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PhD Thesis

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Investigation of household transmission pathways for *Vibrio cholerae* and *Escherichia coli* in Bangladesh

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Abbreviations:

WP	Work Package
WHO	World Health Organization
HH	Household
WGS	Whole Genome Sequencing
mL	Millilitre
PBS	Phosphate Buffer Saline
Ct value	Threshold Cycle value
qPCR	Quantitative Polymerase Chain
DEPC	Diethyl Pyrocarbonate
cfu	Colony Forming Unit
gm	Gram
ORF	Open Reading Frame

Prologue

A multidisciplinary collaborative research project entitled "Combating Cholera Caused by Climate Change" (C5) was funded by a DANIDA FFU Grant in 2012 and conducted between 2013 and 2015 in a low-income area, Arichpur, on the outskirts of Dhaka City, Bangladesh. This project aimed to examine the relative risk of climate change-associated cholera and diarrhea influenced by environmental and household (HH) hygiene behavior in a water-stressed environment (http://cope.ku.dk/research/cholera/). The "C5" project comprised six different work packages (WP) including epidemiological, anthropological and microbiological aspects, as well as modeling of climatic factors influencing cholera incidence. The current study fell under Work Package 5 (WP5) which forms an integral part of the larger, comprehensive "C5" study. This work package analyzed the different contamination routes of fecal *Escherichia coli* and *Vibrio cholerae* within the HH environment (both in-house and outside surroundings) including possible transmission via vectors (fish, flies) and fomites.

Since John Snow's discovery of cholera as a waterborne infection, numerous studies have shown *V. cholerae* transmission via fecal contamination of environmental water sources [1-4]. There are other non-waterborne routes (e.g. direct transmission via food, HH surface contamination or vector associated transmission) which have shown significant importance in transmission of *V. cholerae* [5-8]. Traditional research on cholera transmission and intervention studies have focused on drinking water contamination [1, 9, 10]; therefore, other transmission routes remain understudied. This thesis was conducted to analyze the dynamic factors of different transmission routes of *V. cholerae* and *E. coli*. Some of the routes were already hypothesized but not previously evaluated, and few routes are the empirical findings from this thesis. The overall findings of the WP5 will be joined with the other WP findings to create a bigger impact on battling diarrhea and cholera in Bangladesh.

This PhD work includes the original manuscripts below, which were generated by addressing the research questions and hypothesis.

Manuscript titles (Appendix I)

1. **Hossain ZZ,** Ferdous J, Tulsiani SM, Jensen PKM, Begum A. Quantitative analysis of nucleic acid extraction methods for *Vibrio cholerae* using Real-time PCR and conventional PCR. **Mymensingh Medical Journal** 2018 Apr; 27(2):327-335.
2. Farhana I, **Hossain ZZ**, Tulsiani SM, Jensen PKM, Begum A. Survival of *Vibrio cholerae* O1 on fomites. **World Journal of Microbiology and Biotechnology** 2016 Sep; 32(9):146. doi: 10.1007/s11274-016-2100-x. Epub 2016 Jul 18.
3. **Hossain ZZ,** Farhana I, Tulsiani SM, Begum A, Jensen PKM. Transmission and toxigenic potential of *Vibrio cholerae* in Hilsa (*Tenualosa ilisha*) for human consumption in Bangladesh. **Frontiers in Microbiology** 2018; Jan 30; doi: 10.3389/fmicb.2018.00222.
4. Lindeberg YL, Egedal, K, **Hossain ZZ,** Phelps M, Tulsiani, SM, Farhana I, Begum A, Jensen PKM. Can *E. coli* fly? The role of flies as transmitters of *Escherichia coli* to food in an urban slum in Bangladesh. **Tropical Medicine & International Health** 2017; Nov 9; doi: 10.1111/tmi.13003.
5. **Hossain ZZ,** Sultana R, Tulsiani SM, Begum A, Jensen PKM. Transmission of Diarrheagenic *Escherichia coli* in diarrhea case households in urban Bangladesh. (Draft Manuscript)
6. **Hossain ZZ**, Leekitcharoenphon P, Dalsgaard A, Sultana R, Begum A, Jensen PKM, Hendriksen RS. Comparative Genomics of *Vibrio cholerae* O1 Isolated from Cholera Patients in Bangladesh. **Letters in Applied Microbiology 2018**; Oct ; doi: 10.1111/lam.13046
7. **Hossain ZZ**, Farhana I, Sultana R, Jensen PKM, Begum A. Investigation on Household Contamination of fecal *Escherichia coli* and *Vibrio cholerae* in Bangladesh. (Draft Manuscript)

The findings of the research work were also presented in several international conferences (**Appendix II**):

1. **Hossain ZZ**, Farhana I, Sultana R, Begum A, Jensen PKM. Fecal contamination hotspots in low-income households in Bangladesh. Presented at

'The 18th International Congress on Infectious Disease', March, 2018, Buenos Aires, Argentina

2. **Hossain ZZ**, Leekitcharoenphon P, Dalsgaard A, Sultana R, Begum A, Jensen PKM, Hendriksen RS. Comparative Genomics of *Vibrio cholerae* O1 Isolated from Cholera Patients in Bangladesh. Presented on 'International Conference on Genomics Nanotech & Bio engineering (ICGNB)', May 2017, Dhaka.
3. **Hossain ZZ**, Sultana R, Tulsiani SM, Begum A, Jensen PKM. Prevalence of Diarrheagenic *Escherichia coli* in Case Household Environment in Bangladesh. Presented at 'The 13rd The Asian Congress on Biotechnology 2017', July 2017, Thailand.
4. **Hossain ZZ**, Lindeberg YL, Edegal K, Phelps M, Farhana I, Tulsiani SM, Begum A, Jensen PKM. Can cholera fly? ASTMH 65th annual Meeting, November 2016, Atlanta, USA.
5. **Hossain ZZ**, Farhana I, Tulsiani SM, Sultana R, Jensen PKM, Begum A. Investigation on household contamination of *Vibrio cholerae* in Bangladesh. ASM Microbe, June 2016, Boston, USA.
6. **Hossain ZZ**, Farhana I, Tulsiani SM, Jensen PKM, Begum A. Molecular Analysis and Toxigenic Potential of *Vibrio cholerae* Isolated from Hilsa fish (*Tenualosa ilisha)*, Bangladesh, Presented at 'ASM Microbe, June 2016', Boston, USA.

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Zenat Zebin Hossain

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SUMMARY:

Despite the growing evidence of awareness and intervention programs regarding the transmission risk factors for diarrhea and cholera in Bangladesh, people continue to suffer these diseases frequently. The blending of human behavior with bacterial epidemiological and biological aspects promotes transmission of diarrheal disease in overcrowded low-income settings. Arguments over the incidence of environmental fecal contamination within HHs and its role in the transmission pattern of diarrheal pathogens still continue. This reinforces the need for research to better understand the mechanisms governing person-to-person fecal bacteria transmission in the domestic environment. This thesis intended to connect the dots between fecal contamination pathways and subsequent human-to-human transmission which may trigger local outbreaks of diarrhea leading to epidemics.

The interaction of hygiene with other environmental transmission pathways has not been evaluated as extensively as the role of water. To improve understanding of the complex pattern of transmission, the interdependence of pathways needs to be explored. In this thesis, the author investigated the most high risk areas (other than drinking water) within the HH domain for the prevalence of diarrheal pathogens like *V. cholerae* and *E. coli*, and their potential in the movement of the pathogen along the fecal-oral route. The prospective role of fish and fomites in the transmission of pathogenic *V. cholerae* from the environment to humans was assessed. In addition, the latest genetic shifts of *V. cholerae* strains in Bangladeshi HH case patients and their location in global transmission lineages were explored. The investigation was carried out using methodological validation of microbiological observation of a range of environmental pathways.

In the first manuscript, the author chose a desired nucleic acid extraction method by comparing three such methods based on processing time, cost and the results of further downstream investigation. The boiled template method was found to be the method of choice and was applied for DNA extraction in further investigations conducted under this dissertation. Results of the second manuscript demonstrated that among eight different types of fomites assessed as

persistent vehicles for *V. cholerae*, glass and aluminum surfaces showed the presence of culturable *V. cholerae* for only one hour after inoculation, but cloth and wood showed it for up to four hours. Persistent *V. cholerae* as a viable but non culturable (VBNC) form was quantitatively detected via EMA-qPCR even seven days after inoculation. The result of this study indicated that the fomites contaminated with clinical *V. cholerae* O1 retained their pathogenicity potential for a long period. This pivotal study features the potential of common HH surfaces as a health risk factor associated with cholera transmission.

Another segment of this PhD study (manuscript 3) shed light on the incidence of *V. cholerae* in Hilsa, an anadromous fish, and suggests that Hilsa can act as a transporter of *V. cholerae*, thereby contributing to the *V. cholerae* contamination pathway from the Bay of Bengal in Bangladesh. Eighty one percent of collected fish (freshly caught and market fish) showed the presence of *V. cholerae* and 20% of fish were cholera toxin gene-positive on PCR. Occurrences of *V. cholerae* coincide with the dual cholera outbreak peaks in Bangladesh. Genotypic and phenotypic characterization of the isolated *V. cholerae* strains from fish showed substantial virulence potential. The findings indicate that Hilsa fish could serve as a transmission pathway for *V. cholerae*, thus making kitchens more vulnerable to contamination due to unsanitary fish gutting and cleaning processes in a low-hygiene and limited water resource environment. In manuscript 4, houseflies were followed for fecal *E. coli* transmission to food under field settings where fly-exposed rice showed a significantly higher risk of contamination than covered rice. The findings confirm that fly control may effectively prevent transmission of fecal bacteria to food and subsequent diarrheal diseases.

In manuscript 5, the relationship between diarrhea and fecal *E. coli* contamination was assessed to explore the distribution in the in-house environment. Clinical and environmental samples (surfaces and food) from the same HHs showed the presence of similar pathotypes of diarrheagenic *E. coli*. Results indicate the significant role of human-to-environment or environment-to-human transmission of diarrhea pathogens. The results of manuscript 6 show the

current genomic profile of *V. cholerae* O1 from the diarrhea patients of case HHs. Whole Genome Sequencing (WGS) was utilized to determine the genomic and evolutionary aspects by comparing with diverse spatial and temporal clinical *V. cholerae* O1 strains available to date in the database. The current Bangladeshi strains showed clonal origin from a South Asian epidemic progenitor. The pathogenicity islands, antimicrobial mobile genetic elements (SXT), of our study strains are considerably diverse compared to recent clinical O1 strains from Bangladesh. A similar transmission source of cholera pathogens within the community was indicated by the strict clonality of the isolates. Moreover, results of fecal contamination surveillance for identification of hygiene-induced diarrhea risk areas in low-income HHs of Bangladesh are demonstrated in the seventh manuscript. The study findings indicate that the kitchen area is at high risk of exposure to fecal *E. coli* and *V. cholerae*.

In conclusion, this thesis shows that environmental hygiene and its underlying risk factors may greatly influence diarrhea and cholera transmission in vulnerable populations. Fomite contamination is an important attribute for diarrhea and cholera transmission. The importance of kitchen hygiene has been emphasized to improve sanitation as a means for preventing access of diarrheal pathogens to the HH. The pathways explored in this thesis need proper attention from public health researchers and policy makers to limit the spread of recurrent diarrhea. Distinct intervention strategies should be designed for the multiple transmission pathways.

DANISH SUMMARY

På trods af en markant indsats for at oplyse om kolera og diarrésygdommes smitteveje og –risici i Bangladesh, gennem interventioner og kampagner, optræder sygdommene fortsat jævnligt i befolkningen. I overbefolkede områder med lav gennemsnitsindkomst, hvor menneskelig adfærd blandes med bakterielle, epidemiologiske og biologiske aspekter, er der særlig stor risiko for smitte af diarrésygdomme. Det er fortsat uklart hvilken rolle fækale bakterier i husholdninger spiller for overførslen af diarrésygdomme. Af den grund er der brug for flere undersøgelser, for at skabe en bedre forståelse for de mekanismer, som styrer fækalbakteriers smitte fra person til person inden for husholdningen. Denne afhandling søger at finde sammenhænge mellem fækalbakteriers smitteveje og den efterfølgende smitte fra person til person, som muligvis spiller en markant rolle i lokale udbrud af diarrésygdomme og af epidemier.

Samspillet mellem hygiejne og husholds smitteveje er ikke blevet undersøgt i lige så høj grad som vandets rolle. For at skabe en bedre forståelse for det komplekse smittemønster må den indbyrdes afhængighed mellem smittevejene undersøges grundigere. I denne afhandling har forfatteren undersøgt de faktorer i husstanden (udover drikkevandet), som er mest udsat for at bære diarré-fremkaldende patogener, såsom *V. cholera*og *E. coli*, og deres potentielle rolle i at overføre patogenerne gennem den fækal-orale smitterute. Den mulige rolle som fisk og materiale overflader spiller i smitten af *V. cholerae* fra miljø til mennesker er ligeledes blevet undersøgt. Yderligere har afhandlingen gransket det seneste genetiske skift i *V. cholerae*-stammer, fra patienter fra husholdninger i Bangladesh, samt dets placering i den globaletransmissionslinje.

I manuskript 1 anvendte forfatteren en ekstraktionsmetode for nukleinsyre ved at sammenligne tre sådanne metoder baseret på processeringstid, bekostning og resultaterne fra yderligere nedenstrøm sundersøgelser. 'Boiled template'-metoden blev udvalgt til DNA-udtræk i de resterende undersøgelser. Resultaterne fra manuskript 2 demonstrerede at blandt otte forskellige overflader, som vurderedes til at kunne bære *V. cholerae*, viste glas- og aluminiumsflader

dyrkningsbar *V. cholerae* i kun en time efter inokulation, mens det for stof og træ var op til fire timer. I en genoplivelig, men ikke dyrkningsbar form kunne *V. cholerae* spores kvantitativt med EMA-qPCR i op til syv dage efter inokulation. Studiet indikerer at overfladerne som kontamineredes med klinisk *V. cholerae* O1 vedholdte deres patogene potentiale i lang tid. Studiet er med til at understrege at flader i almindelige husholdninger potentielt har kolera-relaterede sundhedsrisici.

En anden del af afhandlingen (manuskript 3) belyser forekomsten af *V. cholerae*i Hilsa-fisk, og foreslår at Hilsa-fisk, ved at transportere *V. cholerae*-bakterier, kan bidrage til *V. cholerae*-smitteruten fra den Bengalske Bugt i Bangladesh. 81% af de indsamlede fisk (dels friskfangede, dels fra markedet) indeholdt *V. cholerae*, mens 20% var PCR-positive for kolera-toxingener. Der konstateredes et sammenfald mellem indhold af *V. cholerae*på ydersiden af fisken og de sæsonmæssige koleraudbrud i Bangladesh. En genotypisk og fænotypisk karakteristisk af de isolerede *V. cholerae*-stammer fra fiskene viste et betydeligt virulenspotentiale. Studiet indikerer at Hilsa-fisk kan fungere som smittevej for *V. cholerae*, hvilket betyder at køkkenområder må regnes for udsatte for smitte, som følge af den ofte uhygiejniske rensning af fiskene i miljøer med begrænset adgang til vand.

I manuskript 4, blev husfluer undersøgt for deres potentiale i at overføre fækal *E. coli*-smitte til mad. Her viste flue-eksponeret ris en betydelig højere risiko for smitte end overdækket ris. Resultaterne bekræfter at beskyttelse mod fluer kan hjælpe med at forebygge overførslen af fækale bakterier til mad, samt de deraf afledte diarrésygdomme.

Manuskript 5 undersøgte forholdet mellem diarré og fækal *E. coli*-kontaminering i hjemmet. Undersøgelser af kliniske og miljømæssige prøver (overflader og mad) fra samme husstand påviste tilstedeværelsen af diarréfremkaldende *E. coli*-lignende patotyper. Resultaterne indikerer at person-til-miljø- eller miljø-til-person-smitte spiller en betydelig rolle for transmissionen af diarréfremkaldende patogener.

Resultaterne fra manuskript 6 viser den nuværende genomiske profil for *V. cholerae* O1, hentet fra diarré-patienter fra de undersøgte husstandene. Whole GenomeSequencing (WGS) blev anvendt til at bestemme de genomiske og evolutionære aspekter ved at sammenligne *V. cholerae* O1-stammer i en database. Den nuværende Bangladesh-stamme ser ud til at have en klonal oprindelse fra en syd asiatisk epidemi. De patogenesiskeøer, antimikrobielle mobile genetiske elementer (STX), i studiets stammer er væsentlig forskelligartede sammenlignet med nylige kliniske O1-stammer fra Bangladesh. Isolaternesklonalitet indikerer at der findes en lignende smittekilde for kolerapatogener i miljøet.

Endvidere indeholder manuskript 7 resultaterne fra overvågningen af fækal-oral-smitte for at identificere hygiejne-relaterede risikoområder for diarré i husholdninger med lav gennemsnitsindkomst i Bangladesh. Studiet indikerer at køkkenmiljøet er særdeles udsat ift. risiko for fækal *E. coli* og *V. cholerae*.

For at konkludere viser nærværende afhandling at husholds hygiejne og de underliggende risikofaktorer potentielt har en stor indflydelse på diarré- og kolerasmitte i en udsat befolkning. Kontaminering af overflader er en vigtig kilde til diarré- og kolerasmitte. Betydningen af køkkenhygiejne skal understreges, og mht. forebyggelsen af diarrésygdomme kan meget vindes ved at forbedre hygiejnen. Smittevejene som afhandlingen har undersøgt kræver opmærksomhed fra forskere i folkesundhedsvidenskab og politikere, som skal begrænse tilbagevendende tilfælde af diarré. Separate interventionsstrategier, målrettet mod de enkelte smitteruter, bør sættes i værk.

1.0 Introduction

1.1 Background on diarrhea and cholera

1.1.1 Burden

According to the Global Burden of Disease (GBD) study, diarrhea was a leading cause of death among all ages worldwide and responsible for 1.31 million deaths in 2015 [11]. The burden is higher in low and middle-income countries where access to safe drinking water and basic sanitation facilities is limited. Bangladesh, which is a densely populated developing country with the fastest urbanization rate in Asia [12], experienced approximately 20,000 deaths caused by diarrheal diseases, with a mortality rate of 12.4 per 100,000 people in 2015 [11]. Cholera, an acute diarrheal disease, is endemic in Bangladesh, occurring with seasonal regularity at a rate of more than 100,000 cases annually [13].

1.1.2 Definition of diarrhea and cholera

Diarrhea, including cholera, most often results from the ingestion of fecal pathogens via contaminated water or food, or from lack of hygiene. According to World Health Organization (WHO) guidelines, individuals are characterized as having diarrhea when they experience more than three liquid stools per day [14, 15]. Cholera is transmitted by water carrying high concentrations of *V. cholerae*, a Gram-negative bacterium which has both human and environmental stages in its life cycle. The severe form of the disease, cholera gravis, is characterized by passage of voluminous rice water stools, vomiting and dehydration [16]. Severe dehydration leading to hypovolemic shock can cause death in adults as well as children.

1.1.3 Etiology of diarrhea

Etiologies of diarrhea include a wide range of fecal pathogens (bacteria, viruses, and parasites). A prospective case-control study, the Global Enteric Multicenter Study (GEMS), reported that the major attributable etiologies of diarrhea cases in infants and young children

seeking health care were rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli*, *Shigella*, *Aeromonas*, *Vibrio cholerae*, *Campylobacter jejuni*,etc. [17].

1.1.4 Water, sanitation, hygiene and diarrheal diseases

The importance of hygiene and sanitation in connection with excreta-related enteric and waterborne diseases was first highlighted in 1854 after John Snow´s discovery that fecally contaminated water from the broad street pump was the main culprit of the spread of the cholera epidemic in Soho, London [18]. Since then, numerous hygiene and sanitation interventions have been identified to prevent the distribution of stoppable enteric diseases like cholera and diarrhea. Recent systematic review studies found that a 30-40% reduction in diarrheal illness occurred as a result of sanitation improvements such as an improved drinking water supply, sewer connection, etc. [19].

The major transmission routes facilitating fecal pathogen infection of the mouth of a new host were put into a diagram, popularly known as the "F-diagram", which includes fluid (water), food, field (soil or crop), fingers and flies [20]. Multiple effective barriers may break the transmission cycles of pathogens for complete HH sanitation.

1.2 Fecal contamination indicator

All known fecal pathogens cannot be surveyed at one time to test the contamination level in environmental samples. Each pathogen identification needs specific methods of choice and thus respective laboratory facilities. World Health Organization guidelines recommend thermotolerant ("fecal") *E. coli* as the index organism to assess potential fecal contamination from both human and animal origins in drinking water, food and environmental samples [21-24]. These gram negative bacilli were first proposed in 1892 as an indicator of fecal contamination because of their abundance as normal flora in human and animal feces [25]. Although the presence of *E. coli* does not definitively confirm the presence of pathogens in water or food, it does indicate a high chance of recent contact with fecal-borne bacteria and

viruses. *E. coli* has also been used as standard indicator bacterium to assess environmental fecal contamination of HH floors and surfaces in low-income settings [26, 27]. The relationship between diarrhea and indicators of exposure in drinking water and on surfaces has been quantified in previous studies [28, 29].

1.2.1 *E .coli* as a deadly diarrheal pathogen

E. coli is one of the most studied and diverse bacterial species, including highly pathogenic to harmless avirulent, and human to environmental strains. However, enteric *E. coli* is currently one of the leading causes of childhood diarrhea and a public health crisis in developing countries [17]. Based on pathogenesis features and associated virulence factors, enteric *E. coli* has been divided into six categories: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC) [30]. Among these, ETEC was found to be the most pathogenic type, responsible for 1 billion cases of diarrhea annually [31]. The disease outbreak pattern of ETEC in Bangladesh shows a similar biannual periodicity as *V. cholerae* and there are often concomitant outbreaks of both organisms during peak seasons [32]. A new pathotype named cytolethal distending toxins-producing *E. coli* (CTEC) is causing severe diarrhea in young children [33].

The transmission of pathogenic *E. coli* has been linked to changing climatic factors, lack of sanitation facilities, poor hygiene practices, contaminated water and food, malnutrition, etc. Scientists still know little about the transmission of the bacterium in developing world HHs [34]. A study of ETEC infection in Bangladeshi HH contacts showed a high transmission correlation with socioeconomic status [32]. Person-to-person transmission of EHEC was common within the same HHs, with mothers and siblings being affected the most [35]. Infection with EPEC can spread to other hosts by fecal contamination of surfaces, weaning fluids, etc. [36]. High rates of *E. coli* transmission were also observed between humans and livestock [34].

1.3 Cholera is one of the oldest worldwide scourges

Cholera has swept through the continents of the world and claimed millions of lives since the first recorded cholera pandemic in 1817. History traced back to ~500 to ~400 B.C. describes a disease resembling cholera in India, which indicates that this disease occurred before it started being recorded as a pandemic [37]. All the pandemics, except for the seventh, originated on the Indian subcontinent, mostly in the Ganges Delta in Bengal [16]. The first six cholera pandemics occurred between 1817 and 1923. The current (seventh) pandemic, which originated on an Indonesian island in 1961, has been the most explosive pandemic and continues to pose serious health problems on all six continents [37, 38]. Cholera is caused by upper intestinal infection of human host. The virulence factors of the bacteria responsible for this diarrheal illness have been mostly described; however, the factors that could explain the worldwide transmission of the disease are still largely unknown [39, 40].

1.3.1 The cholera bug: *Vibrio cholerae*

V. cholerae is well recognized as normal living flora (autochthonous) in estuarine aquatic systems [41]. This Gram-negative comma-shaped bacterial species has both human and environmental stages in its life cycle. Currently, 206 distinct serogroups have been identified based on their O antigen, but only the O1 serogroup was recognized as a cause of epidemic and pandemic cholera until 1992 [42, 43]. During 1992-1993, a large cholera epidemic associated with the emergence of the first non-O1 serogroup of *V. cholerae,* designated as "O139 Bengal", erupted in India and Southern Bangladesh [44]. Since then, extensive outbreaks have occurred worldwide, mostly caused by these two serogroups (O1 and O139). Other serogroups negative for O1/O139, collectively called "non O1/O139 *V. cholerae*", are responsible for acute cholera-like diarrhea and sporadic diarrhea. *V. cholerae* O1 is further differentiated into two biotypes based on their range of phenotypic and distinct genotypic traits, namely, the classical (CL) and El Tor (ET) biotypes [16, 45]. The CL biotype was responsible for the first six cholera pandemics, and presumably the earlier unrecorded pandemics, and disappeared after the ongoing seventh cholera pandemic caused by the ET biotype started in 1961 [16]. The *V. cholerae* O1

serogroup can be further divided into three serotypes based on agglutination in antisera to type-specific O antigens: Inaba, Ogawa, and Hikojima (very rare).

Generally, acute cholera patients excrete 10^7 to 10^8 colony forming units (cfu) of *V. cholerae* per gm of stool, and even after symptoms end, excretion may continue for one to two weeks without any antibiotic treatment [16, 46]. In severe watery diarrhea cases, adults may excrete up to one liter of stool per hour [47]. Study reports showed that human volunteers required doses of $10^5 - 10^8$ cfu of *V. cholerae* O1 [47] and a relatively short incubation period of two hours to five days to cause diarrhea. However, when *V. cholerae* O1 was ingested with food such as rice and fish, the infectious dose decreased to 10^6 cfu [46] and as low as 10^3 in the absence of stomach hydrochloric acid [47]. One state of infectivity of *V. cholerae* has been described as a "hyperinfectious state" where strains from one infected person are one to two orders of magnitude more infectious when ingested by other individuals in close proximity [48, 49]. Subsequently, low infectious doses of hyperinfectious *V. cholerae* from the environment could infect new hosts [50]. Rehydration therapy using oral or intravenous administration of cholera saline is the first line of treatment during severe dehydration. Azithromycin and ciprofloxacin are commonly prescribed antibiotics for treatment after the reduction of initial fluid loss [51].

1.3.2 Virulence factors

Epidemic cholera is largely induced by the expression of cholera toxin (CT), encoded by the *ctxAB* genes, and the colonization factor involved in intestinal colonization known as toxin coregulated pilus (TCP), which are co-regulated by toxin regulatory protein (ToxR) [52]. The CT encoding genes are located on a 6.9-kb single-stranded DNA filamentous phage (CTX phage) in the toxigenic strains of the *V. cholerae* genome, also known as the "cholera virulence cassette" [53, 54].

The virulence cassette of *V. cholerae* O1 also comprises virulence genes other than CT genes such as the zonula occludens toxin (Zot), which affects the intracellular tight junctions of the small intestine [55], accessory cholera enterotoxin (Ace), that causes fluid secretion in rabbit ileal loops [56] and the core-encoded pilus (Cep), which is an intestinal colonization factor [57]. Other putative virulence factors identified in El Tor strains are hemolysin, with intestinal pore forming ability encoded by the *hlyA* gene; and hemagglutinin protease (HA/protease), which has a role in the environmental transmission of *V. cholerae*. Although these major virulence factors have been exclusively detected in clinical O1 and O139 serogroups, previous reports showed the presence of cholera toxin genes in clinical and environmental non-O1/O139 serogroups [58-60]. Studies have indicated that some of the potential virulence factors such as repeats-in-toxin (RTX), mannose-sensitive hemagglutinin (MSHA), heat-stable enterotoxin (NAG-ST) and the novel type III secretion system (T3SS) and type VI secretion system (T6SS) play a crucial role in the diarrheagenic mechanisms of non-O1/non-O139 serogroups [61-66].

1.3.3 Epidemiology of cholera

Since the beginning of the seventh pandemic in 1961, cholera has remained a major global health threat, particularly in South Asia, sub-Saharan Africa, and Latin America, initially affecting more than 7 million individuals in over 50 countries [67]. The seventh cholera pandemic spread over most of Asia, reached Africa in 1971 and eventually reached South America in 1991,devastating the entire continent, with approximately 10,000 deaths [67]. Coastal regions appeared as the most vulnerable areas for the majority of cholera outbreaks [68, 69]. According to WHO, African countries reported 3,221,050 suspected cholera cases from 1970 to 2011 [70]. Most of these outbreaks were caused by the *V. cholerae* O1 serogroup, until the new epidemic serogroup O139 Bengal emerged in the Bengal Delta region in 1992 [71]. Cholera continues to cause significant damages in public health, with over 2.9 million cases and 95,000 deaths in 69 endemic countries annually [72]. In recent years, outbreaks have increased in populations affected by war, earthquakes, conflicts and

famines. Most recently, an explosive outbreak has occurred in war-torn Yemen where over 600,000 suspected cases have been reported since April 2017 [72].

1.3.4 Cholera: Bangladesh scenario

The coastal regions of Southeast Asia, also called the Bengal Delta region, include Bangladesh and West Bengal in India and have been considered as the native homeland of cholera since the early nineteenth century [73]. Bangladesh is small, low-lying and one of the most densely populated countries in the world. Cholera occurs in Bangladesh both as an endemic disease with annual seasonal outbreaks in pre- and post-monsoon periods, and as epidemics that normally emerge after natural disasters (e.g. flooding events, cyclones etc.) [74]. Despite groundbreaking cholera interventions and diagnostic advancement, Bangladesh still reports more than 100,000 cholera cases annually [13]; however, the true cholera burden is much higher due to underreporting. An estimated 300,000 severe cholera cases each year require hospitalization, according to the diarrheal hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) [67].

1.3.5 *V. cholerae* non-O1/O139:

Until the emergence of *V. cholerae* O139, the clinical significance of *V. cholerae* non-O1 serogroups was not exclusively investigated. Although, these serogroups are ubiquitously found in global riverine and marine environments and are mostly nonpathogenic, a few atypical environmental strains possess cholera toxin genes [61, 75]. Non-O1 and non-O139 strains which contain cholera toxin may cause severe disease [76]. Some non-O1/O139 *V. cholerae* strains produce toxins other than CT and are responsible for acute cholera-like diarrhea, gastroenteritis and a variety of extra-intestinal infections [77-80]. A few pathogenic non-O1 and non-O139 strains, such as O141, O10 and O12, have been associated with outbreaks of gastroenteritis and watery diarrhea [64, 81]. A study during 2002-2010 among hospitalized patients in Kolkata, India showed prevalence rates of 2.2% for *V. cholerae* non-O1/O139 strains in the total isolated strains [82]. Recently, non-O1/O139 strains also

contributed to a high number of cases in the catastrophic Haiti cholera epidemic in 2010 [83]. Hence, the public health risk posed by the non-O1 and non-O139 serogroups of *V. cholerae* cannot be ignored.

1.3.6 Genotypical evolution

Pandemic clones of *V. cholerae* strains have experienced a number of genotypic and phenotypic shifts in the last decade [84]. Repeatedly, existing pandemic clones have replaced previous strains and new strains have arisen via high rates of recombination and lateral gene transfer (LGT) [85]. The current seventh pandemic was caused by serogroup O1 El Tor biotypes of *V. cholerae* which first emerged in Indonesia in 1961and fully replaced the earlier classical strains [86]. This biotype subsequently spread throughout Asia and Africa, where it still causes recurrent epidemics [87, 88]. As Bangladesh is situated at the border of the Bay of Bengal, the hub of the seventh cholera pandemic, the *V. cholerae* strains in this region have become extremely vulnerable to natural selection and genetic influence to evolve as new epidemic strains. After the first representation of genetic changes in the *V. cholerae* O139 genome, new variants of El Tor biotype strains with the classical biotype traits (hybrid strains or Matlab variants) and El Tor strains producing classical toxin,*ctxB*1 (altered strains),emerged in Bangladesh in the late 1990s with more severity [89]. The current outbreaks in most Asian and African countries are mostly attributable to these new variants of *V. cholerae* [45]. The recent increase in disease severity with the genetic change of *V. cholerae* requires current data on genomic features of the ongoing cholera outbreak strains in Bangladesh.

DNA-based genomic fingerprinting methods have been used for many years for the epidemiological study of *V. cholerae*. After the genomic era breakthrough, Whole Genome Sequencing (WGS) technology has become a paramount tool for analyzing the evolution and clonal expansion of global cholera pandemics, due to its holistic robustness and greater resolution compared to conventional fingerprinting methods [90, 91]. Based on single nucleotide polymorphism (SNP) differences, global cholera pandemic strains have been

divided into major phylogenetic lineages and new strains can be compared with global and local epidemic strains [92]. Comparative genomics using a WGS platform paved the way to identify the variations in genomes, particularly the CTX genotypes, virulence factors, mobile genetic elements, etc., of distinct *V. cholerae* strains [93-95].

1.4 Transmission dynamics of *V. cholerae*: Primary and secondary /public and domestic

1.4.1 Primary/Public transmission

Cholera vibrios show two major forms of transmission: primary and secondary. Here, the author intends to designate the transmission routes as "public" and "domestic". Public transmission can occur through the riverine, estuarine and coastal aquatic reservoirs or contaminated water sources, without previous human infection or fecal contamination, and thus is accountable for beginning the initial outbreak. Public transmission is the first transmission to the index case and it may come from any environmental source such as water, fish, shellfish, flies or human carriers from the endemic area. Several physical, biological and climatic parameters are considered to be influential factors for the environmental survival of *V. cholerae* as well as the initiation of cholera epidemics through public transmission of *V. cholerae* [73].

In the aquatic environment, *V. cholerae* thrives in warm temperatures, pH above 4.5 and moderate salinity [96, 97]. In estuarine water, recovery of viable cells was possible for up to 35 days [98]. The environmental persistence of *V. cholerae* in different environmental niches is largely facilitated by its ability to convert into a viable but non-culturable (VBNC) state during unfavorable conditions for growth such as nutrient deprivation, high salinity and temperature, which is also a resistance mechanism to stop predation by heterotrophic protists and bacteriophages [99]. This *V. cholerae* survival strategy was described as the state in which cells are still viable but not culturable in conventional culture media [100]. The VBNC *V. cholerae* can retain its pathogenicity by reverting to a culturable state, which was

demonstrated by challenging human volunteers [101] or passaging through rabbit ileal loops [102, 103].

The pathogen can be transmitted in the environment by a wide range of environmental hosts including suspended zooplankton, aquatic plants, shellfish, and fish [104]. Another *V. cholerae* transmission mechanism that has been suggested is that of migratory water birds dispersing *V. cholerae* as an outcome of their direct consumption of fish and zooplankton [105]. *V. cholerae* attach to various biotic and abiotic surfacesas biofilms, and when attached to living, mobile hosts, biofilm formation could act as a means of persistence during interepidemic seasons, as well as a means of dispersal [106].

1.4.1.1 Seasonality of cholera in Bangladesh

Cholera has an area of endemicity in the Bay of Bengal (in Bangladesh and India) and along coastal areas in South and Central America [107]. Recently, WGS has been used to trace the bacterium behind the latest (seventh) cholera pandemic back to a source in the Bay of Bengal from which it can thrive and spread [108]. Cholera follows a regular seasonal pattern in Bangladesh. Rural coastal villages maintain an annual single peak of cholera cases (March-May), whereas cholera outbreaks show a unique bimodal distribution in the capital city of Dhaka, where the first peak occurs just after the monsoon (September-November), with a smaller second peak in the spring (March-May) [109].

Studies primarily conducted in the Bay of Bengal have revealed the contribution of climatic factors (salinity, temperature) to the cholera seasonality in Bangladesh [110, 111]. Colwell et al. [112] have suggested that in Bangladesh, the colonization in plankton (copepods, phytoplankton) by *V. cholerae* provides a reservoir that assists in *V. cholerae* proliferation in the aquatic environment. *V. cholerae* exist as commensals to zooplankton (notably crustacean copepods) both in their gut and in biofilms on their chitinous surfaces, where they are present throughout the year in Bangladesh [112]. Further, zooplankton blooms are known to occur after the monsoon rains and flooding, when cholera season begins, and this was previously

used to explain the seasonality of outbreaks [113]. Another cholera season early in the year (from January through April) had been thought to occur due to the lower discharge volumes and associated saltwater and plankton intrusion from the Bay of Bengal to the deeper inland rivers providing an optimal environment for *V. cholerae* growth and eventually causing the outbreaks during the dry season [114]. The post-monsoon cholera season is driven by substantial inundation along the major riverbanks of Bangladesh during flooding, which causes an open mixture of sediments and creates ideal conditions for the growth and proliferation of *V. cholerae* to contaminate water sources. However, mathematical modeling by King et al. using cholera data from Bangladesh [115] indicated the greater importance of human-associated non-environmental sources for seasonality than human-independent environmental sources.

1.4.1.2 Hilsa fish as a missing link for cholera transmission in Bangladesh

It is likely that environment is the source of epidemic strains, but the mechanism that enables the spread of *V. cholerae* across water bodies is still not clearly understood [105]. In Bangladesh, environmental free-living waterborne pathogens have been linked to the beginning of cholera epidemics in which the pathogen was distributed from the Bay of Bengal [112]. However, a recent study showed that saline-rich river water conducive to *V. cholerae* growth is commonly avoided as a potential source of drinking water because of its high salinity and the fact that it is less aesthetically pleasing [116]. These findings indicate that contaminated river water may not be the sole reason for cholera infection in Bangladesh. In addition, King et al. [115] have shown by mathematical modeling that the environmental connection is critical to endemicity, and this has led to the consideration of new ideas. Hence, the environmental link in the transmission of *V. cholerae* infections from the coastal region to the central part of Bangladesh is still inexplicable. One hypothesis could be the transmission of *V. cholerae* via migration of the national fish of Bangladesh, the Hilsa fish (*Tenualosa ilisha*), through the rivers from the Bay of Bengal and detours from this route to the kitchens of Dhaka City. The probable role of the Hilsa fish in maintaining cholera endemicity in India

was previously postulated more than 60 years ago, although extensive investigation to validate this hypothesis has not been performed yet [117].

Hilsa, the national fish of Bangladesh, is an iconic component of Bengali cuisine. This anadromous fish follows the route (Figure 1) to inland breeding grounds in Bangladesh from the Bay of Bengal beginning in July and continuing until the October monsoon rains flood the rivers, and again from January to March [118]. Hilsa fish can migrate from 50-100 to up to 1,200 kilometers upstream for spawning [119]. About 60-70% of the Hilsa catch per year is caught during the peak migratory periods [119]. Fish are caught in both the coastal marine regions of the Bay of Bengal as well as the deltaic rivers, and sold in local fish landing centers and in the local markets, from which they are transported to major cities of the country.

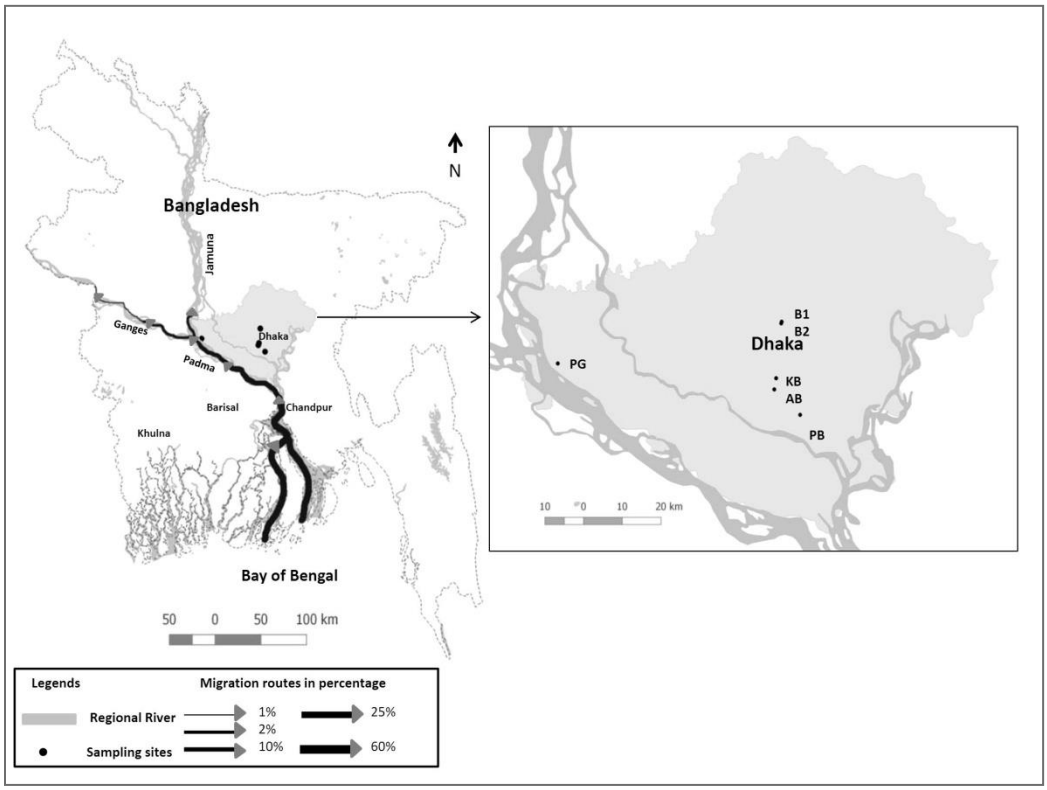

Figure 1: **Migration routes of Hilsa from the Bay of Bengal to Dhaka**, adopted from Ahsan et al. [118]. Sample collection sites for this study are indicated: Boubazar 1(B1), Boubazar 2 (B2), Kawran Bazar (KB), Ananada Bazar (AB), PaglaBazar (PB), and Paturia Ghat (PG).

1.4.2 Secondary/Domestic transmission

When the aquatic reservoirs of *V. cholerae* are triggered by seasonal environmental factors, they begin to increase the burden of public transmission cases and result in rapid spreading of community level or secondary/domestic transmission. Domestic cholera transmission occurs directly from person to person through the fecal-oral route, for example: exposure to fecally-contaminated water, food, surfaces or other fecal-oral routes shared by a cluster of individuals such as HHs [6, 120]. Domestic transmission starts with the infection of a susceptible person caused by direct contact with primary transmission pathways (previous fecal contamination or an infected person). In economically developed countries with proper hygiene and sanitation infrastructure, secondary transmission is rare and the epidemic may occur via primary transmission [120]. In contrast, secondary routes of cholera transmission cause the major outbreaks in poor and developing densely-populated countries [120]. The importance of public transmission is indisputable from the perspective of pandemic disease spread and persistence in the environmental reservoir [121]; however, the direct secondary transmission can play a role in controlling spatial cluster level cholera risk factors [6, 122-125].

Multiple transmission routes may involve in domestic transmission of *V. cholerae* and are influenced by the demographics, socioeconomic conditions, sanitation and hygiene practices of a locality [126]. Most often, water is considered to be the primary source of contamination, following Dr. Snow´s legacy [110, 127]. However, understanding HH environmental transmission is also mandatory for preventing cholera and cholera-like diarrhea because little is known about the exact point of contamination. According to previous investigation, person-to-person transmission within families has often failed to identify a single mode of transmission such as water or food, and points towards other environmental routes [128]. Below is a list of transmission pathways, excluding water, to explain the complex pattern of person-to-person transmission.

1.4.2.1 Domestic Environment

The domestic environment comprises both the area where people live and the surrounding environment. Epidemic spread of *V. cholerae* is associated with movement from the environmental reservoir to the human-to-human pathway and back to the environment [48]. Multiple *V. cholerae* infections commonly occur in the same household. In endemic areas, large numbers of persons, particularly household contacts, remain asymptomatic [16]. A study showed that half of the household contacts of hospitalized cholera patients who shed for ⩾7 days were asymptomatic and few of them showed prolonged shedding [129]. These asymptomatic carriers were infected by either exposure to the primary source of contamination or via secondary transmission pathways in the household domain due to poor hygiene [130]. A study in Dhaka, Bangladesh identified that the risk for cholera infection is >100 times higher for the index patient´s household members during the first week after the patient seeks hospital care [130]. Multiple risk factors for *V. cholerae* infection in the HH have been identified, such as having long-distance water sources, drinking street-vended water, eating dried fish, consuming food prepared by a recently ill food handler, placing dirty hands into stored HH water, improperly treating drinking water, and not washing hands with soap prior to eating food [130-133]. Studies also showed that HH animals, including cows, dogs, and chickens, can be *V. cholerae* O1 carriers [134]. Although transmission of *V. cholerae* through HH water sources and drinking water, and the benefit of water treatment interventions for the prevention of transmission, have been demonstrated in several studies [49, 135, 136], no thorough research on other in-house environmental components which might influence transmission has been conducted yet. Possible environmental fecal contamination exposure routes have been demonstrated in light of the F-diagram in Figure 2. Environmental components such as fields, flies, and raw foods, as well as human carriers, introduce fecal pathogens to the in-house environment. The kitchen and the food consumption area play a primary role in transmitting pathogens to healthy members' mouths.

