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Prevalence and associated risk factors of *Shigella flexneri* isolated from drinking water and retail raw foods in Peshawar, Pakistan

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Abstract: This study was designed to investigate the prevalence and associated risk factors of *Shigella flexneri* isolated from drinking water and retail raw food samples in Peshawar, Pakistan. A total of 1,020 different samples were collected from various areas of Peshawar between January 2016 and May 2017, followed by identification of *S. flexneri* through biochemical, serological, and *16S rRNA* gene sequencing. Potential risk factors associated with the development and spreading of *S. flexneri* infection were also investigated. Overall, 45 (4.41%) samples were positive for *Shigella* species. Among these samples, the predominant species was *S. flexneri* ($n = 44$) followed by *S. boydii* ($n = 1$). Interestingly, *S. sonnei* and *S. dysenteriae* isolates were not found in any sample. The isolation rate of *S. flexneri* in drinking water samples, market raw milk, and fruits/vegetables from Peshawar were 6.47%, 3.5%, and 2.9%, respectively. The phylogenetic reconstruction showed genetic diversity among three clades, as clades I and II have isolates of *S. flexneri* that were circulating within the drinking water, milk, fruits/vegetables, while clade III isolates were recovered from milk samples. Most of *S. flexneri* were detected in June to September. Potential risk factors of *S. flexneri* were water sources contaminated by toilet wastes ($p = 0.04$), surface water drainage ($p = 0.0002$), hospital wastes ($p = 0.01$), unhygienic handling ($p < 0.05$), and transportation of raw food ($p = 0.04$). In conclusion, *S. flexneri* isolates of closely related lineage originating from non-clinical samples might be associated with an increased human risk to shigellosis in Pakistan, as significant numbers of *S. flexneri* were observed in the drinking water and retail raw food samples.

Practical Application: This study demonstrated the presence of *S. flexneri* in drinking water and retail raw food samples which seem to possess a serious threat to public health. Potential sources of food and water contamination should properly be monitored by public health authorities to reduce cases of shigellosis.

KEYWORDS
food, Pakistan, risk factors, *Shigella flexneri*, water

1 | INTRODUCTION

Food- and water-borne illnesses are a major threat to public health and economy worldwide (Rahimi et al., 2017). The World Health Organization (WHO) estimated that annually 600 million populations were affected by foodborne illnesses, whereas 420,000 deaths occurred. Among these, 30% of deaths occurred in under 5 years of age (WHO, 2015a). Annually 2.2 million people die due to water-borne illnesses, among which 1.4 million of these deaths are children (WHO, 2015b). The report described that globally, 2.2 billion people are unable to drink clean water (WHO & UNICEF, 2019).

Approximately 1,100,000 deaths occur annually due to *Shigella* infections worldwide (Mardaneh et al., 2013; Saima et al, 2018). In Africa, the United States, and Asia, 8 million, 500,000, and 91 million cases of *Shigella* infections occur annually, respectively. In Pakistan, 5 million cases were reported, among which 5,094 deaths occur annually (Khalil et al., 2018). Among the four species of *Shigella*, *S. flexneri* is the predominant species in low income countries with a frequency of 60%, while in developed countries only a frequency of 16% was observed (Rahimi et al., 2017). The mode of transmission of *Shigella* spp. occurs primarily through the fecal-oral route either via the consumption of contaminated water and food or direct person to person contact (Dekker & Frank, 2015).

Poor sanitation and the lack of hygienic living conditions lead to foodborne shigellosis in developing countries, which are subsequently difficult to control due to nonexistent or poor reporting systems (Mama & Alemu, 2016; Mukhopadhyay et al., 2012). There is no comprehensive report on the *S. flexneri* in drinking water and retail raw foods in Pakistan. So the present study was carried out to investigate the prevalence and associated risk factors of *S. flexneri* in drinking water and retails raw food in Peshawar, Pakistan.

2 | METHODOLOGY

2.1 | Study area

This study was conducted in Peshawar district, (34°00056.2″ N, 71°34039.8″ E) the capital of the Khyber Pakhtunkhwa province in Pakistan between January 2016 and May 2017. Peshawar district has seen a rapid increase in population 4.269 million (2017), where most of the population that is 2.299 million are living in rural areas as compared to urban areas (1.970 million) with a total area 1257 km^2 (Pakistan Bureau of Statistics, 2018). It has four towns and these towns are further subdivided into different union councils.

