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ORIGINAL ARTICLE

Molecular epidemiology of *Shigella flexneri* isolated from pediatrics in a diarrhea-endemic area of Khyber Pakhtunkhwa, Pakistan

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Abstract

Shigella flexneri is considered as an important causative agent of Shigellosis causing diarrhea in the countries with a low socioeconomic status. No study has been carried out on the molecular prevalence of *S. flexneri* in Khyber Pakhtunkhwa, Pakistan. So this study was designed to evaluate the molecular prevalence of *S. flexneri* and their associated risk factors. A total of 2014 diarrheal stool samples were collected from January 2016 to May 2017 from pediatrics patients of Khyber Pakhtunkhwa followed by identification of *S. flexneri* through biochemical, serological, and molecular methods. The overall prevalence of *Shigella* species was found to be 7.9% ($n = 160$). The predominant *Shigella* specie was *S. flexneri* ($n = 155$, 96.8%) followed by *S. boydii* ($n = 5$, 3.1%). Interestingly, no sample was found positive for *S. sonnei* and *S. dysenteriae*. The majority of Shigellosis cases occurred from June to September. Potential risk factors related with Shigellosis were unhygienic latrine usage, bad hand washing, and consumption of unhygienic food and water, and pipe leakage in the sewage system. In this study, we have observed a high number of Shigellosis cases especially those caused by *S. flexneri*. It is suggested that effective health awareness programs should be organized by the regional health authorities to minimize the magnitude of pediatrics Shigellosis.

Keywords *S. flexneri* · Pediatrics · Molecular epidemiology · Diarrhea · Endemic area · Pakistan

Introduction

Shigellosis is mainly a disease of pediatric in endemic areas of the world with a low socioeconomic status. It is estimated that

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165 million cases of Shigellosis reported per annum worldwide nearly completely restricted to cases in low socioeconomic countries. Approximately 1.1 million death cases are reported from Shigellosis [1, 2]. Due to its low infectious dose allows its rapid transmission via person-to-person contact in the region with the poor hygienic condition and clinical severity of *Shigella* leads a threatening infection especially in children and the immune-comprised person [3, 4] and 69% of Shigellosis cases reported in children under age of 5 years [5, 6]. In Pakistan, the annual morbidity rate of Shigellosis in less than 5 years of age was 22.1 cases per 100,000 people [7].

Shigella genus consists of four species, comprising *S. dysenteriae*, *S. sonnei*, *S. flexneri*, and *S. boydii* [8]. The dissemination of *Shigella* spp. and their serotypes demonstrate discrete regional variations [4, 9]. *S. flexneri* has been the predominate specie in developing countries with a frequency of 16% [9, 10]. *S. flexneri* has classified into more than 19 serotypes which are characterized on the basis of their O antigen on lipopolysaccharides [9]. To establish effective control policies, it is noteworthy to conduct an epidemiological study about *S. flexneri*. It is important to determine the *S. flexneri* rate and distribution among diarrheal patients and also to study the risk

factors to guide disease control interventions. No study has been carried out on the molecular prevalence of *S. flexneri* in Khyber Pakhtunkhwa province of Pakistan. For this reason, the study was conducted to measure the frequency of *S. flexneri* infections and determine the associated clinical manifestations and risk factors in the Khyber Pakhtunkhwa province of Pakistan.

Material and methods

Sample collection and ethical approval

Stool samples were collected both by rectal swab and in a sterile container from each diarrheal patient before any pre-scribed antibiotic treatment in the tertiary care hospitals of Khyber Pakhtunkhwa, Pakistan. Samples were cultured and processed according to Malnutrition and Enteric Disease (MAL-ED) microbiological protocols for enteric infection [11]. The institutional research ethical committee approved the present study. Pre-informed consent was taken from patient's parent or guardian before the collection of samples.

Bacteriological analysis

The stool samples were directly cultured on Xylose Lysine Deoxycholate (XLD) media and aerobically incubated for 18 to 20 h at 37 °C. *Shigella* colonies were identified by standard biochemical tests [12] and API 10S (Bio-Merieux, Marcy l'Etoile, France) as per vendor protocol. The biochemically positive isolates of *Shigella* spp. were further confirmed serological-ly with specific antisera (*Shigella* Polyvalent Agglutinating Sera) by slide agglutination test (Oxoid, Remel Europe Ltd.).

Enrollment procedure and exclusion/inclusion criteria

Children with an age up to 17 years who were visited Out Patient Department (OPD) or admitted in the gastrointestinal wards with a diarrheal disease from January 2016 to May 2107 were included in the study. The following features were considered as inclusion criteria: diarrheal patients visit-ing the hospital within 24 h, three or four loose stool or one bloody stool, and no antibiotic treatment within 3 days before study enrollment, no history of diarrhea or respiratory illness within 7 days of study enrollment. We excluded the patients having diarrhea with a 3 days post history of antibiotic treat-ment. There were no additional exclusion criteria.

Molecular detection of *Shigella* species

The Genomic DNA was extracted by the boiling method as previously described [13] with minor modifications. Fresh bac-terial culture colony was taken and suspended in 20 μL Milli-Q water in a PCR tube and heated at 95 °C for 10 min in the PCR machine. The tube was then centrifuged at 12000 rpm for 5 min and the supernatant was taken as genomic DNA.

16S rRNA gene was amplified by using 2x phire green hot start II PCR master mix (Thermo Fisher CA, 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. [14]. Amplified PCR product of *16S rRNA* gene was subsequently purified using GenElute™ PCR Clean-up Kit (NA1020, Sigma-Aldrich, USA) in line with the manufacturer. 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 purified PCR product was sequenced by Macrogen Inc. (Amsterdam, The Netherland) and obtained sequences were analyzed by clone manager suite 7 and compared with sequences in Gene bank.

Risk factors and clinical finding

Risk factors associated with infections were investigated includ-ing socioeconomic and demographic, nutritional status, and source of availability of drinking water, latrines usage, and the drainage system in or around the household. Furthermore, dis-posal of household garbage and its condition whether good or poor or medium, traveling, food handling and consumption, and case contact with animals 8 days before the onset of illness were also demonstrated. Socioeconomic factors such as family per capita monthly income and occupation were analyzed. Demographic data were also investigated including age, gender, and education of maternal or paternal. Nutritional status such as well-nourished or mal-nourished, exclusive breastfeeding, or partial breastfeeding was recorded. Clinical findings including duration of diarrhea, stool frequency per day, fever, abdominal pain, stool consistency, nausea, and vomiting were examined.

Statistical analysis

Statistical analysis was performed to find factors related with Shigellosis by calculating Pearson's χ^2 test and binary logistic regression (OR). p values less than 0.05 were statistically con-sidered to be significant.

