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EPIDEMIOLOGY OF GIARDIA AND CRYPTOSPORIDIUM IN DOGS AND CATS FROM FAISALABAD, PAKISTAN

Muhammad Adnan Sabir Mughal[1], Muhammad Kasib Khan[1]*, Adeel Ali[2], Tayyaba Bari[3], Faisal Saleem[1], Muhammad Ahsan[2], Abdullah Azeem[1], Mehroz Latif[4], Subayyal Akram[1], Israr Ullah[5] and Hammad-ur-Rehman Bajwa[6]

[1]Department of Parasitology, University of Agriculture, Faisalabad, Pakistan
[2]Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan
[3]University of Veterinary and Animal Sciences, Lahore, Pakistan
[4]Faculty of Veterinary Science, Bahauddin Zakariya University, Multan, Pakistan
[5]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan.
[6]Department of Pathobiology, University of Illinois, USA

Corresponding author: mkkhan@uaf.edu.pk

ABSTRACT

Gastrointestinal (GI) parasites are common across the globe, particularly in underdeveloped and developing countries. The incidence of these parasites is high in semi-urban and rural settings. *Giardia* and *Cryptosporidium* are two main intestinal parasites that infect humans and domestic and wild animals. Dogs and cats play an important role as reservoir hosts in transmitting these parasites to other hosts. There is a lack of published information on molecular research in Pakistan that examines the incidence of these parasites in cats and dogs and associated risk factors. Using a simple random sampling procedure, 384 fecal samples were obtained from cats and dogs in four towns in the District of Faisalabad (as defined by an epidemiological sample size calculation algorithm). Data on the related risk variables of GI protozoal infections were acquired using a pre-designed questionnaire with closed-ended questions. Centrifugal flotation was used to extract parasitic oocysts from feces. From isolated parasite oocysts, DNA was extracted. Genus-specific primers (18S rRNA gene) were used for PCR analysis. *Giardia* spp. was found to be present in 35 of the total samples, with a prevalence rate of 9.11%, while *Cryptosporidium* spp. was found to be present in 15 of the total samples, with a prevalence rate of 3.91%. Lyallpur town has the greatest frequency of *Giardia* spp., 12.22% followed by Iqbal town 10.11%, Madina town 8.42%, and Jinnah town 6.36%. Similarly, Lyallpur town has the greatest incidence of *Cryptosporidium* spp., followed by Jinnah town, Iqbal town, and Madina town (5.56, 3.64, 3.37, and 3.16%, respectively). Animals less than a year old had considerably ($P \leq 0.005$) greater rates of infection with the abovementioned parasites. In the research area, stray dogs were more likely to have the infection as compared to stray cats. In conclusion, in order to stop the spread of these zoonotic diseases, adequate preventative measures are required.

***Keywords*:** Giardia, Cryptosporidium, Protozoa, Epidemiology, PCR

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1. INTRODUCTION

Dogs and cats, human companions often get infections from multiple types of gastrointestinal parasites. Most of these infections show clinical symptoms or some may be asymptomatic for a long time period. Parasitism is a prime importance issue in canines as well. People living in cities often have a close association with one another and sometimes they may live with pets i.e., dogs and cats. They considered them their true loyal friends, but some other benefits have also been provided like emotional and physiological attachment and social development (Janssens et al. 2021). Canines and felines can also serve as a reservoir for a large number of zoonotic parasites like *Giardia*. These parasites can enter into the body of the host through multiple routes, like through mucosa and fecal-oral route. Human serves as a final host for more than 100 parasites. A study revealed that 85% of adult people have parasitism in their bodies (Martinez-Moreno et al. 2007). The environment is contaminated by the shedding of a large number of oocysts or larvae of zoonotic parasites in their feces directly or indirectly by contaminating the food and water which are ultimately ingested by humans and they get infected (Ayinmode et al. 2016).