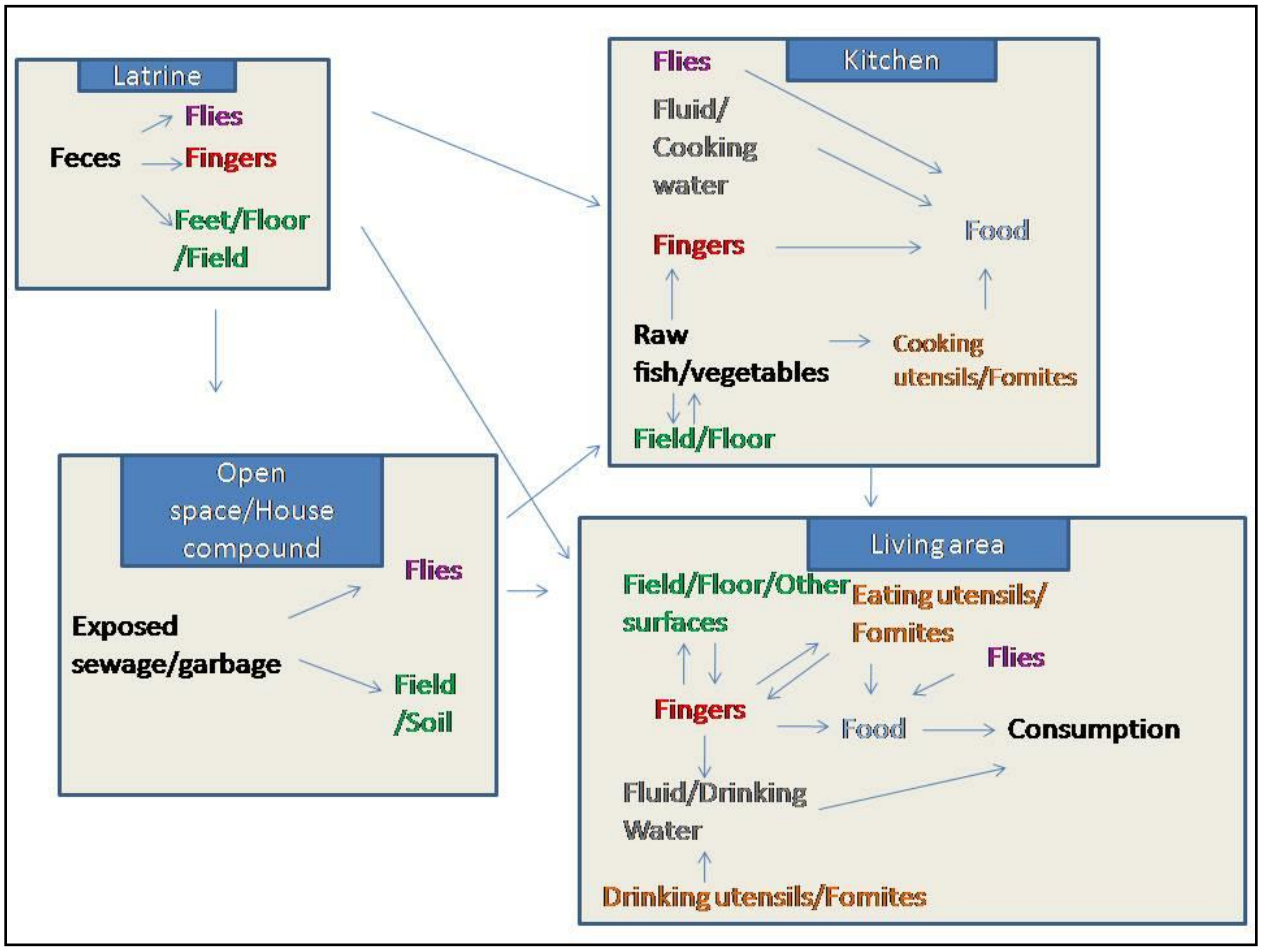

Figure 2: Environmental transmission routes in different household components.

1.4.2.2 Food

Conventionally, water was recognized as the primary vehicle of cholera transmission. Outbreaks associated with the consumption of contaminated food indicated the importance of food as a fecal-oral transmission route [7, 137]. In developing countries, poor hygiene conditions during food preparation, storage at ambient temperature, and eating without further heating may significantly contribute to foodborne transmission of enteric pathogens [128]. The larger impact of food as a transmission vehicle results from the ability of *V. cholerae* to protect itself against intestinal gastric acid. Moreover, food such as cooked rice may act as an ideal culture medium when lukewarm, as it favors the survival and rapid growth of *V. cholerae* O1 [138]. Food with a neutral pH has been reported to serve as a good carrier of

pathogenic *V. cholerae* strains [139]. Tang et al [138] showed a better survival of *V. cholerae* O1 in coffee mixed with condensed milk (pH 6.01) than in plain coffee (pH 4.68). Transmission of an epidemic strain from its location of origin could be possible via food, as toxigenic *V. cholerae* O1 was found to resist and survive refrigeration and freezing temperatures during international shipping [140]. Raw or undercooked seafood was the most common source of foodborne cholera outbreaks, particularly in developed countries (possibly contaminated from its environmental reservoir) [7, 137]. Other foods, such as cooked cereals and vegetables, have also been incriminated in cholera outbreaks [141].

Food is commonly contaminated by food handlers´ hands [142, 143] or the water used in food preparation [16]. Food may also be contaminated by dirty kitchen and eating utensils [144]. Consumption of rice meal left for a long period after cooking was associated with cholera outbreaks in the HHs of Guinea, West Africa [143].

1.4.2.3 Fomites

Fomites or inanimate objects are the important constituents of the common HH items such as toys, doorknobs, clothing and utensils which are shared and frequently touched by a cluster of persons. It has been shown that a minimum of 10^6 bacterial cells can transfer from artificially inoculated fomites to the hand during activities such as turning on/off a kitchen faucet, holding a phone receiver, etc. [145].

Fomites contaminated with *V. cholerae* could serve as an intermediate transmission vehicle to cause infection [128]. It has been speculated that kitchen and eating utensils could be contaminated with *V. cholerae* O1 by cleaning with poor quality water [128]. Fecally-contaminated moist bed-linen and clothing could carry a high load of *V. cholerae* for a longer period [146]. *V. cholerae* may undergo morphological changes (VBNC state) on dry surfaces attributable to nutrient deprivation and less moisture content. Earlier Barua et al [147] using conventional methods, showed that *V. cholerae* could not survive more than one to two days on metal fomites; however, the negative result in laboratory culture media does not guarantee

the absence of *V. cholerae*. Though the contaminated HH surfaces within a HH cluster can facilitate propagation of the bacteria and thus lead to epidemics [6], the question of how long the bacteria persist and retain viability on different HH surfaces remains unanswered.

1.4.2.4 Fish

The role of fish as a *V. cholerae* reservoir is already recognized [148]. Fish can be contaminated with *V. cholerae* from their aquatic reservoir, other environmental sources contaminated by sewage or through poor hygiene in the local market. The bacteria get into the domestic environment when the infected fish are bought for consumption.

Fish have been previously implicated in cholera outbreaks, with the consumption of salt fish, sardines, dried fish and shellfish in different parts of the world [148, 149]. *V. cholerae* was isolated from a species of fish called ‘‘lorna’’ (*Sciaena deliciosa*) that were caught in inshore waters in Peru during a Peruvian epidemic [150]. Normally, people do not consume raw fish in the Southeast Asia region, so fish-borne infections are rare in this area. But fishermen and fish handlers in markets and HHs may possibly be infected with *V. cholerae* due to improper handling practices and poor sanitation. The kitchen environment becomes vulnerable to contamination due to unsanitary fish gutting and cleaning processes [151]. For instance, a cholera case was identified in Germany due to hand contamination during the gutting process and subsequent ingestion of bacteria [152].

1.4.2.5 Flies

The established role of flies as carriers of enteric pathogens and mediators of foodborne transmission of diarrheal diseases has been suggested by the results of numerous laboratory and field studies [153-157]. High fly densities were found to be significantly associated with diarrheal episodes near the area close to a garbage dump [158]. Common houseflies (*Mucosa domestica*) have been previously involved in the dissemination of enteropathogens such as *E.*

coli, *Shigella* spp., *Salmonella* spp., *Helicobacter* spp., *V. cholerae,* rotavirus, *Campylobacter* spp. , *Giardia lamblia* and *Cryptosporidium* spp. [158]. Their random feeding habits on excreta, waste matter and human consumables combined with their morphological structure (presence of hair and sticky pads on the exoskeleton) make this species an ideal mechanical transmitter of pathogens when settling on human food [159, 160].

V. cholerae has been found to be closely associated with many species of arthropods and insects in natural environmental settings [161]. *V. cholerae* has been isolated from the exterior and interior parts of fly bodies [160, 162]. One study indicated that toxigenic *V. cholerae* was acquired by houseflies from exposed human waste from a cholera outbreak area in India, and mechanically disseminated by these flies, thus increasing the possible spread of the outbreak [160]. Even though this is plausible, the biggest gap in knowledge is the lack of evidence of actual fly transmission of *V. cholerae* to food in a real time natural environment.

1.5 Research perspective with current transmission hypotheses

Cholera is one of the oldest diseases in the history of humankind and still continues to threaten millions of people in underdeveloped countries. Being situated near the endemic foci, Bangladesh remains the center of the epidemic disaster. Researchers have been looking for aquatic and HH transmission vehicles of *V. cholerae* for a long time, but the links remain evasive. Literature on person-to-person transmission research in Bangladesh provides indications that infection is caused through HH contacts or contaminated drinking water and food Limited transmission research has been conducted in the actual HH environment and on the real time contamination of HH objects which could trigger a massive outbreak in the overcrowded, hygiene-compromised locality of Bangladesh. Bacterial circulation also needs to be followed for a long period of time to explain the seasonality of prevalence in the HH environment. Moreover, comparative genomics is frequently considered to be the gold standard for perceiving the current local case isolates of *V. cholerae* in a global transmission framework and evaluating in terms of evolution or persistence.

The precise source of fecal bacteria contamination within the HH fostering person-to-person transmission is difficult to identify, as multiple factors may contribute. Knowledge of the burden of fecal contamination, particularly fecal *E. coli* contamination, is vital for demonstrating pathogen transfer from feces to human mouths via these HH components. Little is known either about the HH transmission of fecal *E. coli* in a low-income settlement. In order to develop methods to reduce diarrheal incidence in Bangladesh, and subsequently in all areas of the developing world where diarrhea exists as a public health threat, the issues involved in hygiene-induced risk points must be pinned down.

The factors which influence the transmission routes of diarrheal disease are complex and comprise multiple environmental reservoirs [163]. The importance of transmission routes is focused on drinking water in the existing research hypothesis. Investigation on a combination of barriers to the environmental transmission routes will improve the scope to effectively reduce the diarrheal burden.

2.0 Objectives

This research study was performed with one main objective and six specific objectives to accomplish the overall goal of the project.

2.1 General objective

Investigation of direct fecal contamination routes in the domestic environment with an emphasis on *V. cholerae* and *E. coli*, and evaluation of the genetic features of these pathogens in relation to the epidemiology of diarrhea and cholera.

2.2 Specific objectives

1. Understanding of the survival pattern of *V. cholerae* on environmental surfaces (manuscript 2).
2. Comprehensive analyses of the role of fish as a possible transmission vehicle of *V. cholerae* (manuscript 3).
3. Evaluation of the potential of flies as vectors carrying *E. coli and V. cholerae* to food (manuscript 4 and abstract 4).
4. Analyses of the incidence of diarrheagenic *E. coli* in case HH environments in Bangladesh and their virulence profile (manuscript 5).
5. Investigation of the current genomic profile of *V. cholerae* O1 strains by Whole Genome Sequencing (WGS) (manuscript 6).
6. Assessment of the transmission routes and magnitude of fecal *E. coli* and *V. cholerae* contamination within the HH domain (manuscript 7).

In this thesis, a diverse array of environmental samples was investigated to assess fecal exposure pathways, thus aiming at multiple specific objectives. The first five objectives were designed to

fill the knowledge gaps in order to accomplish the final objective (objective 6) which was to explore HH transmission pathways of fecal pathogens in a prospective study.

As the author aimed to analyze HH surface objects (fomites) for the prevalence of enteric pathogens such as *V. cholerae* in objective 6, objective 1 was developed and met by analyzing bacterial persistence and variability on an array of fomites in the laboratory. An innovative laboratory method was applied along with the traditional culture method for identification of viable but non culturable *V. cholerae* on these fomites.

In objective 2, fish were targeted as a potential transmission vehicle for bringing *V. cholerae* into the HH environment from its natural reservoirs (Figure 2). To accomplish this objective, Hilsa, an anadromous fish, was collected from local markets of Dhaka and Arichpur, and fresh fish near the river were also investigated, over the course of one year. *V. cholerae* prevalence and virulence potential in fish body parts was assessed using molecular microbiological techniques.

In objective 3, flies were investigated to assess their role as imperative transmitters of fecal pollution within the HH domain. To accomplish this aim, rice samples contaminated by flies in the kitchens of low-income urban areas were microbiologically evaluated for the transmission of diarrheagenic *E. coli* and *V. cholerae*.

To meet objective 4, HHs with diarrhea patients were considered, and an attempt was made to identify possible fecal *E. coli* transmission links from patients to the HH environment.

To address objective 5, the genome of pathogenic *V. cholerae* from patients from the study area was sequenced and compared with local and global epidemic strains using the difference in

single nucleotide polymorphisms (SNP). The findings from this study have indicated the evolutionary source of current strains and how they have transferred globally and within the neighborhood.

Finally, to fulfill objective 6, multiple environmental surfaces and leftover foods were followed for fecal *E. coli* and *V. cholerae* contamination in a yearlong surveillance study conducted in the selected HHs of the Arichpur area.

3.0 Summary of the results of the papers and their relation to international state-of-the-art research

This study presents new dynamics of in-house components in efficient transmission of diarrheal pathogens with an emphasis on *V. cholerae* and *E. coli.* The organisms targeted in this PhD work were two gram-negative bacteria. *E. coli* has been assessed as both an index organism for the occurrence of fecal contamination in the environment and a diarrhea-causing pathogen. *V. cholerae* is the predominant cause of the diarrhea burden in Bangladesh and has the potential for pandemic dissemination of disease. Both *E. coli* and *V. cholerae* are highly prevalent diarrheagenic agents in Bangladesh. This environmental contamination study was conducted to explore new facts about direct transmission sources of fecal *E. coli* and *V. cholerae* in urban settings of Bangladesh.

In this section, the author describes a summary of the manuscripts produced to execute the objectives of the PhD work. The relevant methods, results and implications of the findings for each manuscript are demonstrated under the subsections. The details of the methods and results are enclosed in the individual manuscripts and supplementary materials. All the samples for this study were collected in Bangladesh. Sample preparation and examinations were mostly performed at the Environmental Microbiology Lab, University of Dhaka. Part of the analyses for objectives 3 and 5 (manuscripts 4 and 6) were conducted at the University of Copenhagen and the National Food Institute, Technical University of Denmark, respectively.

For environmental surveillance of *E. coli* and *V. cholerae*, the author employed traditional culture methods and basic microbiological techniques on diverse sample types. Arrays of advanced molecular approaches were performed for genomic characterization of bacteria.

Following, the author will first represent the description of the field study area and the overview of the "C5" study protocol. Afterwards, a concise summary of each of the manuscripts to support the study objectives under this PhD study will be provided.

3.1 Sampling site and design of the "C5" study

A field study was set up at Arichpur, a densely packed, rapidly urbanizing community located 15 km north of the capital, Dhaka (Figure 3). The area is situated in Tongi Sub-district and covers an area of only 1.2 km^2 where roughly 129,000 inhabitants live in 29,000 HHs. Although a few high-income families live in multi-story buildings, a large number of low-income families live in this area because of its close proximity to the nearby industrial area and easy access to the capital. Low-income nuclear families mainly dwell in single rooms and commonly more than 10 families living in a compound share water sources, a cooking area and a latrine [164]. Incidences of cholera and other waterborne diseases have been reported in this area, previously [164, 165].

Figure 3: Images of the field site-Arichpur. The upper row from left to right represents the community area and a HH compound; the lower row from left to right represents a typical kitchen and latrine area.

A prospective cohort study set up was established with a partnership between the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and the University of Copenhagen and a field office was set up in the Arichpur community. Primarily 477 low-income HHs were enrolled in Arichpur for the C5 study. Information about socioeconomic status, hygiene practices, water use, etc. were collected at the baseline visit by the field staff from icddr,b for epidemiological analyses in WP 1, and the results will be published elsewhere. Routine visits to each HH for diarrhea surveillance were also performed as part of the study.

3.2 Manuscript 1: Validation of DNA extraction method for quantitative analysis of multiple sample types (Published)

A methodological study was conducted in the laboratory before handling the samples from the field to validate a suitable DNA extraction method for analyzing a high load of samples (manuscript 1). A general and uncomplicated DNA extraction method for diverse sample types eased the load of laboratory work.

3.2.1 Methods

Three DNA extraction methods (boiled template, phenol: chloroform: isoamyl alcohol, and the QiaAmp® mini kit) were applied to four different sample types (pure culture and artificially inoculated food, saline and water samples)and compared by quantitative analysis of *V. cholerae* using real-time PCR and conventional PCR. In addition, sample processing time and cost were compared.

3.2.2 Results and discussion

In manuscript 1, the author designed the experiment to validate an ideal nucleic acid extraction method for identification of toxigenic *V. cholerae* in a high burden of diverse types of samples. DNA extraction is a critical step for positive downstream molecular characterization of any organism because lack of efficient DNA recovery may affect further detection. Results of the study showed that among the four different types of samples analyzed, spiked water samples and spiked rice samples yielded the lowest and highest concentration of DNA, respectively, for all three methods. The possible reason may be that DNA was extracted from only 1 mL of spiked water samples and without filtration which is required for capturing DNA [166]. In an effort to provide an equitable comparison, the same volume (1 mL) of all the samples (water, Phosphate buffered saline (PBS) and rice suspension) was inoculated with the *V. cholerae* O1strain. Despite

lower DNA yield from water samples, we obtained positive threshold cycle (Ct) values via real time PCR, targeting the *ctxA* gene for all extracted DNA, using the three extraction methods. The DNA extracted using the phenol: chloroform: isoamyl alcohol method rendered the lowest Ct values corresponding to a high copy number of the *ctxA* gene in templates for pure culture, spiked water and spiked PBS samples (Table 1). The real time quantitative PCR approach was considered for analyses of DNA quality due to its rapidity, robustness, and specificity in detecting and quantifying target nucleic acid sequences [167]. Moreover, conventional PCR generated detectable agarose-gel bands for all the sample types except the boiled DNA templates for water samples. Detection of boiled DNA templates extracted from contaminated environmental water with a high bacterial load may not affect the positive results in future studies.

Table 1: Comparison of three extraction methods based on DNA concentration, Ct value and quantity obtained in crude culture, spiked water, PBS and rice

Sl	Samples	Ct value			Quantity (copy No./reaction)		
		QiaAmp® Kit	Boiled Template	Phenol : Chloroform : Isoamyl Alcohol	QiaAmp® Kit	Boiled template	Phenol :Chloroform:Isoamyl Alcohol
1	Crude culture of *V. cholerae*	14.92	14.80	14.31	2.55E+06	2.66E+06	3.66E+06
2	Spiked water	26.19	34.19	22.83	5.60E+02	1.607	1.03E+04
3	Spiked PBS	18.08	16.70	16.76	2.28E+05	6.28E+05	5.89E+05
4	Spiked rice	16.7	17.79	19.05	1.79E+06	2.86E+05	1.10E+05

The QiaAmp® kit method was found to have the highest cost per test with the lowest processing time. The boiled template method [168] has a minimal cost as the only reagent required is the DEPC treated water, and the required time for processing is nearly the lowest. Based on the comparative evaluation of the three extraction methods, the boiled template method appeared as the appropriate method with reproducibility, affordability, and low processing time. The

QiaAmp® DNA mini kit would be a suitable tool for all sample types including food and water if budget and time were not limited. This study (manuscript 1) shows important data regarding choosing the ideal DNA extraction method for detection of potential pathogens with public health importance.

3.2.3 Limitations

The study evaluated three DNA extraction methods which employed different mechanisms on four sample types. Although different sample types may present unique challenges for DNA extraction, the author successfully extracted good quality DNA from each sample type using the method of choice: the "boiled template method".

3.3 Manuscript 2: Investigation of the survival pattern of *Vibrio cholerae* on environmental surfaces (Published)

This thesis covers diverse angles of contamination pathways of *V. cholerae* and *E. coli* in the domestic environment. Fomites consisting of materials used in daily life may act as carriers of pathogenic bacteria such as *V. cholerae*. However, the period of persistence and load of bacteria as viable pathogens and in the VBNC state had not been previously established. Thus, the aim of the manuscript 2 was to examine how long *V. cholerae* can survive and persist on fomites such as HH surfaces or daily use items.

3.3.1 Methods

Eight types of fomite materials (cotton cloth, wood, paper, glass, plastic, stainless steel, iron and aluminum) were spiked with cholera toxin possessing clinical *V. cholerae* O1strain N16961. Approximately 10^8cfu/mL of *V. cholerae* were inoculated per cm^2 of fomite. The bacterial

culturability was detected by the drop plate method for up to six hours of spiking. *V. cholerae* from spiked fomites after 6 and 24 hours was enriched for resuscitation in culture media. Due to its ability to reform its cellular morphology and become viable but non culturable (VBNC) on surfaces not beneficial for growth, the viability of *V. cholerae* was determined using ethidium monoazide (EMA), a stain which differentiates between viable DNA and dead cell DNA by inhibiting amplification of dead cell DNA [169]. The DNA was extracted from spiked dry fomites at 2, 4, 6, 24 hour and 7day time intervals and real time quantitative PCR was performed with the EMA-treated DNA. The copy number of viable *V. cholerae* was quantified by standard curve calculation.

3.3.2 Results and discussion

Results revealed that *V. cholerae* growth on fomites, in order from longest to shortest period of time, was as follows: cloth> wood> paper>iron> plastic> steel> glass>aluminum. The culturable *V. cholerae* growth on cloth and wood was detected up to four hours after spiking. *V. cholerae* on non-porous fomites such as metals, glass, etc. lost culturability very rapidly (in approximately one hour).This result showed concordance with previous reports of higher moisture content helping in nutrient capture and subsequent bacterial attachment to porous surfaces more than nonporous surfaces like metals [170, 171]. It has also been demonstrated that the transfer rate of microorganisms is higher and more efficient from nonporous than from porous surfaces to the human hand [145]. All the porous fomites and stainless steel retained a *V. cholerae* load of 10^4-10^5cfu/cm^2 after one hour of inoculation, which is the infectious dose needed to cause cholera when ingested with food. No *V. cholerae* cells from any of the fomites were able to be resuscitated in APW media after 6 and 24 hours of inoculation.

Further quantitative analysis of the VBNC *V. cholerae* in the EMA-qPCR experiment showed that the target *ctxA* gene was detected for up to seven days in all types of fomite samples. A

higher copy number of the gene was detected after seven days on nonporous fomites, such as metals, compared with paper and cloth. After two hours of inoculation, the copy number per reaction had declined to 3-log, and further declined to approximately 1-log after 7 days. Although aluminum lost culturability within the shortest amount of time, it had the highest viable cell fraction detected after 7 days, compared with the other fomites (Figure 4). Correlation analyses revealed that both *V. cholerae* cfu in culture and copy number in EMA-qPCR reduced significantly with time ($p < 0.05$). The author undertook a rapid, sensitive and convenient viable dye-based method combined with quantitative PCR to distinguish between viable and dead *V. cholerae* cells. DNA-intercalating dyes to quantify VBNC cells has previously been applied for a range of bacteria [172, 173]; however, in the current study, this method was used for the first time on environmental samples.

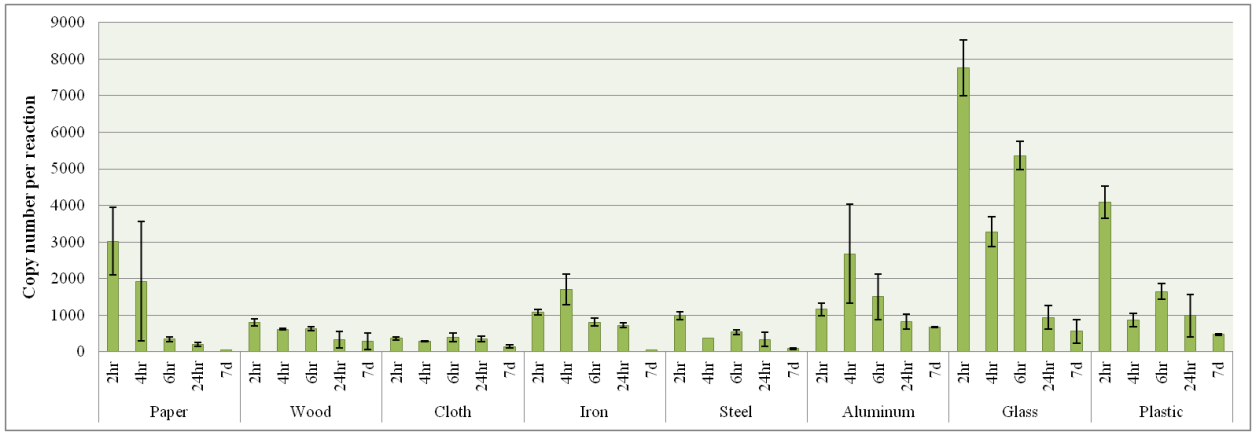

Figure 4: Copy number per reaction bars from quantification of EMA-treated viable *V. cholerae* O1 N16961 from fomites. Error bars in diagram represent standard error from estimated standard deviations of two independent replicates.

The findings from manuscript 2 indicated the presence of persistent pathogenic *V. cholerae* on diverse surfaces of HH materials used for cooking, cleaning and eating, in a laboratory-based experiment. The viability of the persistent *V. cholerae* was confirmed, which makes the result more relevant from a public health perspective. Viable but nonculturable *V. cholerae* may revert

to their infectious form when passing through the human intestine and subsequently start a cholera epidemic [174]. Contaminated surfaces may allow transmission of toxigenic *V. cholerae* to the oral route via direct hand touching or consumption of food and water from the polluted reservoir. The aluminum surface maintained a high copy number of persistent viable *V. cholerae* for a long period of time. As aluminum utensils and equipments are used frequently for daily domestic and hospital activities, effective cleaning measures should be taken to prevent the spread of epidemic *V. cholerae*, particularly during large outbreaks. In an extended EMA-qPCR experiment lasting up to eight weeks, the author found that VBNC *V. cholerae* persisted for four weeks on all the fomites and was subsequently not detected in PCR except on plastic and cloth (the results will be published elsewhere). Viable but non culturable cells were found on cloth and plastic surfaces after seven and eight weeks at room temperature, respectively. Bacterial survival on food processing surfaces such as plastic, wood, etc., or on caretakers´ clothing, may fecally contaminate food and contribute to emerging foodborne disease outbreaks [175]. In a rural Indian village, a cholera outbreak was significantly associated with the use of contaminated utensils in food preparation [144]. Toys have been implicated as a potential fomite for transmission of infectious diseases both in developed and resource-poor countries [176]. Though the transmission role of fomites for *V. cholerae* was contemplated before, an extended microbiological quantitative study of the infectious *V. cholerae* O1 survival pattern on fomites has rarely been conducted. Study results described in manuscript 2 suggested the possibility and prospect of highly touched surfaces and object materials serving as vectors and contributing to the rapid transmission of *V. cholerae*, and also informed of the risk posed by the burden of the bacteria within the HH environment.

3.3.3 Limitations

The study investigated the *V. cholerae* survival pattern for seven days, which may not reflect the longest persistence period. Hence, the author studied the persistence of the VBNC state for an extended period (up to two months), and the results will be published elsewhere.

3.4 Manuscript 3: Assessment of the role of fish (*Tenualosa ilisha*) as a possible transmission vehicle of *V. cholerae* in Bangladesh (Published)

Fish are significant vectors of *V. cholerae* in the aquatic ecosystem and may play a considerable role in dissemination of the pathogen. The role of fish as a transmission vehicle of *V. cholerae* from the coastal area of Bangladesh to inland water, and subsequently to kitchens in urban areas, was not considered before. Therefore, there is a need to analyze the incidence and seasonal prevalence of *V. cholerae* in fish. In manuscript 3, Hilsa, an anadromous fish species, was assessed for the occurrence of *V. cholerae* to present evidence for its possible role as a transmission vehicle. First, the prevalence of *V. cholerae* in Hilsa fish was followed for a period of time, and then the *V. cholerae* population in fish was characterized to observe its virulence potential for causing diarrhea and cholera.

3.4.1 Methods

Fish sampling was conducted for one year; each month, two fish from local markets in and around Dhaka City, including Arichpur, and two freshly caught fish from near the bank of the Padma River, were studied. Details on sample collection and processing are described in manuscript 3. Sample collection points are indicated in Figure 1. In total, 48 fish were collected, and the occurrence of *V. cholerae* was analyzed in different fish body parts (Figure 5). Ice samples for storing fish in the market were also taken for analysis. Virulence factors such as serogroup O1/O139 specific genes and the *V. cholerae* cholera toxin gene were detected via PCR analysis on direct DNA samples.

Figure 5: Collection of fish body parts for analyses. The upper and lower rows depict the collection of four different sample types: (from left to right) upper-outer surface swab, gill, lower-gut, and rectum.

Genomic characterization of *V. cholerae* isolates was performed by detecting 23 virulence and regulatory genes of *V. cholerae* O1/O139 and non O1/O139. Antibiotic susceptibility testing of the isolates was done to perceive the effectiveness of antibiotics on these environmental strains. The potential of the fish strains to cause diarrhea or cholera in humans with contact during handling or cooking was also assessed. Nine *V. cholerae* strains, including *V. cholerae* O1 and non O1/O139 serogroups, were analyzed for pathogenic potential on an established animal model and human cancer cell line, following previously published protocols [79, 177]. Additionally, multilocus sequence typing (MLST) was conducted to construct a phylogenetic tree comparing the clonal relationship between fish and global cholera endemic strains.

3.4.2 Results and discussion

Results revealed that 39 of the 48 total fish taken as samples were confirmed as positive for the *V. cholerae* species specific gene *ompW* by PCR. A total of 216 DNA samples were analyzed from market fish body parts and storage ice and 125 (58%) samples were positive for *V. cholerae*. *V. cholerae* detection was highest in gill (19 of 24 fish) and outer scale (16 of 24 fish) swabs for market and fresh fish, respectively, and lowest in intestines in both types of fish. Market fish (21 of 24) were found to be highly contaminated compared to fresh fish (18 of 24) caught from the river where they migrate from coastal waters. The findings of manuscript 3 suggested *V. cholerae* transmission across both water systems (primary) and the HH environment (secondary) via Hilsa fish in Bangladesh. For assessment of the proliferation of *V. cholerae* from the Bay of Bengal to Dhaka City, situated at the center of the country, both freshwater fish caught from a nearby Hilsa spawning ground and local market fish from Dhaka City were sampled. *V. cholerae* were found in high numbers of fish and, most remarkably, the prevalence pattern showed similarity with the seasonal dual peak phenomenon of cholera in Bangladesh. Previously, flooding and coastal planktonic bloom were found to be contributing factors to seasonal cholera outbreaks in Bangladesh [37, 109]. Our hypothesis on the possibility of Hilsa fish as a transmission vehicle of *V. cholerae* from the Bay of Bengal combines with other suggestions, as the Hilsa migration period is during monsoon flooding when high river discharges influence planktonic intrusion in the river water [119].

The key toxin gene associated with *V. cholerae*, *ctxA*, was detected in 20% (8 of 39) of *V. cholerae*-positive fish by evaluating the direct DNA. Cholera toxin gene was detected in five market fish and three fresh fish in real-time PCR (Table 2). The incidence of pathogenic gene detection was higher in the market fish, which indicates unsanitary handling practices by fishers and vendors, and low storage facility controls in the retail markets prior to fish being purchased by consumers. The population structure of *V. cholerae* from the fish showed the 35 genotypic profiles based on 19 different virulence factors which were identified using PCR. Molecular

characterization based on the virulence profile of the isolates yielded 35 genotypic traits; of these, group-XXIX trait (*rfbO1⁻chxA+mshA+ompU+*) included a large number of isolates (29). Both O1 and non-O1 serogroups of *V. cholerae* strains were isolated, though the clinically important CTX virulence cassette genes were not detected. The author attempted to isolate *ctxA* positive strains from the positive direct samples but was not successful, even with the application of adequate enrichment of the samples. This outcome indicated the complexity of *V. cholerae* isolation from environmental samples as a result of the rapid transition to the metabolically active non-culturable VBNC state in the environment. Aside from cholera toxin genes, the *V. cholerae* strains carried other notorious virulence genes such as *stn/sto*, T3SS genes, *hlyA*, etc., that were related to severe cholera-like diarrhea by initiating pathogenic processes in the infected hosts [178]. Phenotypic expression of the pathogenic potential also indicated that both O1 and non-O1 strains retained positive effects on the experimental human cell line model and rabbit ileal loop. Previously, researchers have also shown that *V. cholerae* strains lacking the cholera toxin gene can cause fluid accumulation in the ileal loop [179]. Moreover, the clonality of the strains explored by MLST analysis suggested the evolution of the fish strains from pathogenic epidemic clones isolated from clinical or environmental origin. More detailed results are available in manuscript 3.

Table 2: Occurrence of toxigenic *V. cholerae* genes in fresh and market fish

Fish types	No. of V. cholerae positive fish/Total (%)	No. of *V. cholerae* positive samples/ Total (%)	No. of V. cholerae O1 positive fish/Total (%)	No. of *V. cholerae* O1 positive samples/ Total (%)	No. of *V. cholerae* O139 positive fish/Total (%)	No. of *V. cholerae* O139 positive samples/ Total (%)	No. of *ctxA* positive fish/Total (%)	No. of *ctxA* positive samples/ Total (%)
Fresh fish	18/24 (75%)	53/96 (55.2%)	6/18 (33.3%)	8/53 (15%)	2/18 (11.1%)	3/53 (5.7%)	3/18 (16.67%)	4/53 (7.5%)
Market fish	21/24 (87.5%)	72/120 (60%)	10/21 (47.6%)	17/72 (23%)	4/21 (19%)	6/72 (8.3%)	5/21(23.8%)	6/72 (8.3%)

Manuscript 3 provided evidence of extreme public health importance associated with Hilsa fish. This comprehensive study showed the presence of pathogenic *V. cholerae* in fish that can provoke not only long-distance dispersal of the pathogen but also contamination risks in the HH environment. Fish can be established as one of the major pathways of pathogen transmission whenever the raw contaminated fish come into contact with different kitchen surfaces and food handlers or when undercooked fish is eaten. As adequate cooking of fish (frying, boiling with spices) is common before intake, lack of fish processing and food preparation hygiene would be key aspects for fish-associated cholera transmission in Bangladesh. These types of cholera transmission mechanisms were also reported elsewhere [151].

3.4.3 Limitations

The small sample size and lack of inclusion of other environmental samples such as river water, plankton, etc. were the few limitations of our sample collection process in this study. Despite the limitations, this study laid a foundation for prospective spatial and temporal studies with a larger sample size to track environmental transmission of *V. cholerae* in Bangladesh.

3.5 Manuscript 4 and Abstract 4: Study of fly transmission of *E. coli* and *V. cholerae* to food in an urban slum area in Bangladesh (Published)

Flies, one of the “F”s of the F-diagram depicting the fecal-oral transmission routes, can spread fecal bacteria to food or water, and this statement has been verified in manuscript 4 within a natural environmental setting. To address objective 3, manuscript 4 and abstract 4 assessed houseflies as a transmission vehicle of *E. coli* and *V. cholerae* to cooked rice in the Arichpur area. The study was carried out over a two month period. This study was conducted as collaboration between the University of Dhaka and a Master´s thesis project in the Department of Public Health, University of Copenhagen. The fieldwork was carried out in collaboration with

icddr,b. All the microbiological analyses were conducted by the author of this dissertation at University of Dhaka.

3.5.1 Methods

Sixty pairs of food containers with cooked rice were positioned on the open ground of kitchen areas in Arichpur community; one container in each pair was left open and designated as the "exposed sample", and the other one was covered with a fly net and designated the "control sample" (Figure 6). The containers were placed in multiple kitchen environments in the study area at a distance between 1 and 26 m from the nearest latrines. The containers were exposed for 30, 60, 90, 120, 150 or 180 minutes and the number of flies landing on the exposed rice was counted. The surface of the rice samples was collected aseptically and homogenized samples were tested for microbiological fecal contamination by quantifying thermotolerant *E. coli* colonies on HiCrome™ m-TEC agar. In addition, molecular detection of diarrheagenic *E. coli* and *Shigella* was conducted using PCR of total DNA samples after enrichment. Detection criteria of diarrheagenic strains were published elsewhere [180, 181]. Statistical analysis was performed for comparison of the contamination level of exposed and control samples and cfu/fly-landing was calculated. Detailed methodology is discussed in manuscript 4.

Figure 6: Set up of paired rice containers in a kitchen in Arichpur

For detection of *V. cholerae* on the paired rice samples, the species-specific outer membrane protein (*ompW*) gene was targeted in the PCR experiment [182]. Positive DNA samples were further tested for the presence of the cholera toxin gene (*ctxA)* in real time PCR, following the previous protocol [183] (abstract 4).

3.5.2 Results and discussion

The results showed that *E. coli* contamination was significantly associated with rice exposure to flies and the chance of contamination was 5.4 times higher than for the rice samples which were not exposed to flies. Forty-two (70%) exposed and 17 (28%) control rice samples were positive for *E. coli* using the culture method. This result demonstrated the effectiveness of the method to elucidate the role of flies in fecal bacterial transmission by evaluating the fly-exposed food. Two different fly species were identified among the captured flies: common houseflies (*Musca domestica*) and oriental latrine flies (*Chrysomya megacephala*). The mean *E. coli* contamination on the uncovered rice samples was 3.1×10^3cfu/gm and an average of

$>0.6 \times 10^3$ cfu/ fly landing were observed for half of the fly landings. The result showed substantial contribution of flies as a food contamination pathway. Molecular characterization of direct DNA samples gave an indication of the diarrhea-causing potential of *E. coli* pathotypes present in the fly-contaminated rice. Virulence genes of four diarrheagenic *E. coli* pathotypes (ETEC, EHEC, EPEC, EIEC) as well as *Shigella* spp. were detected using PCR in 65% (39 in 60) of exposed rice samples. Genes of ETEC strains were found most prevalently (41.7%), which was not consistent with the findings from the latest study in which EAEC was predominantly found in uncovered stored food susceptible to fly landings in rural environments of Bangladesh [184].

For detection of *V. cholerae* transmitted by flies in cooked food, a total of 75 pairs of exposed and control rice containers were examined (abstract 4). Only three exposed samples showed the presence of the *V. cholerae*-specific *ompW* gene in PCR; however, the *ompW*-positive samples did not contain the cholera toxin genes *ctxA* or *ctxB*. All the covered controls as well as the control rice samples before fly exposure were negative. The exposure times for the three positive samples were 30, 120 and 180 minutes, with an average fly-landing intensity of 0.6, 4.4 and 0.8 flies per minute during the three experiments. Although the number of exposed samples on which flies landed and then tested positive for *V. cholerae* was small, our study validates the possibility of diarrhea-causing bacterial transmission through flies.

Table 3: Exposure periods and number of fly landings on the three *V. cholerae*-positive samples

Exposure	**Fly landings**
30 min	6
120 min	505
180 min	138

The method employed in this study is unique in that it is the first time fly contamination of food has been assessed quantitatively in a natural HH environment. This highlights both advantages and challenges of field studies. Previous experiments of quantitative contamination by synanthropic houseflies utilized laboratory equipment and protocols such as green fluorescent protein (GFP)-tagged *E. coli* to contaminate flies, typically in a cage model [185, 186]. A recent study conducted in the rural area of Bangladesh investigated houseflies and food from the same HHs to show the association; however, the researchers did not follow the flies to prove the true burden of fly contamination of food [184]. The studies which only investigate the whole fly bodies would fail to notice the actual bacterial count transferred via fly landing.

3.5.3 Limitations

First of all, 28% of the control rice samples were positive for *E. coli* contamination. It could be argued that some exposed samples could also have been positive due to sources other than flies. It cannot be denied that in an over-crowded field setting where there is natural movement of people and animals, as well as flies sitting on the nets, fecal contamination could not be entirely controlled. However, the same sampling technique and environmental conditions were employed for both control and exposed samples.

Secondly, a limited number of samples showed the presence of *V. cholerae*. This may have happened because of the short sampling period (two months) and the fact that it was the cholera off-peak season. An intensive year-round investigation with a particular focus on the two cholera outbreak seasons will help in better understanding cholera transmission via flies.

3.6Manuscript 5: Transmission of diarrheagenic *E. coli* in the low-income household environment of diarrhea patients in Bangladesh (Manuscript)

It is already recognized that poor hygiene practices may trigger the rapid distribution of pathogens, particularly waterborne diarrheagenic bacteria, in overpopulated urban settings. Manuscript 5 was produced to address objective 4, with an aim to reveal in-house environmental hotspots for diarrheagenic *E. coli* transmission and their involvement in diarrhea. In this study, the author included a subset of diarrhea-reporting HHs for four months under WP 5, in which regular surveillance of selected HHs was carried out over a one year period. The study settings in the Arichpur area were applicable for this objective as well. The particular methods used for this study are described in the following segments. *E. coli* was the target fecal pathogenic bacteria transmitted via the fecal-oral route. Pathogenic *E. coli* in rectal swabs from diarrhea patients were followed concomitantly with HH environmental samples.

3.6.1 Methods

As a component of the "C5" study (WP 2), mobile phones were distributed to 400 enrolled HHs to establish a real-time cholera and diarrhea surveillance system [187]. A call-center was established at the field office and HH caretakers could inform the call-center staff via phone if any HH member was experiencing diarrhea symptoms. A field team visited the HHs within 24 hours of the report and collected rectal swabs from the patients for a rapid dipstick test of *V. cholerae* identification.

To meet objective 4, a concurrent sampling of hotspot swabs (described in detail in Section 3.8), foods, and drinking water was conducted along with rectal swabs of the diarrhea patients from low-income HHs. Field staff were informed of diarrhea via a phone call or during diarrhea surveillance visits to the HHs and all sample collection took place between September 2015 and

December 2015, from eight HHs with diarrhea patients per month. Within 24 hours of learning of the diarrhea, samples were collected (with the patients´ and patients' caretakers´ consent) and transported to the University of Dhaka for further analysis. Sample collection and processing details are shown in Manuscript 5.

Direct DNA from the rectal swab and corresponding HH samples was analyzed by PCR for virulence factors of five major diarrheagenic pathotypes of *E. coli* (ETEC, EHEC, EIEC, EPEC, EAEC) to detect transmission links between the diarrheal incident and the environment. Characterization of isolated pathogenic *E. coli* from multiple sample types was conducted.

3.6.2 Results and discussion

PCR analysis of direct DNA extracted from a total of 245 samples showed that at least one of the virulence-associated genes of diarrheagenic *E. coli* was present in 89 (36%) samples. Genes were distributed in different samples as follows: rectal swab samples (28%, 10 of 36), hotspot samples (44%, 63 of 144), food samples (12%, 4 of 34), and water samples (10%, 3 of 31). Results revealed that 17% of rectal swabs and their HH environmental swabs or "hotspots" were contaminated with virulence genes of the same strains of diarrheagenic *E. coli*. The findings suggest that transmission may occur from infected individuals to the environment or the other way around. More detailed findings are available in manuscript 5.

