. A total of 970 samples were processed for real time PCR based screening of genome corresponding to *cpa* gene (alpha toxin gene) of *C. perfringens.* Only n=96 samples were found positive [9.89% with CI: 95% (8.018- 11.78), p-value< 0.05]. Highest prevalence was found in district "Chakwal" followed by district "Attock" and "Lahore". The villages found positive for *C. perfringens* were evaluated for different risk factors including distance from the main road, animal market and water source; animal density, number of houses in the village and animal and human interaction at the sampling site. Analysis of the data revealed that animal and human interaction was the associated risk factor in most of the districts followed by distance from the water source, animal density and human population of the village. The study concludes that *C. perfringens* is prevalent in the soil of Punjab province and the prevalence varies with several risk factors in different districts. Further studies are required to find out most prevalent type of *C. perfringens* as well as effect of different categorical variables on prevalence of *C. perfringens* in other parts of the country.

Key Words:*Clostridium perfringens*, *cpa gene*, soil, Pakistan

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INTRODUCTION

Clostridium perfringens, a Gram-positive, spore forming, obligate anaerobic microorganism is an Edaphic zoonotic pathogen which is ranked as biosafety level II organism and its enterotoxin is categorized as category B bio-warfare agent by CDC. (CDC Reports, 2006) It produces a variety of toxins which contribute towards its pathogenicity. On the basis of production of four lethal toxins alpha, beta, epsilon and iota *C. perfringens* is classified into 5 types A,B,C,D and E. (Sayeed *et al*. 2010; Ossiprandi and Zerbini. 2013) Type A strain produces alpha toxin, type B produces alpha, beta and epsilon toxin, type C produces alpha and beta toxin, type D produces alpha and epsilon toxin and type E produces alpha and iota toxin. (Baums *et al*. 2004) These toxinotypes can also produce some other toxins like CPE or enterotoxin and CPB2 or beta2 toxin. (Chan *et al*. 2012)

Clostridium perfringens is a frequently isolated pathogen from the gastrointestinal tracts of animals and human beings which has most wide spread distribution in the environment including soil, sewage and marine environment. (O'brien and Melville, 2000; Marks *et al*. 2002; Shimizu *etal*. 2002) It is also widespread in different food items including under cooked meat, canned food, poultry, vegetables and fish. (Doosti *et al*. 2017) The natural source of contamination with *C. perfringens* is feces of animals and man which is transmitted through different types of food and water. (Doosti *et al*. 2017)

Clostridium perfringens makes spores that help it to persist under different environmental conditions like high temperature, toxic chemicals, different radiations and high pressure. (Talukdar *et al*. 2015) The spores of *C. perfringens* are very resistant to heat. These spores withstand temperature as high as 75°C for 15min, can persist in the environment for longer periods and can also resist disinfectants. (Gerba, 2009) It is very difficult to control the transmission of *C. perfringens* because the spores have the potential to remain in the fecal matter and soil for long periods of time. (Baker *et al*. 2010) There is a wide range of animal and veterinary diseases caused by different types of *C. perfringens* including food poisoning, gas gangrene, infectious diarrhea and pigbel in human beings while enterotoxaemia in sheep, cattle, lamb, calves, dogs, cats, swine, bears and horses, lamb dysentery, pulpy kidney disease in lambs and necrotic enteritis in poultry. (Hatheway 1990; Power 1996).

Soil is a complex environment and microorganisms are present diversely in it in separate

micro habitats. (Torsvik *et al*. 1996) Among the microorganisms inhabitant in the soil *C. perfringens* is most frequently isolated. (Hirsh and Biberstein, 2005) Several researchers have isolated *C. perfringens* from soil and incidence as high as 100% have been reported in the soil. (Konishi*et al*. 1981)

Clostridium perfringens has been reported from humans and animals in Pakistan with disease occurrence associated with different resources. It is a prevalent pathogen in the Punjab province of Pakistan. Soil is a major reservoir of *C. perfringens* and plays an important role in its transmission. Since soil can be a potential source of disease transmission as evidenced from studies in other parts of the world. (Voidarou *et al*, 2011) However, there is absolute paucity of any data or evidence from Pakistan. The study was conducted with the objective to find out the prevalence of *Clostridium perfringens* in the soil of different districts of Punjab province of Pakistan. The study gave an insight into the evaluation of different risk factors including distance from the animal market, distance from the main road, distance from the water source, number of animals present at the sampling site, human population and animal human interaction that might affect the prevalence of *C. perfringens* in the soil.