2.2 | Ethics statement

The present study was conducted according to the ethical guide lines of the University of Science and Technology, Kohat. Prior to the start of the study, ethical approval was granted by the institutional research ethical committee human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

2.3 | Sample collection

A total of 1020 samples (340 drinking water, 340 milk, and 340 fruits/vegetables) were randomly collected during January 2016 to May 2017 from different towns of Peshawar, Khyber Pakhtunkhwa, Pakistan. A constant number of samples (60 samples per month) were collected comprising of 20 household drinking water, 20 raw milk, and 20 raw fruits/vegetables samples. Prior to sample collection, household drinking samples from different sources (tanks, pump, well, pond, bore direct source, and tube well direct source) were first allowed to run until the temperature of water was constant and then 250 ml of sample was collected in the dry, leak-proof, and sterilized Duran bottles.

Similarly, retail raw foods include 250 g of each retail raw fruits/vegetables (apple, mangoes, guava, watermelon, melon, grapes, peach, plum, apricot, blackberry, loquat. Mint, cucumber, cabbage, lettuce, carrot, radish, coriander, and tomato) were collected in stomacher bags. Raw milk samples (250 mL) were collected in sterilized Duran bottles from local retailers shops located at various areas in Peshawar city. Before sampling the raw milk container was turned upside down for a few times. Each sample was properly labeled with a code number, subject name, sampling site, date of collection, source of household drinking water, and type of food. All samples were brought to laboratory refrigerator containers and were processed within 4 to 5 hr of collection. The physical properties of drinking

water like temperature, pH, and odor were determined as described earlier (Chapman & Kimstach, 1996). Informed consent was obtained from participants for being included in the study. For this a structured questionnaire was filled (Supporting Information) by 1020 owners of retail shops or households to analyze the risk factors associated with the transmission of *S. flexneri* in retail raw food and household drinking water of the selected area, which includes unhygienic practices during food handling in markets, unhygienic utensils used for carrying and storing food items in markets, vehicles (auto rickshaw, Suzuki, taxi) used for food items transportation, location of livestock from packing and storage areas of food items nearby markets, contamination of water sources like tanks, pump, well, pond, bore direct source, tube well direct source, and physical properties of water like temperature, pH, and odor.

2.4 | Isolation and phenotypic identification

Shigella spp. isolation from food and water was performed according to ISO 21567:2004 (International organization for standardization) (ISO, 2004; Warren et al., 2006) with certain modifications. A sample of approximately 25 g each of fruit/vegetable was shifted to another stomacher bag by using a sterile scalpel and similarly 25 ml water and milk samples was transferred to the stomacher bag in these samples. Then 250 ml of *Shigella* broth containing novobiocin (0.5 μg/ml) wasadded in these samples following 1 min homogenization and was then incubated for 18 to 24 hr at 41.5°C in an anaerobic condition. After incubation, each sample was then streak on MacConkey agar and xylose lysine deoxycholate media (XLD) and again incubated for 18–24 hrs at 37°C. Suspected colonies of *S. flexneri* were then subcultured on XLD plates for 18 to 24 hr at 37°C. Presumptive morphological *Shigella* colonies were confirmed by API 10S analysis (Bio-Merieux, Marcy l'Etoile, France). All isolates were further confirmed by slide agglutination method (El-Gendy et al., 1999).

2.5 | Identification of isolates by *16S rRNA* gene sequencing

Genomic DNA of *Shigella* was extracted as previously described (Carlos et al., 2016) with some modification. Single bacterial colonies were suspended in 20 μl Milli-Q water and subjected to 10 min at 95°C and then centrifuged for 5 min at 12,000 × *g* at room temperature. *16S rRNA* gene amplification was done using PCR with the 2× Phire green hot start II PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA) and the primer sequences of 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and U1492R 5′-GGTTACCTTGTTACGACTT-3′ of 1465 bp were used for identification of *Shigella* spp. (James, 2010). Amplified *16S rRNA* PCR products were subsequently purified by using the GenElute™ PCR Clean-up Kit (Sigma Aldrich, St. Louis, MO, USA) according to guidelines of manufacturer instructions. The volume of 25 μl PCR mixture per reaction was prepared to contain 2× phire green (12.5 μl), forward and reverse primer (each of 0.5 μl), genomic DNA of isolate (2 μl), and nuclease free water (9.5 μl). Thermal cycling was carried out by an initial denaturation step at 98°C for 90 s, followed by 32 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 10 s, and elongation at 72°C for 45 s, which was followed by a final elongation step at 72°C for 55 s in a thermocycler (Senso, Germany).