Results

Prevalence of *S. flexneri*

A total of 2014 samples of diarrheic stool were examined for the existence of *Shigella* bacteria from January 2016 to May 2017. All isolates were confirmed by various

conventional biochemical tests including API 10S, slide agglutination test, and molecular typing (*16S rRNA* Sequencing). The obtained sequences were then compared with the already identified bacterial sequences database using the BLAST (NCBI) online tool and clone manager suite 7. A total of 160 isolate sequencing results have shown close similarity with previously described *Shigella* species giving the isolation rate of 7.9%. The predominant serogroup was *S. flexneri* ($n = 155$ (96.8%)) followed by *S. boydii* ($n = 5$ (3.1%)), interestingly no sample was positive for *S. sonnei* and *S. dysenteriae* (Table 1).

Seasonal distribution

During the evaluating period, Shigellosis presented noticeable seasonal characteristics as most of the cases of Shigellosis occurred in summer that is from June to September. Among 155 cases of *S. flexneri*, 17 (14.78%, $p < 0.05$) cases were observed in July followed by 16 cases (9.24%) in August while the least frequency ($n = 3$, 6.38%) was observed in December. The month-wise distribution of Shigellosis is shown in Table 2.

Risk factors for Shigellosis caused by *S. flexneri*

Various demographic and socioeconomic characteristics were also seen as potential risk factors related to Shigellosis (Table 3). The frequency of *S. flexneri* was diverse in different age groups (Table 3). Patients having 3 to 5 years of age showed high frequency ($n = 51$, 10.36%, $p = 0.01$) of *S. flexneri* infection. The frequency of *S. flexneri*, among males and female, was recorded as 9.16% and 5.66% and respectively, and was statistically significant ($p = 0.003$) as shown in Table 3. Geographical distribution showed that *S. flexneri* was equally prevalent in Pakistani and Afghan population with no significant difference. On the other hand, *S. flexneri* was most frequent in urban areas (8.32%) as compared with rural areas (6.67%) but this distribution was statistically insignificant ($p = 0.17$) as shown in Table 3.

A family's economic status, people with a monthly income less than Pak Rs. 15,000 per month showed an increase risk factor for Shigellosis ($p < 0.05$). Various risk groups including the case in the household, traveling within the country or to foreign country, and time spent outside home setting like country parks, visiting beaches, and farms showed significant association with Shigellosis ($p < 0.05$) as described in Table 3.

Drinking water from the storage tanks increased the risk for Shigellosis ($p < 0.05$) as shown in Table 3. Consumption of boiling water had a shielding effect against *S. flexneri* ($p < 0.05$). Patients that consumed water during recreational activities (e.g., canoeing, swimming) had no significant association with ones not involved in any recreational activities. A significant difference was also observed in an area where there is a water supply problem ($p < 0.05$). Pediatric who were defecated in an open environment nearby the household increased the risk significantly for Shigellosis. People using a shared toilet increased the risk of the disease significantly. Regular hand washing after going to the toilet and before feeding highly reduced the risk for Shigellosis (Table 3).

Consumption of food kept in the kitchen without covering increased the risk for Shigellosis ($p < 0.05$) as compared with food kept in the refrigerator or having a cover on it. Food items like fruits and milk consumed and the location of food items consumed like at a picnic, hotel, street market, and canteen in the last 8 days before the onset of illness showed a significant increase in the risk of disease ($p < 0.05$). Cleaning material i.e. water only for utensils showed a significant difference ($p < 0.05$) as compared with soap or chemical. The contact with animals, animal excreta in households, or visiting/living in a farm or poultry had a statistically insignificant effect on the risk of Shigellosis (Table 3).

Contact with contaminated soil has a significant association with the risk of *S. flexneri* ($p < 0.05$) as compared with the one having no contact with the contaminated soil. Dispose of waste material in open sewage systems and gardens showed a significant association ($p < 0.05$). The leakage of pipes in the sewage system had significantly increased the risk for the spread of *S. flexneri* (Table 3).

The occurrence of Shigellosis was not associated with the caretaker relation to a child, or caretaker education. The frequency of Shigellosis was significantly associated with the number of children per household, cleaning of children after defecation, exclusive breastfeeding, and nutritional status. More than 2 children per household showed a significant increase in the spread of Shigellosis ($p < 0.05$). Childhood diarrheal disease due to *S. flexneri* was significantly associated with the children with improper cleaning of defecation as compared with the one who is always cleaned after defecation. Also, bottle feeding practice was significantly related to the frequency of Shigellosis. The risk of increasing Shigellosis was low among pediatrics whose mothers had exclusive breastfeeding (3.52%) compared with pediatrics that had bottle feeding practice (11.01%). The frequency of Shigellosis was also associated with the nutritional status of the pediatrics. Pediatrics that was well-nourished showed a significantly low risk of Shigellosis (5.95%) compared with mal-nourished pediatrics (10.42%) as shown in Table 4.

Clinical findings

Several clinical findings were observed to have a significant association with Shigellosis like stool frequency ($p = 0.007$), abdominal pain ($p = 0.002$), fever ($p = 0.001$), consistency of stool with blood and mucus ($p = 0.0003$), bloody stool ($p = 0.0003$), and vomiting ($p = 0.001$) as shown in Table 5. Some

Table 1 Molecular identification of clinical isolates of *S. flexneri* using *16S rRNA* sequencing