MATERIALS AND METHODS

Study Area and sampling strategy: Punjab province is the most populous province of Pakistan with a population of 110 million as estimated in 2017 and is the second largest province with respect to area covering 205,344 square kilometers of country. People earn their livelihood mainly through livestock and agriculture. There are total 36 districts in the Punjab province out of which nine districts were selected. These districts include "Attock", "Chakwal", "DG Khan", "Faisalabad", "Gujranwala", "Lahore", "Sahiwal", "Sargodha" and "Sheikhupura". There are total 4850 villages in these nine districts. The districts were selected on the basis of three factors. Firstly, they are representative of major livestock production area as well as agricultural practices in the province, secondly the districts have high incidence of different human and veterinary diseases and lastly there is evidence of many soils borne zoonotic pathogens in the districts.(Shabbir *et al*. 2015; Ahmed *et al*. 2017; Ali *et al*. 2017; Muhammad *et al*. 2017)

From each district 10% of the total villages were selected randomly (Table.1). From nine districts 485 villages were selected. There were total five sampling sites selected from each village. Out of these five sampling sites, four sites were the four corners of each village. Those places were selected for sampling where close human and animal interaction was present like farm area, some households, domestic animal sheds, passages from where animals are transported in and out of village. While one site was that where no animal and human interaction was present or interaction was less frequent. It was labeled as control.

Approximately 1Kg sample was collected from each sampling site in each village. Samples were collected in triplicate from each site and mixed together. The samples were transported to the lab of Microbiology Department of UVAS, Lahore. The samples which were collected from four corners of the village were pooled by taking 50-gram soil from each sample and mixed properly. While the fifth sample, control, was processed as such.

Collection of Risk Factors Data: A questionnaire was designed and was filled at the time of sampling for collecting the information about different risk factors. It covered the information about the farmer i.e., name, contact number, address, union counsel, tehsil and village name, source of irrigation, distance from the main road, total number of animals, number of houses in the village, distance from the animal market, information about the endemic diseases in the area and types of vaccine. GPS receiver was used to measure the geographical coordinates (Longitude and Latitude) at the time of sampling.

Detection of *C. perfringens* in the Soil Sample: 0.25 g of soil was measured from each soil sample, labeled properly and processed for genome extraction with the help of DNA isolation kit i.e. PowerSoil^{R} DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad CA, U.S.A) The kit was used to extract the whole genome as per manufacturer's recommendations. After extraction, the quality and quantity of DNA was determined using Nano Drop spectrophotometer with accuracy of measurement of DNA up to 3700 ng/µL (Thermo Scientific, USA) following the procedure of Haque *et al*. (2003).

The specific and sensitive primers were designed for detection of *C. perfringens* in the extracted genome (F: 5/-TGCACTATTTTGGAGATATAGATAC-3/; R: 5/-CTGCTGTGTTTATTTTATACTGTTC-3/) and probe: FAM-TCCTGCTAATGTTACTGCCGTTGA-TAMRA was used targeting *cpa* gene (alpha toxin gene).

Real time-PCR was optimized and assay was validated for detection of soil borne *Clostridium perfringens* using DNA extracted from ATCC ^{R} 13124 ™ as positive control in CFX96 ™ Real-Time PCR Detection Machine (BIO-RAD, U.S.A.) using different concentrations of probe, primers, BSA, master mix and DNA template (Wang *et al*. 2011) After optimization of the PCR machine for positive control the soil DNA samples were processed for presence or absence of *C. perfringens* on optimized conditions.

