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Certificate

This is to certify that the work incorporated in the thesis entitled "**Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for *Escherichia coli* and *Vibrio cholerae***" was conducted by the PhD student Jannatul Ferdous. Jannatul Ferdous carried out the research work and the write up of the thesis under my guidance and supervision. The thesis meets acceptable standards and can be submitted for evaluation in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the graduate program of Public Health and Epidemiology from the Department of Public Health, University of Copenhagen jointly affiliated with the Department of Microbiology, University of Dhaka.

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PhD Thesis

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Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for *Escherichia coli* and *Vibrio cholerae*

Supervisor: Peter Kjær Mackie Jensen & Anowara Begum

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PhD thesis

Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for *Escherichia coli* and *Vibrio cholerae*

Jannatul Ferdous

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List of Abbreviations

APEC	Avian pathogenic *E. coli*
APW	Alkaline peptone water
BLAST	Basic Local Alignment Search Tool
chxA	Cholix toxin encoding gene
CT	Cholera toxin
C_T	Cycle threshold value
C5	Combating Cholera Caused by Climate Change
CFU	Colony forming units
CI	Confidence interval
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DANIDA	Danish International Development Agency
DEC	Diarrheagenic *E. coli*
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ETEC	Enterotoxigenic *E. coli*

ExPEC	Extra-intestinal pathogenic *E. coli*
GBD	Global Burden of Diseases
HUS	Haemolytic uremic syndrome
hlyA	hemolysins encoding gene A
InPEC	Intestinal pathogenic *E. coli*
JMP	Joint Monitoring Program
LC	Linguistic Complex
LMIC	low- and middle-income countries
MDG	Millennium Development Goal
MF	Membrane Filtration
MFA	Ministry of Foreign Affairs
m-TEC agar	modified thermotolerant *E. coli* agar
mshA	mannose-sensitive hemagglutinin protein subunit A encoding gene
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NMEC	Neonatal meningitis *E. coli*
ompW	outer membrane protein encoding gene W
OR	Odds ratio
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
R^2	Regression coefficient
rtxA	repeats-in-toxin encoding gene A
rtxC	repeats-in-toxin encoding gene C
SDG	Sustainable Development Goals
STEC	Shiga toxin-producing *E. coli*
toxR	transcriptional activator controlling gene
Tm	Melting temperature
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infections
USD	United States Dollars
USEPA	United States of Environmental Protection Agency
UNICEF	United Nations International Children's Emergency Fund
V. cholerae	*Vibrio cholerae*
VBNC	Viable but non-culturable
WHO	World Health Organization
WASA	Water Supply and Sewerage Authority

Preface

A multidisciplinary collaborative research project entitled "Combating Cholera Caused by Climate Change (C5)" was awarded a Grant by the Danish International Development Agency **(**DANIDA) in 2012. The C5 project aimed at examining the contributable risks and effects of climate change for creating a hygiene-compromised water stress environment which induces cholera and diarrheal illnesses (**http://cope.ku.dk/research/cholera/**). The study commenced in January 2013 in a low-income area, Arichpur, on the outskirts of Dhaka City, Bangladesh, and ended in December 2015. A group of researchers and PhD students in epidemiology, microbiology and anthropology, from the University of Copenhagen, University of Dhaka, Bangladesh University of Engineering and Technology, and the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), were involved in the study. The C5 study was comprised of six Work Packages (WPs) and I was in charge of Work Package 4 (WP4). The central role of WP4 was to assess the microbiological quality of drinking water of the study households in the Arichpur area.

This dissertation was conducted to highlight the need for optimizing advanced methodologies for detection of microbiological contamination in drinking water and the contributing factors for contamination of drinking water.

Thus, the PhD work has resulted in the following six original manuscripts:

I. **Quantitative Analysis of Nucleic Acid Extraction Methods for *Vibrio cholerae* Using Real-time PCR and Conventional PCR**. Hossain, Z. Z., Ferdous, J., Tulsiani, S. M., Jensen, P. M., & Begum, A. (2018). Mymensingh Medical Journal, **27**(2), 327-335.

