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Original Article

Detection of *Salmonella* genes in stool samples of children aged 5 years and younger in urban and rural areas of Bangladesh

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Abstract

Introduction: Typhoid incidence in children is higher in urban areas than in rural areas of Bangladesh. This study examined whether healthy urban children harboured higher levels of *Salmonella* genes than healthy rural children.

Methodology: Stool samples from 140 children were studied: 70 from rural areas and 70 from urban metropolitan areas.

Results: The stool samples of urban children contained more *Salmonella* genes (median 4, IQR 3-4) than those of rural children (median 3, IQR 3-4). This suggests that urban Bangladeshi children have more *Salmonella* genes in their guts than rural children. Especially, in those under 12 months of age, the *Salmonella* gene prevalence in urban children was unique. They had more *Salmonella* genes (median 4, IQR 4-5) than rural children in the same age group (median 3, IQR 2.5-4). We also found more *Salmonella* genes in urban children who drank tap water (median 4, IQR 3-5) than in rural children whose water source was tube well water (median 3, IQR 2-4) and boiled pond water (median 3, IQR 3-3.5). However, there was no significant difference of *Salmonella* genes between urban children who drank tap-water and children whose water source was a tube well (median 4, IQR 3-4).

Conclusions: These data suggest that the urban environment, including the drinking water supply system, increases the likelihood of healthy children in urban areas harbouring more potentially pathogenic *Salmonella* organisms in their gut than found in rural healthy children.

Key words: *Salmonella* genes; Bangladesh; water-supply system; urban children; rural children; typhoid.

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Introduction

Typhoid fever is a critical health problem in many developing countries, including Bangladesh. An estimate for 2017 showed 14.3 million cases of typhoid and paratyphoid infections globally [1]. Also, nontyphoidal salmonellosis, which is related with gastroenteritis and sepsis, was estimated at 3.4 million cases in 2015 [2].

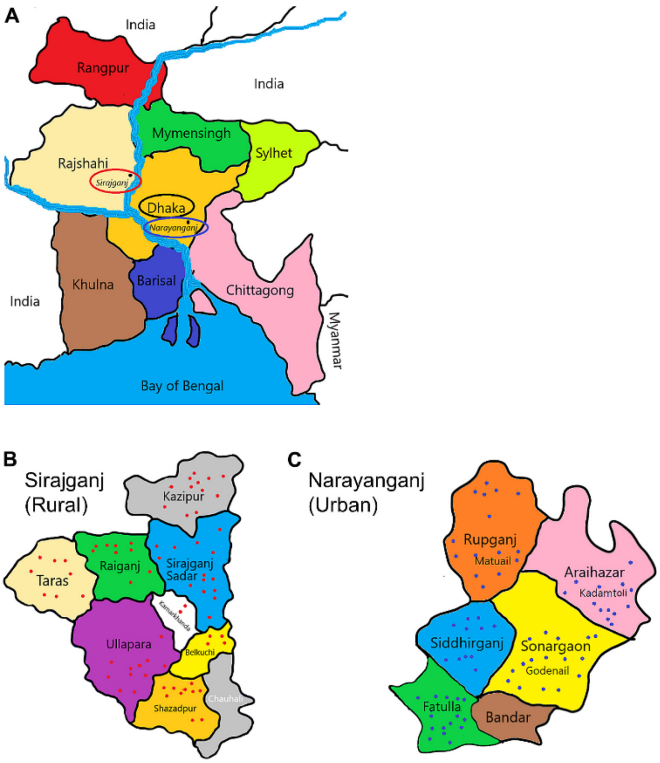
Bangladesh is one of the most densely populated countries in the world with an estimated population of 162.7 million and a population density of 1,103 persons per square kilometre in 2017 [3]. It is a riverine country and routinely faces disasters from monsoon floods and riverbank erosion that cause homelessness and landlessness and compel migration to cities in search of work. In one study, about 10% of the respondents migrated because of a natural disaster and 70% of those migrants remained permanently in urban areas [4]. This in-migration creates huge infrastructure challenges for cities. Around 37% of the Bangladeshi population lived in urban areas in 2018 [5].

Bangladesh has a high incidence of salmonellosis and typhoid fever, which disproportionately affect younger children, with the highest incidence in children < 5 years old [3,6]. The incidence of invasive salmonellosis is high among residents of the densely populated urban communities in Dhaka and in areas adjacent to the Dhaka metropolitan area [7]. This study reported 2.0 typhoid fever episodes (95% confidence interval [CI] 1.5–2.8)/1000 person-years in 2010 using

blood culture system. They also found that the incidence in children < 5 years old was 12-fold higher than the incidence among children ≥ 5 years of age.

In Bangladesh, the incidence of nontyphoidal salmonellosis has historically been higher in metropolitan areas than in rural villages. A study conducted in 1977–1979 found that of 214 isolates from blood and stool from metropolitan Dhaka, 0.3% of 66,341 cultures were positive compared with 12 (0.04%) of 27,265 positive cultures of isolates from rural areas [8-10]. A study of multidrug-resistant pathogenic bacteria in the gut of healthy young children in Bangladesh concluded that the gut of young children, below the age of 5 years, was an important reservoir for pathogenic bacteria [9]. Another study reviewed 19,265 blood cultures from an urban paediatric hospital in Dhaka. Of these, 855 (4.4%) were positive by culture for ST/PTF. The same study found 25 (0.2%) of 15,455 blood cultures from a rural hospital in Mirzapur were positive for *Salmonella* [10]. A 2005 community-based study in an urban slum in Bangladesh reported an overall incidence of salmonellosis of 3.9/1000 person-years and a higher rate in children aged 0–4 years (18.7/1000 person-years) [11]. Another study found a higher incidence in children aged < 5 years (10.5/1000 person-years) with an overall incidence rate of 2.0/1000 person-years [12].

Figure 1. Sample collection areas.



(A) Sirajganj (a rural district in northern Bangladesh, composed mostly of villages; red circle) and Narayanganj (blue circle, an urban city adjacent to the capital city of Dhaka, black circle). (B) Rural sample collection points in Sirajganj district (70 red dots show collection points for 70 stool samples in eight different areas). (C) Urban sample collection points in Narayanganj metropolitan city corporation (70 blue dots where 70 stool samples were collected from five different areas).

Previous studies established that the pathogenesis of enteric fever depends on several factors, including the infecting species and infectious dose. These organisms, when ingested in high doses, survive exposure to gastric acid before gaining access to the small bowel, where they penetrate the epithelium, enter the lymphoid tissue, and disseminate via the lymphatic or hematogenous route [13-15].

Several studies showed a higher incidence of typhoid fever in urban children than in rural children. These studies attributed this higher incidence of typhoid fever in urban children to several factors, including high population density in slum areas, poor sanitary conditions, drinking unsafe water, and ingestion of contaminated food. No study, however, has examined whether the presence of *Salmonella* genes in the guts of children play a role in typhoid fever (i.e., whether the presence of higher numbers of *Salmonella* genes in the intestines of healthy urban children is a predisposing factor for higher incidence of typhoid fever in urban children compared with rural children).