Isolate code	No. of nucleotides	Similarities with reported case of bacteria	Percentage similarity	Coverage	Gene bank accession no.
12	1457	*S. flexneri* Y strain 93-3063	97	89	KT261144.1
19	1460	*S. flexneri* strain FDAARGAS 535	95	86	CP034060.1
27	1437	*S. flexneri* strain FDAARGAS 535	97	98	CP034060.1
31	1460	*S. flexneri* strain FDAARGAS 535	95	86	CP034060.1
35	1405	*S. boydii* strain 59-248	96	91	CP026766.1
44	1457	*S. flexneri* Y strain 93-3063	97	89	KT261144.1
49	1460	*S. flexneri* strain FDAARGAS 535	95	86	CP034060.1
55	1445	*S. flexneri* strain CICC 21534	97	84	KJ643932.1
59	1421	*S. flexneri* strain FDAARGOS 535	98	98	CP034060.1
75	1461	*S. flexneri* 2a strain 2457T	96	87	AE014073.1
80	1431	*S. flexneri* strain FDAARGAS 535	96	97	CP034060.1
92	1460	*S. flexneri* strain FDAARGAS 535	98	87	CP034060.1
93	1421	*S. flexneri* strain FDAARGOS 535	98	98	CP034060.1
97	1460	*S. flexneri* strain FDAARGAS 535	98	87	CP034060.1
101	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
117	1463	*S. flexneri* strain 2016AM-0877	94	93	CP033510.1
119	1460	*S. flexneri* strain FDAARGAS 535	97	80	CP034060.1
120	1440	*S. flexneri* 2a strain ATCC 29903	98	89	CP026788.1
132	1460	*S .flexneri* strain FDAARGAS 535	97	80	CP034060.1
135	1463	*S. flexneri* strain 2016AM-0877	94	93	CP033510.1
149	1460	*S. flexneri* strain FDAARGAS 535	97	80	CP034060.1
152	1415	*S. flexneri* strain FDAARGOS 535	94	98	CP034060.1
162	1463	*S. flexneri* strain 2016AM-0877	94	93	CP033510.1
173	1440	*S. flexneri* 2a strain ATCC 29903	98	89	CP026788.1
175	1440	*S. flexneri* 2a strain ATCC 29903	98	89	CP026788.1
176	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
196	1457	*S. flexneri* Y strain 93-3063	97	89	KT261144.1
202	1458	*S. flexneri* strain 2016AM-0877	96	90	CP033510.1
203	1419	*S. flexneri* strain RSHD96	97	97	KY971022.1
219	1460	*S. flexneri* strain FDAARGAS 535	97	80	CP034060.1
233	1399	*S. boydii* strain 59-248	98	96	CP026766.1
239	1460	*S. flexneri* strain FDAARGAS 535	97	80	CP034060.1
250	1415	*S. flexneri* strain FDAARGOS 535	94	98	CP034060.1
284	1401	*S. flexneri* strain 64-5500	97	92	CP026811.1
286	1426	*S. flexneri* strain 2016AM-0877	98	98	CP033510.1
275	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
310	1401	*S. flexneri* strain 64-5500	97	92	CP026811.1
315	1437	*S. flexneri* strain FDAARGAS 535	97	98	CP034060.1
337	1461	*S. flexneri* 2a strain 2457T	96	87	AE014073.1
349	1447	*S. flexneri* strain FDAARGAS 535	97	98	CP034060.1
361	1458	*S. flexneri* strain 2016AM-0877	96	90	CP033510.1
387	1431	*S. flexneri* strain FDAARGAS 535	96	97	CP034060.1
393	1426	*S. flexneri* strain 2016AM-0877	98	98	CP033510.1
397	1401	*S. flexneri* strain 64-5500	97	92	CP026811.1
415	1401	*S. flexneri* strain 64-5500	97	92	CP026811.1
435	1445	*S. flexneri* strain CICC 21534	97	84	KJ643932.1
422	1431	*S. flexneri* strain FDAARGAS 535	96	97	CP034060.1
444	1462	*S. flexneri* strain 64-5500	95	93	CP026811.1
481	1445	*S. flexneri* strain CICC 21534	97	84	KJ643932.1

Table 1 (continued)

Isolate code	No. of nucleotides	Similarities with reported case of bacteria	Percentage similarity	Coverage	Gene bank accession no.
469	1445	*S. flexneri* strain CICC 21534	97	84	KJ643932.1
500	1421	*S. flexneri* strain FDAARGOS 535	98	98	CP034060.1
507	1388	*S. boydii* strain ATCC 800	95	97	CP026731.1
513	1462	*S. flexneri* strain 64-5500	95	93	CP026811.1
521	1445	*S. flexneri* strain CICC 21534	97	84	KJ643932.1
530	1437	*S. flexneri* strain FDAARGAS 535	97	98	CP034060.1
559	1426	*S. flexneri* strain 2016AM-0877	98	98	CP033510.1
563	1434	*S. flexneri* strain 2016AM-0877	98	98	CP033510.1
577	1462	*S. flexneri* strain 64-5500	95	93	CP026811.1
584	1452	*S. flexneri 2a* strain ATCC 29903	95	94	CP026788.1
617	1462	*S. flexneri* strain 64-5500	95	93	CP026811.1
639	1457	*S. flexneri* Y strain 93-3063	97	89	KT261144.1
656	1452	*S. flexneri 2a* strain ATCC 29903	95	94	CP026788.1
675	1460	*S. flexneri* strain FDAARGAS 535	98	87	CP034060.1
691	1460	*S. flexneri* strain FDAARGAS 535	98	87	CP034060.1
693	1455	*S. flexneri* strain FDAARGAS 535	96	86	CP034060.1
698	1419	*S. flexneri* strain RSHD96	97	97	KY971022.1
727	1402	*S. flexneri* strain RSHD96	97	92	KY971022.1
728	1415	*S. flexneri* strain FDAARGOS 535	94	98	CP034060.1
736	1462	*S. flexneri* strain 64-5500	95	93	CP026811.1
744	1455	*S. flexneri* strain FDAARGAS 535	96	86	CP034060.1
754	1459	*S. flexneri* strain FDAARGOS 535	94	98	CP034060.1
775	1402	*S. flexneri* strain RSHD96	97	92	KY971022.1
786	1391	*S. flexneri* strain 95-3008	87	86	CP026772.1
791	1391	*S. flexneri* strain 95-3008	87	86	CP026772.1
819	1402	*S. flexneri* strain RSHD96	97	92	KY971022.1
837	1410	*S. flexneri* strain FDAARGAS 535	97	87	CP034060.1
853	1410	*S. flexneri* strain FDAARGAS 535	97	87	CP034060.1
876	1410	*S. flexneri* strain FDAARGAS 535	97	87	CP034060.1
880	1402	*S. flexneri* strain RSHD96	97	92	KY971022.1
910	1402	*S. flexneri* strain RSHD96	97	92	KY971022.1
909	1418	*S. flexneri* strain 64-5500	90	97	CP026811.1
927	1451	*S. flexneri* strain 64-5500	95	97	CP026811.1
928	1451	*S. flexneri* strain 64-5500	95	97	CP026811.1
948	1418	*S. flexneri* strain 64-5500	90	97	CP026811.1
965	1465	*S. flexneri* strain 2016AM-0877	96	93	CP033510.1
980	1465	*S. flexneri* strain 2016AM-0877	96	93	CP033510.1
989	1418	*S. flexneri* strain 64-5500	90	97	CP026811.1
999	1398	*S. flexneri* strain RSHD96	98	90	KY971022.1
1003	1412	*S. flexneri* strain RSHD96	96	94	KY971022.1
1004	1412	*S. flexneri* strain RSHD96	96	94	KY971022.1
1007	1449	*S. boydii* strain ATCC 800	96	90	CP026731.1
1009	1433	*S. flexneri* strain RSHD96	95	92	KY971022.1
1027	1431	*S. flexneri* strain FDAARGAS 535	96	97	CP034060.1
1033	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
1068	1440	*S. flexneri 2a* strain ATCC 29903	98	89	CP026788.1
1070	1440	*S .flexneri 2a* strain ATCC 29903	98	89	CP026788.1
1095	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
1111	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1