The 5 μl purified PCR product mixed with 5 μl forward primer/reverse primer was sent for sequencing to Macrogen Inc. (Amsterdam, The Netherland) and 44 obtained sequences of *16S rRNA* gene were analyzed by clone manager suite 7 and compared with sequences in GenBank databases by using the blastn program of the National Centre of Biotechnology Information (NCBI) to identify the closest strains of *S. flexneri* and their sequences similarities. Each nucleotide sequence of the strain were analyzed using the blastn tool and that blast was selected which hit with lowest e-value (expected value) that shows the number of non-chance alignment. To ensure significant blast output cut off value $1e^{-6}$ of e-value was selected.

2.6 | Phylogenetic analysis

To know the phylogenetic relations of strains isolated from drinking water, retail raw milk, and fruits/vegetables with its closest lineages, the sequence of *16S rRNA* gene was aligned using the MUSCLE software program (MX Alignment Explorer). The aligned sequences were inferred using maximum likelihood method implemented in MEGA X 10 software for reconstruction of the evolutionary tree (Kumar et al., 2018). Evolutionary distance matrix was calculated by using Tamura three-parameter model (Tamura, 1992) and an evolutionary tree was reconstructed by neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach.

2.7 | Statistical analysis

Statistical analysis was performed to find factors related with shigellosis by calculating Pearson's X^2 test and OR (binary logistic regression) with 95% CI. The *p*-values of less than 0.05 was considered statistically significant Bivariate analyses were performed using Pearson's X^2 test.

Bivariate analyses were preceded by estimation of correlation between risk factors in a binary logistic regression model. The association between potential risk factors and shigellosis was quantified by odds ratio (OR) with 95% confidence interval (CI). The *p*-values of less than 0.05 was considered statistically significant. Statistical analysis was calculated using MS Excel and VassarStats, an online statistical tool.

3 | RESULTS AND DISCUSSION

3.1 | Prevalence of *S. flexneri* in food and water samples

The present findings provide results of molecular identification and associated risk factors of *S. flexneri* isolated from food and drinking water samples. The overall prevalence rate of *Shigella* species was ($n = 45$) 4.41% in these samples. Interestingly the most predominant species was *S. flexneri* that was identified on the basis of morphological, cultural characteristics, conventional biochemical including API 10S tests, latex agglutination test, and molecular typing (*16S rRNA*). Further confirmation of the isolated *S. flexneri* isolates was carried out by using *16S rRNA* sequencing. The obtained sequencing of 44 nucleotide sequences was analyzed and compared to known bacterial sequences. The evaluated query sequences were compared with database sequences based on query coverage, percentage similarity and e-value. We observed the high percent (95% to 98%) coverage value and percentage similarity (81% to 98%) as shown in Table 1. The sequencing results revealed that these isolates have close similarity with previously reported *S. flexneri* (Table 1).

A higher isolation rate of S. *flexneri* isolates were recorded from study subjects from household water (6.47%, $p > 0.016$) than raw milk and fruits/vegetable samples from the market (3.5 and 2.9%) as shown in Table 2. Interestingly, *S. boydii* was isolated from only one water sample that was collected from household tank source in July 2016 from Union Council 6 (Faqir Abab), Town 1 Peshawar

In the current finding, a high trend in the distribution of *Shigella* species was found in drinking water and food samples. The contaminated water and food are a big source of shigellosis in human beings is commonly characterized by watery diarrhea (passage of watery diarrhea more than twice per day) mixed with blood and mucous. Clinical features include tenesmus, abdominal cramps, and fever. It may lead to a number of serious complications that may be fatal, like hemolytic uremic syndrome (Bardhan et al., 2010). Similar outcomes were observed in different studies carried out in Egypt, Iran, India (Abu-elyazeed et al., 2004; Urvashi & Dutta, 2011), Bangladesh, Indonesia, Vietnam, Pakistan, and China (Von Seidlein et al., 2006). A total of 1020 samples (340 fruits and vegetables, 340 milk, 340 household water) were analyzed, among these 44 positive isolates, 22 (6.4%) isolates were obtained from drinking water, 12 (3.5%) from milk samples, and 10 (2.9%) from fruits/vegetable samples. These results are in agreement with the findings in Nigeria (Onyemelukwe & Njoku-Obi, 1992), but did not corroborate with the findings reported from Ethiopia by (Guchi & Ashenafi, 2010) and Tunis by (Oueslati et al., 2011) where S. *sonnei* was predominant.