Most studies agree that both environment and infrastructure are related to a high incidence of typhoid fever in young urban Bangladeshi children. However, to date, no studies have assessed whether the intestines of urban children harbour more *Salmonella*, even when they are healthy (i.e., not suffering from salmonellosis), than the guts of healthy rural children.

The hypothesis of this study was that in Bangladesh healthy urban children harbour more *Salmonella* genes in their gut as commensal bacteria than rural children. This reasoning was supported by prior studies [8,16], including one that showed children in urban areas were more likely to carry *Escherichia coli* [16].

To assess our hypothesis, we examined stool samples of healthy urban and rural children using molecular detection of *Salmonella* genes by two-step nested PCR to determine the mean number of *Salmonella* genes present in the intestines of the sample children.

Methodology

Stool sample collection

In accord with this study's objective, we collected samples in two settings: the urban district of Narayanganj and the rural district of Sirajganj. The

2011 population densities of the two districts were 4,308 and 1,290 per square kilometre, respectively [17]. In order to ensure representative sampling, we collected samples in five separate administrative areas within Narayanganj (Fatulla, Godenail, Kadamtoli, Matuail, and Siddhirganj) and eight areas of Sirajganj (Belkutchi, Kamarkhand, Kazipur, Rayganj, Sadar, Shahjadpur, Tarash, and Ullapara). Narayanganj is adjacent to Dhaka, a metropolitan city, and has similar sanitary and water-supply infrastructure as well as overcrowding. The study enrolled a total of 140 healthy children aged < 5 years (70 urban and 70 rural). Household selection was random, and samples were accepted only from children who could fulfil all study criteria. We also collected information from each child's parents on the child's eating habits, personal hygiene practices, and the source of the family's drinking water (Figure 1). We began by collecting samples in the rural district of Sirajganj (in May 2016), which was followed by sample collection in the urban district of Narayanganj (in June 2016). During sample collection, healthy children lived at home and did not have any disease/symptoms of gastroenteritis (e.g., vomiting, diarrhoea, fever) or history of taking antibiotics during the prior 6 months. Health workers visited the children at home, obtained informed and written consent from their parents, and collected demographic information such as age, sex, personal hygiene, and dietary habits the day before obtaining samples. The parents collected the samples at home and put them in a refrigerator or in the supplied icebox (if the household lacked refrigeration) at 4°C. The next day, the health workers collected stool samples in the containers provided to the parents, placed them in an icebox, and immediately transported the samples to a laboratory where samples were stored at -20°C until processing for DNA extraction.

DNA extraction

DNA was extracted from 180 to 200 mg of stool samples according to the manufacturer's instructions using NucleoSpin Stool DNA extraction kit (Macherey-Nagel, Düren, Germany). The amount of eluted DNA was quantitatively measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at -20°C. The integrity of DNA was estimated by using 2% (w/v) agarose gel electrophoresis in 0.5X TAE buffer. We mixed 1 µL of Midori Green with 10 µL of extracted DNA, then loaded on the gel. These DNA samples were used for PCR.

Molecular detection by nested PCR

We used nested PCR with a thermal cycler (Veriti 96-well thermal cycler, Applied Biosystems, Foster City, CA) to screen for the presence of *Salmonella* genes in healthy children's stool specimens. In primary PCR, six *Salmonella* gene targets were used for amplification (Table 1). *Salmonella* gene targets included 16S rRNA [18], *Salmonella* pathogenicity island I gene (*hilA*) [19], *Salmonella* enterotoxin gene (*stn*) [20], *invA* gene [21], Fur-regulated gene (*iroB*) [22], and histidine transport operon (*hisJ*) [23]. For primary PCR, we used TopSimple nTaq premix (Enzymomics, Daejeon, Korea) for amplification. Total reaction volume was 20 µL (10 µL of pre-mix and 10 µL of master mix) (forward primer, 1 µL; reverse primer, 1 µL; stool DNA, 1 µL [10 ng/µL]; and ddH_2O, 7 µL). All primers were amplified for 35 cycles (Table 1). For secondary PCR, 1 µL of product from primary PCR was used to amplify the target band with different sets of primers (Table 2). PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30seconds, extension at 72°C for 45seconds, and final extension at 72°C for 10 minutes. Amplification

Table 1. Primary PCR primers used for screening of Salmonella genes.

Target fragment	Primer (5' – 3')	Annealing (°C)	Product size (bp)
16S rDNA	F: TGT TGT GGT TAA TAA CCG CA R: CAC AAA TCC ATC TCT GGA	56	574
iroB	F: TGC GTA TTC TGT TTG TCG GTC C R: TAC GTT CCC ACC ATT CTT CCC	55	606
hilA	F: CTG CCG CAG TGT TAA GGA TA R: CTG TCG CCT TAA TCG CAT GT	62	497
hisJ	F: ACT GGC GTT ATC CCT TTC TCT GGT G R: ATG TTG TCC TGC CCC TGG TAA GAG A	60	497
invA	F: GCT GCG CGC GAA CGG CGA AG R: TCC CGG CAG AGT TCC CAT T	62	389
Stn	F: CTT TGG TCG TAA AAT AAG GCG R: TGC CCA AAG CAG AGA GAT TC	55	260

All primers underwent 35 cycles.

products and sizes were determined by electrophoresis using 2% agarose gels. PCR amplification was considered positive if the amplicon matched the anticipated size and negative if no amplicon was detected (Figure 2). *Salmonella* gene counts were scored according to the number of positive *Salmonella* gene bands shown in the nested PCR using multiple replications of single stool DNA samples and each of the *Salmonella* gene primer pairs. The primer set used for 16s rRNA detection failed to amplify genes of some *Salmonella* organisms but was also negative for many genetically related intestinal bacteria (e.g., *Citrobacter*, *Klebsiella*, *Proteus*, and *Escherichia* species). Some genes including *hisJ*, *iroB,* and *invA* are well-conserved in non-*Salmonella* species but are not detected by *Salmonella*-specific primers. Primer pairs for other factors were not tested for non-*Salmonella* species in other studies [24].

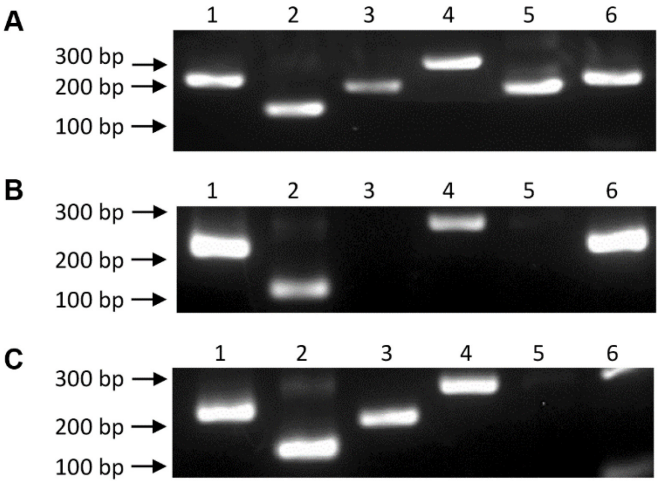
Statistical Analysis

We used the Shapiro-Wilk test to determine whether *Salmonella* gene values were normally distributed. Multivariable logistic regression analysis was used for comparing the effects of multiple factors to the binary-categorized *Salmonella* gene number (detected genes ≤ 3 or detected genes ≥ 4) in rural and urban children (SPSS software version 12.0; SPSS, Chicago, IL, USA). Statistical significance for continuous variables among and between groups was assessed by the Kruskal-Wallis test. The Chi-square test or Fisher's exact test was used to compare the proportion of each factor in rural and urban areas (GraphPad Prism software for Windows, version 5.01). Differences were considered significant at $p < 0.05$.