Drinking water vessel surface swabs were found to be the most contaminated out of all the spots with pathogenic genes of similar strains to those found in the patients. The drinking water from respective HHs was not positive for the same strains found on drinking water pot surfaces, which indicates a different source of contamination, such as transmission from unwashed fingers. Two cutting knife and one latrine door knob swabs were also found to be

contaminated with particular strains of *E. coli* detected in patients from the same house. Two EAEC strains were successfully isolated from both a rectal swab and a latrine door knob from the same HH. To our knowledge, this study is the first to show the direct link between environmental surfaces and diarrhea. Earlier studies generally attempted to observe fecal *E. coli* contamination on environmental surfaces such as knives, latrine floors etc., but association with diarrhea was not investigated [188, 189]. In addition, one rectal swab and its corresponding food sample were positive for the same strains. Diarrhea has been linked to prepared food previously in Bangladesh [190].

ETEC virulence genes were predominantly detected in the rectal swabs of the diarrhea patients. In total, ETEC was detected in six patients' rectal swabs as the solely identified pathotype and in one patient´s rectal swab co-identified with EIEC. Six rectal swab samples showed the presence of similar *E. coli* pathotype genes in the corresponding hotspot and food samples. In two HHs, ETEC genes were detected in both patients' rectal swabs and drinking water vessel swabs. The ETEC pathotype is endemic in Bangladesh [31]. A recent report showed that EAEC was the most frequently found pathogen in infant diarrhea patients in Dhaka [191]. In our study (manuscript 5), a common observation in the Arichpur HHs was that genes of the same pathogenic strains were detected in multiple environmental spots at the same time even though those strains were not detected in the patients' clinical samples (Table 4). For example, ETEC virulence genes were detected in four hotspots in one HH (rectal swab ID- 197) and the patient's rectal swab was negative. The result indicates a common source of contamination within the HH.

Table 4: Presence of virulence genes of diarrheagenic *E. coli* pathotypes in rectal swab and household environmental samples

Rectal swab ID										
SI	ID Number	Patient's Age (Years)	Sex (M=Male F=Female)	Direct Rectal Swab	Cutting Knife	Drinking Water Vessel Surface	Latrine Door Knob	Food Plate	Water Sample	Food Sample
1	R-175	17+	M	ETEC, EIEC		ETEC				
2	R-176	17+	M		ETEC, EHEC	EHEC, ETEC	ETEC,EHEC,EIEC		*	*
3	R-177	11-17	F	ETEC	ETEC,EHEC		ETEC	EIEC		
4	R-178	17+	F		EHEC	ETEC, EIEC			*	
5	R-179	17+	F		EIEC	EHEC	ETEC	ETEC, EHEC,EIEC		ETEC
6	R-180	17+	M		EIEC	EIEC				
7	R-185	0-10	M	EPEC			ETEC			
8	R-186	11-17	M		ETEC					ETEC
9	R-190	17+	F		EIEC	ETEC	ETEC	EHEC,ETEC		
10	R-191	17+	M		EIEC	ETEC	ETEC	EHEC,ETEC		
11	R-196	17+	M					ETEC		
12	R-197	17+	F		ETEC	ETEC	ETEC	ETEC		
13	R-198	17+	M				ETEC	ETEC		
14	R-199	0-10	M	ETEC	ETEC	ETEC				

15	R-200	17+	M		EHEC, ETEC, EIEC		ETEC		*	
16	R-201	0-10	F	ETEC		ETEC,EHEC		EHEC		
17	R-202	17+	F					ETEC,EHEC		
18	R-203	0-10	F				ETEC	EHEC		
19	R-212	17+	F		ETEC		ETEC			
20	R-215	17+	F		ETEC	ETEC			*	
21	R-233	17+	F				EHEC			
22	R-234	17+	F	ETEC		EHEC	EHEC	EHEC		
23	R-235	0-10	F		ETEC,EHEC					
24	R-236	17+	M	EAEC	EHEC	ETEC	EAEC	ETEC		
25	R-237	0-10	M	ETEC	EHEC					
26	R-238	17+	M						*	*
27	R-239	0-10	M		EAEC					
28	R-240	0-10	F							
29	R-246	0-10	F	EIEC						
30	R-248	0-10	M							
31	R-249	0-10	F			EIEC	EIEC			
32	R-250	0-10	F			ETEC			EHEC	
33	R-251	17+	F	ETEC		ETEC			EHEC	ETEC
34	R-252	17+	F			ETEC			EHEC	ETEC
35	R-253	17+	F					EHEC		
36	R-254	17+	F		EHEC					

* Samples not collected due to refusal of caretaker

Blank cells-Not Detected

The author performed both culture-independent multiplex PCR and the traditional culture method for probable molecular detection of *E. coli* pathotypic strains. Further analyses by means of next generation sequencing such as the metagenomic approach will allow more sophisticated and confirmatory indication of environmental pathogenic microbiota related to diarrhea.

3.6.3 Limitations

As the study included diarrhea patients and analyzed for fecal *E. coli* contamination, it does not confirm the exact causative agent of diarrhea, since the only organism which was attempted to be detected was *E. coli*, and other diarrhea pathogens were not examined. However, our results provide an indication of environmental contamination situations within the HH, as well as the origin of the infection.

3.7Manuscript 6: Assessment of the current genomic profile of *V. cholerae* O1 using the Whole Genome Sequencing (WGS) approach (Published)

In addition to fecal *E. coli* contamination, the diarrhea HHs in Arichpur were assessed for cholera incidents. As a sub-study of objective 4, pathogenic *V. cholerae* was isolated from cholera patients in Arichpur HHs to investigate whether or not the whole genomic profile was altered compared to global as well as local epidemic strains, for the purpose of exploring genomic and transmission origin. This investigation addressed objective 5 of this dissertation and comprises manuscript 6. This study extended the scope of the transmission and epidemiological perspective of the cholera pathogens obtained from Arichpur patients by assessing genomic changes using the WGS approach. The sequencing was performed at the National Food Institute, Technical University of Denmark (DTU).

3.7.1 Methods

The genomes of the *V. cholerae* strains from patients were sequenced and several advanced bioinformatics tools were applied for analyses. The webserver tools such as 'MyDbFinder' and 'ResFinder' developed by DTU were employed for identification of genetic alterations in CTX genotypes, virulence factors, pathogenicity islands, mobile genetic elements and acquired antimicrobial resistance genes. Phylogenetic analysis was performed based on genome-wide SNPs of 466 *V. cholerae* global pandemic and outbreak strains. The 'CSI Phylogeny' web tool was used for identification of SNPs. The 'Mpileup' module in SAMTools version 0.1.18 was used to select the SNPs meeting the following criteria: (i) a minimum distance of 10 bps between SNPs, (ii) a minimum of 10% of the average depth and at least 10X, (iii) a mapping quality greater than 30, and (iv) an SNP quality greater than 25 [192, 193]. Both global and local phylogenetic trees were created using the online tool iTOL. All the methods are described in detail in Manuscript 6.

3.7.2 Results and discussion

Three *V. cholerae* strains were isolated from two case patients: two strains (VC-1, VC-3) from case 1 and one strain (VC-2) from case 2. The patients were both female; one was four (patient 1) and the other 22 years old (patient 2). Results of WGS analyses revealed that the strains isolated from Arichpur patients were part of the seventh pandemic serogroup and the virulence backbone resembled the recent El Tor variant trait (standard classical *ctxB* genotype, *ctxB*1) of epidemic strains. The prevalence of this El Tor variant trait was also reported in several Asian countries and in Africa, coinciding with severe morbidity and mortality. Previous reports showed that the amount of cholera toxin produced by El Tor variant strains was much higher than the prototype El Tor strains [194]. Variants of two hallmark genetic markers of the seventh pandemic *V. cholerae* VPI-1 and VSP-2 were observed in all three strains from Arichpur. The strains showed a unique deletion in the Vibrio pathogenicity island-1 (VPI-1) gene cluster

containing three consecutive gene deletions (VC-0819–0821). A variant of Vibrio seventh pandemic island-2 (VSP-2) was found in all three genome sequences compared to the prototype N16961, with a deletion of four open reading frames (ORFs) (VC-0495–0498). Interestingly, the variants were found to be different from the recent pandemic strains in published reports [195] and a shift was observed to prototype seventh pandemic El Tor N16961 isolated from Bangladesh.

Phenotypic expression of the antimicrobial resistance capability of the three strains showed resistance to multiple antibiotics, particularly the resistance conferred by the SXT mobile genetic element. Our result was consistent with Rashed et al.´s study where the researchers showed that the majority of the previous years´ isolates in Bangladesh were predominantly resistant to streptomycin, nalidixic acid, tetracycline, and sulfamethoxazole-trimethoprim [196]. Moreover, reduced susceptibility to ciprofloxacin was observed in rural areas of Bangladesh [196]. Whole genome sequencing analysis also confirmed the presence of multiple antimicrobial genes (AMR) which included AMR genes for aminoglycosides (*str*A/*str*B); sulphonamides (*sul*2); chloramphenicol (*cat*B9); and trimethoprim (*dfr*A1) in VC-2 and VC-3. The VC-1 strain lacked the *sul2* gene which confers resistance to sulphonamides, although the strains phenotypically expressed resistance to sulfamethoxazole. The findings correspond with earlier reports [197, 198]. The absence of the *sul*2 gene in the VC-1 SXT element in SXT variant types has been previously reported [197]. Integrating conjugative elements (ICEs) or SXT elements confer antimicrobial drug resistance in *V. cholerae* by integrating within the host chromosome, and we compared the ICEs of our strains with the *V. cholerae* O1 from the recent cholera outbreak in Haiti (ICE*Vch*Hai1). The SXT elements of Arichpur strains showed variance from ICE*Vch*Hai1 and were most similar to a specific clade (clade 1) of strains from Chandigarh, India isolated in 2009 [199]. The findings indicate recent spread and transfer of SXT elements within common geographical areas.

In manuscript 6, SNP-based phylogenetic analyses provided insights on possible mechanisms of emergence and transmission through a time and region-specific approach. Zero SNP difference in the genomes of the three *V. cholerae* from Arichpur patients was defined by the shortest time interval (11 days) and distance (588m) between the two cases. In the 2010 Haiti outbreak, clinical isolates collected from shortly after the first outbreak showed zero or low nucleotide substitution per SNP site which became seven times larger for 2012 outbreak isolates [200]. Although clonal strains were the possible cholera contributing agent in the two separate HHs, the water sources for the two HHs were different. The findings were consistent with the investigation of Bi et al. which explained that risk factors other than water contamination, such as poor hygiene, may drive spatial clustering of cholera cases in Arichpur [201]. Phylogenetic analysis using the sequences of 38 Bangladeshi clinical strains revealed that Arichpur strains were highly related to recent outbreak strains (the nearest strain was PSC-022, with 18 SNPs difference). Global phylogenetic analysis of SNP differences among 469 global *V. cholerae* O1 strains revealed some interesting findings. With only 11 SNP differences, the closest strain to the Arichpur strains was *V. cholerae* strain S9KCH9, with a coastal origin, which was isolated during a massive outbreak in 2010 in Pakistan (Figure 7) [202]. Both S9KCH9 and PSC-022 strains were found to be closely related, differing in only three SNPs, and both were isolated in 2010, only in different countries. The coastal cities of Pakistan border the Arabian Sea, which eventually meets with the Bay of Bengal south of India. Taken together, the results from the comparative genomic analysis support the hypothesis that the marine ecosystem of the Bay of Bengal or human traveling may be the potential vehicle of transportation of *V. cholerae* O1 in Southeast Asia, causing annual outbreaks [203].

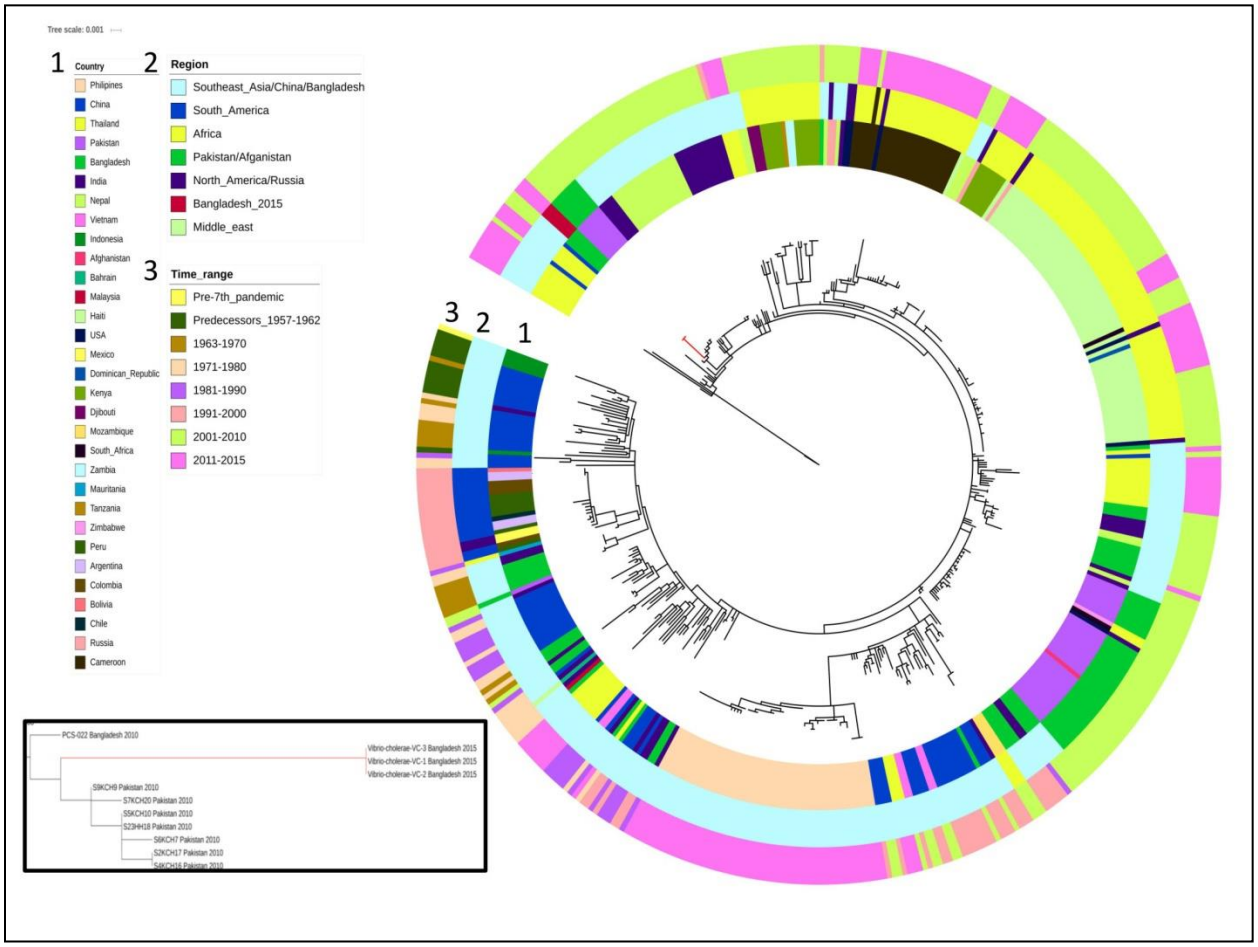

Figure 7: Global phylogenetic tree constructed with 466 database strains based on SNPs (regions are highlighted in colors). The box in the lower left corner is focusing on the position of the three study strains in the global tree.

3.7.3 Limitations

First, despite using a highly robust and specific genome sequencing and phylogentic analysis, only two clinical cases were assessed in this study. In our study, a total of 37 rectal swabs from diarrhea patients were analyzed in the laboratory for the causes of cholera. Only three out of the 37 rectal swabs (8.1%) were positive for *V. cholerae* and confirmed by PCR. We successfully isolated three *V. cholerae* O1 strains from the rectal swabs of two diarrhea cases. However, due

to our low sample size, we included 38 genome sequences of the seventh pandemic *V. cholerae* O1 El Tor clinical strains from Bangladesh for comparison with our current study strains.

Second, the study did not identify the source of infection within the HH via sequencing analysis. Although subsequent environmental samples such as drinking water, food and environmental swabs from the respective patients' HHs were collected and investigated for the presence of *V. cholerae*, this organism could not be cultured from any of the environmental samples.

3.8 Manuscript 7: Assessment of the magnitude of fecal *E. coli* and *V. cholerae* contamination within the household domain (Manuscript)

Touching a range of environmental surfaces and HH objects is a vital part of our daily activities and thus disease transmission may occur via contaminated surfaces. Evidence suggesting the role of surfaces as a transmission pathway is derived from transmission modeling studies, microbiological studies, observational epidemiologic studies, intervention studies aimed at improving the efficacy of cleaning and disinfection, and outbreak reports [204].

As the final part of this thesis, the author undertook a real-time environmental surveillance study (manuscript 7) to investigate the environmental load of fecal bacteria through microbiological observation in the cohort of low socioeconomic HHs. HH environmental surfaces with possible high exposure to fecal contamination and contribution in direct transfer via the fecal-oral route were selected as "hotspots" on which to perform comprehensive analyses. Our intention was to broaden the perspective of the existing fecal-oral pathways in the "F-diagram". The study also provides significant knowledge on the level of fecal contamination on cooked food stored at room temperature for long periods of time. In addition, evaluation of *V. cholerae* contamination

and the virulence mechanism of disease outbreaks in terms of molecular genomic characteristics were the author's major points of interest.

3.8.1 Methods

For this PhD work (WP 5 of the "C5" study), 32 HHs were randomly selected from 477 enrolled HHs for in-depth analyses of *V. cholerae* and *E. coli* HH contamination points, with an emphasis on environmental surfaces and foods. After preliminary observation, four surfaces from the living, kitchen and latrine areas were selected based on frequency of hand contact, and mentioned as 'hotspots'. These were: latrine door knobs, cutting knives for cooking, cleaned food plates for taking meals and water vessels such as mugs and glasses used for drinking (Figure 8). In addition, cooked food samples which had been stored at room temperature or warmer for more than six hours were collected during sampling. In the presence of HH caretakers, samples were collected from each HH by field staff members every six weeks (an average of 16 HHs each month) between November 2014 and December 2015. A total of 668 environmental swab or "hotspot" samples (cutting knife, n=169; food plate, n=165; latrine door knob, n=169; and drinking water vessel surface, n=165) and 137 food samples were collected. All the samples were transported to University of Dhaka to be kept cool in a cool box within four hours of collection. Details on sample collection in the field and processing in the lab are discussed in manuscript 7.

Figure 8: Sample collection from different surface locations and food

The hotspot and food samples were examined for fecal thermotolerant *E. coli* contamination by quantifying cfu in chromogenic selective media (mTEC) for thermotolerant *E. coli*. Fisher's exact test was performed to compare the contamination level in the hotspot locations. A subset of hotspot samples (taken from five of the HHs sampled each month) was further analyzed by PCR to detect the virulence factors of five different diarrheagenic pathotypes of *E. coli*.

V. cholerae prevalence was detected in all the collected samples by targeting the species-specific gene *ompW* in PCR. Direct DNA samples were used in this analysis. From PCR positive samples, *V. cholerae* strains were isolated for further genotypic and phenotypic characterization. For both *E. coli* and *V. cholerae*, seasonal prevalence of contamination was estimated in hotspot and food samples.

3.8.2 Results and discussion

The author chose the four high-touch hotspots on the basis of availability as common items in all study HHs in Arichpur. Results showed that cooking/eating utensils had a higher frequency of fecal *E. coli* contamination than latrine door knobs and the level of contamination from highest to lowest was as follows: food plate> cutting knife>drinking pot>latrine door knob (Table 5). High fecal coliform contamination was observed in moist locations within the house such as the ladle for sink water, the cutting board surface, etc., in earlier studies in Cambodia and Arizona, USA [24]. However, in our study, food plates were found to be the most contaminated (Mean cfu/cm^2 2.53E+02) followed by cutting knives which were apparently clean and dry at the time of sampling. Fisher´s exact test results showed that fecal contamination levels depend on surface locations, such that the chance of fecal contamination is 4.7 times higher (p<0.05)on cutting knives than latrine door knob surfaces. *E. coli* can persist on dry inanimate objects for 1.5 hours – 16 months [205]. The cleaning measures taken by caretakers may not be adequate for maintaining the hygiene standard. The utensils and cutting instruments were not always washed using dishwashing powder or soap; the cutting tool called “boti”, in particular, was found to be mostly only water-washed after daily use in cutting vegetables, fish or other raw items. The author showed in manuscript 5 that in Arichpur diarrhea case HHs, food plate swabs were least contaminated with pathogenic *E. coli* genes, which was not consistent with non-diarrhea HHs. For both types of HHs, cutting knives contained a high load of fecal *E. coli* contamination.

Table 5: Burden of fecal *E. coli* contamination on household surfaces

Hotspot locations	**Mean *E. coli* count (cfu/ cm^2)**
Cutting boti /knife	2.40E+02
Latrine door knob	7.32E+01
Point of use/Drinking vessel surface	1.67E+02
Food plate	2.53E+02

A subset of surface swab samples (a total of 240) was investigated for pathogenic *E. coli* contamination. The *E. coli* species-specific *uidA* gene was detected in 67% of the samples. ETEC genes were the most prevalent of all pathotypes in all four types of hotspots.

The presence of *V. cholerae* in the four hotspots was consistent with the *E. coli* contamination results. The author performed a PCR experiment targeting the *V. cholerae* species-specific *ompW* gene from a direct sample, as the chance of alteration to the VBNC state is high in a dry and nutrient-poor environment. Similar to fecal *E. coli* contamination, the highest prevalence of *V. cholerae* in hotspot samples was on food plate swabs (20% of samples positive). The lowest prevalence was found on latrine door knobs (1.8%). Monthly *V. cholerae* incidence in hotspot samples indicated two peaks: one in May and the second in November. The food plates commonly used by the study HHs in Arichpur were made of steel, aluminum, melamine plastic, glass, etc. Evidence from manuscript 2 also confirmed that the persistence potential of pathogenic *V. cholerae* on aluminum, plastic and glass was higher than on other surfaces after seven days. Although total DNA samples showed positive results for cholera virulence genes in PCR, the isolates from hotspots (12 strains) lacked this gene. Only one hotspot isolate was identified as *V. cholerae* O1. Genotypic characterization of the non-O1/O139 strains demonstrated the presence of toxin genes such as *chxA* and genes for the type 3 secretion system,

providing evidence of their diarrhea-causing potential. The phenotypic expression of the extracellular virulence factors of the isolates was similar to that of the epidemic *V. cholerae* O1 El Tor reference strain.

Manuscript 7 shows significantly higher occurrences of *E. coli* and *V. cholerae* on kitchen and HH utensils than on latrine door knobs in the year-round sampling. Along with latrines, kitchens can become a key zone of pathogen entrance into the domestic environment. In the low-income community of Arichpur, female HH caretakers typically process the raw fish or vegetables while sitting on the kitchen floor. Fecal bacterial transmission may occur inside the house if the caretakers touch or step on the contaminated area. Contaminated cutting knives could act as a transmission vehicle of fecal bacteria spreading a high load of bacteria to fresh produce items which are eaten raw [189]. Cross-contamination of other kitchen utensils could also occur. The author already established the role of fish as a transmission vehicle of *V. cholerae* into the kitchen atmosphere in manuscript 3. Moreover, free roaming of animals like chickens was observed in the HH compounds of Arichpur. Earlier, animal feces was linked to domestic fecal contamination in Bangladesh [206].

The contamination level of four hotspots does not definitively confirm the same amount of intake. Pathogens on surfaces such as eating and drinking utensils can be directly transmitted to food and drinking water for ingestion [207]. In contrast, the same concentration of bacteria on a latrine door knob would probably result in a lower oral transfer, since contact with the mouth is more limited. Further research should be done on daily intake of fecal bacteria from different transmission sources.

The study provides evidence of a high percentage of cooked food contamination with *E. coli* of human origin (41.5%), with 5.45E+02 mean cfu/gm. Seven percent of food showed the presence of *V. cholerae*. *V. cholerae* detection was highest in September (50% of the monthly

collected samples were positive). Food items such as chicken curry and rice with water were found to be highly contaminated (Table 6). This finding indicates direct oral transmission of the high load of fecal bacteria via food. We collected leftover food stored for six or more hours at room temperature, as it was observed earlier in Tanzania that contamination in stored food increases significantly after four hours at ambient temperature, compared with freshly prepared food [208]. In Arichpur, food is normally prepared once daily and consumed by family members throughout the day. Food can be contaminated by serving plates or utensils washed with contaminated water or through cross-contamination in the kitchen environment [190]. We found the highest burden of contamination on food plates, which correlates with the strong possibility of food contamination via utensils in Arichpur HHs. The direct role of fly landings in cooked rice contamination was perceived by the investigation discussed in manuscript 4. Moreover, food handlers´ hand hygiene has serious implications in fecal pathogen transmission [209].

Table 6: *E. coli* count in collected food samples

Type of food	**Range of contamination (Fecal *E. coli* cfu/gm)**	**N (Mean contamination cfu/gm)**
Lentils	0-1,400	14 (293)
Chicken	0-6,640	2 (3,320)
Fried fish	0-60	3 (20)
Fish with vegetables	0-4,240	40 (561)
Rice	0-4,240	29 (616)
Vegetables	0-2,480	34 (109)
Water rice/Panta vat	2,620-6,400	4 (3,565)
Beef	0-40	3 (13)
Mutton	Nil	2 (Nil)
Other	0-2080	6 (710)

3.8.3 Limitations

There are several limitations in the study design characteristics and overall scientific implications of the study (manuscript 7). First, the author selected four hotspots for analysis which cannot indisputably reflect the contamination load of the entire HH. Due to limited sample processing facilities per day, the common HH items and areas characterized as high-touch surfaces available in all HHs, and having fecal bacteria transmission potential, were identified for collection based on prior observation. However, the selected surfaces provide the possibility of enteric pathogen transmission via the fecal-oral route and the findings indicate which HH areas need more attention to prevent rapid transmission in an urban community like Arichpur.

Second, the presence of fecal bacteria such as diarrheagenic *E. coli* and *V. cholerae* on the surfaces does not definitively confirm the transmission of the pathogens. However, researchers have established the transmission pattern of pathogenic bacteria from surfaces to hands and further transmission from hands to mouths [145]. Moreover, a very low infectious dose of several toxigenic bacteria is required in cases such as enterohemorrhagic *E. coli* (EHEC) (10 - 100 cells) and *V. cholerae* O1 or O139 serogroups (10^4cells) [210, 211]. Food and water may acquire the diarrheal pathogens directly through contact with contaminated surfaces (food plates, drinking pots, etc.), and the risk would then increase overtime due to the chance of multiplication during food storage. In addition, longer persistence of the virulent bacteria on environmental surfaces may increase the opportunity of forward transmission to multiple contacts even after resolving the initial infection in a HH environment.

Third, this study was conducted in only one low-income urban community in Dhaka, so it may not be entirely representative of rural HHs in Bangladesh. Since the hygiene and cleaning practices in food preparation and daily activities of the HH caregivers are consistent, the study findings may be applicable for similar community settings.

3.9 Overall limitation of the dissertation

This PhD dissertation overall explored components of the 'F-diagram' (food, flies) and new components (fomites, fish) in natural settings of urban Bangladesh as transmission routes for fecal pathogenic bacteria; other important routes such as fluids (water), fingers (hands), etc. were not included. The author intended to include less-studied pathways with a high potential for fecal pathogen exposure in the context of low socioeconomic HHs in Bangladesh. As a direct ingestion pathway, drinking water has already been extensively studied [212]. Moreover, the effect of hand hygiene and HH compound soil on diarrheal disease transmission was observed previously in Bangladeshi HHs [213].

4.0 Conclusion and future prospects

Despite a few shortcomings, this dissertation pointed out the extreme importance of multiple routes of fecal-oral transmission other than drinking water which were interpreted through methodical examination. The laboratory-based experiment on the survival of pathogenic *V. cholerae* on fomite surfaces showed their potential as reservoirs which can persist for a long period of time and can promote the distribution of *V. cholerae* during inter-epidemic periods. The clinical *V. cholerae* cells retained the cholera toxin gene at a detectable limit in the non-culturable state which allows us to understand the critical public health risk of cholera transmission associated with fomites.

This thesis presents the first comprehensive information on the prevalence of *V. cholerae* in Hilsa fish and explores the possibility of a novel route of transmission from coastal bodies of water to the domestic environment in Bangladesh. The analogous periods of Hilsa migration from sea to freshwater and major cholera peaks in the Ganges Delta region indicate a need to monitor the role of this fish species in the maintenance and transmission of *Vibrio* species. Furthermore, as cholera is primarily a waterborne disease that may require secondary transmission to enhance the epidemic, Hilsa fish could serve as both primary and secondary vehicles.

The empirical evidence from the fly study verifies the direct transmission of *E. coli* and *V. cholerae* by flies to food for human consumption in a field setting. The study suggests the relative importance of fly disease transmission compared to the other routes of the F-diagram, and that flies pose a significant public health hazard similar to direct contamination of water and food. Furthermore, keeping stored food covered can effectively prevent transmission of diarrheagenic bacteria via fly landings in settings similar to those of the study area.

Diarrheagenic *E. coli* contamination has been found on environmental surfaces, particularly surfaces directly related to the human food chain and to diarrhea patients in the same HH. The findings from this dissertation suggest that environmental surfaces play a dominant role as a source of diarrheal disease in low-income urban settings. Community-based extensive molecular surveillance of the HH environment will help in preventing *E .coli* contamination and further suffering due to diarrheal illness. This study also provides the transmission pattern of *V. cholerae* outbreak strains by determining genetic relatedness using WGS. The findings lead to the conclusion that the source of the outbreak cases investigated in this study was common and the causative strains have evolved from recent epidemics in Pakistan. This result could help in predicting new emerging strains for the spread of future outbreaks in Bangladesh.

Finally, a novel investigation of fecal contamination in urban low-income settings showed extensive fecal exposure in different domestic environments. The study findings indicate the vulnerability of the kitchen environment in the HH domain in terms of both *V. cholerae* and fecal *E. coli* contamination. In addition, the research provides knowledge on food safety issues in this urban slum setting. As cholera and diarrhea are still permanent threats to public health in Bangladesh, elucidating the proposed factors contributing to the transmission routes analyzed in this dissertation will be of great utility to the public health analysts and policy makers, enabling an update of the cholera and diarrhea risk factors for early prevention of disease progression in risk groups.

This thesis presents the scope for several future research projects. The research could be extended by selecting more areas in both latrines and kitchens for better comparison of fecal contamination exposure between these two HH locations, given that microbial contamination in latrine areas and hygiene interventions have been previously assessed on several occasions.

Future research on surveillance of complete microbial communities in the HH environment, particularly in the kitchen area, could be performed using a culture-independent metagenomic approach. That would help to understand the burden of both culturable and unculturable fecal

pathogens in different domestic areas as well as to track the source of diarrhea. Further intervention strategies could be designed based on these remaining investigations on kitchen and in-house locations.

5.0 References

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Appendix I:

Manuscripts (1-7)

Manuscript 1

Original Contribution

Quantitative Analysis of Nucleic Acid Extraction Methods for *Vibrio cholerae* Using Real-time PCR and Conventional PCR

Hossain ZZ[1], Ferdous J[2], Tulsiani SM[3], Jensen PM[4], *Begum A[5]

The devastating diarrheal disease cholera is caused by the bacterium *Vibrio cholerae*. Nucleic acid extraction is the primary step for several molecular detection approaches. In order to identify the sources of cholera illness, an efficient, fast and easy DNA extraction method for toxigenic *Vibrio cholerae* is desired which can be applied on diverse type of samples. This methodology developmental setup study was performed in the Environmental Microbiology Lab, Department of Microbiology, University of Dhaka, Bangladesh from March 2015 to May 2015 attempted to compare three DNA extraction methods for efficient detection and quantification of *Vibrio cholerae.* Three nucleic acid extraction methods: (Boiled template, Phenol: Chloroform: Isoamyl alcohol, QiaAmp® mini kit), were assessed for four, routinely tested, templates: crude culture, suspension in water, phosphate buffer saline (PBS), and food by conventional and real time PCR targeting the toxin-coding *ctxA* gene. Finally, the results were compared in context of processing time and overall cost. Cycle threshold (Ct) values were within acceptable parameters by real time PCR (5 to 35 cycles). All the three extraction methods produced sufficient yield of DNA and copy number for detection by real time and conventional PCR. The boiled template method for water samples yielded low amount of DNA in comparison to the other methods, and is therefore sensitive to detect by non-quantitative, conventional PCR only. Despite an overall low detectability from water samples, our comparison reveals that the boiled template method is the most suitable method for high quality and quantity pathogenic DNA particularly in light of limited access to expensive kits and reagents, time constraints, and high sample load.

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Key words: Boiled template, DNA extraction method, Food, Real time PCR, *Vibrio cholerae*

Introduction

Vibrio cholerae, the etiological agent of life threatening disease cholera, is an autochthonous inhabitant of global estuarine and river system[1], though some strains induce severe human morbidity and mortality. Toxigenic *V. cholerae* O1 and O139 produce cholera enterotoxin which is the major virulence factor for epidemic cases of cholera[2,3]. According to the World Health Organization (WHO, 2012), an estimated 1.4 billion of the world's population is at the risk of cholera and among them Bangladesh has the largest population at risk leading to 1.5% case fatality rate (CFR)[26]. Infection due to *V. cholerae* initiates with the intake of contaminated water or food[4,5] and timely detection of causative agent as well as rehydration therapy, is crucial at post-infection.

For rapid and sensitive detection of *V. cholerae*, DNA based conventional PCR methods have been in practice for years[6,7,8,9]. More recently, conventional PCR has been replaced by real-time and probe-based assays such as quantitative PCR[10,11].

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DNA of high quality and quantity yielded during the extraction process is a critical aspect in downstream molecular detection and characterization tests. Sample processing followed by recovery of DNA for PCR involves effective cell lysis and free of PCR amplification inhibitors. Efficient lysis of bacterial cell depends on constitution of cell wall, the physiological condition where the cell is in and the concentration of cell[12,13]. A common DNA extraction method applicable for diverse sample types to detect one species will be essential and beneficial for any laboratory functioning to apply molecular based, pathogen detection methods, particularly in a low-resource, high-throughput setting.

The objective of this study is to present a DNA extraction method for toxigenic *V. cholerae* which is inexpensive, time efficient, easy to modify based on the template and provide accuracy in results for both, qualitative and quantitative PCR methods. To examine existing DNA extraction protocols, several types of samples were chosen for DNA extraction by three different types of extraction methods. The suitability of the methods was compared by downstream amplification using both qPCR, conventional PCR, sample processing time and cost per sample. This study aimed to assist with identifying a desirable DNA extraction method when there is a high turn-over of specimens requiring testing by molecular methods.

Methods

Design of the study

This study was methodology development set up to analyze a rapid, reproducible and effective DNA extraction method, four different types of samples - i) Pure culture of *Vibrio cholerae* in enrichment media, ii) Spiked water, iii) Spiked phosphate buffered saline and iv) Spiked suspension of rice samples with known quantity of *V. cholerae* were subjected to three DNA extraction methods: a) Boiled template, b) Phenol: Chloroform: Isoamyl alcohol and c) QiaAmp® DNA mini kit (Qiagen GmbH, Hilden, Germany). The methods were compared by evaluating DNA concentration, band intensity on agarose gel electrophoresis (qualitative PCR), cycle threshold value (Ct) along with bacterial yield/quantity (quantitative PCR) and the study was performed in the Environmental Microbiology Lab, Department of Microbiology, University of Dhaka, Bangladesh from March 2015 to May 2015.

Bacterial strain and culture conditions

The reference strain used for optimization of this experiment and spiking of the samples was *Vibrio cholerae* O1 biovar El Tor strain N16961 which possesses *ctxA* gene. Typical colonies from overnight culture of this strain from non-selective nutrient agar media were transferred into 10mL of Alkaline Peptone Water (APW) (1L distilled H_2O, 10 gL^{-1} peptone, 10gL^{-1} sodium chloride; pH 8.5). Bacterial cultures in APW were then incubated at 37°C for 24h.

Sample preparation and spiking

Four different types of samples were taken for experiment - i) 1.0mL of enriched *V. cholerae* bacterial culture in APW, ii) 1.0mL of spiked sterilized distilled water, iii) 1.0mL of spiked phosphate buffered saline (PBS) (1.0L distilled H_2O, 10gL^{-1} NaCl, 0.25gL^{-1} KClgL^{-1}, 1.8gL^{-1} Na_2HPO_4, 0.3gL^{-1} KH_2PO_4; pH 7.4) and iv) 1.0mL of spiked rice suspension. Rice suspension was prepared by homogenization of twenty five grams of boiled rice with 225µl of PBS in a Stomacher Lab Blender (Seward Stomacher® 80, Lab Biomaster, UK). To verify the efficacy of the study, the whole experiment was conducted in duplicate. To keep the volume of the studied sample equal, 990µl each of distilled water, PBS and rice suspension was taken and inoculated with 10µl of overnight *V. cholerae* culture from APW (log_8 cfu/mL).

DNA extraction

Three DNA extraction methods were applied to extract DNA from 1 mL pure culture of *V. cholerae* and the spiked samples. These are - Boiled template method[14], Phenol: Chloroform: Isoamyl alcohol method[15] and QiaAmp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instruction. DNA was extracted in duplicate from each sample and compared. For all three methods, 200µl elution solutions were kept for further use.

Measurement of DNA concentration and purity

The efficiency of the extraction methods was evaluated by the amount and quality of the DNA extracted from the samples using Colibri Microvolume Spectrometer (Titertek-Berthold, Berthold Detection Systems GmbH, Germany). Concentrations of DNA were checked by

measuring the absorbance at 260nm (A260) and 280nm (A280). Purity was determined by calculating the ratio of absorbance at 260nm and at 280nm (A260/ A280).Values between 1.8 and 2.0 for A260/A280 indicate low level of contamination with protein and aromatic substances[16].

Real time PCR

Standard curve calibration and quantification of V. cholerae in spiked samples

QiaAmp® DNA mini kit is a widely accepted method of DNA extraction for diverse bacterial species[17,18,19] and was therefore chosen as the method to generate standard curves for this study. The DNA extracted by using QiaAmp® DNA mini kit were applied as template in standard curve experiment for 24 hour enriched culture of *V. cholerae* positive strain and the concentration of the DNA was measured in Colibri Microvolume Spectrometer (Titertek Berthold, Germany). For standard curve calibration, the DNA was diluted in 10-fold series up to 7 log unit and previously published protocol was followed to calculate the number of cells containing specific target genes[20]. The measured concentration of stock bacterial DNA was 19.224ng/µl which is equivalent to 3.48×10^6 *V. cholerae* cells. The empirically calculated exact gene copy numbers for each dilution were entered in the ABI StepOne™ System software (Life Technologies, USA) for in the standard curve set-up.

The standard curve was generated by plotting the duplicate log value of calculated quantity of cell number per reaction versus Ct value (Figure 1). DNA extracted using three different methods from crude culture, spiked water, PBS and rice DNA samples were run in duplicate as unknown template in the standard curve experiment. The quantity of the *V. cholerae* cells were calculated using StepOne™ software v2.2.2.

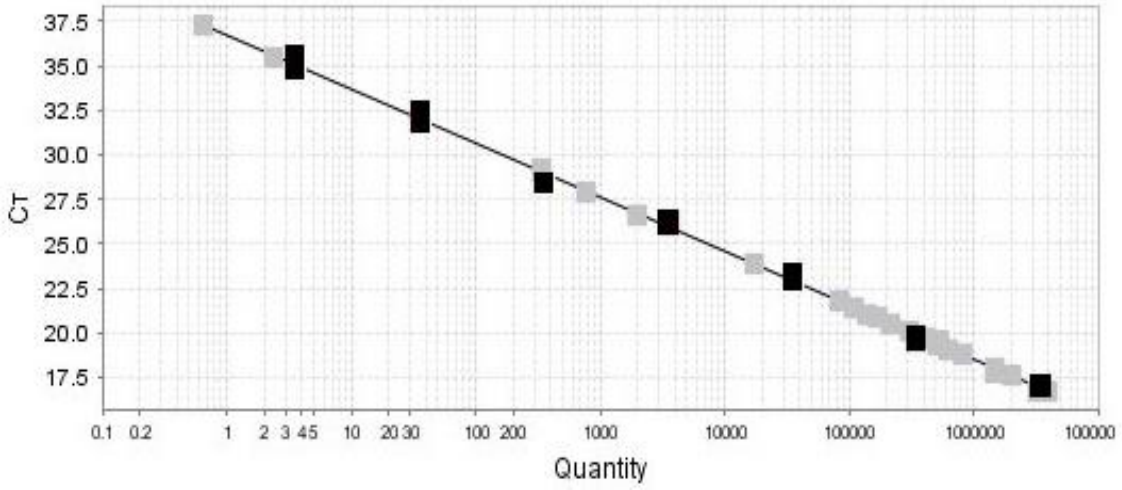

Figure 1: Standard curve for ctxA gene of *V. cholerae* O1 N16961 showing correlation between Cycle threshold (Ct) value with log value of quantity (copy no/reaction) calculated from concentration of serially diluted DNA. ■ Standard ■ Unknown

Reaction protocol of TaqMan q PCR assay

The qPCR flurogenic probe and primers set (Tag Copenhagen A/S, Denmark) for *ctxA* gene detection and quantification were used from a previously described protocol[10]. Sequences of primers and probes are listed in Table I. In short in 25µl reaction mixture there were, 12.5µl 2X TaqMan® Universal Master Mix II with UNG (Applied Biosystems USA, containing AmpliTaq Gold® DNA Polymerase, dNTPs, ROX™ passive reference, Uracil-N glycosylase), 2.5µl of 100nM of each primer, 2.5µl of 250nM probe with 5µl of template.

The qPCR thermal cycling was run on ABI StepOne™ System (Life Technologies, USA) using an initial UNG incubation step at 50ºC for

2.0 minutes and polymerase activation step at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds and a combined anneal and extension step at 60ºC for 1 minute. The other parameters of the machine were set as default for analysis.

Conventional PCR

Reaction protocol

For detection of *ctxA* gene in conventional PCR, the PCR reaction mixture concentrations and conditions were optimized. PCR reactions were conducted in 12.5µl volumes containing 1.0µl 10X PCR buffer including 20mM $MgCl_2$, 0.2µl of 10mM deoxynucleoside triphosphates (dNTP) mix (Thermo Scientific, USA), 0.05µl of 5U Dream Taq DNA Polymerase (Thermo Scientific, USA) per µl and 0.625µl of 25µM each primer (Tag Copenhagen A/S, Denmark). Primers used in this study are noted in Table I.

The PCR reaction cycles was performed as follows: initial denaturation at 95ºC for 5 minutes followed by 95ºC for 1 minute, 55ºC for 45 seconds, 72ºC for 45 seconds with 35 cycles including a final 7 minute extension at 72˚C. For resolving PCR products (band size 308 bp), 1.5% agarose gel in Tris-Acetate EDTA (TAE) buffer was used for electrophoretic separation. The gel was stained in Et-Br staining solution and observed under UV transilluminator (Gel Doc, Bio-Rad, USA). The bands were analyzed with "Quantity One®" software (Bio-Rad, USA). PCR product size was determined using 100 bp DNA size markers (Invitrogen, USA).

The percentage of DNA band density based on intensity pixels was analyzed using ImageJ analysis software pursuing the steps described in the user guide IJ1.46r (http://imagej.nih.gov/ij/docs/guide/user-guide.pdf)

Table I: List of primers and probes used in this study[9,10]

Target gene	Target organism	Assay	Primer	Sequence(5' -3')	Size (bp)	Reference
ctxA	*Vibrio cholerae*	Conventional PCR	ctxA-vct-f	ACA GAG TGA GTA CTT TGA CC	308bp	9
			ctxA-vct-r	ATA CCA TCC ATA TAT TTG GGA G		
ctxA	*Vibrio cholerae*	TaqMan qPCR	ctxA-f	TTT GTT AGG CAC GAT GAT GGA	84 bp	10
			ctxA-r	ACC AGA CAA TAT AGT TTG ACC CAC TAA		
			Probe	FAM-TGT TTC CAC CTC AAT TAG TTT GAG AAG TGC CC-BHQ-1		

Statistical analysis

Ct value mean difference for each method independently of sample type was analyzed by ANOVA /analysis of variance test. Statistical analysis was performed by using SPSS version 22.0 for windows, SPSS Inc. (http://www.spss.com).