3.2 | Phylogenetic analysis

Phylogenetic analysis based on the nucleotide alignment of *16S rRNA* gene sequences were inferred using maximum likelihood method implemented in MEGA X 10 software. The constructed evolutionary tree clearly identifies the relationships among 44 nucleotide sequences of nonclinical isolates of *S. flexneri*. The study revealed that 44 nucleotide sequences were divided into three major clades that is, I, II, III showing similarity with *S. flexneri* as shown in Figure 1. The clade I and II were comprised of isolates of *S. flexneri* isolated from drinking water, retail raw milk, and fruits/vegetables, whereas the clade III composed of only two isolates recovered from milk samples. The results show an interesting association where isolates of nonclinical samples, including drinking water, retail raw milk, fruits/vegetables, were closely related with each other.

The present findings revealed that the evolutionary tree shows close similarity among the *S. flexneri* isolates isolated from a nonclinical source based on their evolutionary dynamics and demonstrate that *S. flexneri* isolates lineage originating from drinking water, retail raw milk, fruits, and vegetables might be related with an increased human risk of shigellosis in Peshawar, Pakistan. Thus, effort should be made to control the shigellosis targeted by improving the drinking water and food safety and quality.

3.3 | Seasonal distribution

The highest prevalence rate of *S. flexneri*, was observed in July (8.33%) followed by September (6.66%), but no statistically significant association was observed with the season (Supporting Information Table S1). Our findings are in agreement with a study carried out in Tunis (Oueslati et al., 2011), which reported that *Shigella* was isolated from raw milk and raw vegetables in Nabeul and Tunisia (Mokhtari et al., 2012). The prevalence rate of shigellosis in food items (raw fruits/vegetables and milk) is high in warmer and humid months, however, the association was not

TABLE 1 Molecular identification of selected retail raw food and water isolates *of S. flexneri* using *16S rRNA* sequencing

Isolate code	No. of nucleotides	Similarities with reported case			
		Bacteria	Percentage similarity	Query cov.	Gene bank accession no.
W1	1442	*S. flexneri* Strain 61–4982	97	91	CP026792.1
W2	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
W3	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
W29	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
W42	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
W57	1452	*S. flexneri* 2a Strain ATCC 29903	95	94	CP026788.1
W68	1448	*S. flexneri* Strain FDAARGAS 535	98	81	CP034060.1
W70	1448	*S. flexneri* Strain FDAARGAS 535	98	81	CP034060.1
W95	1448	*S. flexneri* Strain FDAARGAS 535	98	81	CP034060.1
W115	1449	*S. flexneri* Strain 73339	97	99	MH304308.1
W128	1423	*S. flexneri* Strain FDAARGAS 535	97	87	CP034060.1
W144	1423	*S. flexneri* Strain FDAARGAS 535	97	87	CP034060.1
W146	1445	*S. flexneri* Strain CICC 21534	97	84	KJ643932.1
W170	1449	*S. flexneri S*train 73339	97	99	MH304308.1
W194	1419	*S. flexneri* Strain RSHD96	97	97	KY971022.1
W204	1398	*S. flexneri* Strain RSHD96	98	90	KY971022.1
W217	1423	*S. flexneri* Strain FDAARGAS 535	97	87	CP034060.1
W241	1398	*S. flexneri* Strain RSHD96	98	90	KY971022.1
W263	1419	*S. flexneri* Strain RSHD96	97	97	KY971022.1
W271	1421	*S. flexneri* Strain FDAARGOS 535	98	98	CP034060.1
W314	1442	*S. flexneri* Strain 61–4982	97	91	CP026792.1
W295	1437	*S. flexneri* Strain FDAARGAS 535	97	98	CP034060.1
W333	1461	*S. boydii* Strain 59–248	96	91	CP026766.1
F1	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
F2	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
F83	1445	*S. flexneri* Strain CICC 21534	97	84	KJ643932.1
F97	1465	*S. flexneri* Strain 2016AM-0877	96	93	CP033510.1
F126	1426	*S. flexneri* Strain 2016AM-0877	98	98	CP033510.1
F152	1442	*S. flexneri* Strain 61–4982	97	91	CP026792.1
F166	1431	*S. flexneri* Strain FDAARGAS 535	96	97	CP034060.1
F193	1431	*S. flexneri* Strain FDAARGAS 535	96	97	CP034060.1
F289	1449	*S. flexneri* Strain 61–4982	97	91	CP026792.1
F309	1442	*S. flexneri* Strain 61–4982	97	91	CP026792.1
M1	1457	*S. flexneri* Y Strain 93–3063	97	89	KT261144.1
M2	1410	*S. flexneri* Strain FDAARGAS 535	97	87	CP034060.1
M3	1410	*S. flexneri* Strain FDAARGAS 535	97	87	CP034060.1
M15	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1
M19	1436	*S. flexneri* Strain 2016AM-0877	97	97	CP033510.1
M87	1436	*S. flexneri* Strain 2016AM-0877	97	97	CP033510.1
M129	1421	*S. flexneri* Strain FDAARGOS 535	98	98	CP034060.1
M166	1445	*S. flexneri* Strain CICC 21534	97	84	KJ643932.1
M187	1433	*S. flexneri* Strain RSHD96	95	92	KY971022.1
M234	1433	*S. flexneri* Strain RSHD96	95	92	KY971022.1
M259	1419	*S. flexneri* Strain RSHD96	97	97	KY971022.1
M297	1461	*S. flexneri* 2a Strain 2457T	96	87	AE014073.1