Ethics Statement

The Department of Biomedical Sciences, Seoul National University (SNU) College of Medicine, Republic of Korea, and the Department of Microbiology, Bangladesh University of Health Sciences (BUHS), Dhaka, jointly carried out this study from January 2016 to May 2018. Because the study-children were minors, we obtained written informed consent from their parents, who voluntarily agreed to participate in the study after the study details were explained to them. All personal information has been kept confidential - as per ethics requirements. This study was approved by the ethical review committee of BUHS, Dhaka.

Figure 2. Representative amplification bands of Salmonella genes.



PCR amplification products were loaded onto 2% agarose gels and electrophoresis was performed for band separation. Lane 1, 16s rRNA; lane 2, *Stn;* lane 3, *iroB;* lane 4, *hilA;* lane 5, *invA;* lane 6, *hisJ*. (A) Samples from typhoid cases. (B) Representative samples from healthy rural children. (C) Representative samples from healthy urban children.

Results

Salmonellosis in urban and rural children

We first tested five stool DNA samples from *Salmonella*-culture-positive patients and all samples produced the expected band sizes (16S rRNA, 215 bp; iroB, 201 bp; hilA, 281 bp; hisJ, 231 bp; invA, 197 bp;

Table 2. Secondary PCR primers used for screening of Salmonella genes.

Target fragment	Primer (5'-3')	Product Size (bp)
16s rDNA	F: TTA CCC GCA GAA GAA GCA CC R: GCA TTT CAC CGC TAC ACC TG	215
iroB	F: TCA GCG AAG AGA TGA CCG AC R: GGC GGT AGG CGT TAG AAA GT	201
hilA	F: GAA CAC CAA CCC GCT TCT CT R: AAA ATC CCC ATT TGC GCC AT	281
hisJ	F: TGC TCA TTG CCG AAG GTC TC R: GGA TGC GCT GAT TCC GTC TT	231
invA	F: TCC TTT GAC GGT GCG ATG AA R: ATC GCA ATC AAC AAT GCG GG	197
Stn	F: GCG TAA AAA TCG CCT CCA GC R: CTA TTC ATG CGA TTG GCC GC	132

All primers underwent annealing at 58°C for 30 cycles.

Stn, 132 bp) for every *Salmonella* gene assessed by nested PCR (Figure 2A). Of the 140 stool samples analysed, seven (3 from rural areas [Sirajganj] and 4 from urban areas [Narayanganj]) were negative for 16s rRNA.

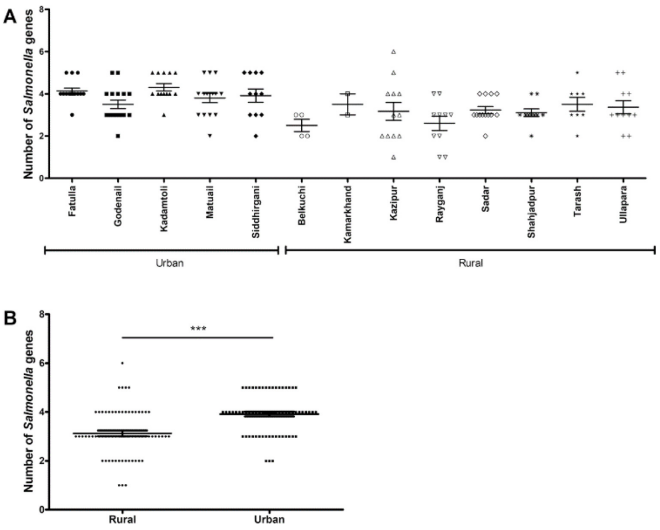
We then used *Salmonella* gene counts (0–6) as determined by positive *Salmonella* gene bands in nested PCR to score all samples (Supplementary Table 1). We found significantly more amplified *Salmonella* genes in urban samples than in rural samples (Figure 3A and Figure 3B). In this study, the stool samples of urban children contained more *Salmonella* genes (mean, 3.92 per sample) than found in rural children (mean, 3.13 per sample).

Drinking-water source and Salmonellosis in children

As water is an important medium of Salmonellosis transmission, we examined the findings for each drinking-water source. Of the rural children studied, 38 (54.3%) drank water from tube wells and 32 (45.7%) consumed boiled pond water. Among the urban children, 55 (78.6%) consumed tap water and 15 (21.4%) water from tube wells (Table 3).

Children who drank tap water had the highest number of *Salmonella* genes (mean, 3.95 per sample), followed by children who drank water from tube wells (urban and rural tube wells, respectively, mean 3.80 and 3.24 per sample) and boiled pond water (mean 3.03 per sample) (Figure 4). These differences suggest that water source may affect the prevalence of *Salmonella* genes. However, in the multivariable analysis, water supply system did not significantly affect the *Salmonella* gene number when other factors were adjusted constantly (Supplementary Table 2).

Figure 3. Children in an urban Bangladesh region have more Salmonella genes than children in a rural area.



(A) Number of *Salmonella* genes detected by nested PCR in samples by collection site. (B) Number of *Salmonella* genes grouped by origin (rural and urban areas). Rural (○), urban (Δ). Data were grouped and analysed by Mann-Whitney test. ***$p < 0.001$.

Personal hygiene and Salmonellosis in children

As Salmonellosis is also spread by food, personal hygiene habits may have an impact of Salmonellosis in children. We, therefore, also sought information about each child's hand-washing habits and whether the household used a pit latrine with or without a slab. Of the rural children, 33 (47.1%) used pit latrines without a slab and did not wash their hands after visiting the

Table 3. Age, dietary habit, water source, and hygiene of children studied.

		Participant Number (n = 70, each group)			Average Gene Number		
		Urban	**Rural**	***p***	**Urban**	**Rural**	***P***
Age	0–12 months	21	16		4.29	2.94	
	13–24 months	15	18		3.67	3.22	
	25–36 months	17	10	0.19	3.94	3.50	< 0.01
	37–48 months	4	11		3.50	2.64	
	49–60 months	13	15		3.69	3.33	
Dietary habit	Breast milk	5	3	0.74 (F)	4.60	3.67	
	Breast milk and soft food	31	34		3.94	3.00	< 0.01
	Normal food	34	33		3.79	3.21	
Water source	Tube well	15	38		3.8	3.21	
	Tap water	55	0	< 0.01	3.95	-	< 0.01
	Boiled pond water	0	32		-	3.03	
Hygiene	Modern WC, wash	55	0		3.95	-	
	Flush toilet/pit latrine, wash	15	37	< 0.01	3.8	3.30	< 0.01
	Pit latrine w/o slab, w/o wash	0	33		-	2.94	

Frequency of age, diet, water source, and hygiene between urban and rural children were compared using chi-square or Fisher's exact tests. Likewise gene number distributions between urban and rural children were assessed by Kruskal-Wallis test. WC, water closet; w/o, without; wash, handwashing.