Results

Results for the performance of the DNA extraction methods are described by quantity of DNA, cost per reaction, time per reaction and quality of DNA. The total processing time for eight samples was 90 minutes for Boiled template method, 270 minutes for Phenol: Chloroform: Isoamyl alcohol method and 70 minutes for QiaAmp® DNA mini kit. Processing cost per sample by boiled template method was the least expensive (0.16 USD/sample) compared to other two methods (Table II).

Table II: Comparison of three DNA extraction methods based on processing time, required reagents, DNA concentration, purity and cost

Extraction method	Cost/ sample (in USD)	Processing time (hr:min)[a]	Reagents	Purity of DNA (A260/A280) of 1 mL *V. cholerae* culture
Boiled template method[14]	0.16	01: 30	DEPC water	1.74
Phenol: Chloroform: Isoamyl alcohol method[15]	1.02	04:30	Solution I : 50mM Tris-HCl buffer (pH 7.5) containing 20% (wt/vol) sucrose Solution II: 50mM NaCl, 1% (wt/vol) sodium dodecyl sulfate (SDS), 200µg of proteinase K/mL Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution TE buffer: (10mM Tris-Cl [pH 7.5], 1mM EDTA)	1.98
QiaAmp® DNA mini kit (Cat. No 51306)	5.04	01:10	Kit, phosphate buffer saline, molecular grade alcohol	2.10

*[a] Time required to process a batch of 8 samples

In spectrometer, Phenol: Chloroform: Isoamyl alcohol method rendered the highest concentration of DNA (average 28.76ng/µl) for pure culture of *V. cholerae*. In water samples, the DNA concentrations remain lower for all three methods compared to PBS and rice (Table III). The spiked rice samples displayed highest concentration of DNA, even more than crude culture in all methods. Additional presence of plant DNA with the spiked bacterial DNA may explain the reason behind the high DNA concentration in rice samples. However, this result did not interfere in our study findings. The purity of the DNA ranged from 1.50 to 3.00 at absorbance level A260/A280. No method showed purity level specifically between 1.80 and 2.00 for all type of samples.

A standard curve was generated with a duplicate of 7-$\log_{10}$ fold dilution series for each of *V. cholerae* DNA templates using estimated copy no. per reaction (Figure 1). The efficiency of the reaction was 110% as calculated by StepOne™ software v2.2.2. The R^2 value of the standard curve was >0.997. The qPCR assay (TaqMan) displayed positive signals for *ctxA* (*V. cholerae*) in all the pure culture and spiked samples. No signal was detected for any template controls (NTC). The StepOne software calculated the quantity of an unknown target from the slope of the standard curve.

Phenol: Choloform: Isoamyl alcohol extraction method depicted lowest Ct values for detection of *ctxA* gene in pure culture, spiked water and spiked PBS samples which are in average of 14.31, 22.83 and 16.75 respectively (Table III). However, for rice suspension samples, the QiaAmp® DNA mini kit extraction method gave the lowest Ct value (average 16.7). The boil template method worked adequately on different kind of samples except for spiked water samples (average Ct value 34.19) which is just above the cut off level (Ct 35). As Ct value increases with a decreasing amount of template, the quantity of cell number changed according to Ct values. The initial cell number/reaction which were calculated as unknown in the pure culture, spiked water, PBS and rice were in average of $\log_6$, $\log_4$, $\log_4$ and $\log_5$ from the DNA templates of Phenol: Chloroform: Isoamyl alcohol extraction method respectively. Boiled DNA template

recovered least amount of DNA from spiked water samples (average $\log_1$ cells/reaction). For all four types of samples (three methods) the cycle threshold values ranged between averages of 14 to 34 which are within detectable limit. All the results on Ct value, quantity are reported in Table III. The P value from ANOVA /analysis of variance test did not show any evidence for difference for Ct value mean for each method regardless of sample type (p = 0.659).

Table III: Comparison of three extraction methods based on DNA concentration, Ct value and quantity obtained in crude culture, spiked water, PBS and rice

Sl	Samples	Concentration (ng/µl)			Ct value			Quantity (copy no/reaction)		
		QiaAmp® Kit	Boil template	Phenol: Chloroform: Isoamyl alcohol	QiaAmp® Kit	Boil template	Phenol : Chloroform: Isoamyl alcohol	QiaAmp® Kit	Boil template	Phenol :Chloroform:Isoamyl Alcohol
1	Crude culture of *V. cholerae*	22.14	23.235	28.76	14.92	14.80	14.31	2.55E+06	2.66E+06	3.66E+06
2	Spiked Water	03.94	02.395	1.47	26.19	34.19	22.83	5.60E+02	1.607	1.03E+04
3	Spiked PBS	06.48	13.845	11.41	18.08	16.70	16.76	2.28E+05	6.28E+05	5.89E+05
4	Spiked rice	42.44	27.32	43.82	16.7	17.79	19.05	1.79E+06	2.86E+05	1.10E+05

Each value corresponds to the mean of two reactions

The band densities given by the gel image of conventional PCR were also evaluated for three extraction methods with different samples. The highest average percentage of band density was found for the PCR products from the crude (17.63%) which were extracted by Phenol: Chloroform: Isoamyl alcohol method. But for spiked PBS and rice, boiling method resulted in highest band density (18.26% and 18% respectively). The boiled DNA templates from water samples rendered 7.36% of mean density which were visually not detectable (Figure 2). Band intensity results are given in Table IV.

Table IV: Mean band intensity (%) compared by three extraction methods evaluated by conventional PCR

Samples	Band intensity (%)		
	QiaAmp® Kit	Boiled template	Phenol : Chloroform: Isoamyl alcohol
1mL culture of *V. cholerae* from APW	15.16	17.21	17.63
Autoclaved spiked water by *V. cholerae*	28.26	7.36	14.38
Spiked PBS by *V. cholerae*	18.24	18.26	16.98
Spiked rice by *V. cholerae*	14.53	18	17.46

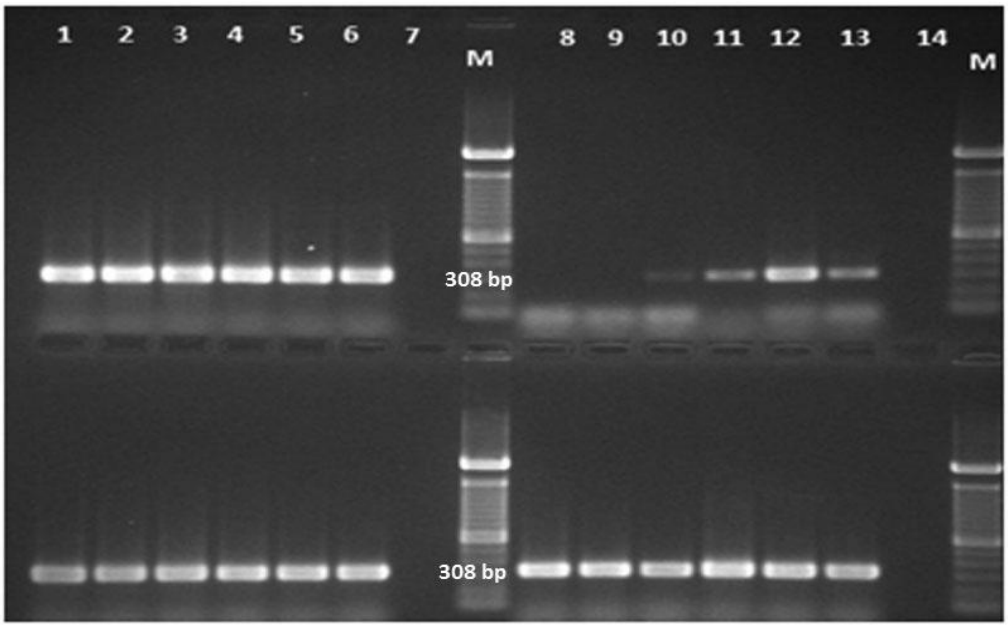

Figure 2: Detection of *V. cholerae ctxA* gene (308 bp) by conventional PCR. Upper gel lane 1-7 crude DNA templates extracted by three methods, lane 1, 2 - boiling, lane 3, 4 - Phenol method, lane 5, 6 kit method, lane 7 - NTC,100 bp Marker (Invitrogen, USA), lane 8-14 spiked water samples, lane 8, 9 - boiling, lane 10, 11 Phenol method, lane 12, 13 - kit method, lane 14 - NTC, Marker, Lower gel lane 1 - 7 spiked PBS samples and lane 8 - 14 spiked rice samples.

Discussion

As molecular methods continue to evolve for detection and quantification of toxigenic *V. cholerae*, the need for rapid processing of samples and recovery of DNA is a crucial step. The result of this study attributed a suitable DNA extraction method if the sample size is large with different specimen types.

In our study, we targeted four sample types (crude culture and *V. cholerae* spiked water, PBS and rice suspension) and observed the applicability of three different extraction methods for *V. cholerae* based on cost, processing time and detection ability in quick PCR based methods.

Previous reports have showed that QiaAmp® DNA mini kit and modifications of boiled template were used for PCR based detection of *V. cholerae* as well as other *Vibrio* species from food, clinical and environmental samples[10,21,22,23,24]. Phenol: Chloroform: Isoamyl alcohol method was used for water samples to detect *Shigella* spp. and as per our knowledge; this method has not been used for quantitative PCR based detection of *V. cholerae*[15].

All the three methods rendered mean DNA concentration values between 20-30 ng/µl for crude culture of *V. cholerae*. The QiaAmp® DNA mini kit showed highest DNA concentration in water and rice samples whilst the boil template method gave the highest for PBS samples. To our knowledge, there is no assessment comparing the DNA concentration of spiked food with crude bacterial DNA. The reason behind the higher DNA concentration level in rice than the crude culture may be the presence of rice DNA together with *V. cholerae* cells.

The Ct values and quantity ($\log_6$ cells/reaction) in crude DNA templates were indistinguishable for all the three methods (Table III). The only significant differences on Ct values and quantity were found for boiled DNA of spiked water samples which gave the lowest cell count with highest Ct value. Factors like low DNA concentrations, presence of PCR inhibitors, persistence of *V. cholerae* in water samples may influence the outcome of microbial quantification by boiled template method. In the conventional PCR assay, the boiled DNA templates

showed no detectable bands for both of the samples. So these results implicate that all the three methods potentially worked on more sensitive and specific quantitative probe based method to detect *V. cholerae* cells from four experimental samples. However the applicability of boiling method for analysis of water samples containing very low concentration of *V. cholerae* DNA using conventional PCR is questionable. Working with highly polluted samples may not affect the results.

From the Table II, the highest cost per test with the lowest processing time was found for kit method. Boiled template method[14] needs minimal cost as the only reagent required is the DEPC treated water and the required time is also lower. The disadvantage of this method is inadequate clean-up of the PCR inhibitors like proteins, lipids and other cellular debris which potentially can decrease PCR efficiency[25]. The sample processing time for Phenol: chloroform method is distinguishably higher because of long incubation times and preparation of solutions, although the cost is almost three times lower than using a commercial kit. The use of phenol may cause problems in inhalation and irritation to skin which involves efficient laboratory protection during work. The QiaAmp® DNA mini kit also requires the use of irritating substances according to manufacturer's instruction.

Following the results of this study, though Phenol: Chloroform: Isoamyl alcohol method showed highest Ct values for three types of samples except rice samples, use of hazardous chemicals and utilization of comparatively more time make this method inappropriate when handling large number of samples. In other words, boiled template is most rapid, reproducible, low cost method with average detection capability in PCR based methods from food and environmental samples. Hence, this may be a method of choice when working within budgetary constraints and require assessing high number of samples on a routine basis. If budget and time is not a limiting factor, QiaAmp® DNA mini kit will be an ideal method of choice for all specimen types.

Acknowledgement

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Manuscript 2

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ORIGINAL PAPER

Survival of *Vibrio cholerae* O1 on fomites

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Abstract It is well established that the contamination sources of cholera causing bacteria, *Vibrio cholerae*, are water and food, but little is known about the transmission role of the fomites (surfaces that can carry pathogens) commonly used in households. In the absence of appropriate nutrients or growth conditions on fomites, bacteria have been known to assume a viable but non-culturable (VBNC) state after a given period of time. To investigate whether and when *V. cholerae* O1 assumes such a state, this study investigated the survival and viable quantification on a range of fomites such as paper, wood, glass, plastic, cloth and several types of metals under laboratory conditions. The fomites were inoculated with an outbreak strain of *V. cholerae* and its culturability was examined by drop plate count method at 30 min intervals for up to 6 h. For molecular detection, the viable/dead stain ethidium monoazide (EMA) which inhibits amplification of DNA from dead cells was used in combination with real-time polymerase chain reaction (EMA-qPCR) for direct quantitative analyses of viable *V. cholerae* at 2, 4, 6, 24 h and 7 day time intervals. Results showed that *V. cholerae* on glass and aluminum surfaces lost culturability within one hour after inoculation but remained culturable on cloth and wood for up to four hours. VBNC *V. cholerae* on dry fomite surfaces was detected and quantified by EMA-qPCR even 7 days after inoculation. In conclusion, the prolonged survival of *V. cholera*e on various household fomites may play vital role in cholera transmission and needs to be further investigated.

Keywords EMA-qPCR · Fomites · Survival · VBNC · *Vibrio cholerae*

Introduction

Cholera, an acute diarrheal disease, poses a serious threat to public health globally particularly in developing countries causing 28,000–142,000 deaths worldwide from a total 1.4–4.3 million reported cases (Ali et al. 2012). Outbreaks of cholera, mostly caused by *V. cholera*e O1 and O139 Bengal which possess cholera toxin gene can take place mostly in overcrowded areas with inadequate sewage treatment infrastructure, water and sanitation facilities and poor hygiene practice (Ali et al. 2015).

The spreading of cholera mainly occurs from ingestion of contaminated water and food from environmental or household source (Glass et al. 1991; Lim 2001; Pollitzer et al. 1959; Sack et al. 2004; Snow 1855). Direct transmission can also result from exposure to contaminated inanimate objects or household surfaces within a household cluster and thus may lead to epidemics (Cholera vaccines: WHO position paper 2010; Glass et al. 1991; Glass and Black 1992; Mandal et al. 2011; Pollitzer et al. 1959; Sugimoto et al. 2014). Previous reports evidenced that *V. cholerae* O1 could survive on contaminated cooking or eating utensils and clothing depending on moisture content (Glass and Black 1992). Conventional culture and enrichment based studies show the survival of *V. cholerae* on various fomites for several days (Barua 1970). However

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these reports have overlooked the viability of *V. cholerae* cells that have lost their ability to grow on culture media.

Vibrio cholerae undergoes morphological changes and enters into a viable but non-culturable (VBNC) state in the environment when conditions are not conducive for active growth and survival (Colwell 2009; Xu et al. 1982). *Vibrio cholerae* in the VBNC state pose a serious health hazard as the bacteria can revert to culturable state as demonstrated by previous studies on human volunteers. Human volunteer challenged with 10^6 to 10^8 *V. cholerae* cells in the VBNC state developed diarrhea within two to several days and culturable form of that *V. cholerae* strain were isolated from their stool (Colwell et al. 1996). Passage of *V. cholerae* through human intestine may give rise to *V. cholerae* with enhanced epidemic potential through providing increased transmissibility (Merrell et al. 2002). Infectious dose of *V. cholerae* shed in human stool in hyperinfectious state show lower infectious dose (around one-fifth) than *V. cholerae* grown in vitro (Butler et al. 2006; Nelson et al. 2008; Zahid et al. 2008). Although fomites possess the potential for cholera transmission, little is known about survivability of *V. cholerae* on fomites, most importantly viable but non-culturable *V. cholerae*.

Inability to assess viability status by routine bacteriological culture method complicates studying health hazards posed by the non-culturable strains. The fluorescent antibody (FA) technique using epifluorescence microscopy is widely used for the detection of viable cells in the natural environment (Huq et al. 1983; Islam et al. 1990, 1993). Nucleic acid based molecular analysis for viability determination is based on the presence of the intact DNA or use of mRNA as a marker of viability or analysis of membrane integrity or cellular activity (Keer and Birch 2003). Both, the microscopic and PCR based techniques lead to difficulty with reproducibility and measurement of accurate number of viable cells (Nocker and Camper 2006). Detection of viability by covalent binding of viable/dead stain ethidium monoazide (EMA) in dead cell DNA in combination with real-time PCR (EMA-qPCR) is a novel quantitative and sensitive approach (Nocker and Camper 2006; Nogva et al. 2003; Rudi et al. 2005). EMA penetrates only dead cells with damaged membrane/cell wall systems after subsequent photoactivation and thus employs inhibition of amplification of DNA from dead cells in qPCR. DNA from dead cells is removed during DNA extraction process along with other cell debris (Nocker and Camper 2006). EMA-qPCR has been developed for direct quantitative analyses of the fraction of viable *E. coli* O157: H7, *Salmonella* spp. and *Listeria monocytogenes* (Nogva et al. 2003). This technique was also applied for enumeration and distinction of *Campylobacter jejuni* in complex samples with mixed bacterial populations (Rudi et al. 2005).

The presence and persistence of *Vibrio cholerae* on fomites was investigated in the current study by examining the survival pattern of *Vibrio cholera*e O1 N16961by culture and molecular methods. These fomites included paper, wood, glass, plastic, cloth, iron, aluminum and steel and were selected due to their presence in common household items such as toys, doorknob, clothing, utensils etc. This study is pivotal to understanding the contamination and potential health-risk posed by common household surfaces. Moreover, in this study, the fraction of viable *Vibrio cholerae* O1 on fomites was quantified by EMA treatment followed by real-time PCR targeting *ctxA* gene for several time intervals.

Materials and methods

Bacterial strain and culture conditions

The clinical strain *Vibrio cholerae* O1 El Tor N16961 possessing *ctxA* gene used in this study was obtained from International Centre for Diarrheal Disease Research, Bangladesh (icddr, b). The strain was grown overnight on TCBS agar (Thiosulfate Citrate Bile Salt Sucrose; Oxoid, UK) at 37 °C. One loopful of culture with 10 µL inoculation loop was inoculated into Alkaline Peptone Water (APW) (1L distilled H_2O, 10 g L^{-1} peptone, 10 g L^{-1} sodium chloride; pH 8.5) and incubated at 37 °C for 22 h. The cells were then harvested by centrifugation for 10 min at 14,000*g* and the bacterial pellet was re-suspended in 10 mL of phosphate buffered saline (PBS). Absorbance of the bacterial suspension at 600-nm wavelength was adjusted to a reading of 0.193, which corresponded to about 1.66×10^8 cfu mL^{-1}.

Preparation and spiking of fomites

In this study, eight fomites were tested for survivability of *Vibrio cholerae* which includes cotton cloth, wood, paper, glass, plastic, stainless steel, iron, and aluminum. The fomites were cut into small pieces (1×1 cm^2). Two pieces of fomites (a total of 36 pieces of each type) were placed in a petri dish and autoclaved. After drying, 50 µL of PBS suspension containing approximately 10^8 cfu mL^{-1} *V. cholerae* were inoculated onto each piece. The fomites were also inoculated with 50 µL of PBS solution without *V. cholerae* culture and used as no-template control. For better recovery, this large volume of culture suspension was used. The fomites were kept in the dark at room temperature for further investigation.

Determination of culturability, resuscitation of *Vibrio cholerae*

Culturability was determined by drop plate count method at 30 min interval up to 6 h. Immediately after inoculation, three pieces of each spiked fomites were picked with sterile forceps and transferred into three separate McCartney bottles, each containing 2 mL 0.85 % normal saline solution. The bottles containing spiked fomites were vortexed vigorously and subjected to ten-fold serial dilution. Thereafter, in total volume of 30 µL, these fomite suspensions from different dilutions were dispensed as 10 µL drops on Nutrient agar (Oxoid, UK). After overnight incubation at 37 °C, the bacterial colonies were counted to calculate colony-forming units per cm^2. The colonies from Nutrient agar were further streaked on to TCBS agar to confirm identity as *V. cholerae*. Randomly selected yellow flat colonies from TCBS agar plates were further analyzed by *V. cholerae* specific PCR targeting outer membrane protein (ompW) (Nandi et al. 2000).

Resuscitation

Spiked fomites after 6 and 24 h incubation were introduced to APW media for resuscitation of viable bacteria. After 24 h incubation at 37 °C, broth culture was streaked on to both TCBS and Nutrient agar media and left for 24 h incubation at 37 °C. After incubation, the plates were examined for the growth of *V. cholerae* by naked eye.

Assessment of bacterial viability

EMA cross-linking assay

Suspension in normal saline was prepared from inoculated fomites at 2, 4, 6, 24 h and 7 day time intervals. The fluorescent stain ethidium monoazide bromide (EMA) (Thermo Fisher Scientific, USA, Cat no-E1374) was prepared for an initial stock solution (5 mg mL^{-1}) by adding nuclease free water and kept in dark at −20 °C until further use. Following the protocol of previous study, EMA solution was added to 1 mL of bacterial suspension in a dark Eppendorf tube (Eppendorf, Germany) to achieve a final concentration of 100 µg EMA/mL for effective photolysis (Nogva et al. 2003). The samples were then incubated for 5 min in the dark and subsequently exposed to light for 1 min by exposure to a 500-W halogen light bulb from a 20 cm distance from the light source. Before light exposure, the sample tubes were chilled in ice to minimize excessive heat (Rudi et al. 2005). The sample suspensions were then centrifuged for 10 min at 14,000 rpm to recover the pellet for DNA extraction.

DNA preparation

DNA was extracted from EMA treated fomite suspension by Boiled template method (De Medici et al. 2003).

Quantification by qPCR

The real time quantitative PCR was employed for amplification of cholera toxin gene *ctxA* of *Vibrio cholerae*. In short, 25 µL reaction mixture was prepared with 12.5 µL 2X TaqMan® Universal Master Mix II with UNG (Applied Biosystems USA, containing AmpliTaq Gold® DNA Polymerase, dNTPs, ROX™ Passive Reference, Uracil-N glycosylase), 2.5 µL of 100 nM of primers sense (5′ TTT GTTAGGCACGATGATGGAT-3′) and anti-sense (5′-AC CAGACAATATAGTTTGACCCACTAAG-3′) (Blackstone et al. 2007), 2.5 µL of 250 nM *ctxA* specific probe (5′-TG TTTCCACCTCAATTAGTTTGAGAAGTGCCC-3′) (Blackstone et al. 2007) 5′ and 3′ labeled with FAM reporter dye and BHQ1 quencher respectively. Five microlitre of extracted DNA was used as template. All primers and probes were purchased from Tag Copenhagen A/S, Denmark. The qPCR thermal cycling was performed on ABI StepOne™ System (Life Technologies, USA) with reaction parameters of initial UNG incubation step at 50 °C for 2 min and polymerase activation step at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The other parameters of the machine were set as default for analysis. Enterotoxigenic *Escherichia coli* strain AN33859 and nuclease free water were employed as negative and no template control respectively.

For relative quantification of viable cells, a standard curve experiment was performed at each thermal amplification reaction. The DNA extracted from *ctxA* positive control stain *V. cholerae* N16961 was used as template. The stock DNA was serially diluted up to $7\log_{10}$ in duplicate. The copy number in reaction template was calculated from measured DNA concentration (37.94 ng to 0.037 pg) as the genome mass is equivalent to concentration. ABI StepOne™ System software v2.2.2 (Life Technologies, USA) was used to generate the standard curve by plotting the duplicate log value of the calculated copy number per reaction versus the cycle threshold (Ct) value (Fig. 1). The limit of detection was restricted to 0.037 pg (equivalent copy number per reaction 1.47). The copy numbers of viable *V. cholerae* in fomites samples were estimated from this experiment under similar reaction conditions.

Statistical analysis

Linear regression analysis (95 % CI) was performed to compare the relationship of cfu with time of fomites. The

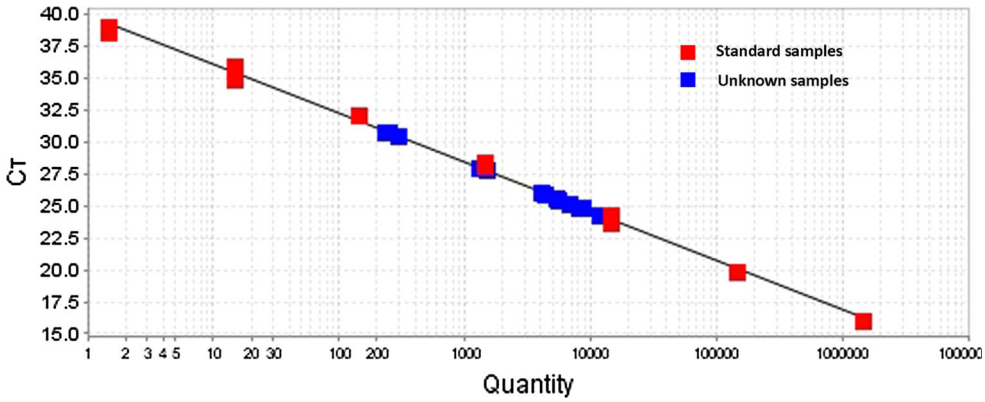


Fig. 1 Cycle threshold versus quantity standard curve in *ctx*A qPCR to quantify viable *V. cholerae* in fomites

calculation was done by using the following formula, the dependent variable (cfu) = Y intercept + regression coefficient (b) × the independent variable (time). Bivariate correlations between cfu and copy number with time interval was also analyzed. The mean differences were considered statistically significant at p value less than 0.05. Graphical and statistical analyses were performed using Microsoft Excel (2007) and SPSS version 16.0 for windows.

Results

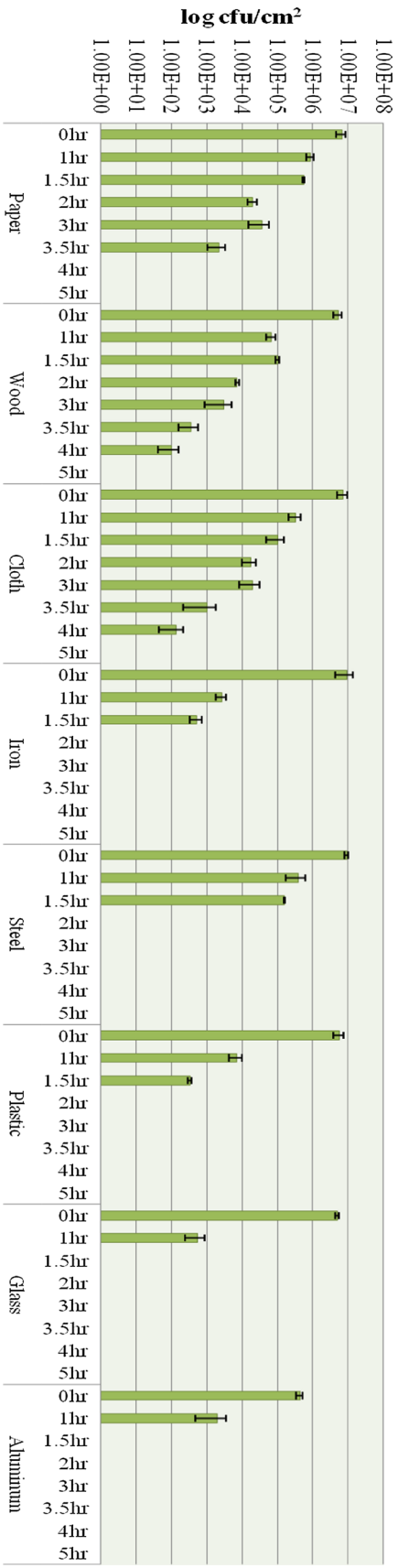
As shown in Fig. 2, *Vibrio cholerae* showed declining culturability during exposure on fomites with time. Among the eight fomites, longest and shortest survival times during which viable growth of *V. cholerae* was found were in ascending order: aluminum, glass, steel, plastic, iron, paper, wood and cloth. *Vibrio cholerae* remained culturable longest on cloth and wood (up to 4 h following inoculation) and shortest (within one hour) on glass and aluminum. A distinct reduction in bacterial numbers of *V. cholerae* from an initial count of 10^6 cfu (0 h after fomite inoculation) was found on per cm^2 of iron and plastic fomites resulting in a decline of 4-log cycle following 1.5 h incubation and thereafter, became non-culturable. After 1.5 h fomite inoculation, *V. cholerae* on stainless steel caused ten-fold reduction and then entered VBNC state. Following one hour exposure to glass and aluminum, cfu of *V. cholerae* declined by 4-log and one-log cycle respectively and finally became non-culturable. *Vibrio cholerae* from none of the fomites resuscitated in APW followed by plating on TCBS after 6 and 24 h inoculation.

EMA-qPCR experiment targeting *ctxA* gene detected and quantified viable cell fraction from dry fomite surfaces at 2, 4, 6, 24 h and even 7 days time intervals. As dead or compromised cells were selectively removed during DNA extraction procedure, DNA from non-viable cells did not affect the results. The efficiency of PCR reactions ranged between 90 and 100 %. The cycle threshold (Ct) values increased proportionally with the decreased number of viable cells (Fig. 3). The results in Fig. 3 showed that copy number per reaction of viable *V. cholerae* exposed on the dry fomite surfaces decreased with time and declined significantly after 7 days. Following 2 h period of fomite inoculation, copy number per reaction was almost 10^3 and reduced further by one-to two log-cycles after 7 days. The ascending order of viable *V. cholerae* copy number per reaction after the 7 day period was: paper, iron, steel, cloth, wood, plastic, glass and aluminum. The linear regression analysis test revealed a significant reduction of cfu count with time (significance, $p < 0.001$, R square value = 0.318, regression coefficient, b = −797,490.801, Y intercept = 2.793E6). The Correlation analysis showed significant negative correlation between both cfu and copy number with time ($p < 0.05$) (Table 1).

Discussion

This study reveals, for the first time, the capacity of *Vibrio cholerae* to enter the VBNC state when exposed to fomites and its ability to resuscitate in suitable enrichment media such as APW. This study also quantified VBNC state-cells by EMA-qPCR. These results therefore enable us to detect and enumerate bacterial load in VBNC state on fomites. A significant finding on its own, this research further provides an avenue to accurately establish infectious doses that lead to infection in humans and identify the true burden of a pathogen within a household. A dose of 10^8 to 10^{11} cfu of *V. cholerae* is required to successfully infect healthy host, though infectious dose may lower to 10^4 to 10^8 when *V. cholerae* ingested with food (Cash et al. 1974a, b; Drasar 1995; Levine et al. 1981; Nelson et al. 2009).

Vibrio cholerae maintained culturability longer (about 4 h) on cloth, wood and paper because the surface of these fomites are porous which might allow bacteria to retain moisture for some more time, therefore might be culturable



◀**Fig. 2** Culturability of *V. cholerae* O1 N16961 from fomites on Nutrient Agar. The *error bars* represent standard error from estimated standard deviations of three independent replicates. *SE values ranges from ± 4,725,815.62 to ± 33.23

(Kramer et al. 2006; Makison and Swan 2006; Taylor et al. 2013; Williams et al. 2005). On the other hand, surface texture of glass, plastic and various metals are smooth which does not offer crevices or passages, hence, bacteria may lose moisture faster and sooner enter VBNC state. Previous studies have showed that plate count from cutting board made up of wood was higher than other metal or plastic boards, even after sufficient cleaning (Abrishami et al. 1994; Milling et al. 2005). Previous studies have reported that transfer rates of microorganisms from hard, nonporous surfaces to hands, eventually to the mouth were more efficient than from porous surfaces (Rusin et al. 2002). Contaminated fomites containing infectious doses of pathogens can serve as reservoirs of bacteria and viruses that can easily transmit to the hands through direct contact, which in turn can be easily transferred to the mouth (Pittet et al. 2006; Rusin et al. 2002).

Culture-independent analysis using EMA-qPCR methodology for viable-cell detection revealed that *Vibrio cholerae* remained viable on fomites even after 7 day. Despite the methodological differences, these findings were consistent with those of previous studies where it has shown the viable persistence of *Shigella dysenteriae* type I, *Campylobacter jejuni* and other waterborne and nosocomial pathogens for several days (Islam et al. 2001; Kramer et al. 2006; Milling et al. 2005; Rollins and Colwell 1986). The reduction of cfu and viable copy number with time among the fomites was not followed gradual pattern which is also reflected in their moderate to weak correlation ($r < 0.8$). Though *V. cholerae* lost culturability faster on glass and aluminum within one hour after inoculation, the fraction of viable cell copy number per reaction after 7 day obtained by EMA-qPCR was higher on glass and aluminum which reveals poor relation between cfu count and copy number per reaction upon exposure to the same type of object under same conditions for defined period of time.

The role of viable and persistent *Vibrio cholerae* on common constituents of daily utensils and equipment of household, food industry and hospitals cannot be overlooked. Glass, plastic and metals are widely used as household utensils, wood and plastic used as cutting boards which would allow entry of *V. cholerae* in human body directly through fecal-oral route which is the main route of entry of *V. cholerae* for causing disease. Survival of *V. cholerae* for extended period of time on these fomites may play a previously undocumented but crucial role in cholera

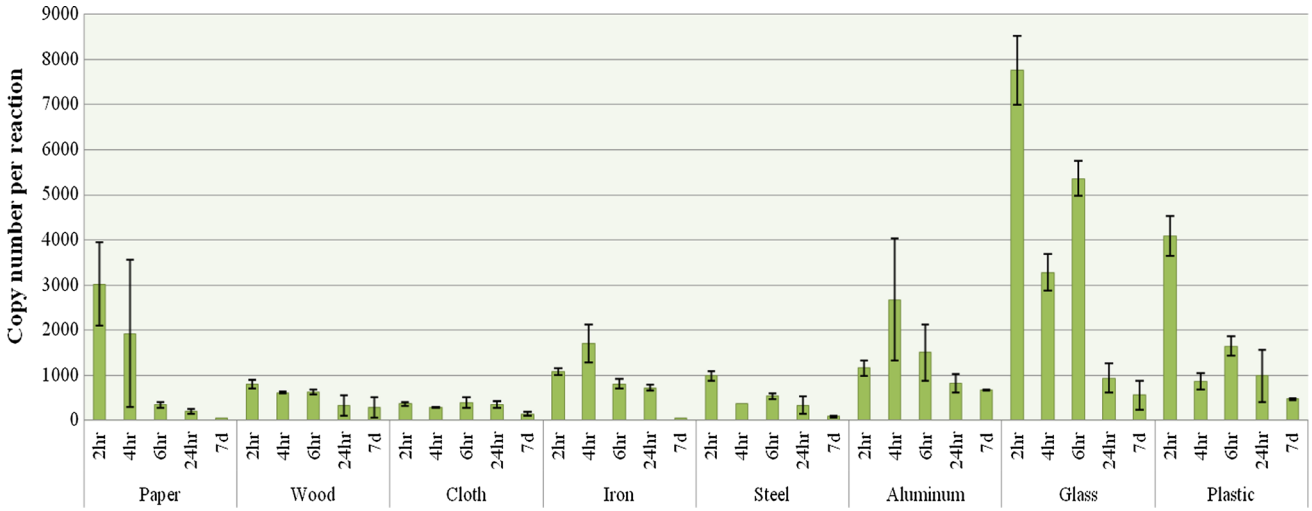


Fig. 3 Copy number per reaction bars from quantification of EMA treated viable *V. cholerae* O1 N16961 from fomites. *Error bars* in diagram represent standard error from estimated standard deviations of two independent replicates. *SE values ranges from ± 1636.03 to ± 8.75

Table 1 Results of correlation analyses

Variables	Pearson correlation coefficient (r)	Significance (two tailed)
cfu count, time	−0.567	0.000
copy number, time	−0.341	0.031

epidemiology because the attenuated stages are capable of reverting to a virulent form and contribute to the transmission of cholera (Mishra et al. 2012). Bacterial survival on paper currency is another potentially serious transmission route due to frequent exchange between individuals (El-Din El-Dars and Hassan 2005; Vriesekoop et al. 2010). Prolonged survival of pathogens on clinical surfaces, medical equipment and health care workers' hand raises patient safety concern and requires proper disinfection to prevent transmission (Noskin et al. 1995; Reuter et al. 2002; Sehulster and Chinn 2003). Surface of food processing area, equipment and utensils used, personal clothing's harboring infectious microorganism pose risk of food contamination resulting in outbreaks of food-borne diseases and economic loss to food industry (Neely and Maley 2000; Noskin et al. 2000; Scott and Bloomfield 1990; Todd et al. 2008, 2009). Moreover, *V. cholerae* clustered in household is an important route of overall transmission of cholera (Eisenberg et al. 2013; Kendall et al. 2010; Sugimoto et al. 2014).

This study shows that *Vibrio cholerae* cells lost culturability within short periods of time (within 4 h) after drying on fomites, though its viability is retained for more than 7 days. Roszak and Colwell suggested that VBNC state prolongs the survival of *V. cholerae* when conditions are less favorable for cell growth and multiplication (Roszak and Colwell 1987). It is always an issue of intense interest where *V. cholerae* persists over time retaining the potential to release and cause human infection between the outbreaks of cholera. *Vibrio cholerae* in the VBNC state on fomites within household could serve as reservoir and facilitates the dissemination of *V. cholerae* during interepidemic period which demands further survival studies for longer period of time.

In conclusion, the fomites contaminated with clinical *Vibrio cholerae* O1 retained the pathogenicity as the cells did not lose the potent cholera toxin gene *ctxA* in the nonculturable state over longer time period. The quantitative data generated from our study can be used for risk assessment and intervention of cholera transmission associated with fomites. The findings from this study will enable public health analysts and policy makers in implementing a modified but more effective hygiene message to the community at large, one that includes hygiene of fomite-surfaces. Further research is required to investigate the risk of infection in a wider range of fomites and the presence of other bacterial pathogens.

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Compliance with ethical standards

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

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Transmission and Toxigenic Potential of *Vibrio cholerae* in Hilsha Fish (*Tenualosa ilisha*) for Human Consumption in Bangladesh

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Fish have been considered natural reservoirs of *Vibrio cholerae*, the deadly diarrheal pathogen. However, little is known about the role of fish in the transmission of *V. cholerae* from the Bay of Bengal to the households of rural and urban Bangladesh. This study analyzes the incidence and pathogenic potential of *V. cholerae* in Hilsha (*Tenualosa ilisha*), a commonly caught and consumed fish that exhibits a life cycle in both freshwater and marine environments in Bangladesh. During the period from October 2014 to October 2015, samples from the gills, recta, intestines, and scale swabs of a total of 48 fish were analyzed. The fish were collected both at local markets in the capital city Dhaka and directly from fishermen at the river. PCR analysis by targeting *V. cholerae* species-specific *ompW* gene revealed that 39 of 48 (81%) fish were positive in at least one of the sample types. Real-time PCR analysis demonstrated that the cholera-causing *ctxA* gene was detected in 20% (8 of 39) of *V. cholerae*-positive fish. A total of 158 *V. cholerae* isolates were obtained which were categorized into 35 genotypic groups. Altogether, 25 O1 and 133 non-O1/O139 strains were isolated, which were negative for the cholera toxin gene. Other pathogenic genes such as *stn/sto*, *hlyA*, *chxA*, *SXT*, *rtxC*, and *HA-P* were detected. The type three secretion system gene cluster (TTSS) was present in 18% (24 of 133) of non-O1/O139 isolates. The antibiotic susceptibility test revealed that the isolates conferred high resistance to sulfamethoxazole-trimethoprim and kanamycin. Both O1 and non-O1/O139 strains were able to accumulate fluid in rabbit ileal loops and caused distinctive cell death in HeLa cell. Multilocus sequence typing (MLST) showed clonal diversity among fish isolates with pandemic clones. Our data suggest a high prevalence of *V. cholerae* in Hilsha fish, which indicates that this fish could serve as a potential vehicle for *V. cholerae* transmission. Moreover, the indigenous *V. cholerae* strains isolated from Hilsha fish possess considerable virulence potential despite being quite diverse from current epidemic strains. This represents the first study of the population structure of *V. cholerae* associated with fish in Bangladesh.

Keywords: fish, Hilsha, *Vibrio cholerae*, transmission, population, pathogenic potential, Bangladesh

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INTRODUCTION

Cholera, caused by *Vibrio cholerae*, remains a major burden in most Asian and African developing countries. Human cholera epidemics have been mostly caused by *V. cholerae* toxigenic serogroups O1 and O139 which express two principal virulence factors, cholera toxin (CT) and the colonization factor known as toxin-coregulated pilus (TCP) (Faruque et al., 1998; Harris, 2012). The other serogroups, collectively referred to as non-O1/O139 serogroups are mostly nonpathogenic, environmental isolates that express other O antigens (Dziejman et al., 2005). However, some non-O1/O139 *V. cholerae* are clearly pathogenic and responsible for acute cholera-like diarrhea (Ramamurthy et al., 1993; Sharma et al., 1998) and a variety of extra-intestinal infections (Morris Jr and Black, 1985). Despite the lack of cholera toxin, a few pathogenic non-O1 and non-O139 strains such as O141, O10 and O12 have caused outbreaks of gastroenteritis (Bagchi et al., 1993; Dalsgaard et al., 1995; Rudra et al., 1996). Recently, genomic analysis has demonstrated that non-O1/O139 strains contributed to the early cholera outbreak in Haiti as the sole pathogen for potentially a high proportion of cases (Hasan et al., 2012). However, studies have indicated that some of the potential virulence factors such as hemagglutinin protease, repeats-in-toxin, mannose-sensitive haemagglutinin, heat-stable enterotoxin, hemolysin and type III secretion system (T3SS) are essential for the diarrheagenic mechanism of non-O1/non-O139 (Nair et al., 1988; Thelin and Taylor, 1996; Rivera et al., 2001; Dziejman et al., 2005). Animal models have been extensively used to study the pathophysiology of diarrhea caused by CT of *V. cholerae* that adheres to human intestinal mucosa and induces an inflammatory response (De and Chatterje, 1953; Singh et al., 2001; Ritchie and Waldor, 2009). Furthermore, recent investigations also suggest that other non-CT virulence factors and inflammatory responses induced by *V. cholerae* independently of CT may contribute to the pathogenesis of cholera (Hodges and Gill, 2010; Chatterjee and Chaudhuri, 2013; Sawasvirojwong et al., 2013). Therefore, both O1/O139 and non-O1/O139 serogroups of *V. cholerae* pose considerable threat to public health.

Bangladesh is an area of cholera endemicity where this disease occurs in seasonal regularity with more than 100,000 cases annually (Lipp et al., 2002; Ali et al., 2015). An annual single peak of cholera cases (March-May) is observed in rural coastal villages, whereas cholera outbreaks maintain a unique bimodal seasonality in the capital city of Dhaka, where the larger peak with the highest number of cases occurs just after the monsoon (September–November), with a smaller peak in the spring (March–May) (Kaper et al., 1995; Faruque et al., 2005; Alam et al., 2011). Major cholera outbreaks primarily originated in the coastal regions of southern Bangladesh, including the initial appearance of O139 Bengal in the coastal areas in 1992, which then spread inland through secondary means (Jutla et al., 2010). Isolation of pandemic strains from the aquatic environment of endemic regions, even during seasonal outbreaks, is rare because toxigenic strains may persist in a non-culturable state (Brayton et al., 1987; Alam et al., 2006). *Vibrio cholerae* may enter into a viable but non-culturable (VBNC) state to persist in the stressed conditions of aquatic environments, in which they may not form colonies on traditional bacteriological culture media (Alam et al., 2007). It is likely that the environment is the source of epidemic strains; however, the mechanism that enables spreading of *V. cholerae* across water bodies from the Bay of Bengal is still not clearly understood (Halpern et al., 2008). It has been suggested that *V. cholerae* proliferates while attached to planktonic bodies, particularly copepods, in aquatic systems (Huq et al., 1983; Colwell, 1996). Migratory water birds and fish have also been linked to *V. cholerae* dissemination between water bodies of western Asia, Europe and Africa (Halpern et al., 2008; Senderovich et al., 2010; Halpern and Izhaki, 2017). In Bangladesh, recurrent cholera infections have been linked to increased environmental concentration of plankton in river delta, although, a recent study has shown the absence of a direct connection between the riverine system and drinking water sources (Grant et al., 2015). Precisely, the transmission of *V. cholerae* between the Bay of Bengal and a major city like Dhaka still remains unknown.