TABLE 2 Prevalence of *S. flexneri* isolated from water, fruits/vegetables, and milk samples

Sample type	Total samples	Positive, *N* (%)	Negative, *n* (%)	X^2	OR	0.95 Confidence intervals		*p*-value
						Lower limit	Upper limit	
Water	340	22(6.47)	318(93.53)	5.74	2.07	1.12	3.79	0.01
Fruits/vegetables	340	10(2.95)	330(97.05)	2.32	0.57	0.28	1.17	0.12
Milk	340	12(3.53)	328(96.47)	0.76	0.58	0.28	1.19	0.38

Note: p value < 0.05 was considered statistically significant. OR stands for Odd Ratio.

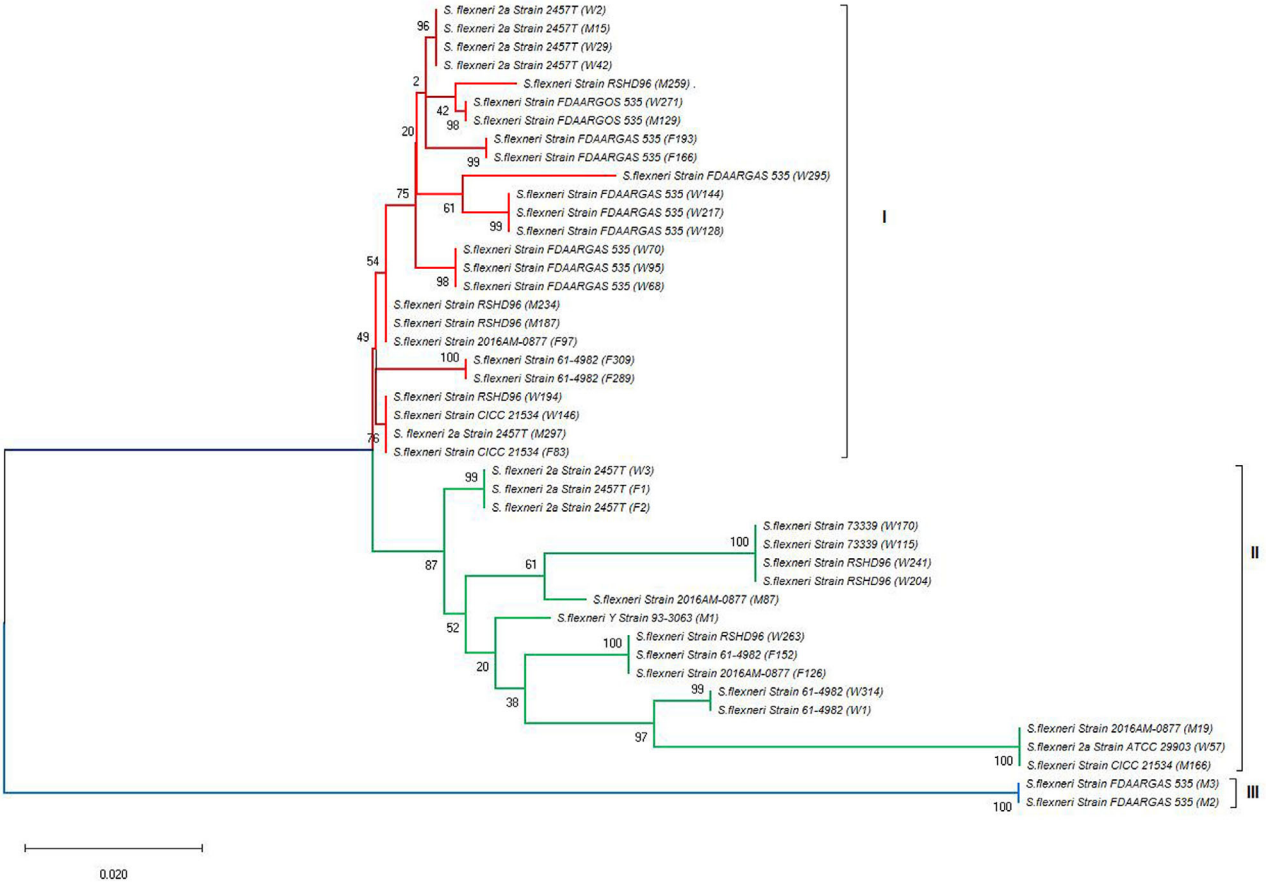

FIGURE 1 Phylogenetic tree based on *16 S rRNA* sequences displaying similarity among *S. flexneri* strains isolated from drinking water and raw foods. The evolutionary history was inferred by using the maximum likelihood method and Tamura three-parameter model. The tree with the highest log likelihood (−2840.37) is shown. The bootstrap values (expressed as percentage of 1000 replicates) in which the associated taxa clustered together are shown next to the branches and was used to assess the tree confidence interval. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3085)). All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 919 positions in the final dataset. Bar 0.005 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA X

significant. This high prevalence of shigellosis might be due to environmental factors and improper practices of handling and eating habits *for example* the intake of uncooked foodstuff in the summer.

3.4 | Risk factors for *S. flexneri* shigellosis

Associated factors for the prevalence of *S. flexneri* infection were shown in Tables 3 to 5, respectively. A higher

TABLE 3 Risk factors associated with *S. flexneri* transmission in retail raw milk samples collected from different region of Peshawar

Variables	Total samples	Positive, *n* (%)	Negative, *n* (%)	*p*- value