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Cryptosporidium and *Giardia* are two main agents that are prevalent in humans, domestic animals, and wild animals causing gastrointestinal diseases of significant public health concern (Savioli et al. 2006; Santin 2020; Razzolini et al. 2020; Ryan et al. 2021). Oocysts of *Cryptosporidium* and *Giardia* at excretion are able to cause infection and are environmentally resistant and can bear harsh conditions like moisture and cooling outside the host (Rinaldi et al. 2008). *Giardia* and *Cryptosporidium* are the members of phylum Protozoa. Giardiasis is a persistent condition that infects the intestines of its host (cats, dogs, domestic animals and humans), resulting in bowel diarrhea having a specific target on duodenum (Ballweber et al. 2010; Mirzaei et al. 2012). In both humans and animals, cryptosporidiosis is brought on by the obligatory intracellular parasite *Cryptosporidium*. Natural infections in dogs and cats can be brought on by *Cryptosporidium (C.) parvum, C. meleagridis*, and *C. canis*. The most often found species in dogs and cats among all of these is *C. canis*. Zoonotic forms of *C. parvum*, *Cryptosporidium meleagridis*, and *C. muris* have also been found in small numbers in dogs and cats (Elmahallawy et al. 2023). Most of the time, *C. canis* infections are asymptomatic, although they can occasionally result in weight loss, diarrhea and malabsorption (Olabanji et al. 2016).

For identification of these parasites multiple methods have been used. Mostly, identification is done through conventional means in which microscope is used for detection. Serological methods like, ELISA can also be used for diagnosis of protozoa (Sinnott et al. 2020; Dessì et al. 2022). For detection of these parasites at species, level molecular detection like PCR is necessary. Molecular methods have more sensitivity and specificity over conventional method in comparison (McHardy et al. 2014).

These selected protozoa are often considered as neglected pathogens in canines. Although, *Giardia* and *Cryptosporidium* have been reported in canines in some areas of Pakistan, however, no data on molecular epidemiology of these parasites in canines in Pakistan has been published yet, indicated the need to design a surveillance of GIT protozoan parasites at molecular level.

2. MATERIALS AND METHODS

2.1. Study Area

The study was performed in major towns of Faisalabad including Iqbal town, Jinnah town, Lyallpur town, and Madina town. The study area was selected due to hot and humid climate which favor the growth of parasite.

2.2. Collection and Processing of samples

The collection of 384 feces samples was done from dogs and cats residing in urban, peri-urban, and rural areas of four towns in district Faisalabad. The sample collection followed a simple random sampling method. An epidemiological method was used to calculate the sample size, taking into account the estimated prevalence of 50%, the confidence interval of 95%, and the accuracy of 5% (Thrusfield 2018). The aim was to obtain a representative sample that would yield reliable estimates of the epidemiology of *Giardia* and *Cryptosporidium* in the study population. To preserve the integrity of the fecal samples, sterile bottles containing 10% formalin as a preservative were used. After the collection of fecal samples, the samples underwent further processing using the centrifugal floatation technique to collect oocysts. The procedure involved stirring the samples in tap water and sieving them through a triple-fold muslin cloth. Subsequently, the samples were subjected to centrifugation, causing the oocysts to float on the surface due to their lower specific gravity compared to the floatation solution used. The oocysts were then collected in specific tubes designated for this purpose. For DNA extraction, 200µL was transferred into Eppendorf tubes from each sample.

2.3. Molecular Investigation

The Stool DNA Isolation Mini Kit (FAVORGEN) was used to extract DNA. 200µL of feces were collected and placed in a 2ml bead tube with 200mg of glass beads. The extracted DNA was stored at -20°C while the other steps of extraction were completed in line with the manufacturer's instructions. The 18S rRNA genes of *Giardia lamblia* and *Cryptosporidium parvum* were amplified through PCR using genus-specific primers (*Cryptosporidium*; Forward Primer: AAGCTCGTAGTTGGATTTCTG; Reverse Primer: TAAGGTGCTGAAGGAGTAAGG, *Giardia*; Forward Primer: GACGGCTCAGGACAACGGTT Reverse Primer: TTGCCAGCGGTGTCCG) to identify a subset of parasites (Abbas et al. 2022). The 20µL PCR reaction mixture contained 5µL of DNA sample, 1µL each of forward and reverse primers, 10µL of the 2X master mixture, and 3µL of distilled water. These amplified products were separated by gel electrophoresis using 1.5% agarose gel. In order to visualize the bands, a gel documentation system was used.

2.4. Questionnaire Development

The questionnaire was constructed to determine the risk factors which may have an association with the prevalence of these parasites in the study area. The risk factors which were included in questionnaire were gender, age, living habitat, feeding pattern, management conditions etc.

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2.5. Statistical Analysis

The SAS statistical package was used to analyze all the prevalence and related determinant data using multiple logistic regression and odds ratio (SAS 2010). The 5% level of significance was applied for statistical significance.