Data Analysis: The results of real-time PCR were compiled in a single Microsoft Excel spreadsheet. Data was analyzed using SPSS version 20.0 (SPSS Inc.,

Chicago, IL, USA) through chi square test using 95% confidence interval and 5% level of significance. The results of physical risk factors were analyzed by calculating Odds ratio. The numeric and categorical variables were analyzed using descriptive statitistics. Table 1,2,3 and 4 present summaries of the statistical analysis.

RESULTS

Prevalence of *Clostridium perfringens* in the Soil: Prevalence of *C. perfringens* in the soil was determined by using Real time PCR based detection. Total 970 samples from nine districts of Punjab were processed for DNA extraction and then Real time PCR based detection using specific primers. Out of these 970 samples, 96 samples were found positive in different districts (Table1). Highest prevalence was found in district "Chakwal" (18%) followed by "Attock", "Lahore", "DG khan" and "Sahiwal". Lowest prevalence was found in district"Sargodha" (6.75%) followed by "Sheikhupura", "Faisalabad" and "Gujranwala". In district "Attock", "45" villages were sampled. Total "90" soil samples including 45 samples and 45 controls were processed for detection of *C. perfringens* genome. Out of 90 samples 12 (13.3%) were positive for *C. perfringens* genome. In district; 'Chakwal" 14 (18%) soil samples," DG Khan" 10 (11%) samples, Faisalabad" 11 (7.4%) samples, "Gujranwala" 12 (8.33%), "Lahore" 7 (12%), "Sahiwal" 11 (10.8%), "Sargodha" 10 (6.75%) while in district "Sheikhupura" 9 (7.62%) soil samples were found positive for *C. perfringens* genome. (Table 1)

The significance of the positive soil samples found through real time PCR was checked by using Chi square test using SPSS software. The prevalence was found significant with a p-value less than 0.05.

Table.1: Prevalence of *C. perfringens* in Selected Districts of Punjab province.

District	Pathogen detection through RT-PCR		Percentage (95% CI)
	Villages	+ve/tested	
Attock	45	12/90	13.33(6.311-20.36)
Chakwal	38	14/76	18.42(9.706-27.14)
DG Khan	43	10/86	11.62(4.853-18.4)
Faisalabad	74	11/148	7.43(3.207-11.66)
Gujranwala	72	12/144	8.33(3.819-12.85)
Lahore	29	7/58	12.06(3.686-20.45)
Sahiwal	51	11/102	10.18(4.481-15.89)
Sargodha	74	10/148	6.756(2.713-10.8)
Sheikhupura	59	9/118	7.62(2.838-12.42)
Total	485	96/970	9.89(8.018-11.78)

Association of Different Risk Factors with Soil borne *C. perfringens* genome: The soil samples found positive for *C. perfringens* were evaluated for different environmental risk factors on the basis of information gathered at the time of sampling. In each district different risk factors were associated with the prevalence of *C. perfringens*. Analysis of these factors showed that the distance of positive village from animal market was significantly associated with the prevalence of *C. perfringens* genome in four districts including district "Attock", "Chakwal", "Gujranwala" and "Sahiwal". In these districts the villages which were located in a distance of less than one kilometer from the animal market showed more prevalence as compared to the villages which were present at a distance of more than one kilometer from the animal market. Distance from the main road was positively associated in five districts including district "Attock", "DG khan", "Sahiwal", "Sargodha" and "Sheikhupura". In these districts the prevalence was high in the villages located near to main road (<500 meters) while prevalence was low in the villages located away from the main road (>500 meters). Distance of the positive village from main water source like river, canal etc was significantly correlated in six districts i.e., "Sahiwal", "Sargodha", "Faislabad", "Gujranwala", "Lahore" and "DG khan". The sampling sites which were located near to some water source (<100 meters) showed more prevalence of *C. perfringens* as compared to the sites away from water source (>100 meters). (Table 2,3,4)