Cloths				
Dirty	220	11(5)	209(95)	0.04
Clean	120	1(0.83)	119(99.16)	0.04
Utensils				
Dirty	226	11(4.87)	215(95.13)	0.05
Clean	114	1(0.88)	113(99.12)	0.05
Gloves				
Dirty	27	3(11.11)	24(88.88)	0.02
Clean	5	0	5(100)	0.66
Not wearing	308	9(2.92)	299(97.07)	0.05
Temperature Controlled (Milk)				
Yes	78	0	78(100)	0.05
No	262	12(4.58)	250(95.42)	0.05
Storage Container for Milk				
Utensils				
Dirty	226	11(4.87)	215(95.13)	0.05
Clean	114	1(0.88)	113(99.12)	0.05
Transportation of food items (Milk)				
Vehicle Contamination				
Yes	152	2(1.32)	150(98.68)	0.04
No	188	10(5.32)	178(94.68)	0.04
Did livestock away from packing and storage areas of food products (Milk)?				
Yes	53	3(5.66)	50(94.33)	0.36
No	287	9(3.13)	278(96.86)	0.36

Note: p value < 0.05 was considered statistically significant.

TABLE 4 Risk factors associated with the hygienic quality of retail raw fruits/vegetables collected from different region of Peshawar

Variables	Total samples	Positive, *n* (%)	Negative, *n* (%)	*p*-value
Cloths				
Dirty	208	9(4.32)	199(95.67)	0.05
Clean	132	1(0.75)	131(99.24)	0.05
Utensils				
Dirty	205	9(4.39)	196(95.6)	0.05
Clean	135	1(0.75)	134(99.25)	0.05
Gloves				
Dirty	172	2(1.16)	170(98.84)	0.04
Clean	24	0	24(100)	0.37
Not wearing	144	8(5.55)	136(94.44)	0.01
Temperature Controlled (Fruits/Vegetables)				
Yes	88	0	88(100)	0.05
No	252	10(3.96)	242(96.03)	0.05
Storage of food items (Fruits/egetables)				
Containers				
Dirty	205	9(4.39)	196(95.6)	0.05
Clean	135	1(0.75)	134(99.25)	0.05
Transportation of food items (Fruit/Vegetables)				
Vehicle Contamination				
Yes	203	9(4.34)	194(95.56)	0.04
No	137	1(0.73)	136(99.27)	0.04
Did livestock away from packing and storage areas of food products (Fruits/Vegetables)				
Yes	63	2(3.17)	61(96.82)	0.9
No	277	8(2.88)	269(97.11)	0.9