Table 4. Multivariable analysis of Salmonella gene based on factors associated with area and hygiene.

Variables		Crude OR	95% CI	Adjusted OR	95% CI
Area	Rural	1	(ref)	1	(ref)
	Urban	4.792	(2.35–9.81)	3.286	(0.93–11.62)
Hygiene	Modern W/C, wash	6.133	(2.38–15.87)	1.867	(0.39–9.07)
	Flush/pit latrine, wash	1.971	(0.79–4.96)	1.4	(0.52–3.8)
	Pit latrine w/o slab, w/o wash	1	(ref)	1	(ref)

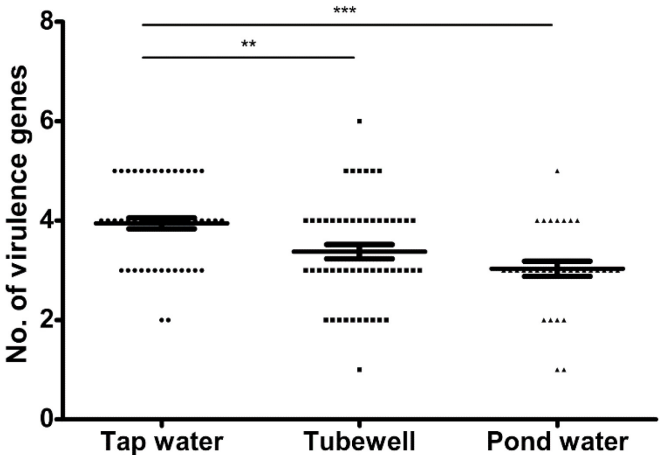
OR: odds ratio, CI: confidence interval. Adjusted OR for *Salmonella* was calculated from multivariable logistic model including area and hygiene simultaneously. WC, water closet; w/o, without; wash, handwashing.

latrine or before eating food and 37 (52.9%) used pit latrines with slabs and washed their hands after defaecation and before eating food; among the urban children, 15 (21.4%) used latrines with flush water and washed their hands after visiting the latrine and before taking food and 55 (78.6%) used modern toilets and washed their hands after defaecation and before eating food. However, hygiene habits did not have a significant impact on the number of *salmonella* genes detected (Table 4).

Salmonellosis in children: Does it vary with age and sex?

Ages of the children studied by locality (rural and urban) were as follows: 0–12 months, 16 rural (22.9%), 21 urban (30%); 13–24 months, 18 rural (25.7%), 15 urban (21.4%); 25–36 months, 10 rural (14.3%), 17 urban (24.3%); 37–48 months, 11 rural (15.7%), 4 urban (5.7%); and 49–60 months, 15 rural (21.4%), 13 urban (18.6%). Median ages of rural and urban children were 27 and 24 months, respectively. Among the rural children, there were 38 girls (54.3%) and 32 boys (45.7%) whereas in the urban group, there were 30 girls (42.9%) and 40 boys (57.1%).

Figure 4. Urban children whose tap water harbours more Salmonella genes.



Number of *Salmonella* genes was compared in groups with different drinking water sources using Kruskal-Wallis test. Rural (○), urban (Δ). **p < 0.01, ***p < 0.001.

We analysed the data by age group (Figure 5). Of interest, children in the under 12-month urban group had significantly higher *Salmonella* gene scores than found in rural samples ($p < 0.001$) (Figure 5A) or in other urban age groups. Urban children under 12 months of age had more *Salmonella* genes (mean, 4.29) than rural children in the same age group (mean, 2.94). By gender subgroup in rural or urban areas, there was no significant difference in numbers of children harbouring *Salmonella* genes (Supplementary Table 2).

Discussion

We collected stool samples in both rural and urban settings to compare the composition of gut microbiota of healthy children in two different living settings. We selected Sirajganj, a rural district in north-eastern Bangladesh, a typical Bangladeshi district (zilla), composed of a small town as its headquarters and several sub-districts (upazillas). Some of the villages have civic facilities such as tube well water and better access to health services; others still rely on traditional water sources (i.e., open ponds). These areas lack municipal sewerage systems and water supplies.

For our urban samples, we selected Narayanganj in the outskirts of the Bangladeshi capital, Dhaka. Some of Narayanganj is on the bank of Shitalakshya River, a tributary of the Brahmaputra River. Samples were collected from children in the Narayanganj City Corporation area, which is relatively well-serviced; however, it is an industrial area and is densely populated. Civic facilities are available, but due to congestion and the mostly unplanned and haphazard development, water lines occasionally are connected accidentally to the sewerage system and supply polluted tap water to most households. As this is an industrial area, many women work, mostly in factories, precluding their ability to breastfeed their children during their long working hours. Instead, they depend on non-breast milk, such as milk from cows or powdered milk. They maintain relatively good levels of personal hygiene and most have access to tap water for drinking.

Many women in rural Sirajganj breastfeed their children at least until the age of 24 months. Rural children receive little protein compared to their urban counterparts and primarily eat carbohydrates and vegetables. A study in 2013 found that 80.6% of the respondent mothers in a Bangladeshi village were housewives [25]. The study also found that 70.7% of those mothers breastfed their children and 75.9% fed colostrum to their babies. Most mothers (92.6%) continued to breastfeed their children even if the child was ill. The study concluded that more rural women breastfed their children than the corresponding national average.

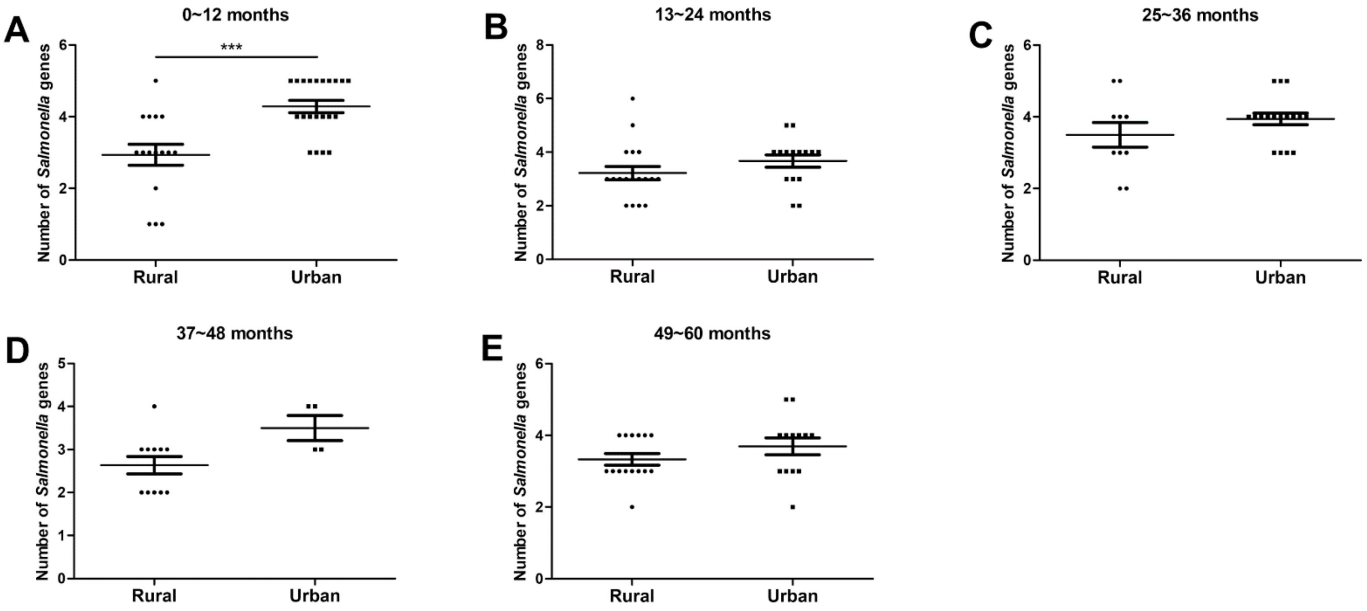
Although our study did not include examining a direct relationship between breast-feeding and less incidence of Salmonellosis, we have examined the existing literature on the same and found that breast-feeding indeed positively affect the incidence of Salmonellosis in children. A 1980 study noted vigorous responses of colostral and breast milk cells against Salmonella spp. [26]. The study concluded that colostral and breast milk cells were demonstrated to be more active against Salmonella than blood neutrophils. Another 2004 study found a strong association between having a liquid diet other than breast milk only and sporadic infant salmonellosis, which suggests that breast-feeding prevents infant salmonellosis [27].