3. RESULTS

3.1. *Giardia intestinalis*

Giardia (G.) intestinalis was recognized in the sample after microscopic analysis. The DNA samples from the positive samples were amplified using primers specific to the genus *Giardia* (18S rRNA). The molecular weight of PCR product was detected to be 623bp (Fig. 1). The overall incidence identified for *G. intestinalis* in district Faisalabad was 9.11% (Table 1). 35 of the 384 fecal samples that were analyzed, determined as positive. A few parameters that were taken into account as related risk factors were host species, age, sex, drinking and eating habits. According to sex, female animals (22/159: 13.84%) had a greater incidence than male animals (13/225: 5.78%) (Table 1). Samples were collected from dogs and cats of various ages. 93 samples were taken from animals less than 1 year of age, 161 samples from 1-3 year of age and 130 samples from more than 3 years of age (Table 1). The prevalence was found higher in animals less than 1 year of age (20/93; 21.51%) followed by animals more than 3 years of age (10/130; 7.69%) and animals with age between 1 to 3 years (5/161; 3.11%) (Table 1). Three categories were established based on the types of water animal drank, one of which included drinking sewage water along with tap water and canal water. The prevalence was found higher in animals drinking water from sewerage (20/106; 18.87%) followed by animals drinking water from canal water (9/118; 7.63%) and animals drinking water from tap water (6/160; 3.75%) (Table 1). As the study was carried out in both dogs and cats so prevalence was noted according to species wise as well. The prevalence was found higher in dogs (25/245; 10.20%) as compared to cats (10/139; 7.19%) (Table 1). Pets were also monitored as compared to stray animals in both species' cats and dogs. Stray animals mostly wander outside and take food from external environment. Prevalence was found higher in stray animals (28/228; 12.28%) as compared to pets (7/156; 4.49%) (Table 1).

3.2. *Cryptosporidium canis*

Cryptosporidium (*C.*) *canis* was recognized in the sample after microscopic analysis. DNA samples from the positive samples were amplified using primers specific to the genus *Cryptosporidium* (18S rRNA). The molecular weight of PCR product was detected to be 435bp (Fig. 2). The overall incidence identified for *C. canis* in district Faisalabad was 3.91% (Table 2). 15 samples were found positive for *C. canis* from 384 examined samples. Associated risk factors which were taken into account include breed, sex, age species and feeding habits.