Prevalence of *C. perfringens* genome was significantly associated with the number of animals present at the sampling site in six districts including "Attock", "Chakwal", "Gujranwala", "Lahore", "Sargodha" and "Sheikhupura". Results showed that more the number of animals (>1000 animals) present at the site of sampling more the prevalence of *C. perfringens*. Human dwelling in the positive villages was significantly associated with prevalence of *C. perfringens* genome in the six districts i.e. "Sahiwal", "Sargodha",

"Faislabad", "Gujranwala", "Lahore" and "DG khan". The villages where more human population was present (no. of houses >300) the prevalence was high as compared to the villages with less human population (no. of houses< 300). The risk factor, human and animal interaction, was significantly associated with the prevalence of *C. perfringens* genome in seven out of nine districts while in two districts the risk factor could not be analyzed as none of the positive sample belonged to the area where no animal and human interaction was present. The sites of sampling where animal and human interaction was present showed more prevalence of *C. perfringens* as compared to the sites with no animal and human interaction. The summary of all the associated risk factors in all districts is given in the table 2, 3 and 4.

Table.2: Risk factors associated with the prevalence of *C. perfringens* in district Attock, Chakwal& DG Khan.

Risk Factor	Criteria	Attock			Chakwal			DG Khan		
		+	-	Odds ratio	+	-	Odds ratio	+	-	Odds ratio
Distance from Animal Market	>1 Kilometer	4	28	1.12(0.3095-4.503)	4	15	1.253(0.3426-4.585)	3	24	0.9286(0.2208-3.905)
	<1 Kilometer	8	50		10	47		7	52	
Distance from Main Road	>500 meters	8	48	1.25(0.3462-4.513)	8	36	0.963(0.2981-3.11)	4	17	2.314(0.5848-9.154)
	<500 meters	4	30		6	26		6	59	
Distance from water source	<100 meters	5	44	0.599(0.1611-1.892)	6	23	0.5417(0.1677-1.75)	5	37	1.054(0.282-3.94)
	>100 meters	7	34		8	39		5	39	
Animal Density	<1000 animals	11	58	3.793(0.4603-31.26)	7	23	1.696(0.5276-5.449)	3	36	0.4762(0.1145-1.981)
	>1000 animals	1	20		7	39		7	40	
No. of houses/village	>300	3	24	0.75(0.1864-3.018)	1	12	0.3205(0.03812-2.695)	7	22	5.727(1.356-24.18)
	<300	9	54		13	50		3	54	
Human and animal interaction	Absent	3	40	3.158(0.7945-12.55)	4	32	2.667(0.755-9.491)	1	42	11.12 (1.341-92.41)
	Present	9	38		10	30		9	34	

Table.3: Risk factors associated with prevalence of *C. perfringens* in districts Faisalabad, Gujranwala &Lahore.

Risk Factor	Criteria	Faisalabad			Gujranwala			Lahore		
		+	-	Odds ratio	+	-	Odds ratio	+	-	Odds ratio
Distance from Animal Market	>1 Kilometer	2	66	0.239(0.0498-1.147)	3	26	1.359(0.243-5.376)	3	37	0.2838(0.0562-1.431)
	<1 Kilometer	9	71		9	106		4	14	
Distance from Main Road	>500 meters	1	47	0.1915(0.02379-1.541)	3	56	0.4524(0.1171-1.747)	4	30	0.9333(0.1889-4.611)
	<500 meters	10	90		9	76		3	21	
Distance from water source	<100 meters	6	59	1.586(0.4619-5.449)	7	71	1.203(0.3632-3.983)	4	25	1.387(0.2815-6.83)
	>100 meters	5	78		5	61		3	26	
Animal Density	<1000 animals	4	66	0.6417(0.1721-2.196)	6	58	1.276(0.391-4.163)	6	27	5.333(0.5986-47.52)
	>1000 animals	7	71		6	74		1	24	
No. of houses/village	>300	8	55	3.976(1.01-15.65)	7	54	2.022(0.6098-6.706)	4	15	3.2(0.6374-16.06)
	<300	3	82		5	78		3	36	
Human and animal interaction	Absent	4	67	1.67(0.4689-5.984)	3	68	3.188(0.826-12.3)	0	29	Not possible
	Present	7	70		9	64		7	22	

Table.4: Risk factors associated with the prevalence of *C. perfringens* in districts Sahiwal, Sargodha and Sheikhupura.