Since Bangladesh is in the tropics, we also considered the possibility that seasonal variation might affect our results. We collected the rural samples in May 2016 and the urban samples in June 2016, thus minimising the collection time gap between the two sample groups. A study found two seasonal peaks of Typhoidal *Salmonella* (*Salmonella enterica* serovar Typhi and Paratyphi) in urban Dhaka in January and February and from August to November. According to the same study, rural Matlab had a single seasonal peak from August to November, but those seasonal peaks were insignificant [28]. Another study show there was no significant seasonal difference between May and June in Asia [29].

This study relied on PCR findings of extracted DNA from stools of healthy children. Isolation of live *Salmonella* from the samples collected would have been desired, but this was not possible as half of our stool samples were collected in remote rural areas, where we lacked laboratory facilities that would have enabled us to isolate live organisms from the samples. This was a limitation of this study.

The study's sample size was also relatively small, which may have been due to the strict sample criterion (clinically healthy children with no history of gastroenteritis and/or antibiotic use in the 6 months prior to the sample collection) that we followed in this study. Thus, finding the right samples was extraordinarily difficult. We also had budgetary limitations in part caused by working in two locations:

Figure 5. Urban Bangladeshi infants aged 0–12 months have more Salmonella genes than older children and all children in rural areas.



Salmonella genes from samples collected in rural and urban areas were sub-grouped according to child's age and reorganized and analysed by Mann-Whitney test. (A) 0~12 months; (B) 13~24 months; (C) 25~36 months; (D) 36~48 months; (E) 49~60 months. Rural (○), urban (Δ). ***$p < 0.001$.

Bangladesh for sample collection and DNA extraction, and South Korea for PCR.

Finally, we were unable to conclusively establish a direct correlation between the higher presence of *Salmonella* organisms in the intestines of urban children, who also had a higher incidence of typhoid fever, compared with rural children. We do anticipate that the study results may be useful in future studies in areas with high prevalence of ST/PTF (*Salmonella* Typhi/Paratyphi). Fast diagnosis of ST/PTF through a non-culture-based method (PCR) would aid the development of preventative measures, including routine screening that can be followed by early treatment - as opposed to time-consuming diagnosis of ST/PTF through traditional culture methods. This, in turn, should have a positive impact on reducing typhoid fever incidence in both urban and rural young children in Bangladesh and in countries with similar milieu.

Culture of organisms (TCM, the traditional culture method) remains the reference standard for identification of bacteria. Although culture remains the preferred diagnostic method for detection of bacteria, it is time-consuming and may fail to isolate a particular organism due to external factors, such as absence of high specificity or sensitivity**.** This study also states that only 40–60% of blood cultures from patients in the early phase of infection are positive [30]. Molecular detection methods, on the other hand, are suitable for rapidly identifying pathogens in human excreta as these methods are highly sensitive. PCR can detect minute quantities of the DNA of specific pathogens through amplification of a defined DNA segment and by discriminating on one reaction between different organisms even if they are closely related [31,32].

We hypothesized that urban children in Bangladesh may harbour more *Salmonella* genes in their intestines than children in rural areas and that this higher level of *Salmonella* might be the reason that urban Bangladeshi children have a higher incidence of typhoid fever than rural children. Our results agree with earlier findings [8,11]: Urban children in Narayanganj have a higher incidence of salmonellosis than rural children in Sirajganj. We also found that the young urban children of Narayanganj (aged 0–12 months) harbour more *Salmonella* genes ($p < 0.05$) than rural Sirajganj children in the same age group. Although urban children aged 13–60 months also harbour more *Salmonella* genes than rural children in the same age group, the difference was significant only for the youngest age group. This finding agrees with earlier studies that found typhoid fever disproportionately affects younger children, particularly children < 5 years old [11,12].

When we looked at children < 5 years old in urban Narayanganj and rural Sirajganj, the difference was not sufficiently strong to reach a definitive conclusion regarding a correlation of greater number of *Salmonella* genes in the intestines of healthy urban children as a predisposing factor for the higher incidence of typhoid fever. Even though we could not conclusively establish a correlation between greater numbers of *Salmonella* in the intestines of healthy urban children and the higher incidence of typhoid fever in urban than rural children, our findings may be a reference point for future studies and enable a definitive affirmative or negative conclusion to this hypothesis.

Of interest, our study found that the urban children in Narayanganj who drank tap water harboured more *Salmonella* genes than the urban children who drank tube well water and their rural counterparts in Sirajganj who drank water from tube wells and ponds (boiled water). Of note, the rural people of Bangladesh now usually drink boiled pond water due to years of awareness campaigns. Of the children studied, 38 of the rural Sirajganj children drank tube well water and 32 boiled pond water. Among the urban children, 55 consumed tap water and 15 tube well water.

In this study, children who drank tap water had the highest number of *Salmonella* genes (average, 3.95 per sample). Urban children who drank tube well water had a mean of 3.80. Rural children who drank tube well water and boiled pond water had means of 3.24 and 3.03 *Salmonella* genes, respectively. These findings are similar to earlier findings [33-35] and reinforce the notion that the lack of safe drinking water remains a major factor in the transmission and distribution of salmonellosis in urban and rural populations, particularly among young children.

A previous study suggested that both contaminated surface water and piped water could amplify the likelihood of waterborne infections among people living in the Dhaka metropolitan area, particularly of salmonellosis [33]. The authors also found that in high-risk areas (overcrowding, poor housing conditions, inadequate sanitation), 72.7% of residents had low quality-of-life (QOL), 18.2% had medium QOL, and only 9.08% had high QOL. The study found that unplanned urbanization, higher population density, and lack of critical urban infrastructure in the Dhaka metropolitan area had a considerable impact on the transmission and distribution of salmonellosis.