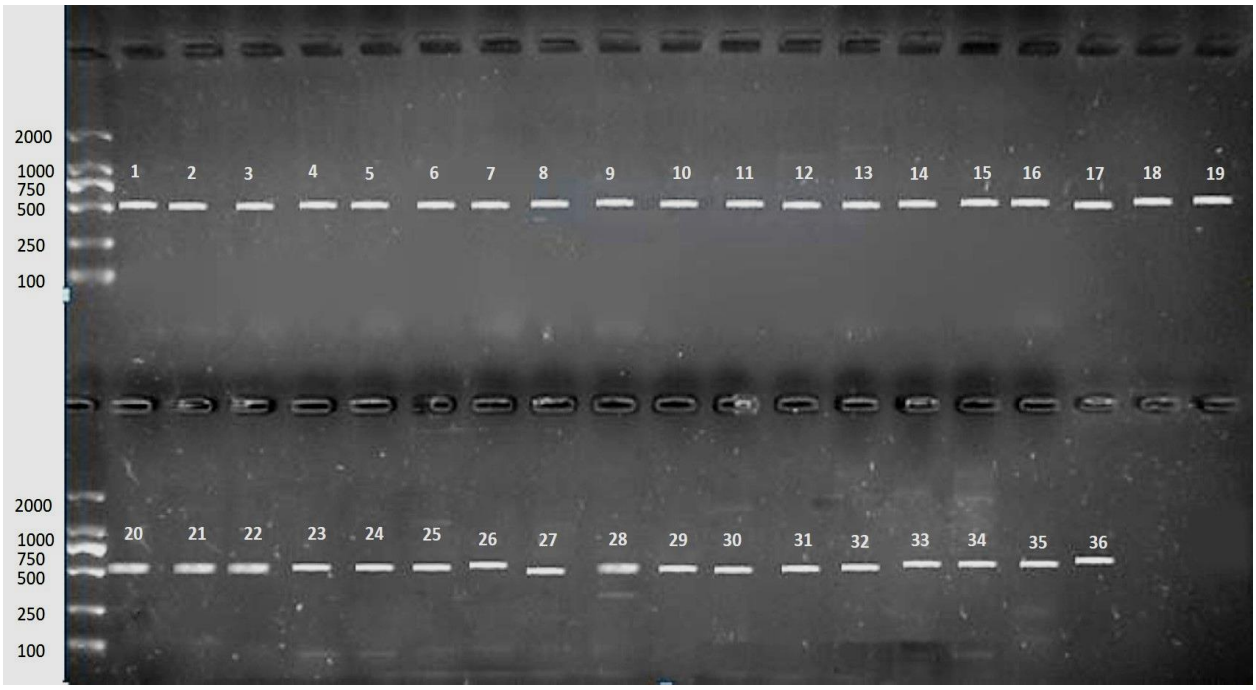

Fig. 1: Gel electrophoresis revealing positive bands of DNA of *Giardia intestinalis* in fecal samples of dogs and cats (M=Molecular marker 2,000 bp; Positive=1-36, Product size=623bp).

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According to sex, female was found highly infected with *C. canis* showing prevalence percentage of 6.29% (10/159) as compared to male which showed prevalence percentage of 2.22% (5/225), respectively (Table 2). Samples were collected from dogs and cats of various ages. 93 samples were taken from animals less than 1 year of age, 161 samples from 1-3 year of age and 130 samples from more than 3 years of age (Table 2). The prevalence was found higher in animals less than 1 year of age (7/93; 7.53%) followed by animals more than 3 years of age (5/130; 3.85%) and animals with age between 1 to 3 years (3/161; 1.86%) (Table 2). Three categories were established based on the types of water animal drank, one of which included drinking sewage water along with tap water and canal water. The prevalence was found higher in animals drinking water from sewerage (9/106; 8.49%) followed by animals drinking water from canal water (4/118; 3.39%) and animals drinking water from tap water (2/160; 1.25%) (Table 2). As the study was carried out in both dogs and cats so prevalence was noted according to species wise as well. The prevalence was found higher in dogs (11/245; 4.49%) as compared to cats (4/139; 2.88%) (Table 2). Pets were also monitored as compared to stray animals in both species' cats and dogs. Stray animals mostly wander outside and take food from external environment. Prevalence was found higher in stray animals (10/228; 4.39%) as compared to pets (5/156; 3.21%) (Table 2).

Table 1: Multiple parameters-based prevalence of *Giardia* in selected towns of Faisalabad

Parameters	Factors	Total Samples Collected (N)	Positive Samples (N)	Prevalence (%)	Odds Ratio (OR)	P Value
Gender	Male	225	22	5.78	_	_
	Female	159	67	13.84	0.3819	0.0086
Age	Less than 1 year	93	20	21.51	0.1177	0.0001
	1-3 years	161	5	3.11	-	-
	More than 3 years	130	10	7.69	0.3871	0.9070
Drinking Water	Canal Water	118	9	7.63	0.4719	0.1656
	Tap Water	160	6	3.75	_	_
	Sewerage	106	20	18.87	0.1675	0.0002
Species	Dog	245	25	10.20	0.6822	0.3271
	Cat	139	10	7.19	-	-
Habitat Based	Pets	156	7	4.49	-	-
	Stray	228	28	12.28	0.3356	0.0123