It was postulated that Hilsha fish (*Tenualosa ilisha*), which migrates between both coastal and up-stream freshwater environments for breeding, might play a role in maintaining cholera endemicity in India (Pandit and Hora, 1951). The authors, however, failed to undertake any direct field or laboratory investigations to validate their hypothesis. Hilsha (*Tenualosa ilisha)* is anadromous in nature, migrating from the Bay of Bengal to inland freshwater through rivers on the Indian sub-continent for spawning, which occurs from July to October and again from January to March (Ahsan et al., 2014) (see **Figure 1**). The upstream migration of Hilsha is associated with the state of sexual maturity, as well as the volume of freshwater discharge from the estuary during monsoons (Bhaumik, 2017). The catch percentage of Hilsha is very high during these migratory periods. Approximately 300,000 tons of Hilsha were caught in inland and marine waters in Bangladesh from 2010 to 2011 (Ahsan et al., 2014). The availability of the fish in local markets also increased during these periods, which results in lower prices. During the remainder of the year and national festivals such as the Bengali New Year, the fish is usually too expensive for poor communities.

In this study, Hilsha fish is analyzed for the first time to be a potential carrier of *V. cholerae*, and for its role as a risk factor in the transmission of *V. cholerae* to humans. The rationale of the current study is to analyze the incidence and seasonal prevalence of *V. cholerae* in Hilsha from both the Padma River, where they have migrated from the Bay of Bengal, and in local markets in Dhaka. The study extensively characterizes the population structure of *V. cholerae* strains isolated from fish, and assesses the pathogenic potential of these strains. In addition, the clonal relationship between environmental and endemic strains was analyzed to discern the understanding of evolutionary history. The study was conducted as a part of a research project funded by the Danish Government (DANIDA) called "Combating Cholera Caused by Climate Change" (C5). The project focuses on the cholera influencing factors by identifying relative risks based on environmental and hygienic issues in Bangladesh (http://cope.ku.dk/research/cholera/).

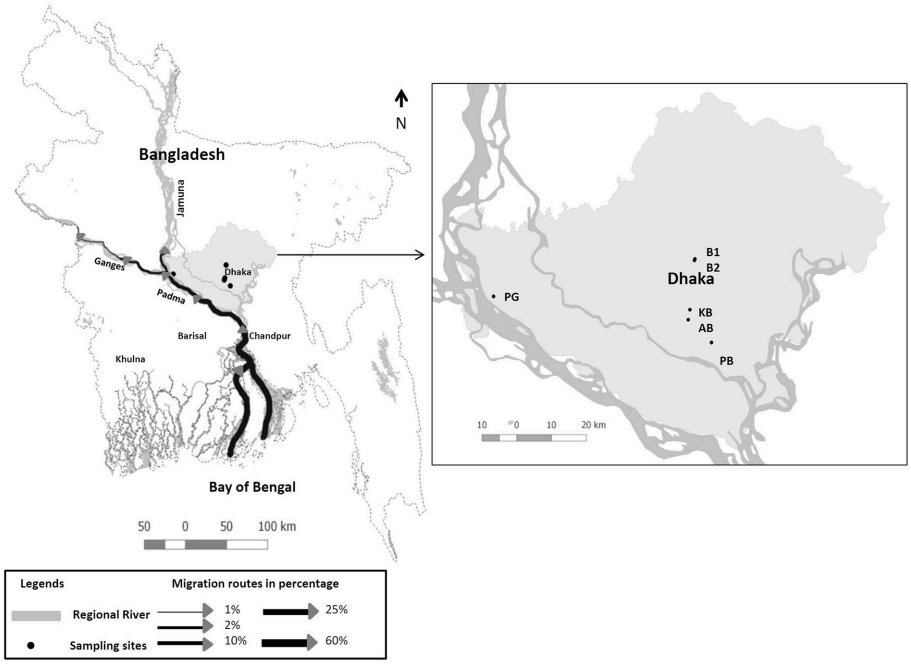

FIGURE 1 | Map showing Hilsha migration routes in Bangladesh adopted from Ahsan et al. (2014) and sample collection sites are indicated: Boubazar 1(B1), Boubazar 2 (B2), Kawran Bazar (KB), Ananadabazar (AB), Pagla bazaar (PB), and Paturiaghat (PG).

MATERIALS AND METHODS

Sample Collection and Processing

Four Hilsha fish were collected each month for a period of 1 year, from October 2014 to September 2015; two "market fish" from local markets in and around Dhaka and two "fresh fish" which were freshly caught near the bank of the Padma River (for a total of 48 fish) **Figure 1**, Supplementary Table 1. All the fish (mean body weight 765 g) were healthy, with bright, shiny appearance and natural odor.

The fresh fish were bought directly from fishermen at the major landing point (Paturia, approximately 80 km away from Dhaka) early in the morning. The fish were caught on the previous night in the lower Padma River between Shariatpur and Chandpur. Each fish was collected in individual sterile collection bags and transported to the University of Dhaka laboratory within 4 h of collection in a cool box maintaining cold condition. Four samples were aseptically taken from each fish:—two slits of fish gills, gut, rectum and an outer swab of scales in phosphate-buffered saline (PBS). For the fish collected from the local market (fish stored on ice), ice samples where the fish were kept frozen were also collected in sterile zip-lock sample collection bags. A total of 8 and 10 samples each of fresh-caught fish and local market fish were analyzed each month for 1 year, for a final total of 96 and 120 samples. Approximately 6 gm of gill, gut and rectum samples were enriched in 60 mL of Alkaline Peptone Water (APW) (1 L distilled H_2O, 10 gL^{-1} peptone, 10gL^{-1} sodium chloride; pH 8.5). One mL of PBS outer swab and storage ice water were transferred to 9 mL of APW for enrichment. All of the samples were incubated at 37°C for 24 h.

Total DNA Extraction and Detection of *V. cholerae* by Polymerase Chain Reaction

Total DNA was extracted from all of the samples using the boiled template method (De Medici et al., 2003). The presence of *Vibrio cholerae* in total DNA of fish samples was confirmed by PCR using the previously published primers (5′-CACCAAGAA GGTGACTTTATTGTG-3′ and 5′-GGTTTGTCGAATTAGCTT CACC-3′) for the outer membrane protein (*ompW*) gene of *V. cholerae* (Nandi et al., 2000).

The PCR was conducted in a thermal cycler (MJ Research PTC-200, USA) using 0.2 mL PCR tube with a reaction volume of 12 μL containing 1 μL of 10X PCR buffer including 20 mM $MgCl_2$, 0.2 μL of 10 mM deoxynucleoside triphosphates (dNTP) mix (Thermo Scientific, USA), 0.05 μL of 5 U Dream Taq DNA Polymerase (Thermo Scientific, USA) per μL, 0.625 μL of 25 μM each primer (Tag Copenhagen A/S, Denmark), 7.5 μL of nuclease-free water and 2 μL of DNA template. The PCR tubes containing reaction mixtures were heated at 95°C for 3 min for complete denaturation of DNA templates. The PCR amplification was carried out for 35 cycles in the following order: initial denaturation at 95°C for 45 s, annealing at 55°C for 45 s, hybridization at 72°C for 45 s, with a final extension at 72°C for 7 min. PCR products (304 bp band size) were then resolved by 1.5% (wt/vol) agarose gel electrophoresis and visualized with a UV transilluminator (Gel Doc, Bio-Rad, USA) after ethidium bromide staining.

The chance of contamination between local market fish and fresh-caught fish was scored by the presence or absence of specific *ompW* targets and was statistically analyzed by Fisher's exact test

for a 2 × 2 contingency table in statistical software R version 3.3.1. Significance was defined as having a p value of less than 0.05.

Bacterial Strains

A total of 158 *V. cholerae* strains were isolated by using conventional cultural media TCBS (Thiosulfate citrate bile-salts sucrose agar). Species identification of all the strains was further confirmed by standard biochemical assays and *V. cholerae* species-specific *ompW* gene target PCR (Nandi et al., 2000; Choopun et al., 2002; Huq et al., 2012).

Molecular Characterization of *V. cholerae* Isolates

Genomic DNA from the isolates was extracted by the boiled template method described earlier. Serological assays and PCR targeting the *rfb* sequences specific for O1 and O139 serogroups were used for further subtyping of all *V. cholerae* isolates. PCR was performed to detect the virulence and regulatory genes of *V. cholerae* O1/O139 and non O1/O139 (Supplementary Table 2). Total DNA samples that were detected as *V. cholerae*-positive were further analyzed for the presence of cholera toxin gene (*ctxA*) and the *rfb* sequences of O1 and O139 serogroups. PCR reactions were performed by using the protocol described previously in the section Total DNA Extraction and Detection of *V. cholerae* by Polymerase Chain Reaction. The primers, probes used in this study are listed in Supplementary Table 2. Real time PCR to detect *ctxA* gene was performed by following the previously published protocol (Blackstone et al., 2007). Positive and negative controls used in PCR experiments are listed in Supplementary Table 3.

rpoB Sequencing

Species identities of representative 36 *V. cholerae* strains were confirmed by nucleotide sequencing of 871 bp fragment of the *rpoB* gene. PCR based amplification and sequence analysis of *rpoB* gene were conducted as described previously (Tarr et al., 2007). For sequencing, BigDye Terminator v3.1 sequencing kit (Applied Biosystems, USA) was used following manufacturer's instructions. Sequence determination was conducted onABI3730XL (Applied Biosystems, USA) system.

Antibiotic Susceptibility Assay

Antibiotic susceptibility of the *V. cholerae* strains was conducted by agar disk diffusion method using commercial disks (Oxoid, UK). The strains were tested for Tetracycline (30 μg), Sulfamethoxazole-trimethoprim (25 μg), Chloramphenicol (30 μg), Kanamycin (30 μg), Neomycin (30 μg) according to the standard guidelines of Clinical and Laboratory Standards Institute (CLSI) (Patel et al., 2014). The zone standards for *Enterobacteriaceae* were used when there were no established breakpoint interpretive criteria for *V. cholerae*. *E. coli* ATCC 25922 was used as quality control strain. The experiment was done in duplicate.

Toxicity Assay

Nine *V. cholerae* strains were studied including 6 *V. cholerae* O1 and 3 non O1/O139 serogroups (**Table 3**) for analyzing pathogenic potential on established animal model and human cancer cell line. Multilocus sequence typing (MLST) method was used to determine the nucleotide changes in housekeeping genes of these 9 isolates compared to existing database (see section MLST below).

Tissue Culture Assay

Culture supernatants of *V. cholerae* strains were tested for cytotoxicity in HeLa cell-line (human cervical carcinoma cell-line). Following previous protocol, the cell-free culture supernatants were prepared by centrifugation and filtration through a 0.22-μm-pore size filter unit (Millex-GS; Millipore Corp., Bedford, Mass; Sharma et al., 1998).

HeLa cells were grown as monolayers in Dulbecco's Modified Eagles' medium (DMEM) (Thermo Fisher Scientific, USA) containing 1% penicillin-streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine serum (FBS). Cells (4.4×10^4/400 μl) were seeded onto 24-well plates and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. Thereafter, 100 μl of the culture supernatant sample was added each well. Cytotoxicity was examined under an inverted light microscope (Olympus, Japan) after 24 h of incubation. The uninoculated Trypticase soy broth and *V. cholerae* O1 El Tor N16961were used as negative and positive control. Duplicate wells were used for each sample.

Rabbit Ileal Loop Assay

Cultures of *Vibrio cholerae* were tested for ileal loop fluid accumulation in adult New Zealand albino rabbits as described (De and Chatterje, 1953). The experiments were performed at International Centre for Diarrheal Disease (icddr, b) Bangladesh in complete accordance with icddr, b ethical guidelines. The protocol was reviewed and approved by icddr, b "Animal Experimentation Ethics Committee (AEEC)." Each test was done in duplicate (in two rabbits). Toxigenic *V. cholerae* N16961 and PBS were used as positive and negative control respectively. *V. cholerae* strains with little or no fluid accumulation in the initial passage were recovered from the ileal loops on nutrient agar plates and subjected to second passage in the same way by using the protocol of Sanyal et al. (1984). This process was repeated until third passage to obtain unambiguous positive response.

Multilocus Sequence Typing

Seven house-keeping genes (*adk*, *gyrB*, *mdh*, *metE*, *purM*, *pntA,* and *pyrC*) were recovered by PCR from all nine strains and the products were sequenced. The primer sequences were extracted from previously published work (Octavia et al., 2013). Homologous sequences from these seven loci were sourced from database entries of whole and partial sequences with the following genome strains and accession nos.- N16961 (Accession No.AE003852); BX330286 (Accession No. ACIA00000000); MZO-3 (Accession No. AAUU00000000); M2552 (KC894993, KC895055, KC895117, KC895179, KC89524, KC895303, KC895365); M2554 (KC894995,

KC895057, KC895119, KC895181, KC895243, KC895305, KC895367); M1619 (KC894986, KC895048, KC895110, KC895172, KC895234, KC895296, KC895358). Sequences for the fish isolates are deposited in GenBank under accession nos. KY619689–KY619697 (*adk*), KY619698–KY619706 (*gyrB*), KY619707–KY619715 (*mdh*), KY619716–KY619724 (*metE*), KY619725–KY619733 (*pntA*), KY629640–KY629648 (*purM*), KY629649–KY629657 (*pyrC*).

A multiple alignment of sequences generated by the study and those extracted from the Genbank database was constructed using MAUVE software package (http://asap.ahabs.wisc.edu/software/). The aligned file was used as input for Bayesian inference of genealogy and recombination events using CLONALFRAME v. 1.2 software following the published method along with model parameters (Didelot and Falush, 2007; Islam et al., 2013). The number of populations was determined by the Markov Chain Monte Carlo (MCMC) simulation of 10,000 iterations which gave the posterior probability of *K* following a burn-in of 10,000 iterations and parameter values were recorded for 10 iterations in the posterior sample. Analysis was repeated three times with same data and parameters, but with distinct starting points and 50% consensus trees were produced by Clonal Frame with a threshold of 0.5. The relative effect of homologous recombination on the genetic diversification of populations was measured by calculating the ratio of recombination and mutation events (r/m) (Guttman and Dykhuizen, 1994).

Ethics Statement

This study was undertaken in accordance with the ethical recommendation of Faculty of Biological Sciences, University of Dhaka, Bangladesh. All the fish for this study were obtained directly from fishermen and fish mongers in local markets selling for consumption. The fish were not alive during the time of collection.

RESULTS

Prevalence of *V. cholerae* in Fish Samples

Of the total of 48 individual fish (216 total DNA samples) collected, 39 (81%) fish were positive for the specific *ompW* gene when assayed for *V. cholerae*. Among the market fish, detection was highest in the gills (19 of 24 fish, 79%), followed by outer scale swabs, recta and intestines. In fresh fish, detection was highest in outer scale swabs (16 of 24 fish, 66.7%), followed by the gills, recta and intestines (**Figure 2**). Seventeen storage ice samples were positive for *V. cholerae* out of 24 (70.8%) market fish by PCR. No local market fish was found to be positive only for ice water. The presence of *V. cholerae* was higher in fish purchased from local markets (21 of 24, 87.5%) by PCR than fish from the river banks (18 of 24, 75%), where the fish were considered positive if any part of the fish was positive. A total of 55% (53 of 96) of fresh fish sample types and 60% (72 of 120) of local fish sample types including ice samples were found to be positive for *V. cholerae*.

The prevalence of *V. cholerae* contamination was statistically compared between the fish types. Statistical analysis using Fisher's exact test yielded that the chance of *V. cholerae* contamination in market fish stored on ice is higher than in fresh fish and showed a significant difference (odds ratio [OR]: 0.41; 95% confidence interval [CI]: 0.2, 0.9; $P = 0.03$). No statistically significant difference was found ($P = 0.6$) when the analysis was done on total individual samples (96 from fresh fish and 120 from local market fish).

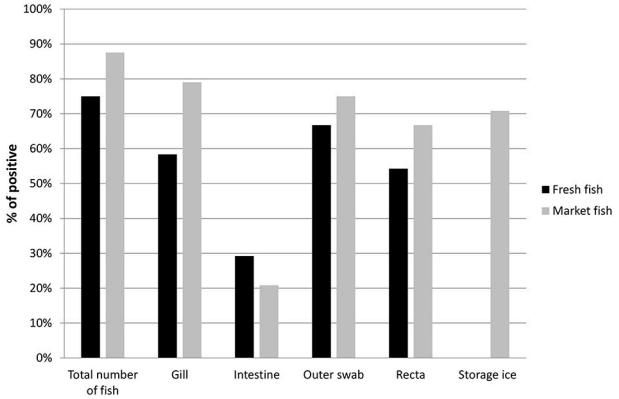

FIGURE 2 | Prevalence of *V. cholerae* in total number of fish (Fresh fish, $n = 24$ and Market fish, $n = 24$) and different fish parts (no ice was collected for any of the fresh fish). Positivity of *V. cholerae* was determined by *ompW* gene target PCR.

Molecular Genotyping of Total DNA and Monthly Incidence

Total DNA samples were extracted from Hilsha fish and ice samples for *V. cholerae* detection and isolation. Among them, 125 (58%) samples tested positive based on the *ompW* PCR. The *ompW* positive total DNA samples were further characterized by targeting genes of toxigenic *V. cholerae*, which yielded 25 positive amplicons (16 fish) for the *V. cholerae* O1 serogroup and 9 (6 fish) for the O139 serogroup. Seven of the *V. cholerae* O1-positive and three of the O139-positive samples were also positive for *ctxA* in real-time PCR which comprised 8% of all *V. cholerae*-positive samples and 20% of all *V. cholerae*-positive fish. Detection of *V. cholerae* O1, O139 and *ctxA* genes was compared between the two types of fish, and 23.8% (5 out of 21) of local market fish were found to be positive for the *ctxA* gene, an amount that was higher than for fresh fish samples (16.67%, 3 out of 18). The results are shown in **Table 1**. The detection of *rfbO1* and *ctxA* gene was confirmed as positive in 11.8% (2 out of 17) and 5.9% (1 out of 17) *ompW* positive storage ice samples respectively and none were positive for *rfbO139* gene. One market fish was found *ctxA* gene positive in ice only, whilst the other parts were negative.

The monthly incidence of *V. cholerae* in fish revealed two annual peaks; one from March to June before the monsoon, followed by a second peak in August-November at late monsoon (**Figure 3**). Detection of DNA from toxigenic *V. cholerae* O1 and cholera toxin peaked in April when 100% (4 of 4) of the fish were positive for both the *rfbO1* and *ctxA* genes. Detection of *V. cholerae* O139 was highest in July and September when 50% (2 of 4) of all the fish were positive for the *rfbO139* gene.

TABLE 1 | Occurrence of toxigenic *V. cholerae* genes in fresh and market fish.

Fish types	No. of *V. cholerae* positive fish/total (%)	No. of *V. cholerae* positive samples /total (%)	No. of *V. cholerae* O1 positive fish/total (%)	No. of *V. cholerae* O1 positive samples/total (%)	No. of *V. cholerae* O139 positive fish/total (%)	No. of *V. cholerae* O139 positive samples/total	No. of *ctxA* positive fish/ total (%)	No. of *ctxA* positive samples/total (%)
Fresh fish	18/24 (75)	53/96 (55.2)	6/18 (33.3)	8/53 (15)	2/18 (11.1)	3/53 (5.7)	3/18 (16.67)	4/53 (7.5)
Market fish	21/24 (87.5)	72/120 (60)	10/21 (47.6)	17/72 (23)	4/21 (19)	6/72 (8.3)	5/21 (23.8)	6/72 (8.3)

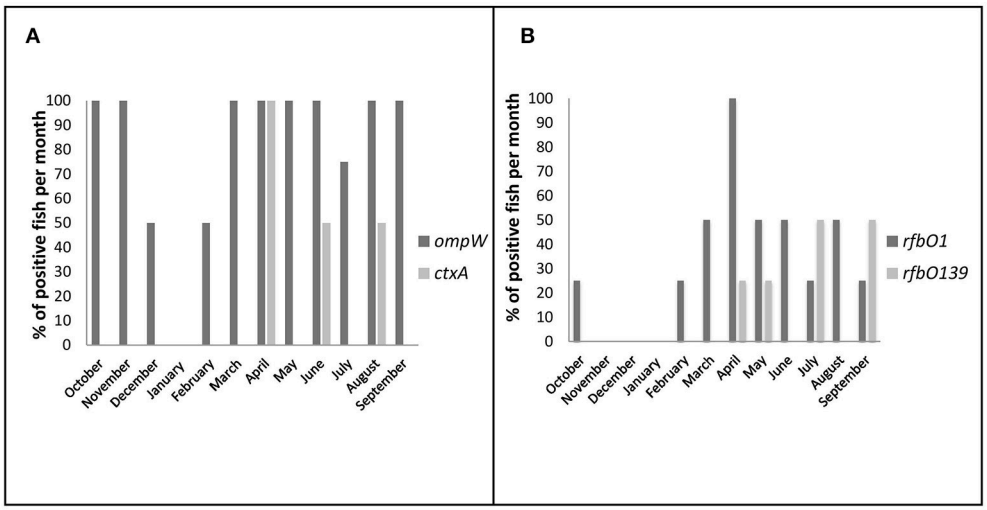

FIGURE 3 | Seasonal variation of total and toxigenic *V. cholerae* prevalence in direct DNA samples (%, bar chart). The fish was considered as positive when any part of the fish was found positive in PCR (including ice samples of market fish). **(A)** Positive fish per month for *ompW* and *ctxA* genes **(B)** Positive fish per month for toxigenic serogroups O1 and O139.

Genotyping of *V. cholerae* Strains

A total of 158 strains isolated from fish were confirmed as *V. cholerae* species by cultural and biochemical tests and *ompW* gene specific PCR. A total of 35 groups of 158 strains were detected using PCR based genotypic characterization (Supplementary Table 2). Twenty-five *V. cholerae* strains were positive for the *rfbO1* gene and 133 strains were negative (non O1/O139), which are included in 13 and 23 groups respectively (**Table 2**). None were positive for the *rfbO139* gene and none of the O1 strains contained cholera toxin genes A and B (*ctxA* and *ctxB*), the toxin-coregulated pilus (*tcp*), colonization factor (*ace*), and core-encoded pilus (*cep*) genes. The highest numbers of isolates (29) in our study were categorized in Group-XXIX with a genotypic trait of $rfbO1^{-}chxA{+}mshA{+}ompU{+}$. The heat stable enterotoxin of *V. cholerae* (*stn/sto*) was present in three out of 25 O1 strains and four out of 133 non-O1 strains. We studied the SXT mobile genetic element that encodes antibiotic resistance in all the strains, and 37 strains (4 O1 and 33 non O1/O139) were positive. Twenty-four (18%) non-O1 strains were positive by PCR for all three genes (*vcsN2*, *vcsC2*, *vopF*) tested for type three secretion system. Sixty-eight percent O1 and 80% non-O1 experimental strains possessed mannose sensitive hemagglutinin pilus (*mshA*). Previous studies show that newly discovered Cholix toxin (*chxA*) is mostly found in non O1 *V. cholerae* (Awasthi et al., 2013). However, approximately 80% of the O1, and 71% of the non O1 strains of this study were positive for the *chxA* gene. The gene of the putative outer membrane protein (*ompU*) was found in 14 (56%) O1 and 62 (47%) non-O1 strains. All the strains were positive for hemolysin hlyA, hemagglutinin protease (HA- protease), toxR, rtxC, and the type six secretion system (vasA, vasH, vasK) PCR. Isolation of *V. cholerae* dropped in January and remained the same for the next 2 months.

A total of 35 representative *Vibrio cholerae* strains of 35 genotypic groups were sequenced to identify those that showed different molecular characteristics. The partial nucleotide sequences of the *rpoB* gene have been evaluated for species identification of *V. cholerae*. The alignment of the study sequences to databases using BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed 99–100% sequence similarity with *V. cholerae* species. Isolate information and accession numbers are listed in Supplementary Table 4.

Antibiotic Sensitivity Test

All the *V. cholerae* strains ($n = 158$) were tested for their antibiotic susceptibility against five commonly prescribed antibiotics. The antibiotic response of the strains revealed that all were uniformly susceptible to Chloramphenicol (100%). Two (1%), 17 (10%), 15 (9%) and 10 (6%) isolates were found

TABLE 2 | Grouping of *V. cholerae* isolates based on genotypic characterization.

Group	No. of Isolates	*hlyA, HA protease, toxR, rtxC,* T6SS	*rfbO139, ctxA, ctxB, cep, tcp, zot, ace*	*rfbO1*	T3SS *(vcsN2, vcsC2, vcsC2)*	*mshA*	*chxA*	*ompU*	*stn/sto*	*SXT*
I	1	+	−	+	−	+	−	−	+	−
II	2	+	−	+	−	+	+	+	+	−
III	1	+	−	+	−	+	−	−	−	+
IV	2	+	−	+	−	+	+	+	−	+
V	1	+	−	+	−	−	+	+	−	+
VI	5	+	−	+	−	+	+	+	−	−
VII	3	+	−	+	−	−	+	+	−	−
VIII	2	+	−	+	−	−	+	−	−	−
IX	4	+	−	+	−	+	+	−	−	−
X	2	+	−	+	−	−	−	−	−	−
XI	1	+	−	−	−	+	−	+	+	−
XII	2	+	−	−	−	+	+	+	+	−
XIII	1	+	−	−	−	−	−	−	+	+
XIV	10	+	−	−	−	+	+	+	−	+
XV	14	+	−	−	−	+	+	−	−	+
XVI	3	+	−	−	−	−	+	−	−	+
XVII	3	+	−	−	−	−	−	−	−	+
XVIII	1	+	−	−	−	+	−	−	−	+
XIX	1	+	−	−	+	+	+	−	−	−
XX	1	+	−	+	+	+	+	+	−	−
XXI	1	+	−	+	+	+	−	−	−	−
XXII	1	+	−	−	+	+	+	+	−	+
XXIII	3	+	−	−	+	+	+	+	−	−
XXIV	6	+	−	−	+	+	+	+	−	−
XXV	1	+	−	−	+	+	+	−	−	−
XXVI	1	+	−	−	+	−	−	+	−	−
XXVII	5	+	−	−	+	+	−	−	−	−
XXVIII	4	+	−	−	+	−	+	−	−	−
XXIX	29	+	−	−	−	+	+	+	−	−
XXX	14	+	−	−	−	+	+	−	−	−
XXXI	7	+	−	−	−	−	−	−	−	−
XXXII	3	+	−	−	−	+	−	+	−	−
XXXIII	6	+	−	−	−	−	+	+	−	−
XXXIV	1	+	−	−	−	−	+	−	−	−
XXXV	16	+	−	−	−	+	−	−	−	−
	Total = 158	158		25	24	123	115	76	7	37

to be resistant to tetracycline, Sulfamethoxazole-trimethoprim, kanamycin, and neomycin respectively. Thirty-five isolates showed antimicrobial drug resistance to at least one of the test antibiotics. All these strains carried the gene for the SXT mobile genetic element. The results are shown in **Figure 4**.

Pathogenicity Assays

Cell-free culture supernatants of nine *Vibrio cholerae* isolates caused morphological changing from cell rounding to cell clumping and cell death of the HeLa cell line. Six of the nine isolates were cytotoxic, three of these (F-44, F-52a, F-91b) induced massive cell death (<1% survival of HeLa cells), indicating the presence of extracellular cytotoxic proteins. The severity of cytotoxicity was relatively less apparent in the supernatants of F-45 and 49d. Three isolates, F-36a, F-47, and F-53 exhibited negligible cytotoxicity with survival of more than 90% of HeLa cells. Morphological changes induced by cell-free culture supernatants of *V. cholerae* were detected by microscopic examinations. Compared to the fish isolates, the positive control *V. cholerae* O1 N16961 in this assay showed a lower cytotoxic effect.

All nine isolates were tested for their ability to cause fluid accumulation in the rabbit ileal loop model. All strains except F-36a had an FA index above the borderline of 0.5, the accepted cut-off for FA in diarrheagenic bacterial strains (Wallis et al., 1986; Islam et al., 2013). Six isolates caused fluid accumulation

(FA index, 0.5–1.8) in the initial passage (**Table 3**). Isolate F-45 showed a positive response after three passages. The control strains N16961 showed a mean fluid accumulation of 1.6 mL/cm.

Multilocus Sequence Typing Analysis

A consensus tree of genealogy was constructed by ClonalFrame software which demonstrates clonality of the population data (**Figure 5**). The fish isolates clustered into four major clades; of which three clades comprised of draft sequences from database as neighbor. One of these groups consists of isolate F-91b, *V. cholerae* strain N16961 and BX 330286, both of the database strains are toxigenic O1 El Tor but interestingly, BX 330286 was isolated from water samples in Australia. Another group comprises of *V. cholerae* strain M2552, MZO-3 and F-32b.The first two strains are clinical non-O1 isolates but the fish strain contains *rfbO1* gene but lacks *ctxA* gene. The third clade comprises F-36a, F-44, and *V. cholerae* strain M1619; the former two fish strains have almost similar virulence gene profile since F-44 only lacks *ompU* gene. Strain M1619, a non-O1/O139 *V. cholerae*, was recovered from environment in Australia and identified as carrying the VPI (*Vibrio* Pathogenicity Island) and CTX phage region. The cluster formation in sets of sequence types predicts the occurrence of recombination or point mutation in different alleles. Our analysis implies recombination events occur more often relative to mutation in study population. In the case of the seven genes analyzed here, the ratio of probabilities of nucleotide substitution through recombination and mutation (r/m) is 0.4 that means recombination induces evolution events 0.4 times higher than point mutations.

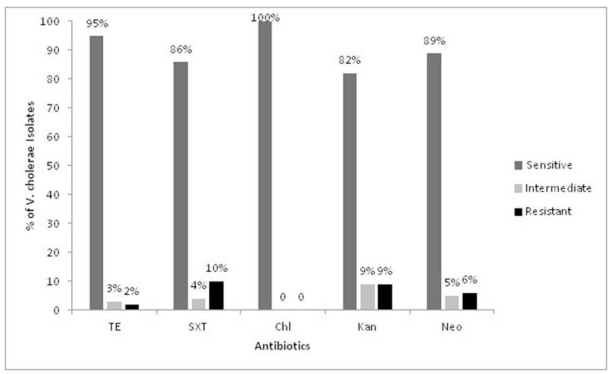

FIGURE 4 | Antibiotic susceptibility pattern of the *Vibrio cholerae* isolates. TE, Tetracycline; SXT, Sulfomethoxazole-trimethoprim; Chl, Chloramphenicol; Kan, Kanamycin; Neo, Neomycin.

DISCUSSION

To the best of our knowledge, this is the only study on *V. cholerae* occurrence in Hilsha, a fish that migrates from cholera-endemic coastal waters upstream to freshwater rivers running through Bangladesh. Here, we show the population dynamics of *V. cholerae* strains isolated from freshly caught and local market Hilsha fish of Bangladesh and map their virulence profile and toxigenic potential. This study is the first to profile virulence related genes of *V. cholerae* in Hilsha fish.

Our results suggest that Hilsha fish may serve as a possible transmission vehicle of *V. cholerae* from the Bay of Bengal to inland Bangladesh due to their unique survival practices, such as migration for breeding and planktonic food. Previously, Bhuyan et al. (2016) reported the role of flooding in the seasonal dissemination of *V. cholerae* from coastal to inland water bodies in India, which corresponds with our investigation as the main period of Hilsha migration is during flooding caused by monsoon rains (Islam et al., 2016). Unlike the study conducted by Bhuyan et al. (2016), river water contamination was not analyzed in this study. Although there was a limited number of fish analyzed in our preliminary study, the data indicate that the occurrence of *V. cholerae* in Hilsha fish maintains similarity with the seasonal regularity of cholera epidemics in Bangladesh. The detection of *ctxA*-positive samples was highest in April. The presence of non-toxigenic O1/O139 and non-O1/O139 in fish may also play a critical role in cholera evolution and transmission, as they share the same environmental habitats as O1/O139 serogroups

TABLE 3 | Results of pathogenicity assays.

Isolate ID (genotypic group)	Fish part from where isolated	*rfb O1*	Cytotoxic effect (survival of HeLa cells)	Rabbit ileal loop assay
				Mean FA index (V/L)*
F-32b (I)	Outer surface	+	Cytotoxic (10–30%)	1.78 (1)
F-36a (VII)	Gill	+	Non cytotoxic (>90%)	0.46 (3)
F-44 (VIII)	Gut	+	Cytotoxic (<1%)	1.29 (1)
F-45 (IX)	Gill	+	Cytotoxic (<40%)	0.67 (3)
F-47 (XXIII)	Storage ice	–	Non cytotoxic (>95%)	0.87 (2)
F-49d (XIX)	Storage ice	–	Cytotoxic (20–50%)	0.53 (1)
F-52a (XXVI)	Rectum	–	Cytotoxic (<1%)	1.44 (1)
F-53 (IX)	Outer surface	+	Non cytotoxic (>95%)	1.73 (1)
F-91b (VIII)	Outer surface	+	Cytotoxic (<1%)	1.25 (1)

**V, Volume of fluid in mL; L, Length of the loop in cm. Number of passages in bracket. Positive cut-off value = 0.5 mL/cm.*

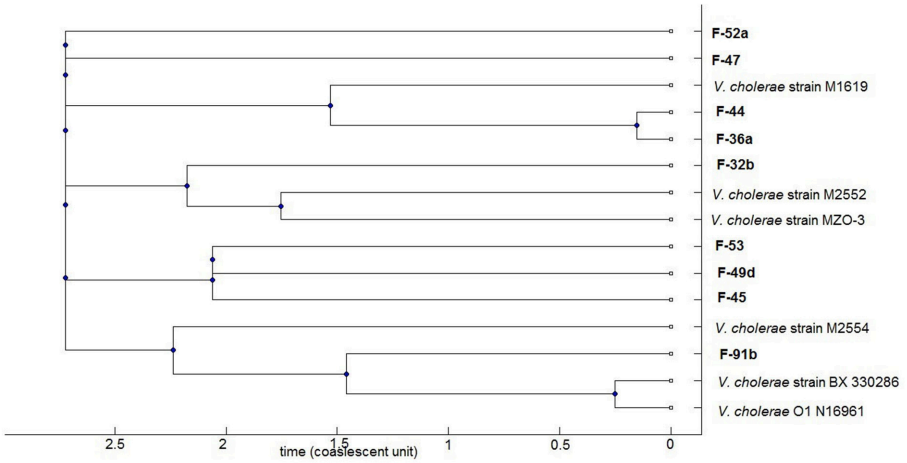

FIGURE 5 | Phylogenomic tree based on concatenated multilocus sequences of the *V. cholerae strains*. The current study *V. cholerae* strains are highlighted as bold which comprises both O1 and non-O1 strains. The remaining strains representing clinical and environmental toxigenic O1 and non-O1 reference strains: strain M1619, environmental toxigenic non-O1; strain M2552, M2554, and MZO-3, clinical non-O1; strain BX330286, environmental toxigenic O1; strain N16961, clinical O1.

(Azarian et al., 2016). A higher occurrence of toxigenic *V. cholerae* genes is seen in the local market fish. A possible explanation for this observation is that unhygienic conditions for fish storage could make the fish more prone to cross-contamination with fecal matter.

Vibrio cholerae has been isolated from other fish species including fresh water *Tilapia* species in Israel, Burkina Faso, and Tanzania (Senderovich et al., 2010; Traor et al., 2014; Hounmanou et al., 2016), from Ayu fish in the rivers of Japan (Kiiyukia et al., 1992), from the species *Rastrineobola argentea* and *Oreochromis niloticus* in Lake Victoria, Kenya (Onyuka et al., 2011), and from ornamental fish in Czech Republic (Rehulka et al., 2015). Reports also demonstrate isolation of *V. cholerae* from marine fish species (Scheelbeek et al., 2009; Senderovich et al., 2010). Furthermore, Mrityunjoy et al. (2013) showed elevated bacterial load in frozen fish collected from Dhaka city in Bangladesh. So far, no studies have been undertaken to investigate the bacterial genomic characteristics isolated from fish of the Ganges Delta region and Bangladesh, where cholera is endemic.

Fish has been indicated as the source of cholera outbreaks in different continents. Cholera has been associated with consumption of raw fish and seafood (McIntyre et al., 1979; Maggi et al., 1997; Forssman et al., 2007). A cholera patient was identified in Berlin, who had become infected while handling and preparing imported fish from Nigeria (Schürmann et al., 2002). Although, fish accounts for approximately 66% of total animal food intake in Bangladesh (Belton et al., 2011), there is no study to our knowledge that examined fish as a transmission risk factor for cholera outbreaks. Hilsha (*Tenualosa ilisha*) is the most important fish species in Bangladesh, which alone contributes to more than 10% of the total fish catch (Ahsan et al., 2014).

A low-income area near Dhaka was selected in which to directly contact households for information on where they purchase their fish. Fishmongers were also asked about the source of their fish. In Bangladesh, fish are normally bought whole without cleaning and taken home to be gutted and cleaned by members of the household themselves. The gutting and slicing of fish normally occurs on the kitchen floor with a water source nearby. Lack of proper hand washing and reusing water for cleaning cutting materials is also observed in overcrowded urban communities with mixed incomes. Factors such as shared cooking areas and inadequate drainage systems lead to susceptibility to cholera infection in these neighborhoods (Wahed et al., 2013). This combination of attributes has been previously reported in a study in Monrovia, Liberia, which suggested a cholera transmission pattern based on the cleaning of fish, rather than its consumption (Scheelbeek et al., 2009).

Vibrio cholerae was isolated from 35 of 40 fish (115 of 125 positive fish specimen types) to test for *V. cholerae* specific PCR. Multiple isolates with different cultural and genotypic properties have been isolated from 6 fish (14 fish specimens) and isolation was not successful for 5 *V. cholerae* positive fish (10 fish specimens). The strains successfully isolated in this study were of nontoxigenic O1 and non-O1/O139 serogroups. None of the O1 isolates carried the genes for the major toxin genes *ctxA* and *tcp*, to which the clinical state of cholera is primarily attributed. Previous studies have shown that the O1 serogroup of *V. cholerae* frequently isolated from the aquatic environment commonly lack cholera toxin genes (Igbinosa and Okoh, 2008). In our study, the presence of the *ctxA* gene in direct DNA samples has been observed, but we have not succeeded in isolating these pathogenic strains. Difficulties in culturing cholera bacteria from environmental samples have been reported in previous studies. However, it has been shown that on average, culturing yielded positive results for only 1% of the environmental samples analyzed during epidemic periods, and rarely during interepidemic periods as cells enter

into a viable but non-culturable (VBNC) state (Huq et al., 1990; Alam et al., 2006; Du Preez et al., 2010; Bhuyan et al., 2016). Fluctuations of environmental factors and the abundance of nontoxigenic isolates in the aquatic system may have an impact on the isolation of pathogenic strains (Mishra et al., 2012). It is noteworthy that small sample size and limited fish collection points could also be limiting factors in this study.

Toxigenic non-O1/O139 serogroups have caused severe cholera-like outbreaks in India and other countries, including Haiti (Rudra et al., 1996; Dalsgaard et al., 1999; Onifade et al., 2011; Hasan et al., 2012). Two toxigenic *V. cholerae* O1 strains, positive for cholera toxin, have been isolated from Tilapia fish gill, harvested in sewage stabilization ponds in Tanzania and 5 O1 strains were isolated from two marine fish in Cochin, India during 2009–2011 (Kumar and Lalitha, 2013; Hounmanou et al., 2016). The presence of pathogenic serogroups O1 and O139 in fish scale samples collected in Mozambique have been detected by direct fluorescent antibody technique but the researchers were unable to culture them (Du Preez et al., 2010). In contrast, non-O1/O139 serogroups are prevalent worldwide in both freshwater and marine fish (Senderovich et al., 2010; Jones et al., 2013; Traor et al., 2014).

PCR based genotypic analysis revealed variability among the isolates, with 35 genotypic profiles comprising of 19 virulence factors (**Table 2**). Virulence factors other than cholera toxin are present in the isolates for example, both O1 and non-O1 strains were found to contain cholix toxin gene (*chxA*), a potent cytotoxin that is capable of halting protein synthesis in eukaryotic cells (Purdy et al., 2010) and the *stn/sto* gene for a heat-stable enterotoxin produced by toxigenic *V. cholerae* and *E. coli* (Rivera et al., 2001). Genes for the type III secretion system (TTSS) were detected in 18% of non-O1/O139 fish isolates. The major role of the TTSS in pathogenesis of non-O1/O139 *V. cholerae* induced diarrhea is already established (Dziejman et al., 2005). Infant rabbits orally inoculated with the wild type non-O1 strain AM-19226, which carries the gene for the TTSS, rapidly elicited a fatal diarrheal disease, and induced disruptions of the intestinal epithelium (Shin et al., 2011). Hemolysin, another virulence factor present in *V. cholerae*, promotes chloride secretion from intact human intestinal mucosa and capable of blood cell lysis in humans (Debellis et al., 2009). Reports indicate strains of non-O1/O139 *V. cholerae* isolates from hospitalized diarrheal patients in Kolkata, India, contained only the hemolysin (*hlyA*) gene, while negative for all other major toxin genes of *V. cholerae* (Chatterjee et al., 2009; Senderovich et al., 2010). The hemolysin gene (*hlyA*) was present in all fish isolates of this study. Another ubiquitously found virulence factor in this study was the type 6 secretion system (T6SS). Unterweger et al. (2012) reported that *V. cholerae* employs T6SS to compete commensal bacteria both in the human intestine and environment. The self-transmissible mobile genetic element termed the SXT element, have a crucial role in transferring antimicrobial drug resistance genes among microbial populations by conjugation (Toma et al., 2005). The SXT element of *V. cholerae* confers resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (Waldor et al., 1996). In this study, 37 fish isolates contain the gene for the SXT element. Among them, 35 isolates showed resistance to at least one of the antibiotics tested, except for Chloramphenicol.

In the absence of CT and major colonization factors, culture supernatants of 4 non-toxigenic O1 (4 of 6 investigated) and 2 non-O1/O139 (2 of 3 investgated) strains showed a positve cytotoxic effect on HeLa cells by a mechanism which remains to be further characterized. Studies with non-O1/O139 strains showed a range of determinants for cytotoxicity, including hemolysins (Coelho et al., 2000), cholix toxin (Jørgensen et al., 2008), and heat stable enterotoxin (Arita et al., 1986). In this study, 4 cytotoxic strains possess *chxA*, 1 possesses *stn/sto* gene, and all the strains ($n = 6$) contained the *hlyA* gene. Eight of the nine strains showed a phenotype (fluid accumulation) in *in vivo* animal models similar to human disease despite the absence of major cholera toxin. Our results showed similar concordance with the previous reports of evoking fluid accumulation in the ileal loop test, despite lacking the CTX virulence cassette in *V. cholerae* O1 (Koley et al., 1999; Rajpara et al., 2013). Two non-O1/O139 fish strains possess a TTSS which mediates human diarrheal disease. Despite the high degree of virulence diversity, some fish strains showed genetic relatedness with pathogenic clones of diverse geographical locations. For example, the nontoxigenic O1 isolate F-91b fell in the same clade with toxigenic O1 N16961 and BX 330286 isolated from Bangladesh and Australia, respectively. These clonal relationships among fish and pandemic strains indicate that Hilsha fish may act as an environmental habitat where new pathogenic strains may emerge their non-pathogenic progenitors.

In conclusion, as cases of cholera in Bangladesh continue to occur, new transmission dynamics and their potential influence on virulence should be monitored. This study presents new data on the prevalence of *Vibrio cholerae* in Hilsha fish, and the possibility of an alternative route of transmission to households (as opposed to drinking water) in Bangladesh. The spectrum of the *V. cholerae* population isolated from Hilsha fish samples was highly heterogeneous, based on genotypic profile analyses. Nevertheless, the *Vibrio cholerae* isolates lacked cholera toxin, yet *in vitro* and *in vivo* activity showed the disease potential of the isolates. Despite the presence of the cholera toxin gene in Hilsha fish samples, isolation of toxigenic strains was not successful. Still it demands close monitoring of the coastal catch of Hilsha fish for cholera transmission and public health awareness to minimize the health risk posed by non-cholera *Vibrio* serogroups.