Table 1 (continued)

Isolate code	No. of nucleotides	Similarities with reported case of bacteria	Percentage similarity	Coverage	Gene bank accession no.
1129	1440	*S. flexneri 2a* strain ATCC 29903	98	89	CP026788.1
1131	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
1142	1448	*S. flexneri* strain FDAARGAS 535	98	81	CP034060.1
1151	1462	*S. flexneri* strain 64-5500	95	93	CP026811.1
1165	1433	*S. flexneri* strain RSHD96	95	92	KY971022.1
1152	1433	*S. flexneri* strain RSHD96	95	92	KY971022.1
1171	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1189	1456	*S. flexneri* strain 2016AM-0877	96	87	CP033510.1
1191	1448	*S. flexneri* strain FDAARGAS 535	98	81	CP034060.1
1172	1447	*S. flexneri* strain 2016AM-0877	95	83	CP033510.1
1204	1419	*S. flexneri* strain RSHD96	97	97	KY971022.1
1209	1436	*S. flexneri* strain 2016AM-0877	97	97	CP033510.1
1217	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
1257	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1290	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1292	1448	*S. flexneri* strain FDAARGAS 535	98	81	CP034060.1
1303	1436	*S. flexneri* strain 2016AM-0877	97	97	CP033510.1
1306	1440	*S. flexneri 2a* strain ATCC 29903	98	89	CP026788.1
1333	1465	*S. flexneri* strain 2016AM-0877	95	87	CP033510.1
1354	1433	*S. flexneri* strain RSHD96	95	92	KY971022.1
1369	1436	*S. flexneri* strain 2016AM-0877	97	97	CP033510.1
1394	1447	*S. flexneri* strain 2016AM-0877	95	83	CP033510.1
1395	1436	*S. flexneri* strain 2016AM-0877	97	97	CP033510.1
1405	1433	*S. flexneri* strain RSHD96	95	92	KY971022.1
1414	1448	*S. flexneri* strain FDAARGAS 535	98	81	CP034060.1
1437	1465	*S. flexneri* strain 2016AM-0877	95	87	CP033510.1
1461	1465	*S. flexneri* strain 2016AM-0877	95	87	CP033510.1
1471	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1474	1436	*S. flexneri* strain 2016AM-0877	97	97	CP033510.1
1507	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1517	1414	*S. flexneri* strain 2016AM-0877	97	99	CP033510.1
1529	1414	*S. flexneri* strain 2016AM-0877	97	99	CP033510.1
1543	1414	*S. flexneri* strain 2016AM-0877	97	99	CP033510.1
1554	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1555	1452	*S. flexneri 2a* strain ATCC 29903	95	94	CP026788.1
1583	1451	*S. flexneri* strain 64-5500	95	97	CP026811.1
1599	1423	*S. flexneri* strain FDAARGAS 535	97	87	CP034060.1
1615	1423	*S. flexneri* strain FDAARGAS 535	97	87	CP034060.1
1626	1423	*S. flexneri* strain FDAARGAS 535	97	87	CP034060.1
1632	1414	*S. flexneri* strain 2016AM-0877	97	99	CP033510.1
1651	1391	*S. flexneri* strain 95-3008	87	86	CP026772.1
1677	1427	*S. flexneri* strain 2016AM-0877	96	96	CP033510.1
1683	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1685	1433	*S. flexneri* strain RSHD96	95	92	KY971022.1
1691	1427	*S. flexneri* strain 2016AM-0877	96	96	CP033510.1
1705	1427	*S. flexneri* strain 2016AM-0877	96	96	CP033510.1
1715	1449	*S. flexneri* strain 73339	97	99	MH304308.1
1781	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
1729	1427	*S. flexneri* strain 2016AM-0877	96	96	CP033510.1

Table 1 (continued)

Isolate code	No. of nucleotides	Similarities with reported case of bacteria	Percentage similarity	Coverage	Gene bank accession no.
1738	1431	*S. flexneri* strain FDAARGAS 535	96	97	CP034060.1
1764	1419	*S. flexneri* strain RSHD96	97	97	KY971022.1
1819	1440	*S. flexneri 2a* strain ATCC 29903	98	89	CP026788.1
1831	1447	*S. flexneri* strain 2016AM-0877	95	83	CP033510.1
1859	1457	*S. boydii* strain ATCC 800	97	83	CP026731.1
1878	1421	*S. flexneri* strain FDAARGOS 535	98	98	CP034060.1
1883	1434	*S. flexneri* strain 2016AM-0877	98	98	CP033510.1
1895	1447	*S. flexneri* strain 2016AM-0877	95	83	CP033510.1
1909	1437	*S. flexneri* strain FDAARGAS 535	97	98	CP034060.1
1946	1442	*S. flexneri* strain 61-4982	97	91	CP026792.1
1968	1461	*S. flexneri* 2a strain 2457T	96	87	AE014073.1
1977	1419	*S. flexneri* strain RSHD96	97	97	KY971022.1
1983	1391	*S. flexneri* strain 95-3008	87	86	CP026772.1

clinical features such as duration of diarrhea ($p = 0.38$), nausea ($p = 0.63$), and headache ($p = 0.11$) had shown no significant association with Shigellosis as shown in Table 5.

Discussion

The present investigation describes a finding on molecular epidemiology and potential risk factors associated with *S. flexneri* isolated from patients having diarrhea in Pakistan. In this study, we found the frequency of *Shigella* species was 7.9% which is consistent with the previous studies [15–19]. There is some previous report which revealed a high prevalence of *Shigella* as compared with the current study [20, 21]. Variation in prevalence rate of Shigellosis with in the same area or among population having the same level of socioeconomic development may be due to the absences of basic social amenities (like personal hygiene, access to clean water for drinking, and safe sewage disposal), socioeconomic status, and research methodologies such as sample size, study population, and design.