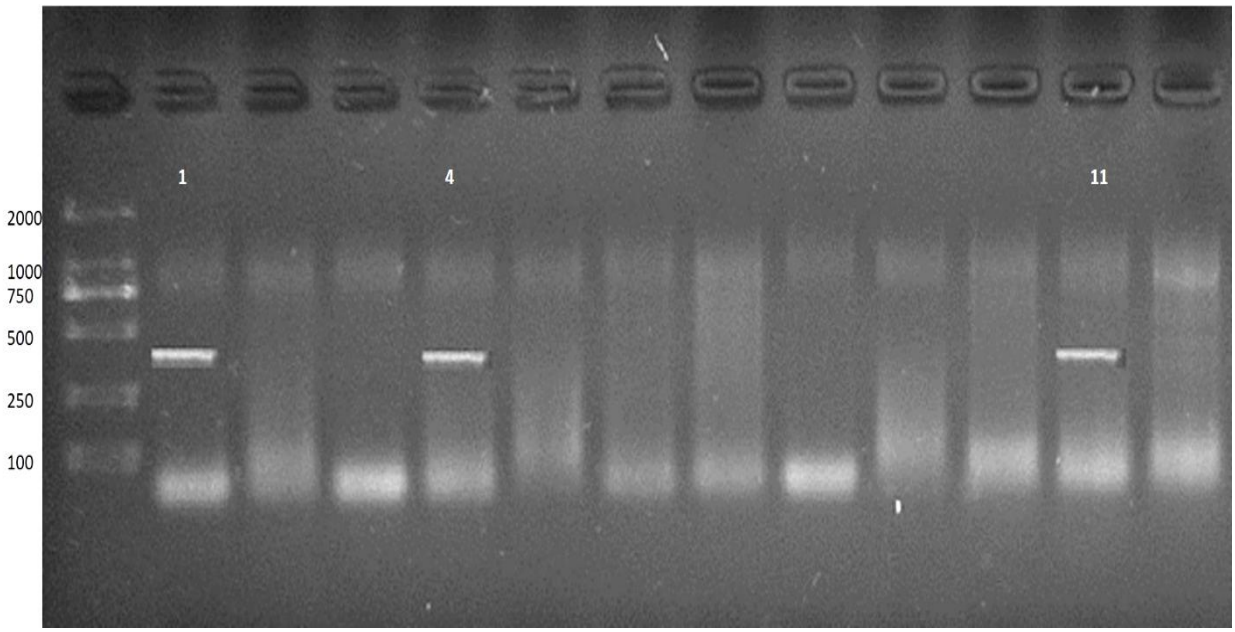

Fig. 2: Gel electrophoresis revealing the positive bands of DNA of *Cryptosporidium canis* in fecal samples of dogs and cats. M=Molecular marker (2,000 bp), Positive=1, 4 and 11, and Product size=435bp.

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Table 2: Multiple parameters-based prevalence of *Cryptosporidium* in selected areas of Faisalabad

Parameters	Factors	Total Number of Collected Samples (N)	Positive Samples (N)	Prevalence %	Odds Ratio (OR)	P Value
Gender	Male	225	5	2.22	_	_
	Female	159	10	6.29	0.3386	0.0523
Age	Less than 1 year	93	7	7.53	0.2333	0.0384
	1-3 years	161	3	1.86	_	_
	More than 3 years	130	5	3.85	0.4747	0.3140
Drinking Water	Canal Water	118	4	3.39	0.3608	0.2438
	Tap Water	160	2	1.25	_	_
	Sewerage	106	9	8.49	0.1364	0.0119
Species	Dog	245	11	4.49	0.6303	0.4370
	Cat	139	4	2.88	_	_
Habitat Based	Pets	156	5	3.21	_	_
	Stray	228	10	4.39	0.7219	0.5591

4. DISCUSSION

Water and food are thought to be essential for the survival of all living things. These are also the point of entrance for several diseases, including bacteria, fungi, viruses, protozoa, and helminths (Magana-Arachchi and Wanigatunge 2020). Overall, protozoans are responsible for a high rate of infection and mortality in both animals and people through spreading a number of diseases (Latif et al. 2020). The World Health Organization (WHO) carried out a number of surveys to identify the prevalence of protozoan infections. These polls were taken in the years 1981, 1986, and 1995 to 2000. Later, scientists updated and evaluated the data. To determine the morbidity, mortality, and infection-related consequences of illness, several research were done. Giardiasis and cryptosporidiosis are the most prevalent infections caused by protozoan parasites on a global scale. Although it predominantly affects underdeveloped nations, *Cryptosporidium* is regarded as the third most common cause of diarrheal mortality in children worldwide (Taghipour et al. 2020). The goal of the current study was to determine the infection burden in dogs and cats, which act as reservoirs for these illnesses. Through PCR, the infection burden was calculated. Since parasitic oocysts are shed by dogs and cats in their feces, contaminating food and water, DNA was isolated from their fecal samples.

In this study, 9.11 and 3.91% of the 384 investigated dog and cat feces samples were positive for *Giardia* and *Cryptosporidium*, respectively. The infection was found ($P \geq 0.005$) higher in female as compared to male animals. Similarly, the rate of infection was significantly ($P \leq 0.005$) higher in animals less than 1 year of age and animals drinking water from canal and sewerage water. Stray dogs were also found to be highly ($P \geq 0.005$) infected with giardiasis and cryptosporidiosis.