Risk Factor	Criteria	Sahiwal			Sargodha			Sheikhupura		
		+	-	Odds ratio	+	-	Odds ratio	+	-	Odds ratio
Distance from Animal Market	>1 Kilometer	6	22	3.674(1.046-13.54)	2	35	0.7357(0.1491-3.63)	1	36	0.2535(0.03053-2.105)
	< 1 Kilometer	5	69		8	103		8	73	
Distance from Main Road	>500 meters	4	24	1.595(0.4288-5.935)	5	53	1.604(0.4432-5.804)	7	50	4.13(0.8207-20.78)
	<500 meters	7	67		5	85		2	59	
Distance from water source	<100 meters	8	47	2.496(0.6224-10.01)	3	55	1.078(0.2477-4.695)	2	37	0.556(0.11-2.811)
	>100 meters	3	44		7	83		7	72	
Animal Density	<1000 animals	2	40	0.2833(0.05795-1.385)	6	62	1.839(0.4967-6.806)	6	57	1.825(0.4344-7.669)
	>1000 animals	9	51		4	76		3	52	
No. of houses/village	>300	7	25	4.62(1.244-17.15)	3	36	1.214(0.298-4.948)	1	44	0.1847(0.02231-1.529)
	<300	4	66		7	102		8	65	
Human and animal interaction	Absent	2	40	3.529(0.7219-17.26)	2	71	4.239(0.8688-20.68)	0	60	Not possible
	Present	9	51					9	49	

DISCUSSION

Several researchers have isolated *C. perfringens* from the soils of different areas since so many years (Yamagishi*et al*. 1964; Matches*et al.* 1974; Li *et al*. 2007; Voidarou*et al*. 2011; Rumah *et al*. 2013). Konishi *et al*, 1981 reported hundred percent incidence of *C. perfringens* in the soil. In Pakistan different studies have been done on *C. perfringens* (Javed *et al*. 2012; Naz *etal*. 2012; Nasir *et al*. 2013; Mohiuddin *et al*. 2016) and its prevalence in different animals like sheep, goats, lambs, deer, camel, poultry etc (Hussain *et al*. 2014;Mohiuddin *et al*. 2016;Maqbool *et al*. 2017;Haq *et al*. 2018). But no studies have been done on the prevalence of *C. perfringens* in the soil of Pakistan. Significant results were obtained when soil samples were analyzed for the presence of *C. perfringens* through Real time PCR with a p-value <0.05, Nagpal *et al* (2015) also found significant prevalence of *C. perfringens* using real time PCR(Nagpal *et al*. 2015). Prevalence of soil borne *C. perfringens* found through Real-Time PCR in this study was "9.89 percent", previously Matches *et al* (1974) reported a prevalence of "4.1 percent" for *C. perfringens* in the soil. The prevalence found in our study is in accordance to the prevalence reported by Haq *et al* in Foals i.e. "18.5 percent" in Punjab province of Pakistan. (Haq *et al*. 2018). Khan *et al* (2015) also reported a prevalence of 2-8 percent in meat samples in Lahore city of Punjab province Pakistan. (Khan *et al*. 2015) Wang *et al* (2011) found a prevalence of "18.2 percent" for type A *C. perfringens* in feces of dairy cattle in China. (Wang *et al*. 2011) In a recent study performed in Saudi Arabia the prevalence of *C. perfringens* 27.2% and 26.47% was found at the animal and herd level in different regions of the country. (Omer *et al*. 2020) The prevalence of 68% was found in commercial poultry of different areas of Balochistan province of Pakistan. (Achakzai *et al*.2020)