Our finding revealed that the most predominant serotype was *S. flexneri* (96.8%) followed by *S. boydii* (3.1%), while no *S. sonnei* and *S. dysenteriae* were isolated in study. The most frequent species in developing and developed countries were found to be *S. flexneri* and *S. dysenteriae* respectively [16, 22]. The findings of our study are in line with the previous studies from different African countries reported *S. flexneri* as a predominant isolates [16, 23–26]. Our finding revealed that *S. flexneri* was found throughout the entire year. The majority

Table 2 Month-wise distribution of Shigellosis cases in Khyber Pakhtunkhwa, Pakistan

Month	Total cases	Positive cases N (%)	Negative cases N (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
January	136	11 (8.08)	125 (91.91)	0.03	1.05	0.55	2.01	0.85
February	238	14 (5.88)	224 (94.11)	1.24	0.72	0.41	1.27	0.26
March	293	20 (6.82)	273 (93.17)	0.36	0.86	0.52	1.40	0.54
April	288	20 (6.94)	268 (93.05)	0.26	0.87	0.54	1.43	0.60
May	244	17 (6.96)	227 (93.03)	0.21	0.88	0.52	1.49	0.64
June	170	15 (8.82)	155 (91.17)	0.33	1.17	0.67	2.05	0.56
July	115	17 (14.78)	98 (85.21)	8.62	2.21	1.28	3.81	0.003
August	173	16 (9.24)	157 (90.75)	0.64	1.24	0.72	2.14	0.42
September	114	11 (9.64)	103 (90.35)	0.64	1.30	0.68	2.48	0.42
October	112	6 (5.35)	106 (94.64)	0.95	0.66	0.28	1.54	0.32
November	84	5 (5.95)	79 (94.04)	0.38	0.75	0.29	1.88	0.53
December	47	3 (6.38)	44 (93.61)	0.12	0.81	0.24	2.65	0.72

p values less than 0.05 were considered as statistically significant

Table 3 Association between risk of Shigellosis and socioeconomic and demographic variables among pediatrics in Khyber Pakhtunkhwa, Pakistan

Variables	Total cases	Positive cases n (%)	Negative cases n (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
Age (year)								
0–2	573	45 (7.85)	528 (92.15)	0.027	1.03	0.71	1.47	0.86
3–5	492	51 (10.36)	441 (89.63)	6.53	1.57	1.10	2.24	0.01
6–8	347	23 (6.62)	324 (93.37)	0.67	0.82	0.52	1.30	0.41
9–11	285	19 (6.66)	266 (93.33)	0.49	0.83	0.50	1.37	0.48
12–14	244	12 (4.91)	232 (95.08)	3.01	0.58	0.32	1.07	0.08
15–17	73	5 (6.84)	68 (93.15)	0.07	0.87	0.34	2.21	0.78
Gender								
Male	1167	107 (9.16)	1060 (90.83)	8.47	1.68	1.18	2.39	0.003
Female	847	48 (5.66)	799 (94.33)	8.47	0.59	0.41	0.84	0.003
Area								
Urban	1250	104 (8.32)	1146 (91.68)	1.80	1.26	0.89	1.79	0.17
Rural	764	51 (6.67)	713 (93.32)	1.80	0.78	0.55	1.11	0.17
Nationality								
Pakistani	1805	139 (7.7)	1666 (92.29)	0.0005	1.006	0.58	1.72	0.98
Afghani	209	16 (7.65)	193 (92.34)	0.0005	0.99	0.57	1.70	0.98
Monthly income (Pak Rs)								
> 10,000	187	3 (1.60)	184 (98.39)	10.76	0.17	0.05	0.56	0.001
10,000–15,000	455	45 (9.89)	410 (90.10)	3.98	1.44	1.004	2.08	0.04
15,000–20,000	630	49 (7.77)	581 (92.22)	0.008	1.01	0.71	1.44	0.92
20,000–25,000	323	30 (9.28)	293 (90.71)	1.37	1.28	0.84	1.94	0.24
> 25,000	419	28 (6.68)	391 (93.31)	0.76	0.82	0.54	1.26	0.38
Risk groups: anyone including the case in the household								
Work as a food handler	182	7 (3.84)	175 (96.15)	4.17	0.45	0.20	0.98	0.04
Have difficulty maintaining personal hygiene	129	4 (3.01)	125 (96.89)	4.09	0.36	0.13	1.008	0.04
Attend or work in a childcare setting (like playgroup, nursery,)	9	2 (22.22)	7 (77.77)	2.68	3.45	0.71	16.79	0.10
Child under 5 years of age	168	20 (11.9)	148 (88.09)	4.57	1.71	1.04	2.82	0.03
Work in healthcare setting (like technician, doctor, nurse,)	58	6 (10.34)	52 (89.65)	0.58	1.39	0.59	3.31	0.44
Raw meat handling in a professional capacity (like chef, abattoir, butcher)	189	14 (7.4)	175 (92.59)	0.02	0.95	0.53	1.61	0.87
Undertake work which involves contact with farm animals	79	3 (3.79)	76 (96.2)	1.75	0.46	0.14	1.48	0.18
Undertake work which involves contact with feces (like lab work, sewage work)	91	13 (14.28)	78 (85.71)	5.82	2.09	1.13	3.85	0.01
None of these	1109	86 (7.75)	1023 (92.24)	0.01	1.01	0.73	1.41	0.91
Before illness any pre-existing long-term medical illnesses (*E. coli* infection)? (like diabetes, heart problems etc.)								
Yes	88	4 (4.54)	84 (95.45)	1.28	0.55	0.20	1.54	0.25
No	1895	151 (7.96)	1744 (92.03)	3.34	2.48	0.90	6.83	0.06
Not sure	31	0	31 (100)	2.62	0	0	0	0.10
Traveling to foreign country								
Yes	40	8 (20)	32 (80)	8.69	3.10	1.40	6.86	0.003
No	1974	147 (7.44)	1827 (92.55)	8.69	0.32	0.14	0.71	0.003
Traveling within the country								
Yes	412	21 (5.09)	391 (94.90)	4.92	0.58	0.36	0.94	0.02
No	1590	134 (5.09)	1456 (91.57)	5.68	1.76	1.10	2.83	0.01

Table 3 (continued)