Another study, based on HPC (Heterotrophic Plate Count), found that all tap water sources of Dhaka city

restaurants were highly contaminated [35]. The HPC of the Dhaka Water and Sewerage Authority was between 1.2 and 5.4 × 10^4 cfu/mL compared with World Health Organization recommended levels of 100–500 cfu/mL [34]. Some 90% of the samples were contaminated with one or more of the three potential pathogenic species—*Vibrio*, *Shigella,* and *Salmonella*.

Overcrowding and unsanitary urban conditions, particularly lack of access to safe drinking water, have been linked to the high incidence of salmonellosis in metropolitan children. One study found that 62.5% of school-age children in Dhaka city who consumed piped water without boiling were positive for *Salmonella* as were 20.8% of those who drank piped water after boiling and 16.7% who drank tube well water [35].

Consumption of contaminated water, vegetables, and fast-food from restaurants also have been identified as major sources of *Salmonella* infection in Dhaka city residents [36]. This study reported that availability of junk food in urban areas, which is not usually found in rural Bangladesh, also contributes to the high incidence of salmonellosis in urban children. The study found that 79.2% of children who ate both home-cooked and junk food were positive for *Salmonella* against 20.8% who were accustomed only to food prepared at home. Another study found that prevalence of typhoid fever was higher among children of school-age groups (66.7%), children who drank unsafe drinking water (58.3%), and those who ate junk food (72.9%) [35].

Conclusions

As a consequence of environmental, infrastructural, and other reasons, the healthy urban children of Narayanganj in the < 5 years age group harboured more *Salmonella* genes in their intestines than the rural children of Sirajganj of identical ages. Further research in this area should focus on why the relationship between prior presence of *Salmonella* and the incidence of typhoid fever differs in urban and rural healthy children. Moreover, there is a need for studies to concentrate on developing preventive measures such as routine screening and early treatment of salmonellosis to reduce mortality and morbidity of young children. Such measures would be particularly effective in developing countries that lack significant investment in public health infrastructure.

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Annex – Supplementary Items

Supplementary Table 1. Multivariate analysis of Salmonella gene based on associated factors.

Factors		Crude OR	95% CI	Adjusted OR	95% CI
Area	Rural	1	(ref)	1	(ref)
	Urban	4.792	(2.35–9.81)	3.328	(0.9–12.32)
Hygiene	Modern WC, wash	6.133	(2.38–15.87)	1.518	(0.29–8.18)
	Flush/pit latrine, wash	1.971	(0.79–4.96)	0.537	(0.07–4.1)
	Pit latrine w/o slab, w/o wash	1	(ref)	1	(ref)
Age	0–12	1.467	(0.55–3.95)	NC	
	13–24	0.833	(0.31–2.29)	NC	
	25–36	2.375	(0.79–7.21)	1.618	(0.49–5.44)
	37–48	0.364	(0.1–1.43)	0.357	(0.08–1.61)
	49–60	1	(ref)	1	(ref)
Dietary habit	Breast milk	1	(ref)	1	(ref)
	Normal food	1.2	(0.62–2.34)	> 999.999	NA
Water supply	Pond water	1	(ref)	1	(ref)
	Tap water	6.815	(2.58–18.03)	0	(0–0)
	Tube well	2.282	(0.9–5.85)	2.524	(0.33–19.8)
Gender	Male	1	(ref)	1	(ref)
	Female	1.007	(0.52–1.96)	1.248	(0.59–2.68)

OR: odds ratio, CI: confidence interval, NC: not calculated. Adjusted OR for Salmonella was calculated from multivariable logistic model including area, hygiene, dietary habit, water supply, age and gender simultaneously. Pit latrine w/o slab, w/o wash indicates children use a pit latrine without a slab and do not wash hands afterwards; flush/pit latrine, wash indicates children use a flush or a pit latrine and wash their hands after visiting latrine and before eating food; modern WC, wash indicates children use modern toilet, wash hands after defecation and before eating food.

Supplementary Table 2. Data for each child in study and detection results of Salmonella genes from stool samples by nested PCR.

Area	Sample No.	Hygiene	Age (months)	Dietary habit	Water supply	Address (city)	Gender	16s	*iroB*	*Stn*	*hilA*	*invA*	*hisJ*	No. of genes	Category
Rural	1	Flush/pit latrine, wash	0–12	Breast milk and soft food	Tube well	Sadar	Female	+	+	+	+	-	-	4	1
Rural	2	Flush/pit latrine, wash	49–60	Normal food	Tube well	Sadar	Male	+	+	-	+	-	-	3	0
Rural	3	Flush/pit latrine, wash	0–12	Breast milk and soft food	Pond water	Rayganj	Male	+	-	-	+	+	-	3	0
Rural	4	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Rayganj	Female	+	-	+	+	+	-	4	1
Rural	5	Flush/pit latrine, wash	25–36	Normal food	Tube well	Sadar	Male	+	-	-	+	-	+	3	0
Rural	6	Pit latrine w/o slab, w/o wash	0–12	Breast milk	Pond water	Rayganj	Female	+	-	-	+	+	-	3	0
Rural	7	Pit latrine w/o slab, w/o wash	37–48	Normal food	Pond water	Rayganj	Female	+	-	-	+	-	-	2	0
Rural	8	Flush/pit latrine, wash	0–12	Breast milk	Tube well	Kazipur	Male	+	-	-	+	-	+	3	0
Rural	9	Flush/pit latrine, wash	0–12	Breast milk	Tube well	Ullapara	Female	+	+	+	+	+	-	5	1
Rural	10	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Tarash	Female	+	+	+	+	-	-	4	1
Rural	11	Pit latrine w/o slab, w/o wash	37–48	Normal food	Pond water	Rayganj	Male	+	+	-	+	-	-	3	0
Rural	12	Pit latrine w/o slab, w/o wash	37–48	Normal food	Pond water	Rayganj	Female	+	-	+	+	-	-	3	0
Rural	13	Flush/pit latrine, wash	25–36	Normal food	Tube well	Sadar	Female	+	-	+	+	+	-	4	1
Rural	14	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Shahjadpur	Female	+	+	+	+	-	-	4	1
Rural	15	Flush/pit latrine, wash	0–12	Breast milk and soft food	Tube well	Kazipur	Male	+	-	+	+	-	-	3	0
Rural	16	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Belkuchi	Female	+	-	-	+	-	-	2	0
Rural	17	Flush/pit latrine, wash	37–48	Normal food	Tube well	Sadar	Female	+	-	+	+	-	-	3	0
Rural	18	Flush/pit latrine, wash	37–48	Normal food	Tube well	Sadar	Male	+	-	-	+	-	-	2	0
Rural	19	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Sadar	Male	+	-	+	+	-	-	3	0
Rural	20	Pit latrine w/o slab, w/o wash	25–36	Normal food	Pond water	Belkuchi	Female	+	+	-	+	-	-	3	0
Rural	21	Pit latrine w/o slab, w/o wash	49–60	Normal food	Pond water	Belkuchi	Male	+	-	+	+	-	-	3	0
Rural	22	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Sadar	Male	+	-	+	+	-	-	3	0
Rural	23	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Sadar	Female	+	+	-	+	-	-	3	0
Rural	24	Flush/pit latrine, wash	25–36	Normal food	Tube well	Ullapara	Male	+	-	-	+	-	-	2	0
Rural	25	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	+	+	-	-	3	0
Rural	26	Flush/pit latrine, wash	49–60	Normal food	Tube well	Tarash	Male	+	+	+	+	-	-	4	1
Rural	27	Flush/pit latrine, wash	49–60	Normal food	Tube well	Tarash	Female	+	-	+	+	+	-	4	1
Rural	28	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Kamarkhand	Female	+	-	+	+	+	-	4	1
Rural	29	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Kamarkhand	Male	+	-	+	+	-	-	3	0