The prevalence found in our study is low as compared to the prevalence found by Elsify *et al* in 2016 who found "41percent" prevalence of *C. perfringens* in the soil (Elsify *et al*. 2016) The reason for high prevalence in their study might be that all the samples were collected from the sheep farm area. Sheep are a big reservoir of *C. perfringens* and continuously shed it in the soil through feces (Uzal and Songer. 2008) while in our study samples were collected from different areas even from the soil where no animals were present. Moreover, they collected soil samples from three provinces of the country while in our study only one province was targeted. There is a lot of difference in the climatic conditions and environmental parameters in different provinces of Pakistan so the prevalence may be different if soil samples are collected from different provinces. Another possible explanation for low prevalence may be that samples were collected from different farm areas and agricultural land where the soil carries different chemicals from disinfectants and agrochemicals that can

act as antimicrobial agent for the pathogen (Edwards *et al*. 1998; Voidarou *et al*. 2011)

Highest prevalence of *C. perfringens* was found in "Chakwal" district (18percent) while lowest in Sargodha district. High prevalence was shown in districts "Attock", "DG Khan", "Sahiwal" and "Lahore". Soil of district "Chakwal" was also found positive for other soil borne pathogens like *B. mallei* (Ali *et al*. 2017) and *F. tulerensis*. (Muhammad*et al*. 2017). In "Chakwal" district distance from the animal market, number of animals in the village and animal and human interaction at the site of sampling were the main risk factors for high prevalence of *C. perfringens*.

In current study the prevalence of *C. perfringens* was correlated with the different risk factors. The results of correlation were different in different districts. In four districts, out of nine districts, including districts Attock, Chakwal, Gujranwala and Sahiwal distance from the animal market (< 1Km) was found to be a positively correlated risk factor i.e., the villages positive for *C perfringens* were located close to the animal market as compared to the villages negative for *C perfringens*. Animals from different areas of the country are brought into the animal market for sale purpose. These animals become source of existence and transmission of different pathogens through the soil. In five districts distance from the main road was a correlated factor i.e., the villages' positive for *C. perfringens* were present in close proximity to the main road (<500m). These districts included Attock, DG Khan, Sahiwal, Sargodha and Sheikhupura. Heavy traffic plays an important role in the persistence and spread of anaerobic soil borne pathogens.

Distance from the water source was found positively correlated with the prevalence of *C. perfringens* in six districts out of nine districts including district DG Khan, Faisalabad, Gujranwala, Lahore, Sahiwal and Sargodha, which shows that distance from the water source has significant impact on prevalence of *C. perfringens*. Previous studies also suggest that *C. perfringens* is widely distributed in fresh water, marine sediments, waste water and river water (Davies *et al*. 1995; Edwards *et al*. 1998) In 6 districts; Attock, Chakwal, Gujranwala, Lahore, Sargodha and Sheikhupura, villages positive for the pathogen had more number of animals present as compared to the villages negative for *C. perfringens*. If a greater number of animals is present in an area there is more possibility of shedding the enteric pathogens like *C. perfringens* in the feces so soil becomes contaminated with pathogen and more chances of soil being positive for *C. perfringens* genome. This is in relevance to the findings of Voidarou *et al* (2011) who found out that if the soil is contaminated with the fecal matter, then there are more chances of presence of *C. perfringens* spores in the soil. In six districts human population was also significantly correlated to the prevalence of *C. perfringens* i.e. the positive villages had a greater number of houses as compared to the negative villages. These districts include DG Khan, Faisalabad, Gujranwala, Lahore, Sahiwal and Sargodha. In seven districts animal and human interaction was significantly related to the presence of *C. perfringens* genome while in two districts Sheikhupura and Lahore all the positive samples were from the sites where close human animal interaction was present so the statistical significance could not be checked. Overall ratio of samples positive for *C. perfringens* was high for the sites where animal and human were present in close proximity as compared to the sites with no animal human interaction. Mostly *C. perfringens* is not considered as a zoonotic pathogen but it can be transmitted to farm workers and animal handlers from animals. (Songer. 2010).

Conclusion: *C. perfringens* is highly distributed in the soil of nine districts of Punjab province of Pakistan. Odds of all the six risk factors are positively associated with the prevalence of *C. perfringens* genome in most of the districts. The pathogen is more prevalent in the areas with close human and animal interaction.

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