Variables	Total cases	Positive cases n (%)	Negative cases n (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
Not sure	12	0	12 (100)	1.006	0	0	0	0.31
Time spent outside home setting or usual work like visiting country parks, farms beaches, etc.								
Yes	1125	102 (9.06)	1023 (90.93)	6.73	1.57	1.11	2.21	0.009
No	786	52 (6.61)	734 (93.38)	2.11	0.77	0.54	1.09	0.14
Not sure	103	1 (0.97)	102 (99.02)	6.91	0.11	0.01	0.80	0.008
Food kept in home								
Refrigerator	1245	98 (7.87)	1147 (92.12)	0.14	1.06	0.75	1.49	0.70
Without cover	222	9 (4.05)	213 (95.94)	4.65	0.47	0.23	0.94	0.03
With cover	547	48 (8.77)	499 (91.22)	1.23	1.22	0.85	1.74	0.26
Food items handled, brought, or consumed in household in the past 8 days before disease								
Beef	229	18 (7.86)	211 (92.13)	0.009	1.02	0.61	1.71	0.92
Fruit	268	29 (10.82)	239 (89.17)	4.24	1.56	1.71	2.38	0.03
Fish	40	4 (10)	36 (90)	0.30	1.34	0.47	3.81	0.58
Vegetables	305	21 (6.88)	284 (93.11)	0.33	0.86	0.53	1.40	0.56
Chicken	152	15 (9.86)	137 (90.13)	1.09	1.34	0.76	2.35	0.29
Milk	261	12 (4.59)	249 (95.40)	4.05	0.54	0.29	0.99	0.04
Not sure	759	56 (7.37)	703 (92.62)	0.17	0.93	0.66	1.30	0.67
Location of food items consumed in the past 8 days								
Picnic	63	9 (14.28)	54 (85.71)	3.97	2.06	0.99	4.25	0.04
Restaurant	81	7 (8.64)	74 (91.35)	0.10	1.14	0.51	2.52	0.74
Hotel	139	17 (12.23)	122 (87.76)	4.32	1.75	1.02	2.99	0.03
Canteen	561	28 (4.99)	533 (95.00)	8.01	0.54	0.35	0.83	0.004
Street market	761	70 (9.19)	691 (90.8)	3.88	1.39	1.001	1.93	0.04
No	409	24 (5.86)	385 (94.13)	2.41	0.70	0.44	1.09	0.12
Consumed water in the past 8 days								
Pump	435	32 (7.35)	403 (92.64)	0.09	0.93	0.62	1.40	0.76
Tank	144	22 (15.27)	122 (84.72)	12.54	2.35	1.44	3.83	0.0003
Bore	450	29 (6.44)	421 (93.55)	1.27	0.78	0.51	1.19	0.25
Tube well	645	48 (7.44)	597 (92.55)	0.08	0.94	0.66	1.35	0.76
Well	315	18 (5.71)	297 (94.28)	2.06	0.69	0.41	1.14	0.15
Pond	20	4 (20)	16 (80)	4.30	3.05	1.007	9.24	0.03
Bottle	3	1 (33.33)	2 (66.66)	2.77	6.02	0.54	66.86	0.09
Spring	2	1 (50)	1 (50)	5.04	12.06	0.75	193.83	0.02
Using of boiling water								
Yes	167	5 (2.99)	162 (97.01)	5.66	0.34	0.14	0.86	0.01
No	1847	150 (8.12)	1697 (91.87)	5.66	2.86	1.15	7.08	0.01
Case activities before onset of illness								
Water was swallowed in the course of recreational activities (like canoeing, swimming)								
Yes	253	21 (8.30)	232 (91.69)	0.14	1.09	0.68	1.77	0.69
No	1761	134 (7.60)	1627 (92.39)	0.14	0.90	0.56	1.47	0.69
Water supply problem								
Yes	763	73 (9.56)	690 (90.43)	6.05	1.50	1.08	2.09	0.01
No	1251	82 (6.55)	1169 (93.44)	6.05	0.66	0.47	0.92	0.01
Washing hands after going to toilet								
Yes	803	41 (5.10)	762 (94.89)	12.61	0.51	0.35	0.74	0.0003

Table 3 (continued)

Variables	Total cases	Positive cases n (%)	Negative cases n (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
No	1211	114 (9.41)	1097 (90.58)	12.61	1.93	1.33	2.79	0.0003
Washing hands before feeding								
Yes	574	25 (4.35)	549 (95.64)	12.61	0.45	0.29	0.71	0.0003
No	1440	130 (9.02)	1310 (90.97)	12.61	2.17	1.40	3.38	0.0003
Cleaning material for utensil								
Water only	37	9 (75.67)	28 (24.32)	14.67	4.03	1.86	8.70	0.0001
Soap	1573	123 (7.81)	1450 (92.18)	0.15	1.08	0.72	1.62	0.69
Chemicals	404	23 (5.69)	381 (94.30)	2.85	0.67	0.42	1.06	0.09
Toilet type								
Private	349	21 (6.01)	328 (93.98)	1.67	0.73	0.45	1.17	0.19
Shared	971	89 (9.16)	882 (90.83)	5.70	1.49	1.07	2.08	0.01
No	694	45 (6.48)	649 (93.51)	2.18	0.76	0.53	1.09	0.13
Latrine usage								
No	1189	105 (8.83)	1084 (91.16)	5.26	1.50	1.05	2.12	0.02
Yes	825	50 (6.06)	775 (93.93)	5.26	0.66	0.46	0.94	0.02
Contact of the case 8 days before illness								
Contact with animals								
Hen	125	8 (6.4)	117 (93.6)	0.31	0.81	0.38	1.69	0.57
Cattle/cow	84	9 (11.25)	71 (88.75)	1.48	1.55	0.76	3.16	0.22
Goat	84	7 (8.75)	73 (91.25)	0.13	1.15	0.52	2.55	0.71
No	1721	131 (7.57)	1598 (92.42)	0.24	0.89	0.56	1.40	0.62
Visit/live in a farm/poultry								
Cattle/buffalo farm	47	5 (10.63)	42 (89.36)	0.60	1.45	0.56	3.72	0.43
Poultry farm	35	2 (5.71)	33 (5.71)	0.18	0.72	0.17	3.06	0.66
No	1931	147 (7.61)	1784 (92.38)	0.09	0.88	0.39	1.95	0.75
Contact made with raw animal product/organ								
Market	75	10 (13.33)	65 (86.66)	3.48	1.90	0.94	3.75	0.06
Butcher	28	2 (7.14)	26 (92.85)	0.01	0.92	0.21	3.91	0.91
Abattoir	20	1 (5)	19 (95)	0.20	0.62	0.08	4.72	0.64
No	1891	142 (7.50)	1749 (92.49)	1.52	0.68	0.37	1.25	0.21
Case have any contact with								
Contaminated soil	314	33 (10.50)	281 (89.49)	4.14	1.51	1.01	2.27	0.041
Agriculture field soil	267	7 (2.62)	260 (97.37)	11.15	0.29	0.13	0.62	0.0008
Garbage's associated soil	304	31 (10.19)	273 (89.80)	3.15	1.45	0.96	2.19	0.07
Manure	189	9 (4.76)	180 (95.23)	2.52	0.57	0.28	1.14	0.11
Sewage waste	249	30 (12.04)	219 (87.95)	7.57	1.79	1.17	2.74	0.005
No	691	45 (6.51)	646 (93.48)	2.07	0.76	0.53	1.10	0.14
Dispose of waste water								
Open sewage system	1425	97 (6.80)	1328 (93.19)	5.422	0.66	0.47	0.94	0.01
Pond	29	3 (10.34)	26 (89.65)	0.290	1.39	0.41	4.64	0.58
Garden	79	15 (18.98)	64 (81.01)	14.75	3.01	1.66	5.41	0.0001
Other (Nala)	481	40 (8.31)	441 (91.68)	0.34	1.11	0.76	1.62	0.55
Leakage in pipe								
Yes	274	36 (13.13)	238 (86.86)	13.22	2.06	1.38	3.06	0.0002
No	1120	80 (7.14)	1040 (92.85)	1.08	0.84	0.60	1.16	0.29