Rural	30	Pit latrine w/o slab, w/o wash	25–36	Normal food	Pond water	Tarash	Male	+	+	+	+	+	-	5	1
Rural	31	Pit latrine w/o slab, w/o wash	37–48	Normal food	Pond water	Tarash	Male	+	-	+	-	+	-	3	1
Rural	32	Pit latrine w/o slab, w/o wash	25–36	Normal food	Tube well	Tarash	Female	-	-	+	+	-	-	2	1
Rural	33	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Ullapara	Female	+	-	+	+	+	-	4	1
Rural	34	Flush/pit latrine, wash	49–60	Normal food	Tube well	Sadar	Female	+	-	+	+	-	-	3	0
Rural	35	Flush/pit latrine, wash	49–60	Normal food	Tube well	Sadar	Female	+	-	+	+	-	+	4	1
Rural	36	Flush/pit latrine, wash	49–60	Normal food	Tube well	Sadar	Male	+	-	+	+	+	-	4	1
Rural	37	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Sadar	Female	+	-	-	+	+	-	3	0
Rural	38	Flush/pit latrine, wash	49–60	Normal food	Tube well	Ullapara	Female	+	-	+	-	+	-	3	0
Rural	39	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Tarash	Female	+	-	+	-	+	-	3	0
Rural	40	Flush/pit latrine, wash	49–60	Normal food	Tube well	Ullapara	Male	+	-	+	+	+	-	4	1
Rural	41	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	-	+	-	-	2	0
Rural	42	Flush/pit latrine, wash	49–60	Normal food	Tube well	Ullapara	Male	+	-	+	+	-	-	3	0
Rural	43	Pit latrine w/o slab, w/o wash	37–48	Normal food	Pond water	Rayganj	Male	+	+	+	+	-	-	4	1
Rural	44	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	+	+	-	-	3	0
Rural	45	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	-	+	+	+	4	1
Rural	46	Pit latrine w/o slab, w/o wash	37–48	Normal food	Tube well	Rayganj	Female	-	-	+	+	-	-	2	0
Rural	47	Flush/pit latrine, wash	25–36	Normal food	Tube well	Ullapara	Male	+	-	+	+	-	-	3	0
Rural	48	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Ullapara	Male	+	+	+	+	+	-	5	1
Rural	49	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Ullapara	Male	+	-	+	+	-	-	3	0
Rural	50	Pit latrine w/o slab, w/o wash	49–60	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	+	+	-	-	3	0
Rural	51	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Ullapara	Female	+	-	-	+	-	+	3	0
Rural	52	Flush/pit latrine, wash	49–60	Breast milk and soft food	Tube well	Ullapara	Male	+	-	-	+	-	-	2	0
Rural	53	Flush/pit latrine, wash	49–60	Normal food	Tube well	Kazipur	Female	+	-	+	+	+	-	4	1
Rural	54	Pit latrine w/o slab, w/o wash	49–60	Normal food	Pond water	Shahjadpur	Female	+	-	+	+	-	-	3	0
Rural	55	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	+	+	-	-	3	0
Rural	56	Pit latrine w/o slab, w/o wash	49–60	Breast milk and soft food	Pond water	Shahjadpur	Male	+	-	+	+	-	-	3	0
Rural	57	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Rayganj	Male	+	-	-	-	-	-	1	0
Rural	58	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Rayganj	Female	+	-	-	-	-	-	1	0
Rural	59	Flush/pit latrine, wash	0–12	Breast milk and soft food	Tube well	Kazipur	Female	+	-	-	-	-	-	1	0
Rural	60	Pit latrine w/o slab, w/o wash	13–24	Breast milk and soft food	Tube well	Kazipur	Male	+	-	-	+	-	-	2	0
Rural	61	Flush/pit latrine, wash	0–12	Breast milk and soft food	Pond water	Shahjadpur	Female	+	-	-	+	-	+	3	0
Rural	62	Flush/pit latrine, wash	37–48	Normal food	Tube well	Kazipur	Male	+	-	-	+	-	-	2	0
Rural	63	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Kazipur	Male	+	-	-	+	-	-	2	0
Rural	64	Flush/pit latrine, wash	37–48	Normal food	Tube well	Kazipur	Male	+	-	-	+	-	-	2	0
Rural	65	Pit latrine w/o slab, w/o wash	0–12	Breast milk and soft food	Pond water	Belkuchi	Male	+	-	-	+	-	-	2	0
Rural	66	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Kazipur	Female	+	+	+	+	+	+	6	1
Rural	67	Flush/pit latrine, wash	25–36	Normal food	Tube well	Kazipur	Female	-	+	+	-	+	+	4	1
Rural	68	Pit latrine w/o slab, w/o wash	37–48	Normal food	Pond water	Tarash	Male	+	+	+	-	-	-	3	0
Rural	69	Flush/pit latrine, wash	25–36	Normal food	Tube well	Kazipur	Male	+	+	+	+	+	-	5	1
Rural	70	Flush/pit latrine, wash	25–36	Normal food	Tube well	Kazipur	Female	+	+	+	-	+	-	4	1
Urban	1	Modern WC, wash	25–36	Normal food	Tap water	Siddhirganj	Female	+	+	+	+	+	-	5	1
Urban	2	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Siddhirganj	Male	+	+	+	+	-	+	5	1
Urban	3	Modern WC, wash	25–36	Normal food	Tap water	Siddhirganj	Female	+	-	+	+	-	-	3	0
Urban	4	Modern WC, wash	25–36	Normal food	Tap water	Siddhirganj	Female	+	+	+	+	-	-	4	1
Urban	5	Modern WC, wash	49–60	Normal food	Tap water	Siddhirganj	Male	-	+	+	+	+	-	4	1
Urban	6	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Siddhirganj	Female	+	-	+	-	-	-	2	0
Urban	7	Modern WC, wash	0–12	Breast milk	Tap water	Siddhirganj	Female	+	+	+	-	-	-	3	0
Urban	8	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Siddhirganj	Male	+	+	+	+	+	-	5	1
Urban	9	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Siddhirganj	Female	+	+	+	-	+	-	4	1
Urban	10	Flush/pit latrine, wash	25–36	Normal food	Tube well	Matuail	Male	+	+	+	-	+	-	4	1
Urban	11	Flush/pit latrine, wash	0–12	Breast milk	Tube well	Matuail	Female	+	+	+	+	+	-	5	1
Urban	12	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Matuail	Male	+	+	+	+	+	-	5	1
Urban	13	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Matuail	Female	+	+	+	-	+	-	4	1
Urban	14	Flush/pit latrine, wash	49–60	Normal food	Tube well	Matuail	Male	+	+	+	-	+	-	4	1
Urban	15	Flush/pit latrine, wash	25–36	Normal food	Tube well	Matuail	Male	+	+	+	-	+	+	5	1
Urban	16	Flush/pit latrine, wash	49–60	Normal food	Tube well	Matuail	Male	+	-	+	-	+	-	3	0
Urban	17	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Matuail	Male	+	-	+	+	+	-	4	1