AUTHOR CONTRIBUTIONS

ZH designed and carried out the study in the laboratory, analyzed the results and wrote the original draft. IF collected the samples, carried out the laboratory work and participated in acquisition of data. ST participated in critical reviewing and editing of original draft. PJ and AB conceived of the study and contributed to the

revision of the draft and final approval of the version to be published. PJ was the principal supervisor of the project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00222/full#supplementary-material

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Manuscript 4

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Can *Escherichia coli* fly? The role of flies as transmitters of *E. coli* to food in an urban slum in Bangladesh

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Abstract

OBJECTIVE To investigate the transmission of faecal bacteria by flies to food under natural settings.
METHODS Over a period of 2 months, paired (exposed and non-exposed) containers with cooked rice were placed on the ground in kitchen areas in an urban slum area in Dhaka, Bangladesh, and the numbers of flies landing on the exposed rice were counted. Following exposure, the surface of the rice was microbiologically and molecularly analysed for the presence of *Escherichia coli* and genes of diarrhoeagenic *E. coli* and *Shigella* strains.
RESULTS Rice was at greater risk ($P < 0{\cdot}001$) of being contaminated with *E. coli* if flies landed on the rice than if no flies landed on the rice (odds ratio 5·4 ($P < 0{\cdot}001$, 95% CI: 2·5–11·7). Mean contamination in exposed rice samples ($n = 60$) was 3·1 × 103 CFU/g (95% CI: 2·2 × 103–4·0 × 103). Furthermore, for approximately half of the observed fly landings, the average CFU per fly landing was >0·6 × 103 CFU. Genes of diarrhoeagenic *E. coli* and *Shigella* species were detected in 39 of 60 (65%) of exposed rice samples. Two fly species were identified: the common housefly (*Musca domestica*) and the oriental latrine fly (*Chrysomya megacephala*).
CONCLUSION Flies may transmit large quantities of *E. coli* to food under field settings. The findings highlight the importance of implementing control measures to minimise exposure of food to flies to ensure food safety. Fly control measures should be considered for the prevention of diarrhoeal diseases caused by *E. coli*.

keywords flies, transmission, food safety, faecal bacteria, *Escherichia coli*, *Shigella*

Introduction

Ever since Wagner and Lanoix [1] published the F diagram in 1958, flies have been perceived as playing an important role in faecal-oral pathogenic transmission. Synanthropic filth flies, such as the houseflies (family *Muscidae)* and blowflies (family *Calliphoridae*), are by their reproductive and feeding habits in contact with faecal matter, waste and human consumables [2–4]. They are found in high densities in urban areas with unsanitary conditions, and studies on fly biology have shown that individual flies can cover large areas travelling up to 7 km over a period of 8 days [5]. Flies can carry human pathogens on the exoskeleton, legs, in mouthparts and in the intestinal tract [2]. Pathogens can then be transmitted by detachment from the exoskeleton, faecal deposition or regurgitation [6]. Many enteropathogens such as *Campylobacter, Cryptosporidium,* diarrhoeagenic *Escherichia coli, Giardia lamblia,* Norovirus, Rotavirus, *Salmonella, Shigella* and *Vibrio cholerae* have been isolated from flies in the field [6–12]. Many of these pathogens are amongst the leading causes of the 499 000 annual deaths in children below 5 years of age [13]. In urban slums in Bangladesh, a prevalence of 7 per 100 persons of diarrhoea in children under five has been reported, although this number is most likely an underestimation [14]. The mechanism of transmission of faecal contamination by flies to food has only been demonstrated under controlled, laboratory settings. To our knowledge, there is an absence of studies conducted in field to investigate the direct role of flies as vectors of faecal bacteria onto food.

Previous studies examining the potential of flies as vector of faecal bacteria can be divided into four categories: (i) Vector-based studies where faecal bacteria have been isolated from flies caught in field [7, 8, 11, 12, 15]; (ii) transmission-based laboratory studies where house flies that had been fed or exposed to faecal bacterial inoculations were capable of transmitting the bacteria onto food and surfaces [12, 16–19]; (iii) observational cohort studies (two such studies found a positive correlation between

fly density and diarrhoeal incidence in urban and rural households in India [8,20], and in Bangladesh, periods of peaks in fly density are followed by a peak in diarrhoea amongst toddlers [21]); (iv) intervention studies, where fly control measures implemented in a specific area reduced fly density and diarrhoeal disease incidence in comparison with control areas where no such measures were implemented [22–24].

Observational and intervention studies suggest that fly control could be a tool for diarrhoeal disease management, but this is based on the assumption that flies can transmit pathogens to food outside laboratory conditions. The lack of transmission-based studies field is in contrast to the numerous vector-based and transmission-based laboratory studies. This study aims to address this knowledge gap.

The objective of this study was to investigate and quantify the possible transmission of faecal bacteria by flies to food for human consumption in a natural setting and to detect possible diarrhoeagenic *E. coli* and *Shigella* strains transmitted by flies. The study was part of a Danish Aid (DANIDA) funded research project 'Combating Cholera Caused by Climate Change' (C5), a study focusing on the interaction between water quantity, hygiene and diarrhoeal disease in an urban slum in Dhaka, Bangladesh.

Materials and methods

Study area and period

The study was conducted in Arichpur (23°53′03·9″N 90°24′31·5″E), an urban slum of 1·2 km^2 in northwest Dhaka, Bangladesh, with a population density of 100 000 per square km [25]. Typically, 10–15 families live in a compound sharing water sources, cooking and latrine facilities [25], which are commonly located within a radius of 20 m (authors observation). Previous studies in Arichpur have indicated underdeveloped sanitation infrastructure [25]. In the area, flies have easy access to faecal matter and are found in high densities in the open-air cooking areas.

Data for this study were collected twice a week from 2 November 2015 to 30 December 2015, after the end of the monsoon season, when temperatures ranged from 16 to 28 degrees Celsius and the weather was dry (<25 mm rainfall) [26].

Ethical approval

Permission to work in each specific location was given by oral informed consent from the household. This study was approved by the University of Copenhagen, Denmark; the C5 study was approved by the Ethical Review Committee of the International Center for Diarrhoea Research, Bangladesh (research protocol number PR-14006).

Exposure and collection of rice samples

The experiment was conducted in, or close to, the communal cooking areas of the compound. The location for the experiment was selected based on observed presence of a large number of flies. Rice, which in Bangladesh is often stored after cooking and reserved without reheating, was selected as a food template for the study [27]. Chinigura aromatic sticky rice was purchased in the local market and cooked on the morning of the sampling day in a rice cooker without salt according to local recipe. Distilled sterile water was used for the purpose. Using a sterile spoon, 120 ml of cooked rice was transferred to each of ten sterile plastic containers ('Partex Lock & Safe', 450 ml, 121 cm^2), an even surface was made on the rice and the containers were closed with lids for transportation to the field. An additional spoonful (approximately 20 g) of rice was taken directly from the rice cooker as a control of contamination, put in a sterile plastic zip bag and placed in a cool box (4 °C).

For the experiment, two rice containers without lids were placed at the same time 1 cm apart on the ground in the cooking area (Figure 1). The pair included one container with exposed rice and one control container covered with a sterile insect net (13 × 13 cm cut from a non-insecticide-treated mosquito bed net purchased at a local market), fixed with a sterile rubber band. The containers were left for exposure for 30, 60, 90, 120, 150 or 180 min. The number of fly landings on the exposed sample during the exposure time was counted by a trained research assistant. The distance to the closest latrine was in the range of 1·5 to 26 m. At the end of the exposure time, a sterile spoon was used to scrape off the surface of the rice to collect a spoonful of rice (approximately 20 g) from each rice container, which was put in sterile plastic bags. All samples were transported in a cool box (4 °C) to the microbiology laboratory at Dhaka University for handling on the same day.

Microbiological analysis of faecal contamination

Each collected rice sample was mixed with sterile phosphate buffer saline (PBS) to make a 1:10 dilution and homogenised in a stomacher (Seward Stomacher® 80, Lab Biomaster, UK) for bacteriological analysis. Enumeration of thermotolerant *E. coli* was performed by

Figure 1 Exposure experiment. The pictures show the setting for the exposure experiment; a typical kitchen area and the placement of rice containers.

spreading 500 µl of homogenised sample onto HiCrome™ m-TEC Agar (Sigma-Aldrich, Fluka, India) followed by incubation at 44.5 °C. After 24 h, deep pink or purple coloured colonies were counted as *E. coli* based on manufacturer's instructions.

Molecular identification of diarrhoeagenic *E. coli* and *Shigella*

Enrichment for DNA extraction and PCR was done on all control and exposed samples by adding 1 ml of homogenised rice sample to 9 ml nutrient broth (OXOID, UK) followed by incubation at 37 °C. After 24 h, 1 ml of the nutrient broth incubated sample was taken for total DNA extraction and purification by boiling according to previously published techniques [28]. Conventional multiplex PCR for detection of diarrhoeagenic *E. coli* and *Shigella* was conducted using previously described primers (Table 1) and conditions [29,30]. The ipaH primer was added to the eight primers described by Nguyen *et al.* [30] to detect EIEC/*Shigella* not harbouring the *ial* gene [29]. PCR reactions were performed in a thermal cycler (MJ Research PTC-200, USA) with a total reaction volume of 12·5 µl containing 1 µl 10× PCR buffer including 20 mM $MgCl_2$, 0·2 µl of 10 mM deoxynucleoside triphosphates (dNTP) mix (Thermo Scientific, USA), 0·05 µl of 5U Dream Taq DNA Polymerase (Thermo Scientific) per µl, 0·625 µl of 25 µM of each primer, 2·5 µl of template DNA and nuclease-free water up to 12·5 µl.

Amplicons were resolved for identity by 1·5 % (w/v) agarose gel electrophoresis in 1× Tris-acetate-EDTA buffer. The products were visualised by an UV transilluminator (Gel Doc; Bio-Rad, USA) after staining with ethidium

Table 1 Virulence marker genes and primers for detection of diarrheagenic *Escherichia coli* and *Shigella*

Virulence marker gene	Primer	Primer sequence	Amplicon size (base pairs)
eltB	LT	5′-TCTCTATGTGCATACGGAGC-3′ 5′-CCATACTGATTGCCGCAAT-3′	322
estA	ST	5′-GCTAAACCAGTA$^{G}_{A}$GGTCTTCAAAA-3′ 5′-CCCGGTACA$^{G}_{A}$GCAGGATTACAACA-3′	147
vt1	VT1	5′-GAAGAGTCCGTGGGATTACG-3′ 5′-AGCGATGCAGCTATTAATAA-3′	130
vt2	VT2	5′-ACCGTTTTTCAGATTTT$^{G}_{A}$CACATA-3′ 5′-TACACAGGAGCAGTTTCAGACAGT-3′	298
eaeA	eae	5′-CACACGAATAAACTGACTAAAATG-3′ 5′-AAAAACGCTGACCCGCACCTAAAT-3′	376
bfpA	bfpA	5′-TTCTTGGTGCTTGCGTGTCTTTT-3′ 5′-TTTTGTTTGTTGTATCTTTGTAA-3′	367
ial	SHIG	5′-CTGGTAGGTATGGTGAGG-3′ 5′-CCAGGCCAACAATTATTTCC-3′	320
*ipaH**	ipaH*	5′-TGGAAAAACTC GTGCCTCT-3′ 5′-CCAGTCCGTAAATTCATTCT-3′	422
pCVD	EA	5′-CTGGCGAAAGACTGTATCAT-3′ 5′-CAATGTATAGAAATCCGCTGTT-3′	630

Virulence marker genes and primers previously described by Nguyen *et al.* (2005) and *Lüscher & Altwegg (1994).

bromide solution. *E. coli* MGL-IC1, NF 9422, 2V, MG1214C2, AE 3171 strains were used as positive control for ETEC, EHEC, EIEC (harbouring both *ial* and *ipaH*), eEAEC and EPEC, respectively. The strains were provided by International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and Environmental Microbiology Laboratory of University of Dhaka. Nuclease-free water was used as no template control.

The minimum identification criteria for diarrhoeagenic strains based on the presence of virulence genes were as follows: the presence of *eltB* and/or *estA* for ETEC, the presence of *vt1* and/or *vt2* for EHEC (additional presence of *eaeA* for typical EHEC), *bfpA* and *eaeA* for typical EPEC (the presence of only *eaeA* for atypical EPEC), the presence of *ial* or *ipaH* for EIEC or *Shigella* and the presence of *pCVD* for EAEC.

Collection, identification and microbiological analysis of flies

Only on the first sampling day, flies for species identification were captured using a sterilised sweep net. The collected flies were put in a sterile plastic bag and placed in the refrigerator for 30 min for immobilisation. The flies were transferred to a bottle with 75% (v/v) ethanol for transportation to Denmark. Identification was undertaken by the Natural History Museum of Denmark, University of Copenhagen, using published taxonomic keys [31,32].

Statistical analysis

Contamination on exposed and control samples was analysed by conditional logistic regression (P value <0·05 was considered statistically significant) stratified by matched sample and controlling for distance to latrines and duration of exposure. CFU/fly landing was calculated by dividing the CFU from the total rice sample by the number of fly landings on the rice. Statistical analysis was performed in IBM SPSS Statistics 22 and R version 3·2·3.

Results

Exposure and collection of rice samples

A total of 66 paired exposed and control rice containers were sampled. Five pairs were excluded from further analysis either because chickens stepped in the exposed rice

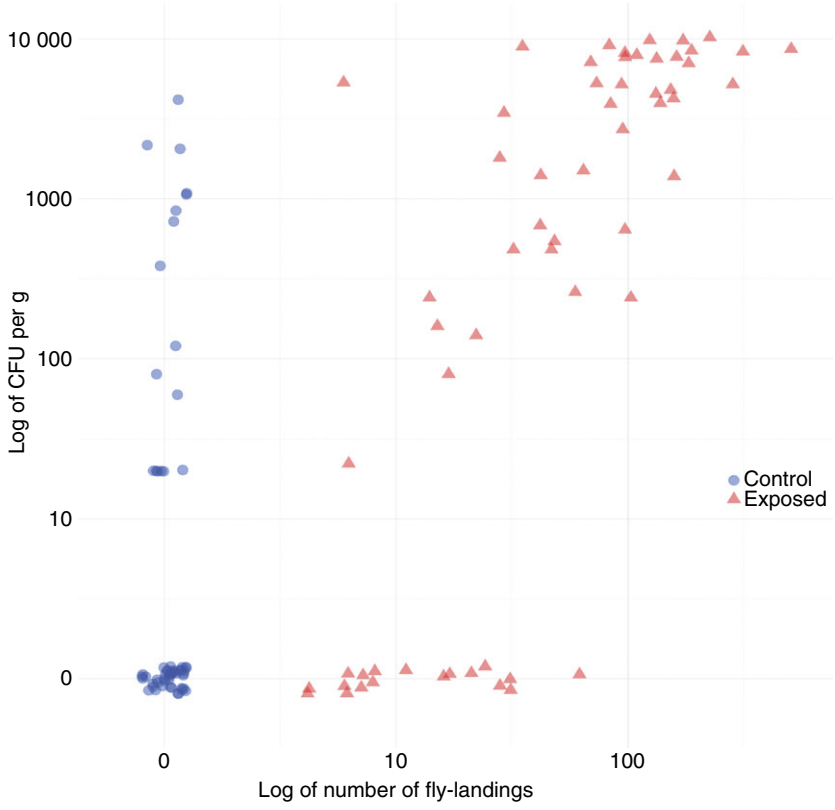

Figure 2 Fecal contamination on rice samples. *Escherichia coli* contamination on the exposed and control samples (n = 60 paired samples) presented as CFU/g (colony-forming bacterial units per gram of sample). x- and y-axis are in log scale and small amount of random noise was added to better visualise data with low x- and y-axis.

container or because no flies landed on the exposed rice. The remaining 60 pairs were included in the final data set. The number of sample pairs in the different time exposure groups was as follows: 30 min (n = 29), 60 min (n = 7), 90 min (n = 2), 120 min (n = 18), 150 min (n = 2) and 180 min (n = 2). Samples were collected at 11 locations, each location was visited one to five times, when one to four sample pairs were placed at the same time.

Microbiological analysis

All rice samples taken directly from the rice cooker were sterile. *E. coli* contamination in exposed and control samples is shown in Figure 2. A total of 42 (70%) exposed samples were positive for *E. coli* (range: 22 to $10{\cdot}2 \times 10^3$ CFU/g) and 18 (30%) were negative. A total of 17 control samples (28%) were positive for *E. coli* (range: 20–$4{\cdot}2 \times 10^3$ CFU/g) and 43 were negative (72%). Conditional logistic regression showed a significant difference ($P < 0{\cdot}001$, $\chi^2 = 17{\cdot}97$) between *E. coli* contamination (CFU/g>0) and no *E. coli* contamination (CFU/g = 0) on the exposed vs. the control samples. The odds of *E. coli* contamination on the rice were 5·4 times ($P < 0{\cdot}001$, 95% CI: 2·5–11·7) greater if flies landed on the rice than if no flies landed on the rice. The mean contamination in the exposed samples (n = 60) was $3{\cdot}1 \times 10^3$ CFU/g (95% CI: $2{\cdot}2 \times 10^3$ to $4{\cdot}0 \times 10^3$), in the control samples (n = 60) it was $0{\cdot}2 \times 10^3$ CFU/g (95% CI: $0{\cdot}0 \times 10^3$ to $0{\cdot}4 \times 10^3$) and the difference in means between the two groups was $2{\cdot}9 \times 10^3$ CFU/g (95% CI: $1{\cdot}9 \times 10^3$ to $3{\cdot}8 \times 10^3$). In the exposed group, the mean contamination in the total sample (20 g), which is equivalent to approximately one tablespoon of cooked rice was $61{\cdot}7 \times 10^3$ CFU (95% CI: $43{\cdot}4 \times 10^3$ to $80{\cdot}0 \times 10^3$). In total, 4831 fly landings were counted on the 60 exposed samples. Half of the fly landings was in samples with an average of $>0{\cdot}6 \times 10^3$ CFU per fly landing.

Table 2 Diarrheagenic strains in rice exposed to flies

Strain	Number of samples (%)
Atypical EPEC	11 (18.3)
EHEC	4 (6.7)
Typical EHEC	2 (3.3)
EIEC/*Shigella*	2 (3.3)
ETEC	25 (41.7)

Number of samples with diarrheagenic *Escherichia coli* or *Shigella* strains and proportions (%) out of the total sample number (n = 60). Identification criteria are as described by Nguyen *et al.* (2005) and Lüscher & Altwegg (1994).

Molecular identification

Genes belonging to diarrhoeagenic strains of *E. coli* and *Shigella* were detected in 39 (65 %) of the 60 exposed rice samples. In five samples, two genes of two different strains of diarrhoeagenic *E. coli* were detected. The identified strains are shown in Table 2.

Fly identification and microbiological analysis of flies

Of the 59 flies that were captured for identification, 48 were common houseflies (*Musca domestica*) and 11 were oriental latrine flies (*Chrysomya megacephala)*.

Discussion and conclusion

With the odds of *E. coli* contamination on the rice being 5·4 ($P < 0{\cdot}001$, 95% CI: 2·5–11·7) greater on exposed rice versus non-exposed, the study indicates a direct role of flies in the transmission of faecal bacteria to food and a higher risk of faecal contamination on food if flies land on it, than if no flies have landed on it.

The mean contamination on the exposed rice samples was $3{\cdot}1 \times 10^3$ CFU/g (95% CI: $2{\cdot}2 \times 10^3$ to $4{\cdot}0 \times 10^3$). However, many samples had no contamination despite having up to 62 fly landings, suggesting that many flies may not have *carried E. coli* bacteria. This accords with previous vector-based studies that have reported varying bacterial carriage amongst flies [8,11,12].

Although the average of *E. coli* carriage per fly landing calculated in the present study does not account for *E. coli* carriage variability in flies and therefore might represent a very simplified description of the true variability, the fact that half the fly landings occurred in samples with $>0{\cdot}6 \times 10^3$ CFU per fly highlights the importance of the fly pathway compared to the other pathways in the F diagram. A laboratory study reported an average of 1×10^3 CFU *E. coli* transmitted per fly landing [17], which is within the range of the average CFU/fly landing observed in the present study. Microbiological and molecular analysis of the presence of *E. coli* on captured flies could further have supported our findings and provided a direct evidence that *E. coli* carried by flies are responsible for the contamination of exposed rice. This was beyond the scope of the study and is suggested as future research.

A relatively large number of controls (17 of 60, range: 20 to $4{\cdot}2 \times 10^3$ CFU/g) was contaminated by *E. coli*. Samples were placed directly on the ground in small kitchen areas with a lot of movement of both people and animals and dust or water drops could fall on both exposed and control rice. Flies landing on the net covered

rice could defaecate or regurgitate through the netting, thereby contaminating the unexposed rice, but this was not measured in the current setup. These sources of contamination could not be eliminated given the circumstances of a field study in this specific location. However, both control and exposed rice samples were equally exposed to these sources of contamination; therefore, the difference in contamination between the two groups can likely be explained by landing of flies on the exposed rice. It should be kept in mind that the some of the reported bacterial contamination on the exposed rice in the present study may be due to other sources than flies.

Conversely, it is possible that some of the negative findings for *E. coli* contamination were the result of contamination missed by the sampling technique, which would bias our results. However, the strength of the association between the exposure status and the presence of *E. coli*, and the fact that the same sampling technique was used on both exposed and unexposed samples, suggests that this limitation would not change our underlying conclusions.

The number of rice containers at each site and number of revisits at each site varied due to size of kitchen and to limit the nuisance for the individual families. This results in a source of variation in the study. However, we suggest that as we were able to compare cooked rice samples on which flies could land against samples that were protected from flies, while controlling for several potential contextual confounders, our central conclusions are not invalidated by this limitation.

Molecular identification in the present study provides knowledge of strains of diarrhoeagenic *E. coli* and *Shigella* present in the environment and transmitted by flies in this specific location. Several genes of diarrhoeagenic strains were detected. The dominant strain was ETEC which was detected in 41·7 % (n = 25) of the exposed rice samples, which is one of the most prevalent pathogens causing diarrhoea in children under five in the world [13]. EAEC, EHEC, EPEC, ETEC and *Shigella* have previously been isolated from flies in vector-based studies [7,8,11,12,33]. The differentiation between EIEC and *Shigella* by molecular methods was beyond the scope of this study as evident in the presentation of results.

To summarise the findings, the study shows that flies attracted to cooked rice and provides empirical evidence of transmission of *E. coli*, and possibly other faecal pathogens, by flies to food for human consumption in a field setting. Even though findings in this study are area and time specific and cannot directly be transferred to other settings, it is an important finding that supports the theory that flies can act as direct vector of diarrhoeagenic pathogens. This suggests that other enteropathogens previously isolated from flies in field could also be transmitted to food through the same pathway.

The result of this study suggests that protecting the rice by covering it with an insect net is an effective preventive measure to reduce faecal contamination. These findings support previous research indicating that fly control measures can reduce diarrhoeal incidence [22–24]. Control measures to reduce fly numbers and flies' access to food such as fly nets and fly traps need to be implemented to ensure food safety.

The relative importance of fly transmission compared to other pathogen routes of transmission needs to be investigated. The importance of distance from latrine facilities to kitchen areas, the quantity of flies at the sampling sites and relating diarrhoeal cases and abundance of flies is suggested for future research. This knowledge is important for risk analysis and for adopting and designing prevention strategies, especially when time or resources limit the number of possible interventions. This study brings new knowledge that can be useful in practical settings for diarrhoeal disease management strategies and provides a foundation for further research on the role of flies in the spread of diarrhoeal disease.

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Manuscript 5

Transmission of Diarrheagenic *Escherichia coli* in dirrhea case households in urban Bangladesh

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Abstract

Prevalence of pathogenic *E. coli* contamination in diarrhea case household environments of low-income urban area in Bangladesh was not elucidated before. In the current study, both patients' clinical samples and in-house environmental samples were collected from the case households in Dhaka city, Bangladesh for investigation of diarrheagenic *E. coli* burden. Direct DNA samples were examined for virulence genes characteristic of five major dairrheagenic *E. coli* pathotype (ETEC, EHEC, EIEC, EPEC, EAEC) by PCR. The cultured *E. coli* strains were analyzed for virulence typing. Results showed that genomic presence of diarrheagenic *E. coli* was detected in 36% (89 of 245) of direct DNA samples. The frequency rate of virulent genes of *E. coli* from rectal swab, household swab, food, water samples were 28% (10/36), 44% (63/144),12% (4/34), 10% (3/31) respectively in PCR analysis of direct DNA. Six rectal swab samples and associated hotspot, water and food samples showed the presence of similar *E. coli* pathotype genes and drinking water vessel surface samples were most contaminated. One EAEC strain was commonly found in both clinical and latrine door knob swab. Contamination of household hotspot appeared to play a significant role in the transmission of diarrheagenic *E. coli* to infected person. This study data suggests high-risk areas for diarrheagenic *E. coli* contamination within low-income case household environment in Bangladesh.

1. Introduction

Diarrheal diseases was a significant cause of death among all ages in 2015 and is still a health problem world-wide particularly in developing countries [1]. Transmission of diarrheagenic pathogen mostly occurs in domestic environment of low-hygiene environment [2]. Curtis et al [3]described different ways of transmission of diarrhea causing pathogen: human to human via the environment, human to human multiplying in the environment, human to animal to human via the environment, animal to human via the environment. The "F-diagram" of Wagner & Lanoix intensively demonstrated how five vectors (food, finger, fly, field, and fluid) played role in the transmission of diarrheagenic pathogen in domestic environment [4]. Although improving domestic hygiene successfully reduced diarrheal disease burden however, identifying the key routes of transmission is essential to promote effective hygiene interventions [3]. The major pathogen transmission routes suggested in "F-diagram" have been evaluated in multiple study settings previously [5, 6]. Microbial transmission via drinking water and food has been found associated with childhood diarrhea in low-income group [7, 8]. Lindeberg *et al* [9] showed how flies, the well-known vector of diarrheal pathogen, played role in fecal *E. coli* contamination of

food in poor community. Pickering and colleagues showed that improved latrine sanitation did not affect household fecal contamination level and thus occurrences of diarrheal illness [10]. In developing countries, in-house and kitchen hygiene was found significantly protective against diarrhea [11, 12].

Despite the fact that fomite or surface is not included as a pathogen transmission pathway in "F-diagram", the potential of environmental surfaces subject to daily touching in the transmission of human diarrheagenic pathogen has already been established [13]. Contaminated kitchen utensils has been suggested before as source of contamination in children's food and drinking water [14]. Cutting tools in kitchen may become contaminated from fish which carries the pathogen [15]. Children may become infected by mother's hand which acquired contamination from infected household surfaces in low-income urban communities [16]. The fact that how surface areas in different locations of household react in diarrheal outbreak situation as fecal contamination risk factor, was not investigated.

The Global Disease Burden study, 2015 has been conducted to find the etiology of diarrheal disease related deaths in Bangladesh and *Escherichia coli* was one of the dominant causative agents [1]. *E. coli* is a genetically heterogeneous group of bacteria whose members are typically non pathogen and live in the intestinal tract of humans and animals as normal micro-flora However, acquisition of virulent factors enables certain subsets of this bacterium to cause intestinal and extra intestinal disease. Currently these organisms are classified in six categories, but this may be increased with time. These categories include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adhering *E. coli* (DAEC) [17]. Cytolethal distending toxin (encoded by *cdt* genes) producing *E. coli* has also been reported with childhood watery diarrhea [18]. ETEC is major etiologic agent of diarrhea in developing countries that account for 500,000 deaths annually and this pathogen is endemic in Bangladesh [19]. GBD study identified EPEC along with ETEC as significant enteropathogen for causing diarrhea in under -5 diarrhea deaths in Bangladesh [1]. EAEC is the one of the most frequent contributors of infant diarrhea in Bangladesh [20].

Previously, Ercumen et al [21] assessed domestic fecal contamination in rural areas of Bangladesh. The authors' did not explore any diarrhea case household. In this study, diarrheagenic *E. coli* transmission pathways in low-income household environment and associated diarrhea cases have been characterized for contamination level in urban community. In addition, diarrheagenic virulence properties of *E. coli* strains have been determined.

2. MATERIALS AND METHODS

2.1. Study Population

The current study was a part of prospective household diarrhea surveillance in a low-income urban area Arichpur (population density 10^5 per km^2), Dhaka, Bangladesh. The area holds approximately 29000 households. Many nuclear families in this community share a single room

dwelling. It is common that an average of 10-15 families share communal stoves, latrines and water source [22]. The majority of this community dwellers work as garments or industrial worker.

2.2. Sample Collection and Processing:

A surveillance study was conducted in Arichpur area under "Combating cholerae caused by climate change, "C5" project funded by Danish International Development Agency (DANIDA) project and 477 low-income households (HHs) were enrolled. A monitoring office had been set up in this area for the collection of samples. The current study was conducted with a subset of diarhhrea households. Eight diarrhea HHs per month between September, 2015 to December, 2015 were selected for investigation. Diarrhea patients were characterized by the occurrence of three or more, loose, liquid or watery stool or at least one bloody loose stool in a 24-h period. Diarrhea cases were reported by patients or caretakers over phone or during regular household visit. Four frequently touched surfaces (water vessel used for drinking water, cutting knife, door knob and food plate) which were obtainable in all HHs have been selected for sampling as 'hotspots". Rectal swab samples of the patients and corresponding household hotspot swab samples, leftover food and drinking water samples have been collected by trained staff from the respective house within 24 hour of reporting. Two HHs reported diarrhea infection of two members simultaneously. Each rectal swab counted as one sample unit (rectal swab and associated HH samples) for the ease of comparison. All the samples were immediately stored at refrigerator in the field office and transferred in a cool box to the Environmental Microbiology Lab, University of Dhaka for further analysis. Total number of samples collected is shown in **Table 1**. Individual collection procedure for each sample type is given below.

Rectal swab:

Adult patients have been explained first about the procedure of taking self rectal swabs. The sterile cotton swabs were first soaked with Phosphate Buffered Saline (PBS) and swabs were taken by inserting this wet swab by inserting approximately 2 cm (1 cm for children under 2) into the rectum and rotating 2 times. Afterwards the swab stick has been inserted into a tube containing 3 mL PBS.

Hotspot swabs:

First sterile cotton swabs were immersed into PBS tube. Around 10 cm^2area was swabbed and the swab stick was returned to the PBS tube for transportation. For the surfaces which holds uneven shapes (drinking vessel, cutting knife, door knob), complete surface area was swabbed.
Both rectal and hotspot swabs in PBS tube were vortexed thoroughly for 1 min and 500 micro liter (µL) was added to 2 ml nutrient broth (OXOID, UK) for overnight incubation at 37˚C.

Drinking water:

About 100 mL of drinking water samples were collected aseptically in sterile water collection bottle. One ml of water sample was added to 10 ml of NB and left for overnight incubation at 37˚C.

Food:

Leftover food samples which were kept in room temperature for 6 hours or more were collected during the visit of the field staff. Approximately 25 gm of solid food were taken with sterile spatula to a sterile zip lock bag. For liquid food such as lentil soup, 25 mL of samples were collected. Ten gm of food sample was weighed aseptically into a sterile stomacher bag. Large portion were cut into smaller pieces before blending. Small amount of nutrient broth (OXOID, UK) was added to the bag and blended thoroughly in a Stomacher Lab Blender (Seward Stomacher 80, Lab UK). After blending, additional nutrient broth was added to bring the total amount added to 90 ml (1:10 dilution). For liquid food, 10 ml of sample was added to 90 ml of nutrient broth to make 1:10 dilution. Samples were incubated at 37˚C for 18-24 hour.

Table 1: Distribution of different the sample types

Sample type	Sample number
Rectal swab sample	36
Hotspot Sample (cutting knife, latrine door swab, drinking water vessel, food plate)	144
Water Sample	31
Food Sample (collected at the same time)	34

2.3. Ethical approval:

Informed consents were taken from the household caretakers at the time of enrolment in the study. Patients or their guardian were also asked for written consent before rectal swab collection. The study (Research protocol# PR-14006) was approved by the Ethical Review Committee (ERC) of International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B) and Faculty of Biological Science, University of Dhaka.

2.4. Total DNA extraction

DNA extraction was done using boil template method according to Medici et al [23] with minor modifications.

2.5. Detection of diarrheagenic *E. coli* by Multiplex PCR

Total 245 DNA of the samples have been extracted directly from the enriched samples and for the presence of the characteristic virulence genes of diarrheagenic *E. coli* virotypes by performing single and multiplex PCR as described previously [24, 25]. The positive controls used in this study are listed in supplementary table 1 and the specific primer information is listed in supplementary table 2 and. For resolving PCR products, 1.5% agarose gel were used melted in Tris-Acetate EDTA (TAE) electrophoresis buffer. Upon electrophoretic separation, gel was stained in Et-Br staining solution and was observed with a UV transilluminator (Gel Doc, Bio-

Rad, USA). Photographs were taken and bands were analyzed with "Quantity One®" software (Bio-Rad, USA). PCR estimated using 100 bp DNA size markers (Invitrogen, USA).

2.6. Isolation and identification of bacteria

E. coli isolates have been screened out from clinical and household samples after enrichement. *E. coli* isolates were identified by culturing on selective media such as MacConkey and Eosine Methylene Blue (EMB) agar (OXOID, UK) and further biochemically confirmed by methyl-red test, oxidase test, indole test, citrate test. All the isolates of *E. coli* were harvested and stored in trypticase soya broth (OXOID, UK) containing 30% glycerol at -80ºC for further analysis. The isolates were characterized as diarrheagenic pathotype by performing PCR experiment described in 2. 5. Additional detection of the genes for CTEC virotype (*cdt*I II, III and IV) was conducted for *E. coli* isolates. Primer sequence information is given in supplementary table 3.

3. Results

3.1. Patient information:

Twenty two out of 36 patients were adult in age (17+) and 11 patients were child (0-10 years).Twenty two patients visited local pharmacy to buy drugs such as oral saline and metronidazole, two patients sought medical help from private doctors in response to the episode of diarrhea. None of the patients were admitted to hospitals.

3.2. Analysis of *E. coli* virulence genes in total DNA and association of diarrhea with in-house samples

PCR analysis of 245 samples demonstrated that at least one of the virulence-associated genes of diarrheagenic *E. coli* was present in 89 (36%) samples (**Table2**). These genes were found among rectal swab samples (28%, 10 of 36), hotspot samples (44%, 63 of 144), food samples (12%, 4 of 34), water samples (10%, 3 of 31).It has been observed that 17% (6 out of 36) rectal swab and the associated household samples showed the presence of similar virulence factors specific for *E. coli* pathotypes (Table 2).The prevalence order of the associated environmental samples with rectal swabs of similar pathotypic genes is as follows: drinking water vessel> latrine door knob> cutting knife> food plate>food (none of the corresponding household samples were missing in this analysis). All the samples collected from only three HHs were found negative for *E. coli* virulence genes.

Table 2: Presence of virulent genes of diarrheagenic *E. coli* in direct rectal swab sample and corresponding hotspot sample.

Rectal swab ID										
SI	ID Number	Patient's age (years)	Sex (M=Male F=Female)	Direct Rectal Swab	Cutting Knife	drinking water vessels urface	Latrine door knob	food plate	water sample	food sample
1	R-175	17+	M	ETEC, EIEC		ETEC				
2	R-176	17+	M		ETEC, EHEC	EHEC, ETEC	ETEC, EHEC, EIEC		*	*

3	R-177	11-17	F	ETEC	ETEC, EHEC	ND	ETEC	EIEC		
4	R-178	17+	F		EHEC	ETEC, EIEC			*	
5	R-179	17+	F		EIEC	EHEC	ETEC	ETEC, EHEC ,EIEC		ETEC
6	R-180	17+	M		EIEC	EIEC				
7	R-185	0-10	M	EPEC			ETEC			
8	R-186	11-17	M		ETEC					ETEC
9	R-190	17+	F		EIEC	ETEC	ETEC	EHEC,ETEC		
10	R-191	17+	M		EIEC	ETEC	ETEC	EHEC,ETEC		
11	R-196	17+	M					ETEC		
12	R-197	17+	F		ETEC	ETEC	ETEC	ETEC		
13	R-198	17+	M				ETEC	ETEC		
14	R-199	0-10	M	ETEC	ETEC	ETEC				
15	R-200	17+	M		EHEC, ETEC, EIEC		ETEC		*	
16	R-201	0-10	F	ETEC		ETEC, EHEC		EHEC		
17	R-202	17+	F					ETEC,EHEC		
18	R-203	0-10	F				ETEC	EHEC		
19	R-212	17+	F		ETEC		ETEC	ND		
20	R-215	17+	F		ETEC	ETEC			*	
21	R-233	17+	F				EHEC			
22	R-234	17+	F	ETEC		EHEC	EHEC	EHEC		
23	R-235	0-10	F		ETEC, EHEC					
24	R-236	17+	M	EAEC	EHEC	ETEC	EAEC	ETEC		
25	R-237	0-10	M	ETEC	EHEC					
26	R-238	17+	M						*	*
27	R-239	0-10	M		EAEC					
28	R-240	0-10	F							
29	R-246	0-10	F	EIEC						
30	R-248	0-10	M							
31	R-249	0-10	F			EIEC	EIEC			
32	R-250	0-10	F			ETEC			EHEC	
33	R-251	17+	F	ETEC		ETEC			EHEC	ETEC
34	R-252	17+	F			ETEC			EHEC	ETEC
35	R-253	17+	F					EHEC		

36	R-254	17+	F		EHEC					

*means sample not collected due to refusal of caretaker
Blank cells- Not Detected

Among the 10 *E. coli* positive rectal swabs, 6 swabs were found exclusively positive for ETEC virulent genes. Co-infection was detected in case of rectal swab sample of one patient; both EIEC and ETEC virulent genes were found positive in R-175.

Three HHs with ETEC positive patients were also found positive with ETEC genes in multiple environmental samples. For example, the HH with rectal swab ID R-199 showed ETEC virulent gene positive for patient, cutting knife and drinking water pot surface.R-201 have been found ETEC virulent gene positive for both patient and drinking water vessel surface. Rectal swab from one patient (R-246) was found positive for EIEC (Table 2). However, any of the corresponding household hotspot, food or water was not positive for EIEC. Individual hotspot sample such as latrine door knob, food plate, drinking water vessel surface, cutting knife have been found positive for EIEC. Although, no patient's sample has been found positive for EHEC, environmental samples from 20 household were positive for EHEC genes. One HH showed the presence of EAEC in both patient's swab (R-236) and the associated latrine door knob swab. It has been observed that the same virulent gene was present in two or more hotspots. In case of R-197, the same virulent gene was present in all four hotspot samples.

3.3. Prevalence of diarrheagenic *E. coli* isolates

Total 50 isolates of *E. coli* were isolated from household samples. The sources of the isolates were; 29 isolates from rectal swab samples, 20 isolates from hotspot samples, 1 isolate from food samples.

3.4. Characterization of virulence genes profile of *E. coli* isolates

Among the 50 *E. coli* isolates, 20% (10 out of 50) strains were toxigenic. The prevalence of isolate of diarrheagenic *E.coli* pathotypes in different sample types has shown in table 3. The most frequently identified diarrheagenic *E. coli* were EAEC and CTEC (3 isolates each type). Two EAEC strains have been isolated from patient's rectal swab and latrine door knob swab of same house.

Table 3: Prevalence of the different toxigenic diarrheagenic *E. coli* strains isolated from different sample source

Source of the sample	Number of toxigenic strains (Total 10)	Diarrheagenic*E.coli* categories	Genes positive
Rectal Swab	06	EPEC(01), ETEC(02),EAEC (02),	*eaeA, eltB,estB, pCVD,cdt*I

Sample		CTEC(01)	
Hotspot Sample	03	CTEC (02), EAEC (01)	*cdt*I, *pCVD*
Food Sample	01	EHEC (01)	*vt*2

4. Discussion

This study provides insight into a range of environmental reservoirs of pathogenic *E. coli* in low-income households in relation to diarrhea incidences. We studied *E. coli* contamination in clinical samples from diarrhea patients along with household surfaces, drinking water and food to explore the diarrhea transmission pathways. Results showed higher prevalence of diarrheagenic *E. coli* genes in hotspot swabs similar to in clinical samples from patient. Lower frequency of contamination was observed in water and food samples.

Fifteen percent patients (rectal swabs) showed association with household sample contamination *by E. coli*. Among the different sample types, drinking vessel surface contamination was highest with similar *E. coli* virulence factors found in patients' rectal swab from same house. Luby et al [7] suggested an increased risk of domestic fecal contamination through hands with the presence of child patients with diarrhea in households. They also indicated that the occurrences of drinking water contamination happened after diarrhea infection. Frequent touching of common household surfaces could transmit pathogens to healthy individual as it has been found that caregiver hands of diarrhea patient can carry diarreal pathogens including pathogenic *E. coli* [26]. Cleaning of kitchen and eating utensils does not always confirm the absence of pathogens. In our study apparently cleaned and dried food plates and drinking pots were found positive for pathogenic *E. coli*. Our results consistent with a study findings in peri-urban area of Peru that showed the presence of fecal indicator bacteria on cleaned tableware in 80% of households even after cleaning with soap [27]. Using bleach solution and boiled or filtered water for cleaning utensils and washing hands in diarrhea households could be an alternative hygiene intervention.

Eating utensils are an intermediary pathway of fecal pathogen transmission to food. Direct contact with floor surfaces could be another way of transmission of fecal pathogens to eating utensils in poor communities. Low-income residents in Arichpur normally take the food sitting on the floor in their single room dwelling. Study in Myanmar reported significant association between intake of food placed on the floor with persistent diarrhea in children [28]. ETEC virulent genes have been found in both patients and cutting knife swabs from same households of Arichpur. Earlier report showed that *V. cholerae* contamination could spread from fish to cutting knife during processing in kitchen [15]and that eventually could lead to cholera outbreak [29]. In our study, pathogenic EAEC strains have been recovered from a patient and door knob of the latrine used by him. Presence of enteropathogens in latrine door handle has been observed where hygiene rules were not practiced properly [30].

In our study, *E. coli* virulence genes have been detected in cooked food samples in three households and from one household both food and patient's rectal swab were found positive for ETEC pathotype. The same household showed the presence of ETEC gene on drinking pot surface. The result indicates the circulation *E. coli* pathogen via multiple routes in the household which eventually spread to food for consumption. A multipathway exposure assessment model describes a combined network of fecal pathogen transfer from environment to human where same vehicle such as hands, may interact with different transmission routes in highly contaminated urban environments[31]. Some households in Arichpur showed several hotspot contaminations with same *E. coli* pathotype which was not detected in the diarrhea patient. The result suggested the contamination may occur from same source within the domestic environment.

Although the diarrhea patients in our study experienced moderate to severe diarrhea, none of them required hospitalization. Majority of the rectal swab and environmental total DNA samples showed the presence of ETEC virulence genes in this study. A systemic review conducted by Walker et al [32] which demonstrated ETEC and *V cholerae* O1/O139 as leading causes of hospitalization. Besides, a study conducted in Dhaka between the years 2008-09 reported detection of the molecular marker of EAEC in majority of the infant diarrhea cases [33]. Commonly cytolethal distending toxin (cdt) positive *E. coli* strains from Bangladesh also possessed the virulence factors of major diarrheagenic pathotypes particularly EPEC [34, 35]. The cdt positive *E. coli* strains in our study did not possess other diarrheagenic *E. coli* toxin markers. However, isolation of this type of cdt positive strains have been reported recently in diarrhea patients from Mexico [18]. *E. coli* molecular markers were not detected in 72% of the patients in our study which indicated diarrhea may be caused by other bacterial, viral or parasitic agents. Although, a limitation of our findings includes that only pathogenic *E. coli* has been targeted as diarrheal pathogen as it would be inconceivable to follow multiple pathogens in household environment simultaneously. However, the results indicate the presence of diarrheagenic pathogen in the environmental components of the households and how they can be transmitted to the mouth of healthy individuals.

In conclusion, this study provides an overview on how contamination in household environment maintains association with the diarrheal agents in the urban community. Our results indicate that not only *E. coli* but also other enteropathogens could also be transmitted through these environmental pathways. Although, this study could not confirm about the initiation of the contamination (pre or post diarrhea) in the in-house environment, contaminated domestic pathways could play as source of transmission to other members of the households. Efforts should be given in hygiene interventions on surface sanitation to reduce diarrhea in highly contaminated urban community.