Previously, numerous researchers used simple microscopic techniques, serological methods, and molecular methods to identify these parasites in the feces of dogs and cats (Barutzki and Schaper 2003; Epe et al. 2010; Sotiriadou et al. 2013; Aziz and Al-Barwary 2019). There are cases of *G. intestinalis* all around the world. *Giardia* prevalence rates in well-cared dogs, pups, and dogs in kennels were found to be 10, 36–50 and 100%, respectively, in a survey to identify the disease in Australia's canine population (Raza et al. 2018). Numerous molecular investigations on the molecular identification of these protozoa have previously been carried out. Research found the genetic sequence of parasite from dog feces that was tested positive for *G. intestinalis*. Direct microscopic approach was used to determine that the samples were positive. Amplified Glutamate Dehydrogenase (GDH) Gene was used in the PCR process. Using Giardia DNA as a template, a positive PCR result of 220bp was found. Except for a few sequences with 1–15bp differences, all diagnostic dog sequences were confirmed to be similar. All positive samples included the dog-specific genotype Assemblage D, which also demonstrated zoonotic potential (Abe et al. 2003). To compare the various cryptosporidiosis diagnostic techniques, numerous researches were done. Simple microscopic methods revealed 8% prevalence, coproantigenic methods 7%, and molecular approaches as 6% only. It was discovered that *C. canis* and *C. parvum*, with respective prevalence's of 3.64 and 1.28%, infect dogs. Dogs with diarrhea were shown to have a higher chance of developing cryptosporidiosis than dogs without diarrhea (Taghipour et al. 2020).

Nesting PCR was utilized as a diagnostic method in a comparative molecular investigation on dogs and cats carried out in Germany utilizing GDF3 and GDB5 as primers. By using this procedure, 19 cat samples and 81 dog samples were analyzed, and only two cats and five dogs were determined to be *Giardia*-infected. Sequence analysis revealed that Assemblage A was present in all positive isolates. Only two dogs, aged 1 and 6 years, were co-infected with *Escherichia coli* and *Campylobacter jejuni* among the tested dog population. In these samples, no additional infections were found (Sotiriadou et al. 2013). Different serological methods have been employed

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in the past to diagnose these parasites. Research was done on dogs to identify *Cryptosporidium* using a serological test. Enzyme Linked Immunosorbent Assay technology was used to detect an 18.5% prevalence (Olabanji et al. 2016).

5. Conclusion

In conclusion, *Giardia* and *Cryptosporidium* infections were identified more frequently in female animals than in male animals owing to immune-compromised conditions during pregnancy. The higher infection incidence in younger animals under one year of age is attributable to the existence of inadequate immunity and parasite resistance. As pond water is contaminated with feces and other contaminants, animals drinking from it were infected more than those drinking from tap water. Similarly, stray animals had a greater infection rate due to exposure to a polluted environment. The findings of this study might aid in the development of effective control methods to combat gastrointestinal parasites, notably *G. intestinalis* and *C. canis*. In the future, a large-scale study would be required to clarify the infection status in different agro-climatic zones of Punjab, Pakistan.

Author's Contribution

Muhammad Adnan Sabir Mughal has completed the research work under supervision of Dr. Muhammad Kasib Khan. Abdullah Azeem, Mehroz Latif, Muhammad Ahsan and Israr Ullah assisted in collection of samples from selected areas of Faisalabad. Muhammad Adnan Sabir Mughal, Adeel Ali, Tayyaba Bari and Faisal Saleem completed write up process and then the manuscript was reviewed by Subayyal Akram, Hammad-ur-Rehman Bajwa and Dr. Muhammad Kasib Khan.

Conflict of Interest

All authors have no known conflict of interests, including competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ORCID

Muhammad Adnan Sabir Mughal https://orcid.org/0000-0001-8854-6064
Muhammad Kasib Khan https://orcid.org/0000-0001-6050-4321
Adeel Ali https://orcid.org/0009-0008-4469-5832
Tayyaba Bari https://orcid.org/0009-0009-9179-9033
Faisal Saleem https://orcid.org/0009-0000-4937-6891
Subayyal Akram https://orcid.org/0009-0002-9041-2553
Hammad-ur-Rehman Bajwa https://orcid.org/0000-0001-6908-7659
Abdullah Azeem https://orcid.org/0000-0002-1188-4345
Mehroz Latif https://orcid.org/0009-0004-6215-0765
Muhammad Ahsan https://orcid.org/0009-0002-5714-8324
Israr Ullah https://orcid.org/0009-0005-9995-6620

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