Urban	18	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Matuail	Male	+	+	+	-	+	-	4	1
Urban	19	Flush/pit latrine, wash	13–24	Breast milk and soft food	Tube well	Matuail	Male	+	+	+	-	+	-	4	1
Urban	20	Flush/pit latrine, wash	0–12	Breast milk and soft food	Tube well	Matuail	Female	+	-	+	-	+	-	3	0
Urban	21	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Kadamtoli	Male	+	-	+	+	+	-	4	1
Urban	22	Modern WC, wash	0–12	Breast milk	Tap water	Kadamtoli	Female	+	-	+	+	+	+	5	1
Urban	23	Modern WC, wash	0–12	Breast milk	Tap water	Kadamtoli	Male	+	-	+	+	+	+	5	1
Urban	24	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Kadamtoli	Female	+	-	+	+	+	+	5	1
Urban	25	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Kadamtoli	Male	+	-	+	+	+	-	4	1
Urban	26	Modern WC, wash	37–48	Normal food	Tap water	Kadamtoli	Male	+	+	+	-	+	-	4	1
Urban	27	Modern WC, wash	25–36	Normal food	Tap water	Kadamtoli	Male	+	+	+	-	+	+	5	1
Urban	28	Modern WC, wash	25–36	Normal food	Tap water	Kadamtoli	Male	+	-	-	+	+	+	4	1
Urban	29	Modern WC, wash	25–36	Normal food	Tap water	Kadamtoli	Male	+	+	-	+	+	-	4	1
Urban	30	Modern WC, wash	0–12	Breast milk	Tap water	Kadamtoli	Male	+	+	-	+	+	+	5	1
Urban	31	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Godenail	Male	+	-	+	+	+	+	5	1
Urban	32	Modern WC, wash	25–36	Normal food	Tap water	Kadamtoli	Male	+	-	-	+	+	-	3	0
Urban	33	Modern WC, wash	25–36	Normal food	Tap water	Kadamtoli	Male	+	-	+	+	+	-	4	1
Urban	34	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Kadamtoli	Female	+	-	+	+	+	-	4	1
Urban	35	Modern WC, wash	49–60	Normal food	Tap water	Godenail	Male	+	-	+	+	+	-	4	1
Urban	36	Modern WC, wash	49–60	Normal food	Tap water	Godenail	Male	+	-	+	-	+	-	3	0
Urban	37	Modern WC, wash	25–36	Normal food	Tap water	Godenail	Male	+	+	+	-	-	-	3	0
Urban	38	Modern WC, wash	49–60	Normal food	Tap water	Godenail	Male	+	-	+	-	+	-	3	0
Urban	39	Modern WC, wash	49–60	Normal food	Tap water	Godenail	Male	+	+	+	-	+	-	4	1
Urban	40	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Godenail	Female	+	-	+	-	+	-	3	0
Urban	41	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Godenail	Male	+	-	+	-	-	-	2	0
Urban	42	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Godenail	Male	+	+	-	-	+	-	3	0
Urban	43	Modern WC, wash	37–48	Normal food	Tap water	Godenail	Female	+	-	+	-	+	-	3	0
Urban	44	Modern WC, wash	49–60	Normal food	Tap water	Godenail	Male	+	+	+	-	+	-	4	1
Urban	45	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Godenail	Female	+	-	+	-	+	-	3	0
Urban	46	Modern WC, wash	25–36	Normal food	Tap water	Godenail	Male	+	-	+	+	+	-	4	1
Urban	47	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Godenail	Male	+	-	+	+	+	+	5	1
Urban	48	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Godenail	Female	-	-	+	+	+	+	4	1
Urban	49	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Godenail	Male	-	-	+	+	-	+	3	0
Urban	50	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Fatulla	Female	-	-	+	+	+	+	4	1
Urban	51	Modern WC, wash	49–60	Normal food	Tap water	Fatulla	Female	+	-	+	+	+	+	5	1
Urban	52	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Fatulla	Female	+	-	+	+	+	+	5	1
Urban	53	Modern WC, wash	49–60	Normal food	Tap water	Fatulla	Female	+	-	+	+	-	+	4	1
Urban	54	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Fatulla	Female	+	-	+	+	-	+	4	1
Urban	55	Modern WC, wash	13–24	Breast milk and soft food	Tap water	Fatulla	Male	+	-	+	+	-	+	4	1
Urban	56	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Fatulla	Male	+	+	+	-	-	+	4	1
Urban	57	Modern WC, wash	25–36	Normal food	Tap water	Fatulla	Female	+	-	+	+	-	+	4	1
Urban	58	Modern WC, wash	37–48	Normal food	Tap water	Fatulla	Female	+	-	+	+	-	+	4	1
Urban	59	Flush/pit latrine, wash	49–60	Normal food	Tube well	Matuail	Female	+	-	+	-	-	-	2	0
Urban	60	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Fatulla	Female	+	-	+	+	+	-	4	1
Urban	61	Flush/pit latrine, wash	37–48	Normal food	Tube well	Matuail	Female	+	-	+	-	+	-	3	0
Urban	62	Flush/pit latrine, wash	49–60	Normal food	Tube well	Matuail	Male	+	-	+	-	+	-	3	0
Urban	63	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Siddhirganj	Male	+	-	+	-	+	-	3	0
Urban	64	Modern WC, wash	25–36	Normal food	Tap water	Fatulla	Female	+	-	+	+	+	-	4	1
Urban	65	Modern WC, wash	25–36	Normal food	Tap water	Fatulla	Female	+	-	+	-	+	-	3	0
Urban	66	Modern WC, wash	0–12	Breast milk and soft food	Tap water	Siddhirganj	Female	+	+	+	-	+	+	5	1
Urban	67	Modern WC, wash	25–36	Normal food	Tap water	Fatulla	Female	+	+	+	-	+	-	4	1
Urban	68	Modern WC, wash	25–36	Normal food	Tap water	Fatulla	Male	+	-	+	+	+	-	4	1
Urban	69	Modern WC, wash	49–60	Normal food	Tap water	Fatulla	Male	+	+	+	+	+	-	5	1
Urban	70	Flush/pit latrine, wash	0–12	Breast milk and soft food	Tube well	Matuail	Male	+	+	+	-	+	-	4	1

+; gene-specific band positive, -; gene-specific band negative. Pit latrine w/o slab, w/o wash indicates children use a pit latrine without a slab and do not wash hands afterwards; flush/pit latrine, wash indicates children use a flush or a pit latrine and wash their hands after visiting latrine and before eating food; modern WC, wash indicates children use modern toilet, wash hands after defecation and before eating food. Under Category, children were categorized by the numbers of genes detected: 0, 1–3 genes detected; 1, 4–6.