5. References

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442 **Supplementary Table 1.** Reference isolate for toxigenic gene

Name of genes	**GenBank accession No.**
vt1	KY319038
vt2	KY221829
eaeA	KY073237
bfpA	KY221831
Ial	KY221830
Pcvd432	KY243935
estA,	KY221833
eltB	KY221832
Cdt I	E6468/62

443

444 **Supplementary Table 2**: Primers used in the multiplex PCR

Virulence Factor	**Virotype**	**Primer Sequence (5' to 3')**	**Reference**
Invasion Plasmid Antigen (*ipaH*)	EIEC	TGG AAA AAC TCA GTG CCT CT CCA GTC CGT AAA TTC ATT CT	[25]
Heat Labile Enterotoxin (*elt*)	ETEC	TCT CTA TGT GCA TAC GGA GC CCA TAC TGA TTG CCG CAA T	[36]
Heat Stable Enterotoxin (*est*)	ETEC	GCT AAA CAA GTA GAG GTC TTC AAA A CCC GGT ACA GAG CAG GAT TAC AAC A	[37]
Verotoxin 1 (*vt* 1)	EHEC	GAA GAG TCC GTG GGA TTA CG AGC GAT GCA GCT ATT AAT AA	[38]
Verotoxin 2(*Vt*2)	EHEC	ACC GTT TTT CAG ATT TTG ACA CAT A TAC ACA GGA GCA GTT TCA GAC AGT	[39]
Intimin (*eaeA*)	EHEC	CAC ACG AAT AAA CTG ACT AA AAT G AAA AAC GCT GAC CCG CAC CTA AAT-	[40]
Invasive-associated locus(*ial*)	EIEC	CTG GTA GGT ATG CTG AGG CCA GGC CAA CAA TTA TTT CC	[41]

Bundle Forming Pillus (*bfpA*)	EPEC	AAT GGT GCT TGC GCT TGC TGC GCC GCT TTA TCC AAC CTG GTA	[42]	445 446 447
pCVD432 *	EAEC	CTG GCG AAA GAC TGT ATC AT CAA TGT ATA GAA ATC CGC TGT T	[43]	

*Primers amplify a fragment of the plasmid pCVD43

Supplementary Table 3: Primers used to detect cdt producing *E. coli*

Primer	Primer Sequence (5´to 3´)	Annealing Tm	Amplicon Size	Reference
CDT-I (*cdt* 1)	TGG TGA GAA TCG GAA CTG CAT TCC ATC AGG TTT GTC	56˚C	418 bp	[44]
CDT-II (*cdt*II)	AAT CCC TAT CCC TGA ACC GTT CTA TTG GCT GTG GTG	56˚C	542 bp	[44]
CDT-III (*cdt* III)	AAACAGGACGGTAATAATGAC TAATA GTGATCTCCTTCCATGAAAATA TAGT	59˚C	2230 bp	[45]
CDT-IV (*cdt* IV)	CCTGATGGTTCAGGAGGCTGGT TC TTGCTCCAGAATCTATACCT	60˚C	350 bp	[46]

Manuscript 6

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ORIGINAL ARTICLE

Comparative genomics of *Vibrio cholerae* O1 isolated from cholera patients in Bangladesh

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Significance and Impact of the Study: *Vibrio cholerae* has frequently experienced genetic changes with rapid evolution of pandemic clones in the Ganges Delta region. Whole genome sequencing can reveal genetic information of current pathogenic *V. cholerae* in Bangladesh which includes cefotaxime genotypes, virulence factors, altered antimicrobial resistance pattern as well as mobile genetic element compared to global pandemic strains. This study data could be used in planning future surveillance strategies in Ganges Delta region by informing new epidemiology of current outbreak strains.

Keywords
antimicrobial resistance, Bangladesh, MLST, SNP, *Vibrio cholerae* O1, whole genome sequencing.

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Abstract

Whole genome sequencing was utilized to investigate the genomic profile of *Vibrio cholerae* O1 strains, isolated from symptomatic patients in a low-income urban area of Dhaka, Bangladesh. Comparative genomics using bioinformatics tools were applied to identify major virulence factors, biotype and antimicrobial resistance genes in three *V. cholerae* O1 strains (VC-1, 2 and 3) isolated from two case patients. A phylogenetic SNP (single nucleotide polymorphism)-based analysis was conducted to infer the relatedness to *V. cholerae* O1 strains isolated elsewhere. The *V. cholerae* strains were the El Tor variant carrying *ctxB*1 (standard classical genotype). SNP-based global phylogeny revealed that the three isolates were strictly clonal and the closest neighbouring genomes were epidemic clones of *V. cholerae* O1 isolated in 2010 from cholera patients in Pakistan. All strains harboured the integrase gene of the SXT element (int_{SXT}), antimicrobial resistance genes for aminoglycosides, phenicol, sulphonamide and trimethoprim except VC-1 that lacked sulphonamide resistance genes. The multilocus sequence typing (MLST) revealed that the strains belonged to sequence type, ST69. The study provides knowledge on current genetic traits of clinical *V. cholerae* O1 circulating in urban household clusters of Bangladesh which may help in predicting emergence of new pandemic strains in Bangladesh.

Introduction

Cholera has claimed millions of lives globally since the spread of the first pandemic from the Indian subcontinent. Despite groundbreaking cholera interventions and diagnostic advancement, cholera cause both endemic and epidemic diseases in Bangladesh (Harris *et al.* 2008).

In urban slum areas of Dhaka, 4 per 1000 people become affected with cholera annually and the number is increasing due to rapid urbanization (Chowdhury *et al.* 2011). Clinical cholera cases in Bangladesh are entirely attributed to the O1 serogroup of *V. cholerae* with occasional sporadic outbreaks caused by the O139 serogroup (Chowdhury *et al.* 2015b). Although the Classical biotype

of *V. cholerae* O1 (*ctxB*1 genotype) was extinct from this region since 1992 (Faruque *et al.* 1993; Chowdhury *et al.* 2015a), new variants of El Tor biotype strains producing classical toxin, *ctxB*1 (altered strains), emerged in Bangladesh in late 1990s (Nguyen *et al.* 2009). The 'altered' biotype strains have replaced the *ctxB*3 genotype of the seventh pandemic El Tor strains since 2001 and continued to be the cause of severe dehydration and morbidity in hospitalized patients (Nair *et al.* 2006; Chowdhury *et al.* 2015a; Rashid *et al.* 2016).

In this study, whole genome sequencing (WGS) combined with the use of bioinformatics tools were used to determine the genomic and evolutionary characteristics of three *V. cholerae* O1 strains collected from two patients in Dhaka city, Bangladesh. Furthermore, comparative genomic analysis was conducted with spatial and temporal clinical *V. cholerae* O1 strains available from the public domain to determine the genetic relatedness.

Results and discussion

The distance between case 1, a girl of 4 years, and case 2, a girl of 22 years was 588 m and the households used different water sources.

Two *V. cholerae* strains were isolated from case 1, i.e. VC-1 and VC-3. The patient was reported to have consumed untreated tap water from a closed roof tank supplied with groundwater. In addition, one *V. cholerae* strain was isolated from case 2, i.e. VC-2. The patient had consumed untreated communal tap water at home provided from the Dhaka city corporation (Water Supply and Sewerage Authority, WASA). Subsequently, a number of relevant environmental samples such as drinking water, food and environmental swabs from the respective patients' households were investigated for the presence of *V. cholerae* but were found negative in culturing.

The three *V. cholerae* strains belonged to the seventh pandemic serogroup O1 (*rfbV-O1*), serotype Inaba, biotype El Tor variant, ST69, and carried the standard classical *ctxB* (*ctxB*1) and El Tor *tcpA* genotype ($ctxB^{CC}rstR^{ET}tcpA^{ET}$) (Son *et al.* 2011; Siriphap *et al.* 2017). All three strains harboured 10 major virulence-associated genes normally seen in clinical serogroup O1 strains (*ctxA*, *ctxB*, *zot*, *ace*, *tcpA*, *hlyA*, *mshA*, *rtxA*, *ompU* and *toxR*) (Finkelstein and Mukerjee 1963; Singh *et al.* 2002; Hasan *et al.* 2013) except for the *ace* gene which was not detected in VC-3. The genes for type III secretion system, cholix toxin (*chxA*) and heat-stable enterotoxin (*stn*), were absent in the strains. The three *V. cholerae* strains harboured similar genetic backbone of seventh pandemic El Tor variant trait which has been the highly dominant type in Bangladesh in recent years (Chowdhury *et al.* 2015a). Since the emergence of the *ctxB*1 genotype in early 1990s, a similar temporal change occurred in Bangladesh after 2008 when classical genotype *ctxB*1 shifted to the Haitian genotype *ctxB*7 and *ctx*B1 returned again in 2013–2014 (Rashid *et al.* 2016).

The strains showed a unique deletion in the Vibrio pathogenicity island-1 (VPI-1) gene cluster containing three consecutive gene deletions (VC-0819–0821). Variant of Vibrio seventh pandemic island-2 (VSP-2) was found in all the three genome sequences compared to the prototype N16961 with a deletion of four ORFs (VC-0495–0498). Similar variants were isolated in India before 2010 (El Ghany *et al.* 2014; Imamura *et al.* 2017). After 2010, another variant with a larger deletion spanning from ORF VC0495 to VC0512 (CIRS101 type VSP-2) became predominant in Kolkata, India, and eventually replaced other types (Imamura *et al.* 2017).

The evolution through the larger deletions may occur due to significant genetic rearrangement within the two loci, at the 3′ end of the VC0498 and VC0511 of the genomic backbone of the island and also the event may be more human host specific (Taviani *et al.* 2010). In Bangladesh, the most recent report from 2004 to 2007 on VSP-2 variant of El Tor strains showed a higher prevalence of the CIRS101 variant (Taviani *et al.* 2010). Our results indicate recent occurrence of genetic shift in VSP-2 which is more similar to prototype seventh pandemic El Tor N16961. Previously, the frequent rearrangements of the variants of VSP-2 islands were also occurring in India and Bangladesh but the explanations for such rearrangements are still unclear (Taviani *et al.* 2010; Imamura *et al.* 2017).

All three strains were phenotypic susceptible to chloramphenicol (CHL), tetracycline (TET), azithromycin (AZM), neomycin (NEO), gentamicin (GEN) and cefotaxime (CTX) and resistant to ciprofloxacin (CIP), streptomycin (STP), nalidixicacid (NAL), trimethoprim (TMP), sulfamethoxazole (SMX) and ampicillin (AMP) (VC-3 intermediate). Multiple acquired antimicrobial resistance (AMR) genes were determined of which VC-2 and VC-3 showed identical profile harbouring AMR genes for aminoglycosides (*str*A/*str*B); sulphonamides (*sul*2); CHL (*cat*B9) and TMP (*dfr*A1). The strains did not show phenotypic resistance to CHL which is in agreement with a recent report showing that the *cat*B9 gene cassette was associated with very low level of AMR to CHL (Kumar *et al.* 2017). VC-1 contained the same resistance genes as VC-2 and VC-3 except for the *sul*2 and *str*A genes. The occurrence of one genetic event may result in the deletion as *sul*2 and *str*A genes locate right next to each other in the SXT element. Comparison of the specific genomic region in SXT element of VC-1, 2 and 3 with *V. cholerae* 2010EL-1786 further confirmed the absence of the *sul*2 gene in VC-1 (Fig. 1). The locus deletion was found

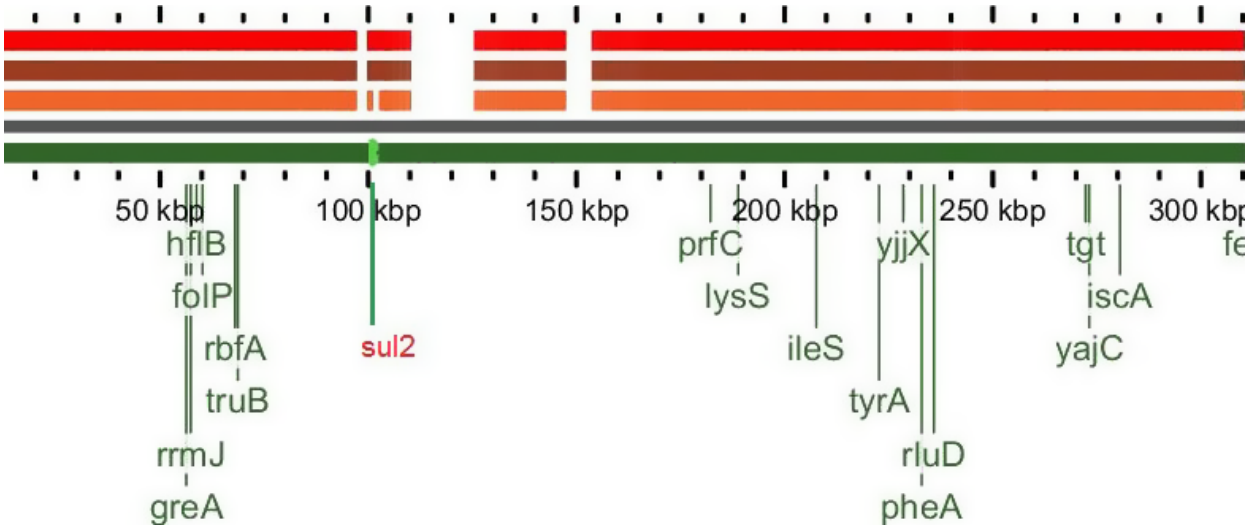

Figure 1 Genomic variation of the three *Vibri cholerae* strains indicating *sul*2 gene using *V. cholerae* 2010EL-1786 chromosome one as reference (■, VC-3; ■, VC-2; ■, VC-1; ■, *V. cholerae* 2010EL-1786). [Colour figure can be viewed at wileyonlinelibrary.com]

within 102·017 to 102·832 bp in VC-1 as compared to *V. cholerae* 2010EL-1786 genome. Although absence of *sul*2 gene in the VC-1 SXT element is unusual, such as *sul*2 gene deletion in SXT variant types has been reported (El Ghany *et al.* 2014; Siriphap *et al.* 2017). In 2009, only 1 in 38 *V. cholerae* strains had the deletions of the *sul*2 gene in a study of hospitalized patients in Chandigarh, India (El Ghany *et al.* 2014).

All the strains contained the SXT element (*int*SXT gene) and lacked the integrase genes of class 1 integron (*int*I gene). Our results corroborate previous findings in Asia and Africa (Iwanaga *et al.* 2004; Sambe-Ba *et al.* 2017; Siriphap *et al.* 2017). The NAL- and CIP-resistant strains conferred amino acid substitutions in *gyr*A (Ser83-Ile) and *par*C (Ser85Leu) but did not harbour the fluoroquinolone resistance *qnrVC1* gene. The genomic organization of the integrating conjugative element (ICE) of the *V. cholerae* strains showed an allelic profile similar to the reference ICE*Vch*Hai1 except the 21 common deletions including *flo*R in loci VC1786ICE6-9, VC1786ICE14, VC1786ICE21-29, VC1786ICE49-53, VC1786ICE83 and VC1786ICE85 (Table S5). VC-3 contains VC1786ICE10 loci, a putative transposase, but it was absent in the other two strains. The strains conferring resistance to TMP harboured the *dfr*A1 gene and lacked *dfr*A18 which reveals the similarity of our strains with ICE*Vch*Ind5 (Spagnoletti *et al.* 2014). The Bangladesh SXT elements have highly structural similarity except for a few additional allelic deletions with the clade 1 isolates from Chandigarh, India, isolated in 2009 which clustered with earlier outbreak strains of *V. cholerae* from India, Bangladesh and Nepal (El Ghany *et al.* 2014). Similarity with the Indian O1 strains indicates recent acquisition of the SXT element within strains from the same geographical areas.

The SNP analysis revealed the three *V. cholerae* strains being strictly clonal with zero SNP between them in comparison to the identified 390 SNPs identified among the 38 Bangladeshi strains. SNP accumulation depends on temporal signature and approximately 3·3 SNPs per year can be accumulated in the *V. cholerae* core genome (Mutreja *et al.* 2011). The three strains were isolated only 11 days apart and from local cholera outbreaks with two households located in the same community, which likely explains the lack of SNP differences. In Haiti, *V. cholerae* O1 strains from the outbreak in 2010 showed no polymorphic sites, whereas strains from Haitian patients in 2012 had 195 SNPs (Azarian *et al.* 2014). Moreover, the identical *V. cholerae* O1 clones found in the two case households and the separate water sources used by the households indicate a contamination source other than water supported by earlier reports of cholera cases in Arichpur neighbourhood (Bi *et al.* 2016). We have found differences in virulence and AMR gene profiles between VC-1 and VC-3 from the same patient; although there was no SNP difference. Moreover, superintegrons of the three strains did not show any diversity. Genetic diversity of *V. cholerae* strains from same patient may occur due to co-infection with multiple environmental strains (Rashed *et al.* 2014). As the three strains were clonal, there would be a possibility of circulation of similar outbreak strains in a short time span and between inter and intra person-to-person transmission.

The local phylogenetic tree showed that the isolates clustered into two major groups based on temporal

patterns: one cluster included strains isolated during the period 1969–2000 and the second cluster included strains isolated during the period 2001–2010 (Fig. 2). The three *V. cholerae* O1 strains of this study branched from the group of 2001–2010 strains with their closest ancestral strain being PSC-022 isolated in 2010 which differed with 18 SNPs.

The SNP-based global phylogenetic tree revealed that the 469 *V. cholerae* O1 strains were discriminated by 4141 high-quality SNPs. Our Bangladeshi strains formed a monophyletic branch within a clade consisting of epidemic clones of *V. cholerae* O1 El Tor, isolated from Karachi, Pakistan, during a major cholera epidemic in 2010 (Fig. 3). The Pakistani clones included in the clade named PSC-1 were of coastal origin and formed a unique clade in the wave 3 of seventh pandemic lineages (Shah *et al.* 2014). We found 11 SNP differences between strain VC-2 and the closest neighbouring genome (S9KCH9) (Table S4) which suggests that the strains are closely related. The second nearest genomes of VC-2 with 12 SNPs difference were strains S7KCH20 and PCS-022 isolated from patients in Pakistan and Bangladesh respectively. Interestingly, S9KCH9 and PCS-022 genomes differ with only three SNPs suggesting a clonal origin of the *V. cholerae* O1 strains associated with cholera outbreaks in 2010 in Bangladesh and Pakistan.

In conclusion, the current Bangladeshi strains showed a clonal origin from Southeast Asian epidemic progenitor and possess the genetic backbone of current ongoing epidemic *V. cholerae* O1 in Bangladesh with considerable

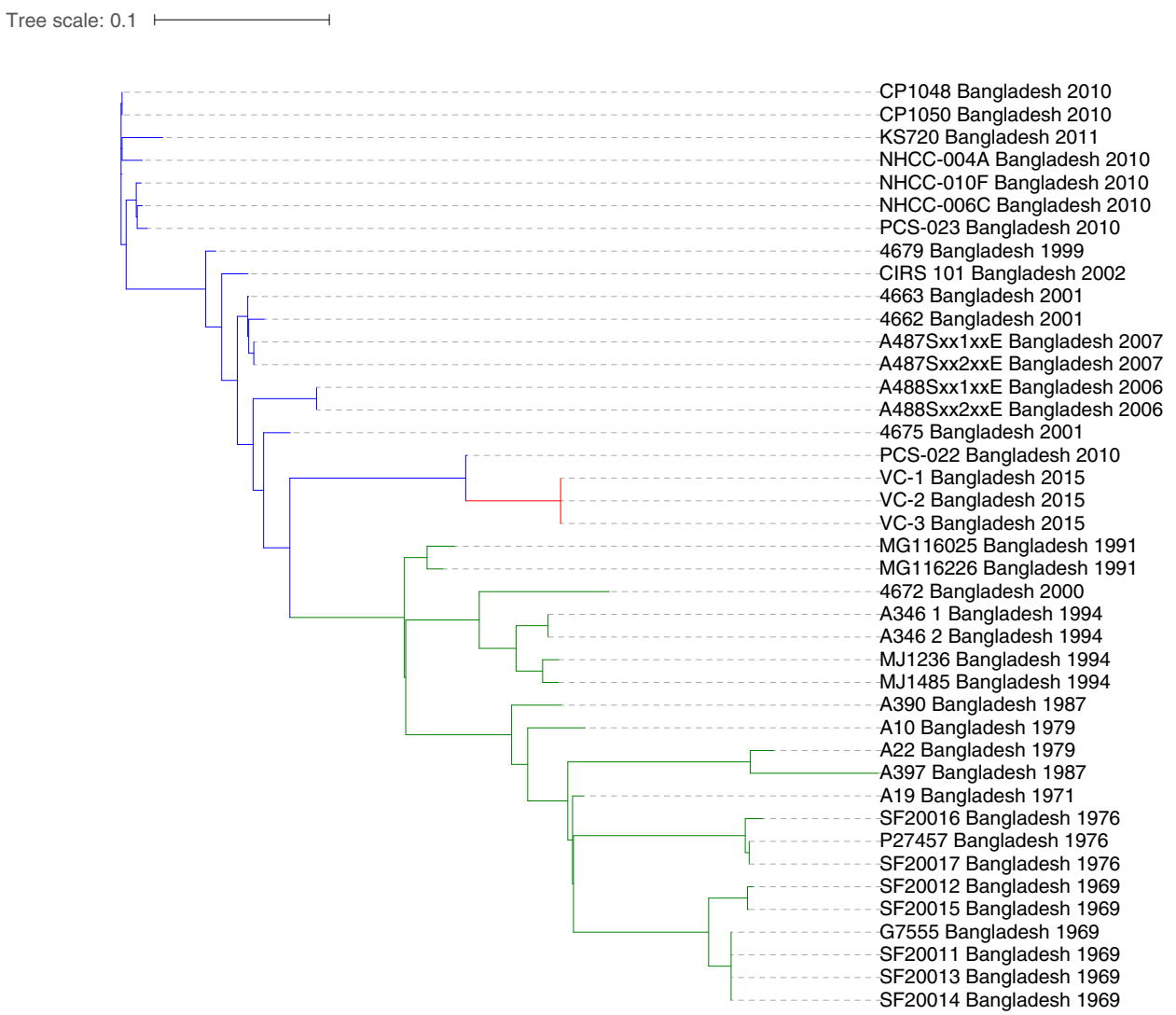

Figure 2 SNP-based local phylogenetic tree constructed including 38 database strains (Branches are highlighted in colours according to years: ■, Bangladesh 2015; ■, Bangladesh 2001–2010; ■, Bangladesh 1969–2000). [Colour figure can be viewed at wileyonlinelibrary.com]

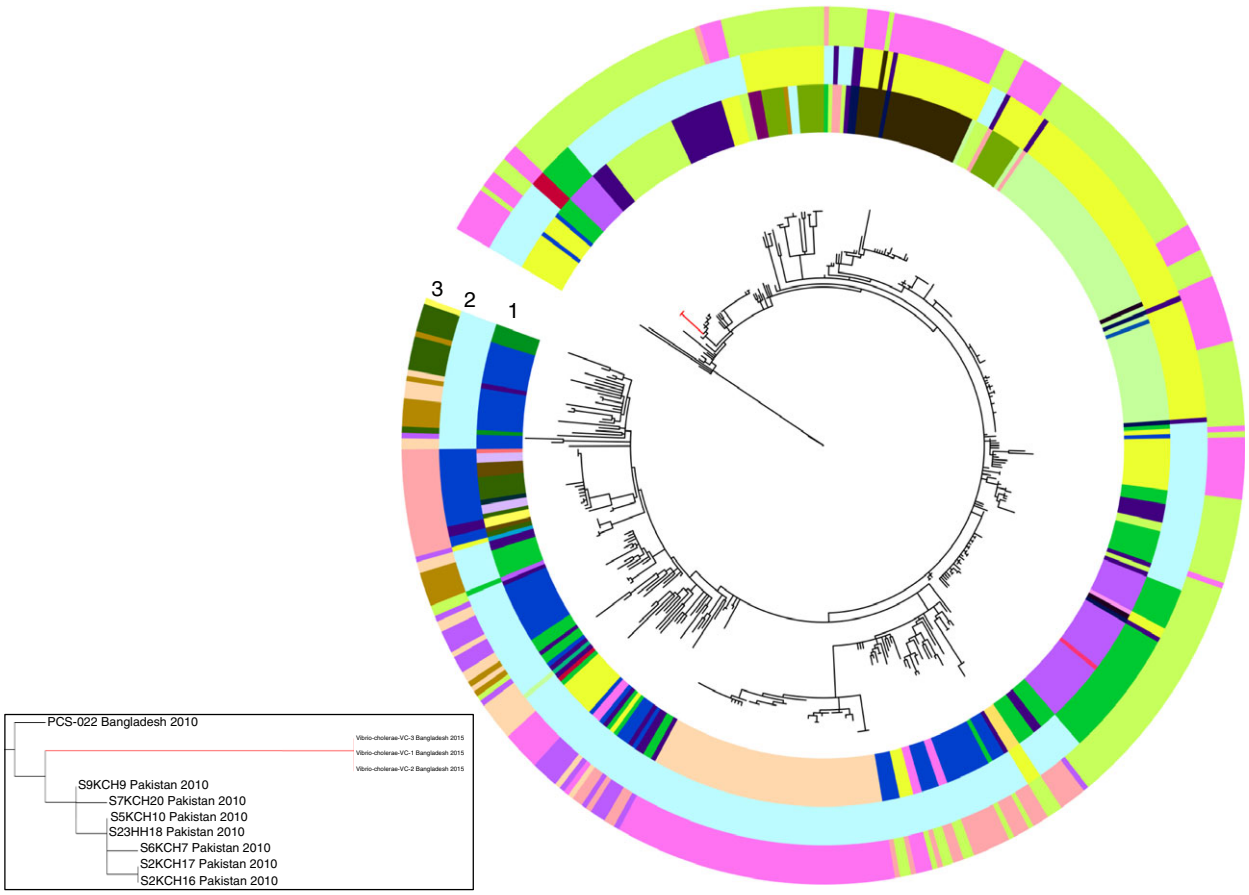

Figure 3 Global phylogenetic tree constructed with 466 database strains based on SNPs (Regions are highlighted in colours). The box in the lower left corner is focusing the position of the three study strains in the global tree. 1. Country (, Philippines; , China; , Thailand; , Pakistan; , Bangladesh; , India; , Nepal; , Vietnam; , Indonesia; , Afghanistan; , Bahrain; , Malaysia; , Haiti; , USA; , Mexico; , Dominican Republic; , Kenya; , Djibouti; , Mozambique; , South Africa; , Zambia; , Mauritania; , Tanzania; , Zimbabwe; , Peru; , Argentina; , Colombia; , Bolivia; , Chile; , Russia; , Cameroon) 2. Region (, Southeast Asia/China/Bangladesh; , South America; , Africa; , Pakistan/Afghanistan; , North America/Russia; , Bangladesh 2015; , Middle East) 3. Time range (, Pre-7th pandemic; , Predecessors 1957–1962; , 1963–1970; , 1971–1980; , 1981–1990; , 1991–2000; , 2001–2010; , 2011–2015). [Colour figure can be viewed at wileyonlinelibrary.com]

diversity in the pathogenicity islands and SXT elements. Our phylogeny results suggest that current *V. cholerae* O1 strains evolved from recent outbreak strains. Considering the global distribution, our study supports the hypothesis that Bengal Delta region remains the origin ground of cholera and persistent infection occur by either new epidemic clones or re-emergence of previously existing clones in this region. This study provides new insights on cholera epidemiology in Bangladesh using WGS technology together with advanced and easily accessible bioinformatics tools. WGS is a more effective, robust and precise method than other molecular approaches for continuous monitoring and possible control of the spread of new epidemic *V. cholerae* O1 clones in Bangladesh, the land which still holds the title as the home of cholera.

Materials and methods

Ethics

The study was approved by the Ethical Review Committee of International Centre for Diarrheal Disease Research, Bangladesh. An informed consent was obtained from the caretakers of the households, household respondents and patients for collection of rectal swab, both survived.

Collection of epidemiological data

During 4 months period, 37 rectal swabs were taken by trained field staff in case of watery diarrhoea reported by household caretaker and demographic, socioeconomic

and clinical data were obtained in a brief interview with patients or respondents (in case of child).

Isolation, identification and antimicrobial susceptibility testing

The rectal swabs were cultured for *V. cholerae*, confirmed by PCR for the *ompW* gene (Nandi *et al.* 2000) and 3 of 37 (8·1%) rectal swabs found positive. In October, 2015, three *V. cholerae* O1 strains were isolated from rectal swabs of two diarrhoea cases identified during random visits by field staff and a report via choleraphone (mobile phones distributed to households for diarrhoea reporting) (Sengupta *et al.* 2013). The distance between the two households was measured by a global positioning system. Suspected *V. cholerae* strains were isolated from rectal swabs by using standard biochemical, serological assays and a species-specific PCR (Nandi *et al.* 2000; Huq *et al.* 2006). According to the standard guideline described by the Clinical and Laboratory Standards Institute (CLSI), antimicrobial susceptibility testing to STP (10 μg ml^{-1}), CIP (5 μg ml^{-1}), CHL (30 μg ml^{-1}), TMP (5 μg ml^{-1}), SMX (25 μg ml^{-1}), NAL (30 μg ml^{-1}), TET (30 μg ml^{-1}), AZM (15 μg ml^{-1}), AMP (10 μg ml^{-1}), NEO (30 μg ml^{-1}), GEN (10 μg ml^{-1}) and CTX (30 μg ml^{-1}) (OXOID, Hampshire, UK) was performed by agar disk diffusion method (CLSI 2016). *Escherichia coli* ATCC 25922 was used for quality control.

Whole genome sequencing

The DNA was extracted from the three isolates for WGS as earlier described (Siriphap *et al.* 2017). Raw sequence data were deposited to Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession no.: PRJEB18714. The raw reads were *de novo* assembled using SPAdes 3·7·0 (Bankevich *et al.* 2012). The genomic data are included in Table S1.

In silico bioinformatics analysis

The web-server tool MyDbFinder 1·1 (https://cge.cbs.dtu.dk/services/MyDbFinder/) and associated database (Table S1) were used to analyse the assembled *V. cholerae* genome sequences to identify the species-specific gene, serogroup-specific genes, the biotypes-specific genes, putative virulence genes, *ctxB* genotype-specific genes, pathogenic islands and the specific gene of the seventh pandemic strain with a selected threshold of 98% identity (Siriphap *et al.* 2017).

The AMR genes were detected using ResFinder web server (ver. 2·1, 80% threshold for %ID/60% minimum length) (Zankari *et al.* 2012). blast atlas tool (https://server.gview.ca/) was used to further compare the strains with reference *V. cholerae* 2010EL-1786, accession no. NC_016445·1 (contain *sul2* gene). The SXT element, the class 1 integron and the presence of mutations in the DNA gyrase (*gyr*A gene) and in the DNA topoisomerase IV (*par*C gene) were determined using MyDbFinder and associated database (Table S2) (Kaas *et al.* 2016).

The genotypic profile of AMR determinates was compared with the genome of the 2010 Haiti *V. cholerae* O1 ICE, ICE*Vch*Hai1(JN648379) and *dfr*A18 gene of SXTMO10 (AY034138) by using MyDBFinder (threshold, 95% identity). The MLST sequence types (ST) for *V. cholerae* strains were determined using the mlst tool (ver. 1·8) (https://cge.cbs.dtu.dk/services/MLST/).

Phylogenetic analysis

High-quality single nucleotide polymorphisms (SNPs) of the three O1 *V. cholerae* genomes from Bangladesh were identified using the pipeline CSI Phylogeny 1·4 available from the CGE website (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). Raw read data and assembled genomes from 466 *V. cholerae* global pandemic and outbreak strains obtained from the European Nucleotide Archive and GenBank were also analysed to determine genome-wide SNPs and a global phylodynamic tree was constructed to map the Bangladeshi strains in a global context as previously published (Kaas *et al.* 2016). Moreover, a phylogenetic tree was inferred using SNPs differences in 38 genomes of the seventh pandemic *V. cholerae* O1 El Tor strains from Bangladesh. Raw reads were mapped to the published completed reference strain N16961 (acc. no.: NC_002505 1) as earlier described (Delcher *et al.* 2002; Kaas *et al.* 2016). The sequence information of 466 global strains and the specific SNP differences are included in the Supplementary Tables S3 and S4. The phylogenetic trees were created and visualized using online tool iTOL (ver. 3) (https://itol.embl.de/).

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Conflict of Interest

None declared.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1 Genomic sequence data, virulence profile and occurrence of antimicrobial resistance genes in the three *Vibri cholerae* strains.

Table S2 The accession numbers of the genes in this study.

Table S3 Sequence information of 466 global *Vibri cholerae* strains.

Table S4 SNP differences in the global *Vibri cholerae* strains.

Table S5 Comparison of ICEVchHai1 gene loci with the three *Vibri cholerae* strains.

Manuscript 7

Investigation on household contamination of fecal *Escherichia coli* and *Vibrio cholerae* in Bangladesh

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Abstract:

Diarrheal illness often related to fecal pathogen exposure via drinking water. Little is known about the fecal contamination risk areas within the household and especially in the kitchen environments in overcrowded low-income setting. Current study intended to analyze the hotspots in a household to identify the fecal contamination transmission routes. Fecal contamination was surveyed in routine swabs from four household identified hotspots: cutting knife, food plate, latrine door knob, and drinking water pot surface among 32 households for 1 year in every 6 weeks period in a low-income area, Arichpur, Dhaka. Moreover, 137 left over food samples were taken. All the samples were analyzed for total thermotolerant *Escherichia coli* count and the presence of *Vibrio cholerae* by molecular method. Fisher's exact test was used to compare the fecal contamination level in different surface locations. A subset of samples was assessed for the presence of diarrheagenic *E. coli* by PCR. Results revealed that *E. coli* contamination level was highest on food plates with a geometric mean (GM) of 3.08 cfu/cm^2 and the least contaminated site was latrine door knob (GM=0.06 cfu/cm^2). Food samples were found heavily contaminated with fecal *E. coli* (GM=13.32 cfu/gm, with counts ranges from 0 to 6400 cfu/gm). The level of fecal contamination is 4.7 times higher (p<0.05) in cutting knife than latrine door knob surfaces and 0.3 times lower (p<0.05) in latrine door knob than drinking water pot surfaces. *V. cholerae* contamination was also most predominant in food plate swabs. Genes of enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) were detected in surface samples among them ETEC was the most prevalent (46% samples were found positive).This study presents new dynamics of in-house components in transmission of fecal bacteria via food and kitchen utensils thus, proves the vulnerability of the kitchen environment of low-income urban settings in Bangladesh. Results of the study will enable an update of the diarrhea risk factors for early prevention of disease progression in risk groups.

Key words:

Fecal contamination, Household Transmission, *Vibrio cholerae*, Bangladesh

Introduction

In Bangladesh, diarrheal epidemics including cholera with higher incidence and increased severity of disease have been observed due to rapid urbanization during recent years in the capital city, Dhaka (Chowdhury et al., 2011; Paul et al., 2016). Enteric pathogens could transmit through multiple environmental reservoirs in communities with unsafe fecal waste management (Julian, 2016).The primary routes of enteric pathogen exposure within household were clearly depicted by the commonly known 'F-diagram' with five "F"s namely food, flies, fingers, field (soil), and fluids (water) (Wagner and Lanoix, 1958). Examination of fecal bacterial burden in household environment of Bangladesh showed fecal contamination in drinking water (Boehm et al., 2016; Ercumen et al., 2015), soil, hands and food in rural areas (Ercumen et al., 2017). Fly transmission of fecal *E. coli* in the urban setting of Bangladesh were also assessed (Lindeberg et al., 2018). Also in a recent study in rural Bangladesh, Pickering et al (Pickering et al., 2018) showed positive association between fecal *E. coli* concentration in child hand rinses and diarrhea, while the other environmental routes (stored drinking water, pond water, child courtyard soil, flies, and food) were not associated. There is a lack of investigation on surfaces of household objects as house transmission routes of fecal contamination particularly in highly populated urban areas.

Environmental surfaces of households in an overly populated, poor sanitation area could be contaminated by the frequent contact with infected members and thus foster person to person bacterial transfer to healthy individuals (Sinclair and Gerba, 2011). A study at a child care center in USA reported the increased diarrheal risk in relation to the surface contamination with fecal bacteria(Laborde et al., 1993). In low-income communities where chances of pathogen exposure are extremely high, accurate measurement of fecal contamination load on risk-prone environmental surface reservoirs is very crucial. In figure 1, multiple environmental transmission routes in low-income households are depicted more comprehensively by taking the "F-diagram" into consideration. In low-income urban areas of Bangladesh latrines and kitchens are generally situated outside and separate from the living rooms of multiple families. Pathogen could spread from house compound to food consumption area via several environmental and non-environmental routes.

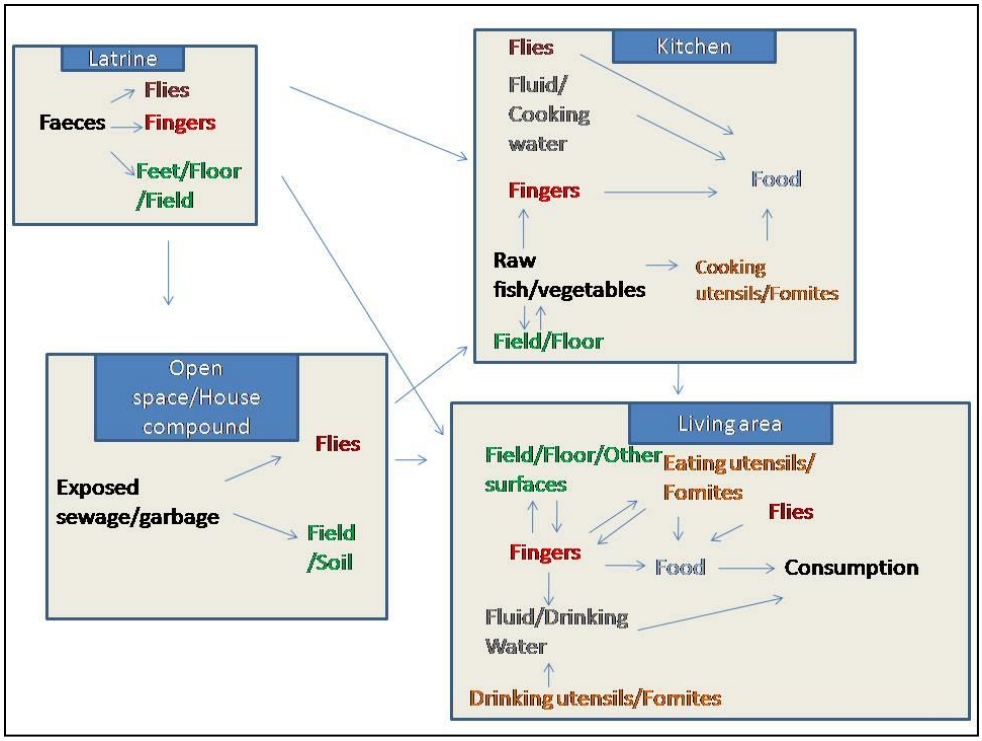


Figure 1: Environmental transmission routes in different household components

Fecal contamination level has been measured by means of quantifying *Escherichia coli* as fecal indicator in the highly polluted low-income urban areas elsewhere (Berendes et al., 2017; Pickering et al., 2012; Robb et al., 2017). In Bangladesh, *V. cholerae* infection is most frequent in the hospitalized diarrhea patients in the urban cities (Chowdhury et al., 2011). Surfaces from different components of domestic area contain range of bacterial prevalence which may differ by country-wise social and personal behavior of human (Robb et al., 2017; Sinclair and Gerba, 2011). Reports showed that communal sources of contaminated food and water or surfaces frequently touched by infected person may contribute fecal-oral transmission of *V. cholerae* causing cholera outbreaks within households in Bangladesh (Sugimoto et al., 2014; Weil et al., 2009).

To identify high risk areas (hotspots) for fecal contamination with potential public health threat in low-income urban community households of Bangladesh, we performed a prospective study to quantity fecal *E. coli* in food and surfaces which are highly exposed to human contact. The prevalence of *V. cholerae*, the etiologic agent of cholera, was also explored. Such information is vital for early prevention of diarrhea by generating awareness in sanitation practices.

Method and materials:

Sampling area:

The study area Arichpur, Tongi is located northwest of capital city Dhaka (GPS coordinates23°53'03.9"N 90°24'31.5"E). The area is surrounded by several industrial establishments and garments factories which make this 1.2 square kilometer area over populated with 29,000 households low income households. Typically, an average of 10-15 nuclear families living here share common cooking areas, latrines and water supply (Azman et al., 2015).

Sample collection and processing:

The study was conducted as a part of the collaborative diarrhea surveillance project "Combating Cholera Caused by the Climate Change, C5" in Arichpur focusing on household hygiene and risk behaviors. Field staffs from International Center for Diarrheal Disease Research (icddr,b), Bangladesh conducted community visit and sample collection. Sample processing and microbiological analysis were carried out at University of Dhaka.

Socio-economic and demographic information were collected from the households enrolled in the project. Hygiene behavior and water use information were collected from the household caretakers during a baseline survey and monthly visits which will be reported in detail elsewhere. In a routine survey in every 6 weeks during November 2014 to December 2015, total 668 environmental swab samples or "hotspot" samples among 32 households (HHs) (average 16 HHs each month) were collected from 4 spots of direct exposure within household domain: - cutting knife (n=169), food plate (n=165), latrine door knob (n=169), drinking water vessel surface (n=165). The hotspots were selected after preliminary observations on the basis of frequency of hand contact and availability to each HH. The utensils used for direct drinking of water (glass, mug) were assigned as "drinking water vessel" and cleaned food plates for taking meal as "food plate". Surface samples were obtained by swabbing approximately 10 cm^2 area with sterile cotton swab stick soaked with Phosphate Buffer Saline (PBS) and the swab sticks were returned to the tube containing 3 ml of PBS. Moreover, 137 leftover food samples have been taken in sterile plastic bags. Approximately 25 gm of solid food were collected with sterile spoon. In case of liquid food, 25 ml of liquid were taken in sterile centrifuge tubes. All the stored foods were collected during sampling that have been resting at room temperature or warmer for more than 6 hours. All the samples were kept at 4˚C in a sterile container and transferred to the laboratory within 4 hours of collection.

Processing of swab samples: Hotspot swabs in the PBS solution were first vortex thoroughly for 1 min and then 100 micro liter (µL) of the sample was used for spread plate technique on mTEC agar (m-TEC ChromoSelect Agar, SIGMA-ALDRICH, Germany). After incubation in 44.5˚C for 24 hour, enumeration of thermo tolerant *E. coli* was done. For enrichment of *V. cholerae*, 0.5

ml of the sample was added to 5 ml Alkaline Peptone Water (APW) (OXOID, UK) and incubated overnight at 37˚C.

Processing of Food Samples: Approximately 25 gm of food sample was weighed aseptically and blended by mixing with PBS in a Stomacher Lab Blender (Seward Stomacher 80, Lab UK). Additional PBS was then added to make 1:10 dilution. For liquid food, 10 ml of sample was added to 90 ml of PBS. Enumeration of *E. coli* and enrichment steps were performed as described above.

Ethical clearance:

The study was approved by the Ethical Review Committee (ERC) of International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B) under the research protocol number# PR-14006. Both verbal and written consent were taken from household caretaker at the time of enrollment and sample collection.

Nucleic acid extraction:

Total nucleic acid was extracted from the samples by using boiled template method (De Medici et al., 2003). All the samples were analyzed to detect the presence of *V. cholerae* by targeting specific *ompW* gene in PCR assay (Nandi et al., 2000). Prevalence of toxigenic *V. cholerae* was also studied by targeting toxigenic serogroups specific gene PCR. Real-time PCR was performed to detect cholera toxin gene *ctxA* according to the previously published protocol (Blackstone et al., 2007).

Specific PCR targeting *uidA* gene of *E. coli* was performed on a subset of samples. Hotspot swab samples from 5 HHs collected each month were selected randomly for this analysis. The positive DNA samples were further analyzed for pathogenic genes of diarrheagenic *E. coli* and *Shigella* spp. All the primer sequences and target genes are shown in Supplementary Table 1.

All the positive control strains used to analyze *E . coli* and *V. cholerae* are listed in Supplementary Table 2.

Isolation and genotypic characterization of *V. cholerae*:

Isolation of *V. cholerae* was conducted from *ompW* PCR positive samples and confirmation was done by cultural, biochemical and serological methods (Huq et al., 2012). Genomic markers for toxigenic *V. cholerae* O1and non-O1 strains were used in PCR to characterize the isolates.