Table 3 (continued)

Variables	Total cases	Positive cases n (%)	Negative cases n (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
Not sure	639	39 (6.29)	581 (93.70)	2.49	0.73	0.50	1.07	0.11
Condition of sewage system								
Good	398	25 (6.28)	373 (93.71)	1.39	0.76	0.49	1.19	0.23
Medium	968	93 (9.60)	875 (90.39)	9.58	1.68	1.20	2.35	0.001
Poor	648	37 (5.70)	611 (94.29)	5.30	0.64	0.43	0.93	0.02

p values less than 0.05 were considered as statistically significant

number of cases of Shigellosis occurred July to September; this may be due to warm and humid weather that occurs from June to September in Pakistan which provides good conditions for bacteria to grow that easily leads to contamination of food [27–29].

The gender of pediatrics was significantly related with Shigellosis. Males were suffered more than females because females often stay close to their parents especially mothers and/or to play indoor with more hygienic toys whereas boys tend to move around and touch unhygienic objects in the surrounding ground. This may be related to the fact that during infancy, male needs to build a larger muscle mass as compared with girls. Subsequently, they may require more micronutrients and are thus at high risk of a damaging balance, comprising deficiency of zinc and vitamin A. This susceptibility might increase the risk of Shigellosis in males [30]. These findings

Table 4 Variant analysis of diarrheal risk factors among pediatric < 5 years old in Khyber Pakhtunkhwa, Pakistan

Variables	Total samples	Positive n (%)	Negative n (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
Care taker relation to child								
Mother	964	92 (9.54)	872 (90.45)	3.47	2.55	0.92	7.11	0.06
Father	82	3 (3.65)	79 (96.34)	3.10	0.36	0.11	1.17	0.07
Guardian	19	1 (5.26)	18 (94.73)	0.33	0.55	0.07	4.21	0.56
Care taker education								
Illiterate	490	43 (8.77)	447 (91.22)	0.06	0.94	0.62	1.44	0.80
Primary	364	40 (10.98)	324 (89.01)	2.62	1.42	0.92	2.17	0.10
Secondary	161	12 (7.45)	149 (92.54)	0.56	0.78	0.41	1.47	0.45
Above	50	1 (2)	49 (98)	3.14	0.19	0.02	1.44	0.07
No. of children/house								
1	213	16 (7.51)	197 (92.488)	0.73	0.78	0.44	1.37	0.39
2 to 5	689	73 (10.59)	616 (89.4)	5.94	1.81	1.11	2.95	0.01
> 5	163	7 (4.29)	156 (95.7)	5.22	0.40	0.18	0.90	0.02
Cleaning of children after defecation								
Always	320	30 (9.37)	290 (90.62)	0.07	1.06	0.67	1.67	0.78
Sometimes	558	40 (7.16)	518 (92.83)	4.86	0.62	0.40	0.95	0.02
No	187	26 (13.9)	161 (86.09)	6.61	1.86	1.15	3.01	0.01
Exclusive breast feeding								
Yes	284	10 (3.52)	274 (96.47)	14.24	0.29	0.15	0.57	0.0001
No	781	86 (11.01)	695 (88.98)	14.24	3.39	1.73	6.62	0.0001
Nutritional status								
Well-nourished	336	20 (5.95)	316 (94.04)	5.61	0.54	0.32	0.90	0.017
Mal-nourished	729	76 (10.42)	653 (89.57)	5.61	1.83	1.10	3.06	0.017

p values less than 0.05 were considered as statistically significant

Table 5 Clinical manifestations of Shigellosis in pediatrics in Khyber Pakhtunkhwa, Pakistan

Variables	Total samples	Positive n (%)	Negative n (%)	Chi-square	OR	0.95 confidence intervals		p value
						Lower limit	Upper limit	
Duration of diarrhea								
1–5 days	1406	113 (8.03)	1293 (91.96)	0.76	1.17	0.81	1.70	0.38
≥ 6	608	42 (6.9)	566 (93.09)	0.76	0.84	0.58	1.22	0.38
Stool frequency per day								
1–4 times	506	25 (4.94)	481 (4.94)	7.22	0.55	0.35	0.85	0.007
≥ 5 times	1508	130 (8.62)	1378 (91.37)	7.22	1.81	1.16	2.81	0.007
Abdominal pain								
Yes	1492	131 (8.78)	1361 (91.21)	9.52	1.99	1.27	3.12	0.002
No	522	24 (4.59)	498 (95.40)	9.52	0.50	0.32	0.78	0.002
Fever								
Yes	1165	109 (9.35)	1056 (90.64)	10.72	1.80	1.26	2.57	0.001
No	849	46 (5.41)	803 (94.58)	10.72	0.55	0.38	0.79	0.001
Consistency								
Watery	916	49 (5.35)	867 (94.65)	13.02	0.52	0.37	0.75	0.0003
Mixed (blood + mucus)	1098	106 (9.66)	992 (90.34)	13.02	1.89	1.33	2.68	0.0003
Nausea								
Yes	783	63 (8.04)	720 (91.95)	0.22	1.08	0.77	1.51	0.63
No	1231	92 (7.47)	1139 (92.52)	0.22	0.92	0.66	1.28	0.63
Vomiting								
Yes	714	33 (4.62)	681 (95.37)	14.71	0.46	0.31	0.69	0.0001
No	1300	122 (9.38)	1178 (90.06)	14.71	2.13	1.43	3.17	0.0001
Blood in stool								
Yes	1098	106 (9.65)	992 (90.34)	13.02	1.89	1.33	2.68	0.0003
No	916	49 (5.34)	867 (94.65)	13.02	0.52	0.37	0.75	0.0003
Headache								
Yes	419	40 (9.54)	379 (90.45)	2.55	1.35	0.93	1.98	0.11
No	1595	115 (7.21)	1480 (92.78)	2.55	0.73	0.50	1.07	0.11

p values less than 0.05 were considered as statistically significant

are comparable with the study reported in Egyptian pediatrics where males affected more than females from Shigellosis and some studies from Iran and Pakistan described that gender had no association with Shigellosis [17, 29, 31].