Note: p value < 0.05 was considered statistically significant.

isolation rate was observed in unhygienic clothes (5%, 4.32%), utensils (4.87%, 4.39%), gloves (11.1%, 1.16%), and vehicle (4.3%, 1.3%) associated with transportation of raw milk and fruits/vegetable samples as compared with clean utensils, gloves, and vehicle, respectively. A higher frequency rate was also reported in an unhygienic storage container (4.87%, 4.39%) used for the transportation of raw milk and fruits vegetable. So, these risk factors are significantly associated with the *S. flexneri* ($p < 0.05$). Various epidemiological data sets showed that poor personal hygiene is often associated with foodborne shigellosis outbreaks around the world (Lewis haw et al., 2007; Warren et al., 2006). The contamination of food with shigellosis might, however, also be due to the use of unclean water for washing and sprinkling the fruits and vegetables to keep them fresh. Furthermore, numerous investigators have determined that several shigellosis outbreaks had been associated with the intake of uncooked or raw foods (Kuo et al., 2008). In addition, previous studies also reported that consumption of unhygienic food significantly contributes to shigellosis in low socioeconomic countries (Lewis haw et al., 2007; Mensah et al., 2002).

While isolation of *S. flexneri* was higher if the location of livestock was near the packing and storage areas of food products as compared to when livestock was far away from packing and storage area, there was no significant association with the risk of diarrheal disease caused by *S. flexneri*, which is very likely because *Shigella* infection is not a zoonotic disease.

The frequency of *S. flexneri* isolated in household water source was 6.4%. These results are in agreement with a study conducted in Bangladesh (Rahman et al., 2011), where a significant number of *Shigella* species were isolated from surface water. A previous study conducted in Khyber Pakhtunkhwa, Pakistan (Ahmed et al., 2003) detected a 71% prevalence rate of *Shigella* species in drinking water which is significantly higher compared to our study. The presence of *S. flexneri* in different drinking

TABLE 5 Risk factors associated with *S. flexneri* transmission in water samples collected from different region of Peshawar

Variables	Total samples	Positive, *n* (%)	Negative, *n* (%)	*p*-value
Sources of Water				
Tanks	203	19(9.4)	184(90.6)	0.008
Pump	23	1(4.35)	22(95.65)	0.66
Well	56	1(1.78)	55(98.22)	0.11
Pond	11	1(9.09)	10(90.91)	0.71
Bore direct source	22	0	22(100)	0.2
Tube well direct source	25	0	25(100)	0.17
Temperature				
18 to 20 °C	16	2(12.5)	14(87.5)	0.31
21 to 23 °C	11	1(9.09)	10(90.91)	0.71
24 to 26 °C	90	2(2.22)	88(97.78)	0.05
27 to 29 °C	120	11(9.16)	109(90.83)	0.13
30 to 32 °C	79	1(1.26)	78(98.73)	0.03
33 to 35 °C	24	5(20.83)	19(79.16)	0.003
pH				
6.4 to 6.7	31	1(3.22)	30(96.78)	0.44
6.8 to 7.1	27	1(3.70)	26(96.3)	0.54
7.2 to 7.5	78	1(1.28)	77(98.72)	0.03
7.6 to 7.9	163	17(10.43)	146(89.57)	0.004
8.0 to 8.3	41	2(4.88)	39(95.12)	0.65
Appearance				
Clear	206	15(7.28)	191(92.72)	0.45
Not Clear	134	7(5.22)	127(94.78)	0.45
Smell				
Odour	79	8(10.12)	71(89.87)	0.13
Odourless	261	14(5.36)	247(94.63)	0.13
Ways of Contamination				
Toilet waste	35	5(14.28)	30(85.72)	0.04
House drainage other than toilet waste	71	1(1.40)	70(98.60)	0.05
Animal wastes	41	2(4.87)	39(95.12)	0.65
Animal body parts	63	2(3.17)	61(96.82)	0.23
Industrial waste	3	0	3(!00)	0.64
Surface water drainage	25	6(24)	19(76)	0.0002
Hospital waste	20	4(20)	16(80)	0.01
No contamination	82	2(2.44)	80(97.56)	0.08

Note: p value < 0.05 was considered statistically significant.

water sources showed that it might be due to the drinking water contamination with fecal material, poor sanitary conditions, or leakage of pipes that increase the chance of mixing sewage water with drinking water thereby providing a good condition for the spread of diarrheal disease.