Phenotypic characterization:

Hemolytic activity: The strains were streaked on blood agar plates (Blood agar base, OXOID, UK) containing 5% sheep blood to obtain isolated colonies. The inoculated plates were incubated at 37ºC for 24 hr. Strains which showed clear zones around colonies were reported as hemolytic.

V. cholerae El Tor strain N16961 and *E. coli* DH5α were used as positive and negative control in this assay.

Serum resistance: Serum resistance test was employed by following the previous protocol (Bier et al., 2013; Moll et al., 1979). Isolates that showed growth in the presence of 60-80% human serum were classified as serum resistant, 20-40% as intermediate resistant and 0-10% human serum as sensitive respectively. *V. cholerae* El Tor strain C6706 and *Escherichia coli* DH5α were used as positive and negative control respectively.

Hemagglutinin assay: The hemagglutinin activity was carried as described previously with minor modifications (Rajpara et al., 2013). Ten percent human RBC was used for this assay.

Protease activity: Protease activity was determined as per previously published protocol (Honda et al., 1987; Rajpara et al., 2013). Zones with diameter of 10-12 mm were classified as high protease activity (+++), 8-10 mm as intermediate activity (++) and 6-8 mm as low activity (+). In this study, proteinase K and Phosphate buffer saline were employed as positive and negative control respectively.

Statistical analysis:

Data of fecal *E. coli* count from surface locations and food samples were used compute mean cfu using Microsoft Excel 2007. Comparison of fecal contamination among different household surface locations was analyzed. To compare between two types of surfaces and calculate *p* value Fisher's exact test was performed using the statistical software package 'R' version 3.3.1. The variables were coded as fecal *E. coli* positive if the count>0 cfu/ cm^2 and fecal *E. coli* negative if the count= 0 cfu/ cm^2) and the difference was indicated significant when $p<0.05$.

Results:

Demographic and socio-economic information:

More than 94% of the families living in the selected HHs were nuclear families and single room dwellers. The cooking stoves were shared among 2-19 HHs. Baseline and monthly survey on hygiene behavior revealed that plates and utensils to be used for the next meal appeared not visibly clean in 6 out of 32 HHs of this study.

Fecal *E. coli* contamination:

The highest fecal *E. coli* contamination level was observed on food plate surfaces (2.53E+02 mean cfu/cm^2) followed by cutting knife surface (**Table 1**). Although cutting knife had the maximum fecal *E. coli* count (2.88E+03 cfu/cm^2). All the food plates were cleaned after last meal with or without cleaning materials such as dishwashing soap and were found mostly in dry condition. The cutting knife was usually found not clean at the time of swab collection as the knife was already used for cutting for daily cooking. The latrine door knob was least contaminated with the mean cfu/cm^2 count of 7.32E+01. Both food and food plates from same HH were found contaminated with *E. coli* for 36 samples. Monthly prevalence of fecal *E. coli* on

surface swabs showed no specific seasonal patterns; mean cfu was highest in October (252 cfu/cm^2) and lowest in January (52 cfu/cm^2) (**Figure 2**).

Fisher's exact test was used to compare the fecal contamination level in different surface locations (**Table 2**). Results revealed that the odds of fecal contamination is 4.7 and 4.34 times higher (p<0.05) in cutting knife and food plate respectively than latrine door knob surfaces and 0.3 times lower (p<0.05) in latrine door knob than drinking water vessel surfaces. There is no significant difference on the level of fecal contamination when compared drinking water pot vs. food plate surfaces and food plate vs. cutting knife surfaces (p>0.05). Results indicated that fecal *E. coli* contamination level differs on household surface locations.

Table 1: Burden of fecal *E. coli* contamination on household surfaces

Hotspot locations	**Mean *E. coli* count (cfu/ cm^2)**
Cutting boti /knife	2.40E+02
Latrine door knob	7.32E+01
Point of use/Drinking vessel surface	1.67E+02
Food plate	2.53E+02

Table 2: Comparison of fecal *E. coli* contamination level in four different hotspots

	p value	CI (95%)	Odds ratio
Cutting knife vs. latrine door knob	3.91E-07**	2.48, 9.17	4.71
Latrine door knob vs. drinking water vessel	6.53E-04**	0.18, 0.65	0.34
Drinking water vessel vs. food plate	0.2	0.37, 1.21	0.67
Food plate vs. cutting knife	0.9	0.5, 1.68	0.92

Drinking water vessel vs. cutting knife	0.1	0.3, 1.12	0.62
Food plate vs. latrine door knob	1.63E-06**	2.3, 8.4	4.34

Leftover food samples were found highly contaminated with fecal *E. coli* (mean cfu/gm 5.45E+02, with counts ranges from 0 to 6400 cfu/gm). Ten different types of cooked food item were mainly collected as remaining food after the last meal or stored in warm temperature after cooking (**Table 3**). Mostly contaminated foods were cooked chicken curry with the cfu count ranges from 0 to 6640 cfu/gm and rice with water with the cfu count ranges from 2620 to 6400 cfu/gm. Cooked mutton showed no fecal contamination. Seasonal distribution of fecal contamination revealed that in May, the contamination was highest (mean count 1218 cfu/gm) and in November it was lowest (mean count 4 cfu/gm) (Figure 2).

Table 3: Fecal *E. coli* count in collected food samples

Type of food	**Range of contamination (Fecal *E. coli* cfu/gm)**	**N (Mean contamination cfu/gm)**
Lentil	0-1400	14 (293)
Chicken	0-6640	2 (3320)
Fish fry	0-60	3 (20)
Fish with vegetable	0-4240	40 (561)
Rice	0-4240	29 (616)
Vegetable	0-2480	34 (109)
Water rice/Panta vat	2620-6400	4 (3565)
Beef	(0-40)	3 (13)
Mutton	Nil	2 (Nil)
Other	0-2080	6 (710)

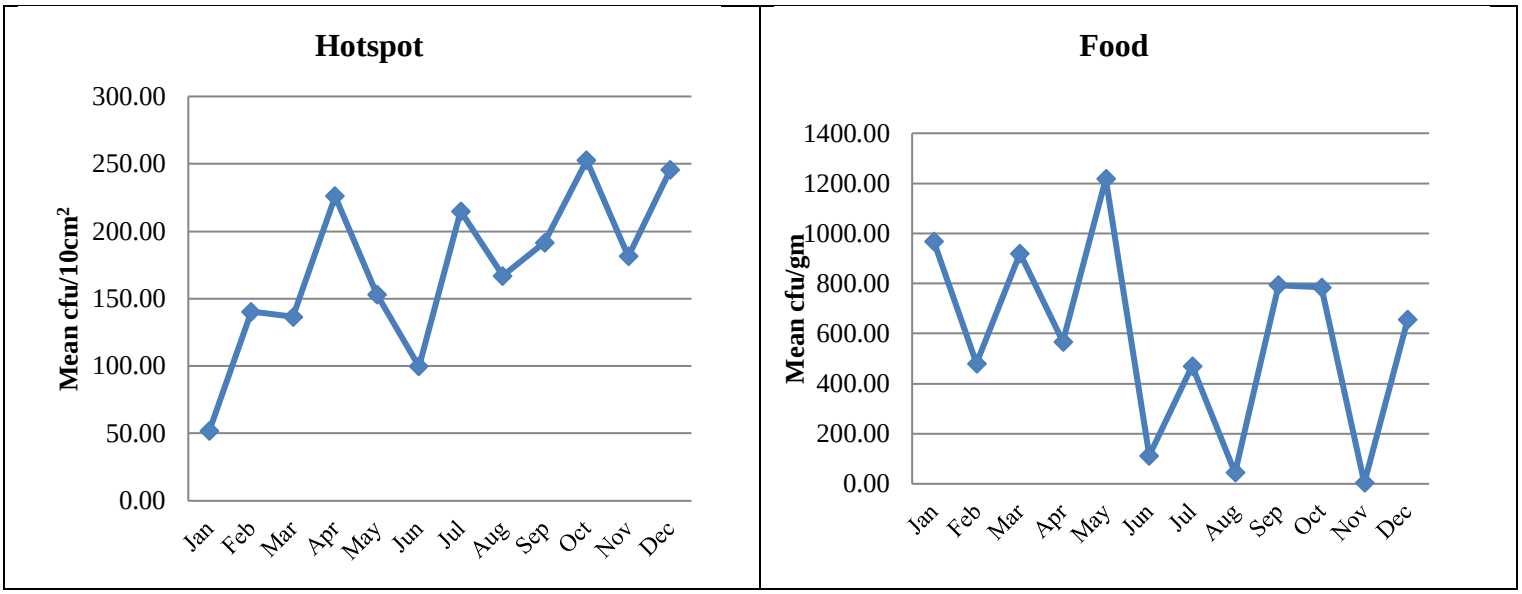


Figure 2: Monthly prevalence of *E. coli* in hotspots and food samples

Total 240 surface swab samples from 5 HHs each month (total 60 HHs) were analyzed for the presence of the molecular marker of *E. coli* (*uidA*) and 67% of the samples showed the positive results. Further PCR analysis was conducted for the virulence genes of diarrheal pathotypes of *E. coli* and *Shigella* spp. (Figure **3**). Distribution of pathogenic genes in four spots showed ETEC genes were highly prevalent in drinking vessel surface (40% , 24 samples positive out of 60), EHEC in latrine door knob (6.7%, 4 samples positive out of 60), EIEC in both cutting knife (8.3%) and latrine door knob (8.3%) and EPEC in latrine door knob (6.7%) (**Figure 3**).

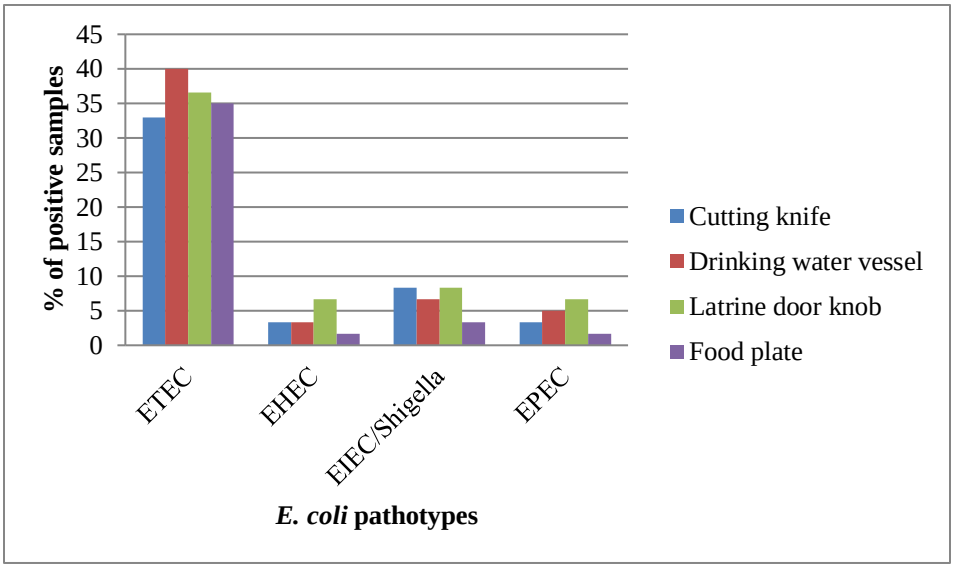
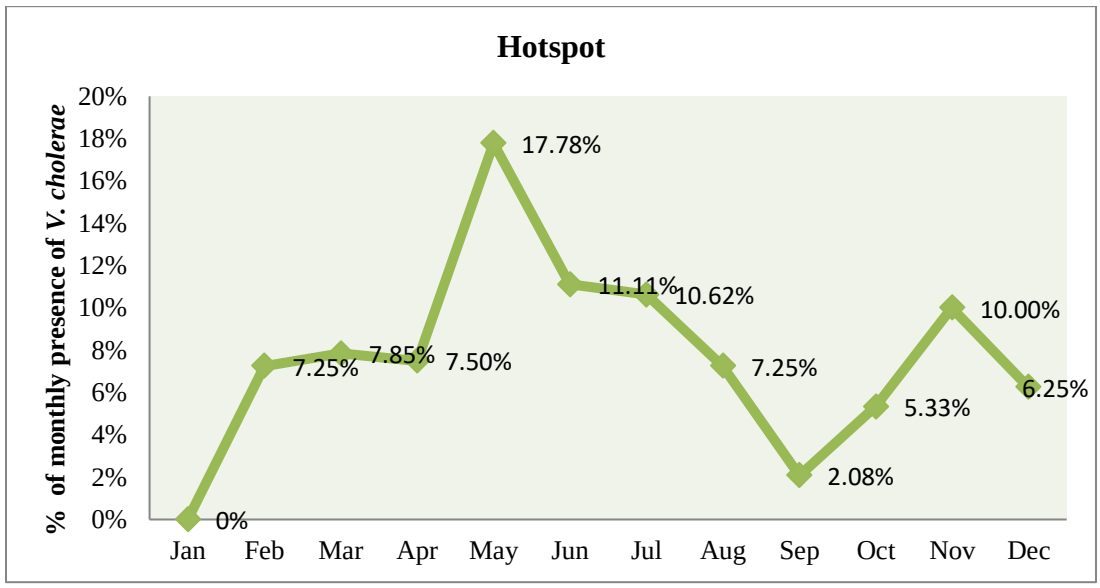


Figure 3: Genomic distribution of diarrheagenic *E. coli* in hotspot swabs. Sample size for each type of hotspot swab is 60 (n=60).

Prevalence of *V. cholerae* contamination:

Total 668 hotspot samples were analyzed by *V. cholerae* species-specific *ompW* gene PCR. Among them most predominant was food plate swabs with 33 samples positive out of 165(20%), followed by water pot (14 of 165, 8.5%), knife (6 of 169, 3.6%), latrine door knob (3 of 169, 1.8%).In total 137 food samples were analyzed, only 10 found positive in PCR.

Further characterization of *ompW* positive hotspot total DNA for virulence genotype revealed the presence of *rfb O1* (12.5%), *rfbO139* (6.25%), *ctxA* (0.9%) and *cep* (10.4%) genes. Only one food total DNA sample was positive for *rfbO1*gene. Monthly distribution of *V. cholerae* in surface samples showed two peaks in the months of May and November (**Figure 4**). For leftover food samples, the highest peak of *V. cholerae* contamination was found in September.



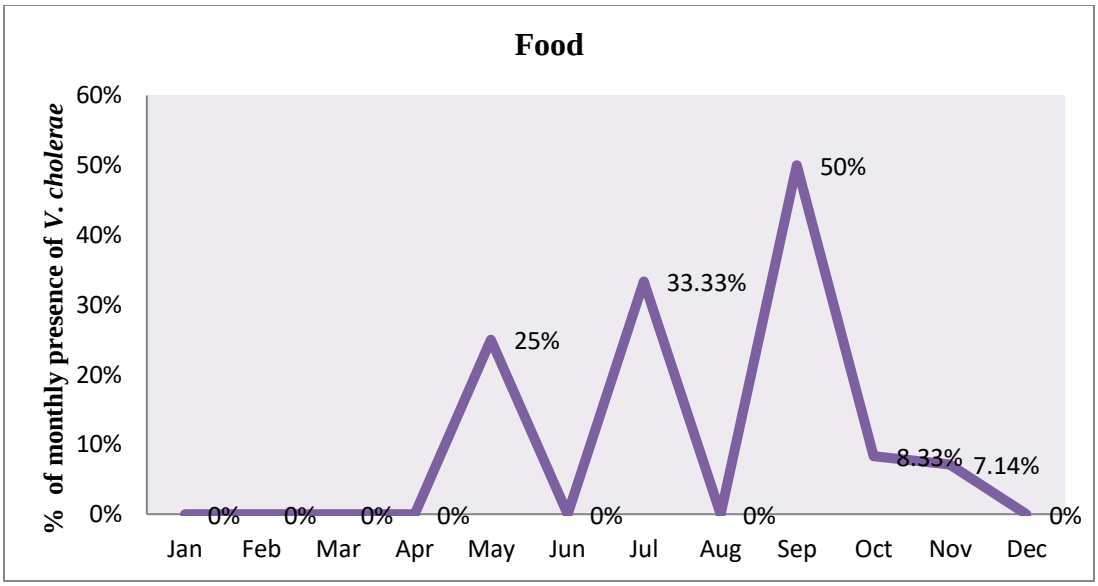


Figure 4: Monthly prevalence of *V. cholerae* in hotspot and food samples

Of the 12 hotspot and 2 food *V. cholerae* strains isolated from household samples, 8 (66.6%) of them were harvested during time period of May-June. One strain was confirmed as *V. cholerae* O1 and others as non-O1/ O139 by PCR based analysis. The O1 strain lacked *ctxA*, *B*, *zot*, *tcp*, *cep*, *ace*, *ompU* genes but possessed other regulatory and virulent genes like *toxR*, *rtxC*, *hly*, *msh*, *HA/protease* (Supplementary Table 3). Genes for type three secretion system were detected in two non-O1/O139 isolates. All the strains showed hemolysis on sheep blood agar and proteolysis on 1.5 % skim milk agar including clinical strain *V. cholerae* El Tor N16961. Only 2 hotspot *V. cholerae* strains were sensitive to human serum. The current strains did not exhibit any hemagglutinin activity on human erythrocytes.

Discussion:

Although the F-diagram of fecal pathogen transmission already showed five major routes (fluid, food, field, finger, flies), current study explored domestic surfaces as new non-water borne routes of fecal contamination inside low income community households. Fecal bacterial load in leftover food samples were assessed to investigate post-cooking/eating contamination. Our results showed that high frequency of thermo tolerant *E. coli* was detected in eating and food preparation utensils. It has been suggested that environmental high risk areas can be identified by monitoring indicator bacteria (Reynolds et al., 2005). According to Henroid et al (Henroid Jr et al., 2004) and ISO 4832:2006 (ISO, 2006), the standard mean log coliform count for food contact surfaces in is <1.0 log cfu/cm^2. The three food contact surfaces (food plate, cutting knife, drinking pot) analyzed in this study showed higher cfu count for *E. coli* than <1.0 log cfu/cm^2. Although *E. coli* is one of the members of the broad group of coliform, the actual coliform count in these household surfaces would be considerably higher than the standard limit.

Significantly high incidence of *E. coli* contamination in food preparation utensils may be attributable to lack of proper cleaning and sanitation practices. For example, contamination of the hands of food handler and used water for cleaning was found associated with *E. coli* contamination of food-serving utensils (Gil et al., 2014; Kusuma et al., 2012). Global disease burden study for water, sanitation and hygiene also indicated the use of contaminated cleaning water as a transmission route of fecal-oral pathogens (Prüss et al., 2002). Houseflies could also readily contaminate eating utensils with enteric pathogens (Levine and Levine, 1991). Cross-contamination of fecal pathogens in food via cutting board and cutlery was observed in another study (De Jong et al., 2008). From our study, it can also be speculated about cross-contamination between food and food utensils as the results provide evidence of fecal contamination in both food and food plate in same household. In a previous study in Bangladesh, author showed the possibility of *V. cholerae* contamination of cutting materials attributable to processing of contaminated fish (Hossain et al., 2018).

The *E. coli* count was lower in latrine door knob compared to other surfaces from kitchen and in-house area. There is a chance of rapid air-drying of the commonly metal made door knobs after contact with hand and therefore, the bacterial ability to survive on theses surfaces may be reduced (Fuster-Valls et al., 2008). Studies also indicated that metals like copper kills bacteria highly rapidly (Zeiger et al., 2014). Several studies in both developed and developing countries have observed lowest contamination with indicator bacteria in toilets and highest contamination in kitchen (Ojima et al., 2002; Rusin et al., 1998; Sinclair and Gerba, 2011). Russin et al showed that toilet seat was the least contaminated area in the house whilst kitchen was highly contaminated (Rusin et al., 1998). Virulence genes of ETEC were highly predominant in the household environment of Arichpur. ETEC is most common diarrheagenic pathotype of *E. coli* in Bangladesh (Qadri et al., 2007) and has been found in the household environment (Ahmed et al., 2013).

High prevalence of *V. cholerae* has also been detected in the swabs of food utensils, although the number of the cultured bacteria was low. In a laboratory experiment, it has been shown that high copy number of *V. cholerae* remained viable for up to 7 days on fomites such as plastic, glass, Aluminum which are the main constituents of food utensils, though they had lost the culturability within 2 hours of inoculation (Farhana et al., 2016). Nontoxigenic O1 and toxigenic non O1/O139 serogroups of *V. cholerae* strains have been isolated from hotspot and food samples. The phenotypic expression of virulence potentials of the isolates was similar as compared to the epidemic diarrheal strain *V. cholerae* O1 El Tor. Seasonal prevalence of *V. cholerae* in hotspot swabs was in accord with dual cholera seasonality in Bangladesh (Hashizume et al., 2010). This result indicates surface components of households may act as a component of annual transmission of *V. cholerae* or they may introduce the bacteria.

Although low numbers of food samples were positive for *V. cholerae*, high *E. coli* contamination was observed in our study. Contaminated food can act as direct fecal-oral transmission route for enteric pathogens. Food can be contaminated with fecal pathogens if it is not properly cooked or stored (Newman, 2005), by contaminated kitchen utensils (Gil et al., 2014), contact with dirty hands of food handlers or children (Ngure et al., 2013), and landing of vectors such as flies on open food (Lindeberg et al., 2018). Sometimes leftover foods have been reheated before consumption. That would cause bacterial cell death to some extent. In Bangladesh, food is normally cooked well by either boiling, frying or baking before consumption. Varieties of spices have been used in cooking of fish, meat and vegetable curries. A remarkable contaminated food source has been recognized which is 'Water rice' or locally called as 'Panta vat'. Occasionally during summer season, clean water is added to leftover cooked rice to prepare the fermented 'Panta vat' and store for a period of time until the next meal. Cooked rice preserved in warm temperature may provide ideal growth environment for fecal *E. coli* and *V. cholerae* (Kolvin and Roberts, 1982; Lee et al., 2006) and adding contaminated water to rice could further increase the survival and growth rate of enteric pathogens. In west Bengal, India, there was report of cholera outbreak in the village after preparation of 'panta vat' with contaminated pond water (Biswas et al., 2014).

To the best of authors' knowledge, this was the first study to survey fecal indicator and *V. cholerae* in domestic environment of Bangladesh. Despite the shortcomings, our study findings suggest that apparently cleaned surfaces which have direct contact with food and drink was major source of fecal contamination in low-resource settings. These results show serious susceptibility of the kitchen hygiene and cleaning regimes which can induce cross-contamination between other primary transmission pathways and environmental surfaces. This knowledge is significant to prevent direct or person to person transmission of acute diarrheal pathogens.

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Supplementary Table 1: Primer and target gene information for PCR

Target gene	Target Organism	Sequence	Amplicon size (bp)	Reference
rfb O1	*V. cholerae*	F-TCTATGTGCTGCGATTGGTG	638	(Goel et al., 2007)
		R-CCCCGAAAACCTAATGTGAG		
rfb O139		F-AGCCTCTTTATTACGGGTGG	449	(Hoshino et al., 1998)
		R-GTCAAACCCGATCGTAAAGG		
ctxB		F-GGTTGCTTCTCATCATCGAACCAC	460	(Olsvik et al., 1993)
		R-GATACACATAATAGAATTAAGGAT		
cep		F-GCTACATGTTTAGCTCACTG	251	(Bhattacharya et al., 2006)
		R-TTTAGCCTTACGAATTAAGCC		
ace		F-TAAGGATGTGCTTATGATGGACACCC	309	(Kumar et al., 2009)
		R-CGTGATGAATAAAGATACTCATAGG		
tcp		F-CGTTGGCGGTCAGTCTTG	805	(Goel et al., 2007)
		R-CGGGCTTTCTTCTTGTTCG		
zot		F-TCGCTTAACGATGGCGCGTTTT	947	(Singh et al., 2001)
		R-AACCCCGTTTCACTTCTACCCA		
***vcsN2*(T3SS)**		F-CAACACCTTCAAAGCCTTG	848	(Awasthi et al., 2013; Shin et al., 2011)
		R-GCGAGCTCCAATTGAAAC		
***vcsC2*(T3SS)**		F-GGTCTCATAGACACTACG	589	
		R-ACGATGCTATGGGGTATG		
***vopF*(T3SS)**		F-GGAAATTCGCCAAGGTGTA	839	
		R-CAAAACCGTCCATACAAGG		
ctxA		F-TTTGTTAGGCACGATGATGGAT	84	(Blackstone et al., 2007)
		R-ACCAGACAATATAGTTTGACCCACTAAG		
		Probe-**FAM**-TGTTTCCACCTCAATTAGTTTGAGAAGTGCCC-**BHQ1**		
		R- TCA TCG CAC CGT CAA AGG AAC C		
eltB	Enterotoxigenic *E. coli*(ETEC)	F-TGG AAA AAC TCA GTG CCT CT R-CCA GTC CGT AAA TTC ATT CT	322	(Inoue et al., 1993)
estA		F- GCT AAA CAA GTA GAG GTC TTC AAA A R- CCC GGT ACA GAG CAG GAT TAC AAC A	147	(Moseley et al., 1983)
vt1	Enterohemorrhagic *E. coli* (EHEC)	F- GAA GAG TCC GTG GGA TTA CG R- AGC GAT GCA GCT ATT AAT AA	130	(Pollard et al., 1990)
vt2		F- ACC GTT TTT CAG ATT TTG ACA CAT A R- TAC ACA GGA GCA GTT TCA GAC AGT	298	(Lindqvist, 1997)
eaeA	Enteropath	F- CAC ACG AAT AAA CTG ACT AA	376	(Svenungsson et

	ogenic *E. coli* (EPEC)	AAT G R- AAA AAC GCT GAC CCG CAC CTA AAT		al., 2000)
bfpA		F- AAT GGT GCT TGC GCT TGC TGC R- GCC GCT TTA TCC AAC CTG GTA	367	(Yatsuyanagi et al., 2002)
pCVD432	Enteroaggregative *E. coli* (EAEC)	F- CTG GCG AAA GAC TGT ATC AT R- CAA TGT ATA GAA ATC CGC TGT T	630	(Pereira et al., 2007)
ial	Entroinvasive*E. coli* (EIEC)/*Shigella* spp.	F- CTG GTA GGT ATG CTG AGG R- CCA GGC CAA CAA TTA TTT CC	320	(Frankel et al., 1990)
ipaH		F- TGG AAA AAC TCA GTG CCT CT R- CCA GTC CGT AAA TTC ATT CT	422	(Lüscher and Altwegg, 1994)

Supplementary Table 2: Positive control strains used in this study

Organism name	Collection or isolation number*	Source
V. cholerae O1	N16961	Clinical
V. cholerae O1	C6706	Clinical
V. cholerae O139	NIHC0270	Clinical
V. cholerae non-O1	4460	Clinical
ETEC	mgl-IC1	Clinical
EHEC	BH 29	Clinical
EPEC	AE 3171	Clinical
EAEC	1214C2	Clinical
EIEC	H2	Clinical

*The positive control strains were obtained from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B).

Supplementary Table 3: Genotypic traits of *V. cholerae* strains isolated from environmental and food samples

Strain ID	Source	Regulatory and virulence associated genes															
		O1 rfb	***O139 rfb***	***ctxAB***	***tcp***	***zot***	***ace***	***cep***	***ompU***	***rtxC***	***toxR***	***mshA***	**T3SS**	**T6SS**	***chxA***	***hlyA***	***HA-protease***
HS/I-1	**Food plate**	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+
HS/I-2	**Food plate**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
HS/I-3	**Food plate**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
HS/I-4	**Drinking water pot**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
HS/I-5	**Food plate**	+	–	–	–	–	–	–	+	+	+	+	–	+	+	+	+
HS/I-6	**Drinking water pot**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
HS/I-7	**Drinking water pot**	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
HS/I-8a	**Food plate**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
HS/I-8b	**Food plate**	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
HS/I-9	**Food plate**	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+
HS/I-10	**Food plate**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
HS/I-11	**Food plate**	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+

F/I-1	**Rice**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+
F/I-2	**Rice**	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+

Appendix II:

Conference Abstracts (1-6)

Abstract 1: Fecal contamination hotspots in low-income households in Bangladesh

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Conference: The 18th International Congress on Infectious Disease March, 2018, Buenos Aires, Argentina

Type: Oral Presentation

Keywords: Fecal contamination, Household Transmission, Bangladesh

Background:

Diarrheal diseases continue to be major causes of morbidity and mortality in developing countries. Infectious Diarrhea is often related to fecal pathogen exposure via drinking water. Little is known about the other fecal contamination hotspots within the household and especially in the kitchen environments in overcrowded low-income setting. Current study intended to perform quantitative analysis of fecal contamination in food and domestic surfaces.

Methods & Materials:

Fecal contamination was surveyed in routine swabs from four household environmental sites: cutting knife (n=169), food plate (n=165), latrine door knob (n=169), and drinking water pot surface (n=165) among 32 households for 1 year period in a low-income area, Arichpur, Dhaka. Moreover, 137 left over food samples were taken. All the samples were analysed for total thermotolerant *Escherichia coli* count and the presence of *Vibrio cholerae* by molecular method. Fisher's exact test was used to compare the fecal contamination level in different surface locations. A subset of samples was assessed for the genomic presence of diarrheagenic *E. coli* by PCR.

Results:

Results revealed that *E. coli* contamination level was highest on food plates with a geometric mean (GM) of 3.08 cfu/cm^2 and the least contaminated site was latrine door knob (GM=0.06 cfu/cm^2). Food samples were found heavily contaminated with fecal *E. coli* (GM=13.32 cfu/gm, with counts ranges from 0 to 6400 cfu/gm). The level of fecal contamination is 4.7 times higher (p<0.05) in cutting knife than latrine door knob surfaces and 0.3 times lower (p<0.05) in latrine door knob than drinking water pot surfaces. *V. cholerae* contamination was also most predominant in food plate swabs. Genes of enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic

E. coli (EHEC) and enteropathogenic *E. coli* (EPEC) were detected in surface samples among them ETEC was the most prevalent (46% samples were found positive).

Conclusion:

This study presents new dynamics of in-house components in transmission of fecal bacteria via food and kitchen utensils thus, proves the vulnerability of the kitchen environment of low-income urban settings in Bangladesh. Results of the study will enable an update of the diarrhea risk factors for early prevention of disease progression in risk groups.

Abstract 2: Comparative genomics of *Vibrio cholerae* O1 isolated from cholera patients in Bangladesh

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Conference: International Conference on Genomics Nanotech & Bio engineering (ICGNB), , May 2017, Dhaka

Type: Poster Presentation

Key words: *Vibrio cholerae*, Cholera, Whole Genome Sequencing

AIM: Cholera remains an endemic disease in Bangladesh and recently, the severity of the disease has significantly increased in urban area since the emergence of the new variant of *Vibrio cholerae* O1 El Tor. In this study, Whole Genome Sequencing (WGS) was utilized to investigate the current genomic profile of *V. cholerae* O1 strains, isolated from symptomatic patients in the low-income urban area of Arichpur, Dhaka, Bangladesh.

METHODS: During October 2015, three *V. cholerae* O1 strains (VC-1, 2 and 3) were isolated from rectal swabs of two patients living in households 588 m apart. One of the two patients was co-infected with two *V. cholerae* strains (VC-1 and VC-3). Major virulence factors, biotype and antimicrobial resistance genes were identified by WGS. A global phylogenetic tree was inferred using genome wide SNPs (Single Nucleotide Polymorphism) analysis.

RESULTS: All the *V. cholerae* strains were El Tor variant of *Vibrio cholerae* O1 carrying *ctxB*[1] (standard classical genotype). SNP based global phylogeny revealed that these isolates are strictly clonal and the closest neighboring genomes were epidemic clones of *V. cholerae* O1 from Pakistan isolated in 2010. All strains harbored the *integrase* gene of the SXT element *(int$_{SXT}$)*, antimicrobial resistance genes for aminoglycosides, phenicol, sulphonamide, and trimethoprim except VC-1 that lacked sulphonamide resistance genes. The *in silico* MultiLocus Sequence Typing (MLST) revealed that these strains belonged to sequence type 69.

CONCLUSION: The study provides knowledge on current genetic traits of clinical *V. cholerae* O1 circulating in urban household clusters of Bangladesh which may help in predicting emergence of new pandemic strains in Bangladesh.

Abstract 3: Prevalence of diarrheagenic *Escherichia coli* in case household environment in Bangladesh

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Conference: The 13rdThe Asian Congress on Biotechnology 2017, July 2017, Thailand.

Type: Poster Presentation

Key words: *E. coli*; Diarrhea; Bangladesh

Diarrheagenic *Escherichia coli* have been contributed a significant role to the global burden of diarrheal diseases. In this study, the prevalence of diarrheagenic *E. coli* was studied in households of diarrhea patients in Arichpur, Dhaka city, Bangladesh. During 4 months period, 40 rectal swabs from patients in 32 different households and swabs from 4 spots (cutting knife, latrine door knob, drinking glass and food plate swab), food and drinking water samples were collected from each household. Direct DNA samples were examined for virulence genes characteristic of enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and cytolethal distending toxigenic *E. coli* (CTEC) by PCR. The cultured *E. coli* strains were analyzed for virulence typing and multilocus sequence typing (MLST). The presence of the virulence genes of diarrheagenic *E. coli* were detected in 33% (13 of 40) rectal swab samples, 53% (74 of 140) household swab samples, 8% (3 of 37) food samples and 6% (2 of 34) water samples in PCR analysis of direct DNA. Among 50 *E. coli* isolates from rectal swabs and environmental samples, 20% (10 out of 50) strains were diarrheagenic (3 EAEC, 2 ETEC, 1 EPEC, 1 EHEC, 3 CTEC). MLST analysis of the toxigenic strains showed multiple STs (Sequence Type) with most dominant type was ST 10 (2 strains) and 4 strains showed various

novel STs. The phylodynamic tree constructed by MLST data showed that 9 toxigenic strains clustered with the clinical diarrheagenic database strains. The data suggesting high-risk areas for diarrheagenic *E. coli* contamination within case household environment emphasizes designing interventions for in-house sanitation and hygiene infrastructure in Bangladesh.

Abstract 4: Can Cholera fly?

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Type: Poster presentation

The role of the housefly in the fecal-oral transmission route has been almost non-disputed. Previous studies have established flies as potential transmitters of diarrheal pathogens to humans. Furthermore, bacterial pathogens including *Vibrio cholerae,* have been isolated from the exoskeleton, legs, mouthparts and intestinal tract of flies. Despite an obvious link, no studies to date have confirmed transmission of *Vibrio cholerae* to food via flies in a real kitchen environment.

To investigate the potential for food contamination by flies, food samples with and without fly exposure were examined. The study was carried out over two month's period in real life environmental settings in an overcrowded urban slum area in Dhaka, Bangladesh. Paired exposed and non-exposed (covered with a fly net) containers with cooked local sticky rice were placed on the ground in open air kitchen areas and the numbers of flies landing on the exposed rice were counted. After exposure, the surface of the rice was scraped off and analyzed for the presence of *V. cholerae* DNA by targeting detection of outer membrane protein (*ompW*) genes using molecular methods.

Of a total of 75 pairs of rice samples, *V. cholerae* specific *ompW* genes were detected in three of the exposed rice samples. The *ompW* gene is highly conserved marker for this species complex environmental adaptation of the organism may be linked to the expression of these genes The exposure time for the three positive samples were 30, 120 and 180 minutes with an average fly landing intensity of 0.6, 4.4 and 0.8 flies per minute during the three experiments. The non-exposed rice samples before fly exposure were also examined for *ompW* gene of *V. cholerae* and not detected by PCR.

The findings are to our knowledge the first study that indicate *V. cholerae* contamination in prepared food may occur via fly transmission. Storage of contaminated prepared food, a practise that is normal in Bangladesh where water is added to the leftover rice and eaten the following

morning, may allow infectious dose of *V. cholerae* to multiply and can initiate sporadic outbreaks.

Abstract 5: Investigation on household contamination of *Vibrio cholerae* in Bangladesh

Zenat Zebin Hossain[1,2*], Israt Farhana[2], Suhella MohanTulsiani[2,3], Rokaia Sultana[1], Peter Kjær Mackie Jensen[2, 3], Anowara Begum[1]

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Conference: ASM Microbe, June 2016, Boston, USA.

Type: Poster presentation

The role of in-house transmission on the incidence of *Vibrio cholerae*, the deadly waterborne pathogen, is still not developed. The aim of the current study was to investigate possible contamination routes in household domain for effective cholera control in Bangladesh.

To examine the prevalence of *V. cholerae*, routine swabs from four hotspots (cutting knife, latrine door knob, drinking water pot and food plate surface) and leftover food samples were collected. The cohort of 22 low income households has been studied for every 6 weeks between November 2014 and December 2015, from an urban area of northwest Dhaka, Bangladesh. Molecular genotypic and phenotypic traits of *V. cholerae* strains were assessed.

Total 660 hotspot samples were analyzed by *V. cholerae* species-specific *ompW* gene PCR. Among them most predominant was food plate swabs with 37 samples positive out of 163 (22.69%), followed by water pot (17 of 163, 10.43%), knife (6 of 167, 3.6%), latrine door knob (5 of 167, 2.9%). In total 137 food samples were analyzed, only 10 found positive in PCR. Further characterization of *ompW* positive hotspot total DNA for virulence genotype revealed the presence of *rfb O1, rfb O139*, *tcp*, *cep* genes but lacked the major cholera toxin genes *ctxA* and *ctxB*. Only one food tDNA sample was positive for *rfb O1* gene.

Of the 12 hotspot and 2 food *V. cholerae* strains isolated from PCR positive samples, 8 (66.6%) were harvested during time period of May-June. One hotspot strain was confirmed as *V. cholerae* O1 and others as non-O1/ O139 through PCR based analysis. The O1 strain lacked cholera toxin genes but possessed other regulatory and virulent genes like *toxR*, *rtxC*, *hly*, *msh*, *HA/protease*. Genes for type three secretion systems were detected in two non-O1/O139 isolates. All the strains including clinical *V. cholerae* El Tor strain N16961, showed hemolysis and proteolysis activity but none of them exhibited any hemagglutinin activity on human erythrocytes.

The study findings indicate that *V. cholerae* contamination is mostly originated in and around kitchen area rather than latrine area. Contaminated food and water supply may be the reason

behind this relatively high presence of virulence factors in food plates and water pots. Direct exposure routes of disease transmission should be a major consideration in cholera prevention policies.

Abstract 6: Molecular Analysis and Toxigenic Potential of *Vibrio cholerae* Isolated from Hilsa fish (*Tenualosa ilisha)*, Bangladesh

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Conference: ASM Microbe, June 2016, Boston, USA.

Type: Poster presentation

Exposure to contaminated fish may upsurge the virulent strains of *Vibrio cholerae*, the deadly human pathogen in the households of rural and urban Bangladesh. Since *V. cholerae* spreading was reported from the Bay of Bengal, this study hypothesized that Hilsha (Tenualosa ilisha), a marine and fresh water fish may serve as a transmission vehicle of potential emerging epidemic causing strains.

We studied 9 toxigenic *V. cholerae* strains isolated from Hilsha fish including 6 *V. cholerae* O1 and 3 non O1/O139 serogroups for virulence associated genotype and their pathogenic potential on animal model and human cancer cell line. The study also analyzed clonality by genetic fingerprinting and *rpoB* gene sequencing.

The *V. cholerae* O1 strains possessed diverse virulence genes but lacked some major toxin genes like *ctxA*, *tcp* etc. The non O1/O139 strains harbored genes for type III (T3SS) and type VI secretion systems (T6SS). Eight of the nine strains showed survivality up to 10% sodium chloride in broth culture which indicates their coastal origin. All nine isolates were able to accumulate fluid in rabbit ileal loops. Cell free culture supernatant of three O1 and two non O1 strains caused distinctive cell death in established HeLa cell line. Diverse polymorphic patterns were revealed in Random amplified polymorphic DNA (RAPD) fingerprinting except two non O1 isolates (I-49d and I-52a) showed considerable correlation in band patterns. Similar restriction fragment length polymorphism (RFLP) profile of *groEL*-I of *V. cholerae* chromosome 1 in all strains indicated homogeneity in species level. Hierarchical cluster analysis by β subunit of RNA polymerase gene (*rpoB*) sequencing showed that theses isolates did not cluster together and also distinct from clinical and environmental toxigenic strains.

Our results indicate the indigenous *V. cholerae* strains associated with Hilsha fish possesses considerable virulence potentiality despite being quite diverse from current epidemic strains. In a cholera epidemic-prone region like Bangladesh, surveillance research on this fish species is vital to control the evolution of outbreak strains of *V. cholerae*.

Appendix III:
Co-authorship declarations of the manuscripts

DECLARATION OF CO-AUTHORSHIP

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Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Quantitative analysis of nucleic acid extraction methods for Vibrio cholerae using Real-time PCR and conventional PCR

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	**(A,B,C)**
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	B
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

**Benchmark scale of the PhD student's contribution to the article*		
A. refers to:	*Has contributed to the co-operation*	*0-33 %*
B. refers to:	*Has contributed considerably to the co-operation*	*34-66 %*
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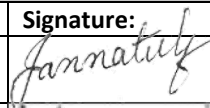
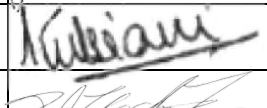
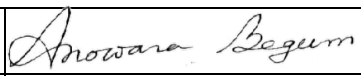
4/11/2018	Jannatul Ferdous	M.S	
5/11/2018	Suhella M. Tulsian	PhD	
8/11/2018	Peter Kjær Mackie Jensen	PhD	

4/11/2018	Anowara Begum	PhD	

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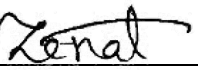
Date: 4/11/2018 **PhD student:** 	**Date:** 8/11/2018 **Principal supervisor**: 

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Title of PhD thesis:
Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Survival of Vibrio cholerae O1 on fomites

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	**(A,B,C)**
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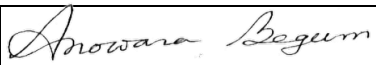
4/11/2018	Anowara Begum	PhD	

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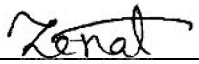
Date: 5/11/2018 **PhD student:** 	**Date:** 6/11/2018 **Principal supervisor**: 

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Title of PhD thesis:
Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Transmission and toxigenic potential of Vibrio cholerae in Hilsha (Tenualosa ilisha) for human consumption in Bangladesh

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	**(A,B,C)**
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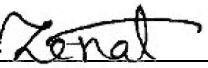
Date: 6/11/2018 **PhD student:** 	**Date:** 5/11/2018 **Principal supervisor**: 

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Title of PhD thesis:
Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Can E. coli fly? The role of flies as transmitters of Escherichia coli to food in an urban slum in Bangladesh

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	B
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Title of PhD thesis:
Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Transmission of Diarrheagenic Escherichia coli in dirrhea case households in urban Bangladesh

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	**(A,B,C)**
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
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Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Comparative Genomics of Vibrio cholerae O1 Isolated from Cholera Patients in Bangladesh

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
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Title of PhD thesis:
Investigation of household transmission pathways for Vibrio cholerae and Escherichia coli in Bangladesh

This declaration concerns the following article:
Investigation on household contamination of fecal Escherichia coli and Vibrio cholerae in Bangladesh

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	**(A,B,C)**
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
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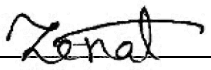
Date: 4/11/2018 **PhD student:** 	**Date:** 8/11/2018 **Principal supervisor**: 

Appendix IV:

Permission to use Figure 1

ড. এম. নিয়ামুল নাসের
অধ্যাপক
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This is to declare that Ms. Zenat Zebin Hossain, PhD student of Department of Microbiology, University of Dhaka has been granted permission to use the below referenced figure for the purpose of journal publication. Ms. Hossain is the first author of the article entitled "Transmission and toxigenic potential of *Vibrio cholerae* in Hilsha Fish (*Tenualosa ilisha*) for Human Consumption in Bangladesh". The details of the requested figure are as follows-

Figure number and name: 3.11 Hilsa Migration in Bangladesh
Page: 58
Book title: Migration, Spawning Patterns and Conservation of Hilsa Shad in Bangladesh and lndia.
Publisher: Academic foundation New Delhi, India

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Professor and author