The present study revealed that *S. flexneri* was more frequently found in age groups below 5 years as compared with above 5 years of age which may be due to the fact that mother proper care and attention on the children below 5 years become difficult because they are very mobile and play unsupervised within the open and unhygienic environment. These findings are in parallel with other studies carried out in China, Iran, and Brazil where Shigellosis was common in pediatric less than 5 years of age [15, 19, 32]. A decrease in the number of cases among the age group > 5 years may be due to a stronger immune system against the pathogen.

We found that pediatric from urban residences were more prone to report Shigellosis, than pediatric from rural communities. This study was diverse from a previous

study carried out in DebreBirehan Town in North East of Ethiopia and Sheko district in Southwest of Ethiopia [33, 34]. This result might be accredited to various factors, comprising unhygienic food in markets, water supply problems, and congested areas with the improper sewage system.

One of the findings of our study showed that traveling factor significantly increases the risk of Shigellosis either within the country or to foreign country or even time spent outside the home like visiting park and beaches this may be due to the fact that *S. flexneri* has transmitted through an oral-fecal route either by contaminated hands, drinking water or food.

We also observed that risk for Shigellosis was independently related with the numerous factors including a family having low monthly income, household unhygienic environment, and contact with feces, insufficient sanitation, and poor hygiene. In Pakistan, poverty is one of

the leading problems due to low income; many people cannot have enough money to purchase a refrigerator for food storage. Furthermore, unhygienic environmental conditions with the existence of flies and cockroaches also significantly increase the risk of Shigellosis.

The present study revealed that food kept without covering to be a strong risk factor for Shigellosis that might be contaminated with cockroaches and flies. The present study also showed that consumption of raw fruits and vegetables to be a strong risk factor for Shigellosis might be the children have consumed the raw fruits and vegetables without cleaning with water. Similarly, the food items consumed in the hotel, canteen, and street market and at picnic spot have significantly increased the risk of diarrhea caused by Shigellosis which may be due to the fact that children consumed food with dirty hands or may be food consumed from this area are contaminated with *S. flexneri*. The present study indicated a significant difference between sources of drinking water and the frequency of Shigellosis. In contrast, a study reported from Mbour, Senegal did not significantly correlate source of drinking water and the threat of diarrhea [35]. A study reported from Ethiopia showed that sources of water are a significant environmental predictor of diarrheal illness [36].

We also observed that the consumption of water without boiling was significantly related with the occurrence of Shigellosis. Washing hands after using the toilet and before a meal, and cleaning of utensils with soap and chemicals showed the strongest independent shielding effect, that decreasing the risk of Shigellosis. Our study is comparable with others that reported a reverse association between hand washing and Shigellosis [37]. The present study confirms that an insufficient supply of water and poor sanitation are a major threat to diarrhea caused by *Shigella*. Unclean water supply for drinking purposes has been reported as a principle cause of outbreaks of Shigellosis worldwide [38].

Similarly, our study revealed that diarrhea occurrence was significantly associated with sharing the bathroom with other household. We found that the use of toilets had a significant protective effect in decreasing the risk of Shigellosis; this may be because the people who defecated outside the house contaminated the environment which increased the transmission of Shigellosis. Earlier studies reported that sharing toilets increases the threat of diarrhea caused by *Shigella* which is according to our findings [17, 35]. Our study showed that factors like contact with sewage waste, contaminated soil, and disposal of waste in open sewage system and garden, poor condition of sewage system and leakage of pipes had a significant association with Shigellosis this might be improper disposal of waste and low-quality drainage and sewerage system in Khyber Pakhtunkhwa, Pakistan which favor the spreading of Shigellosis. Our finding is in line with the previous study in northern Israel [39].

We have observed that children with exclusive breastfeeding have less frequency to develop *Shigella* diarrhea as compared with ones with a bottle feeding. This may be probably because the breast milk contains essential components that make the immune system stronger as compared with bottle milk. This finding is in line with a study conducted by Nisar in Pakistan [17].

We observed that Shigellosis was not significantly associated with caretaker education which is in parallel to a study conducted in Egypt by Abu-Elyazeed [29] and is in contrast with the result reported by Ghaemi in Iran and Nisar in Pakistan [17, 19]. In this finding, Shigellosis was related significantly with the presence of 2 to 5 children per family. This is in line with the study reported by Mengistie in eastern Ethiopia [40]; this may be because of the inability of the parents to give proper care to a large number of progenies.

Similarly, nutritional status also had a significant association with Shigellosis; this may be probably mal-nourished have weak immunity as compared with well-nourished that has a strong immunity. Our findings revealed that among clinical finding, stool containing blood and mucus was significantly associated with diarrhea caused by Shigellosis. These findings are in agreement with the findings of Nisar in Pakistan and Ghaemi in north Iran where abdominal, tenesmus, fever, and blood in stool were usually found during Shigellosis [17, 19] though there is a contradictory result which showed that majority of children suffering from Shigellosis did not have a bloody stool and fever [29]. Stool frequency greater than 5 times per day was significantly associated with *Shigella* infection. These outcomes are in parallel with the findings of Ghaemi et al. [19] and were in contrast with the previous study [17].

Conclusion

In conclusion, our study revealed that Shigellosis remains a significant health concern among Pakistani children living in Khyber Pakhtunkhwa, Pakistan. The most frequently identified specie was *S. flexneri*. Several factors were found that were statistically associated with Shigellosis among pediatrics namely children age, gender, season, traveling, latrine-sharing, more childrens per house, improper hand washing after attending the toilet; no hand washing before taking a meal; unsafe storage of food, water sources, sewage waste, disposal of wastewater, condition of sewage system, and breastfeeding. Our findings recommend a clear message on what interventions and prevention of Shigellosis should be focused on. Breastfeeding has to be promoted to all mothers, and there should also be proper sanitation in urban areas, delivery of safe water, waste disposal, and the raise of hygienic measures, and especially washing hands should be improved. Furthermore, effective health awareness programs should be organized by the regional

health authorities to minimize the magnitude of Shigellosis among children in the region.

Authors' contributions Muhammad Qasim, Iqbal Nisa, Arnold Driessen, Jeroen Nijland, and Fazli Bari designed the study, carried out the experiments, analyzed the data, and drafted the manuscript. Muhammad Qasim, Iqbal Nisa, and Jeroen Nijland helped in data analysis and results interpretation. Muhammad Qasim, Arnold Driessen, Jeroen Nijland, Fazli Bari, Mohammad Haroon, Hazir Rehman, Nusrat Yasin, Taj Ali Khan, Mubbashir Hussain, and Waheed Ullah critically reviewed and commented on the manuscript.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

Ethical approval   All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. In addition, the institutional research ethical committee approved the present study.

Informed consent   Informed consent was obtained from all patients/guardian for being included in the study.

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