According to our findings water from water tanks, tube well source (9.4%), and ponds (9.09) is an effective source of transmission of shigellosis to humans, as it showed a higher isolation rate of *S. flexneri* as compared with pumps (4.35%) and wells (1.78%) and depicted a significant increase in the risk of spreading shigellosis ($p = 0.008$) compared to other sources (Table 5) this could be due to leakage of pipe anywhere on the way from source to the household tanks which result in mixing of sewage water with drinking water.

High frequency (20.83%, 10.43%) of *S. flexneri* in water samples was observed in the temperature range from 33°C to 35°C and pH range 7.6 to 7.9, respectively. Furthermore, it has been reported that the physical properties of the water sources, including clear appearance and odor were

more likely to be positive for *S. flexneri* following with unclear and odorless water samples, but have no significant association with the risk of shigellosis.

Given that contaminated water may be a common factor in infection of shigellosis in human beings, our findings showed that the number of *S. flexneri* isolates were higher in contaminated water from different sources (toilet waste, house drainage other than toilet waste, animal waste, surface water drainage, and hospital waste), which results in a significant risk of shigellosis ($p < 0.05$ and $p < 0.01$) (Table 5).

4 | CONCLUSIONS

In this study, *S. flexneri* isolates originating from nonclinical samples might be associated with the increased human risk to shigellosis in Peshawar, Pakistan as a significant number of *S. flexneri* were observed in the drinking water and retail raw food samples. The intake of raw fruits and vegetables without washing, a poor sanitary system, and improper water supplies treatment can increase the risk of contamination by *S. flexneri*. Most of the milk, delivered in the town to the customer, was managed under unhygienic conditions in containers and at temperatures that are not properly controlled. Most of the stockholders have limited awareness and knowledge regarding the handling and contamination of raw milk and the influence of the milk-borne pathogens on public health. The sources of *S. flexneri* in the uncooked milk might be from poor sanitation practices, contaminated water, containers, and clothes of milk handlers themselves. Therefore the present study suggests that, for controlling shigellosis, special measures are needed in order to improve hygiene for example safe food handling and processing, properly washing of fresh vegetables and fruits before consumption, uses of chlorinated or boiling water, avoiding surface water for drinking, appropriate sanitation condition, and flies control are the key practices to improve the quality of drinking water and food that can be helpful from a public health point of view. The present study has a great consequence on public health as it will assist the government agency to regulate and monitors the clean drinking water distributions and scrutinizes food supplies and its processing in order to control the outbreak. The regional and local administration should emphasis on the availability of safe drinking water for the community and they should increase the awareness regarding communicable diseases, which would significantly improve the control of the shigellosis in the area. However, the endowment of resources to improve such infrastructure to the peoples of a country like Pakistan is a complex issue and will take time to resolve; hence concentrating the scientific thrust towards the advance of a safe and affordable multivalent vaccine may be the dire need of the era.

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AUTHOR CONTRIBUTIONS

Muhammad Qasim: Conceptualization, Methodology, Supervision, Validation, Formal analysis, Data Curation, Visualization, Writing-Review & Editing. Iqbal Nisa: Validation, Investigation, Formal analysis, Writing-Original Draft, Visualization, Methodology. Arnold Driessen: Conceptualization, Methodology, Supervision, Visualization, Resources, Writing-Review & Editing, Validation, Formal analysis. Jeroen Nijland: Supervision, Visualization, Resources, Writing - Review & Editing, Validation, Formal analysis, Methodology. Rafiullah: Methodology, Visualization, Resources, Validation, Writing - Review & Editing. Anwar Ali: Methodology, Visualization, Resources, Validation, Writing - Review & Editing. Munazza Raza Mirza: Writing - Review & Editing. Mirza Ali Khan: Writing - Review & Editing. Taj Ali Khan: Writing - Review & Editing. Abdullah Jalal: Writing - Review & Editing. Hazir Rahman: Writing - Review & Editing.

CONFLICTS OF INTEREST

No conflict of interest was declared by the author.

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