II. **Optimization and Validation of Real Time PCR Assays for Absolute Quantification of Toxigenic *Vibrio cholerae* and *Escherichia coli***. Ferdous, J., Hossain, Z. Z., Tulsiani, S., Rashid, R. B., Jensen, P. K. M., & Begum, A. (2016). Tropical Biomedicine, **33**(4), 641-651.

III. **Development and Validation of a Novel Real-time Assay for the Detection and Quantification of *Vibrio cholerae***. Rashid, R. B., Ferdous, J., Tulsiani, S., Jensen, P. K. M., & Begum, A. (2017). Frontiers in Public Health, **5**, 109.

IV. **Comparative assessment of fecal contamination in 'improved' piped-to-plot communal source and point-of-drinking water**. Ferdous, J., Sultana, R., Rashid, R. B., Begum, A., & Jensen, P. K. (2019) (Draft to be submitted).

V. **The fecal origin of pathogenic *E. coli* in 'improved' piped-to-plot communal source and point-of-drinking water of a low-income urban community, Bangladesh**. Ferdous, J., Rashid, R. B., Begum, A., & Jensen, P. K. (2019) (Draft to be submitted).

VI. **A Comparative Analysis of *Vibrio cholerae* Contamination in Point-of-Drinking and Source Water in a Low-Income Urban Community, Bangladesh**. Ferdous, J., Sultana, R., Rashid, R. B., Tasnimuzzaman, M., Nordland, A., Begum, A., & Jensen, P. K. (2018). Frontiers in Microbiology, **9**, 489.

The findings of the PhD work were also presented in the following international conferences:

I. **Standard Curve Quantification for Bacterial DNA Using a Real-time PCR Assay**. Ferdous, J., Hossain, Z. Z., Tulsiani, S., Jensen, P. K. M., Begum, A. Poster session at International Conference on Emerging Challenges in Biotechnology, Human Health and Environment (ECBHE-2014) in Indore, India, Dec. 2014.

II. **Prevalence of Virulent *Escherichia coli* Belonging B1 Phylogroup in Municipal Water Supply in Dhaka, Bangladesh**. Ferdous, J., Rashid, R. B., Tulsiani, S., Saima, S., Jensen, P. K. M., Begum, A. American Society for Microbiology Abstracts. Poster session at the ASM Microbe 2016, Boston, MA, June 2016.

III. **A Comparative Study of Cholera Transmission via Source of Supply and Point of Drinking Water at the Households**. Ferdous, J., Rashid, R. B., Tasnimuzzaman, M., Tulsiani, S., Jensen, P. K. M., Begum, A. Oral presentation at American Society for Microbiology (ASM Microbe 2017, New Orleans, LA, June 1-5, 2017). Session Type- Symposium. Main Session 421- Bugs in Water and their Associated Recent Outbreaks.

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This achievement of this PhD work would not have been accomplished without the enormous support and help from mentors, colleagues, friends and family members.

I would like to express my gratitude to the eminent Professor Sirajul Islam Khan (retired), the founder of Environmental Microbiology Laboratory, University of Dhaka for offering me this PhD position to conduct the study.

I am very thankful to my two of the supervisors Anowara Begum, Peter Kjær Mackie Jensen for their invaluable support and encouragement throughout the PhD. I feel very lucky to have a supervisor like Peter for his super supportive nature and super prompt response in any issue. His continuous guidance and vital feedbacks through his expertise within the subject area, helped me immensely in framing and improving manuscripts as well as this dissertation. Peter contributed indispensably to utilize my microbiological knowledge for the broader perspectives of public health throughout the study. Accordingly, I am deeply grateful to my supervisor, Anowara Begum for providing me enormous support and freedom to conduct the research work in the Microbiology field.

I would like to thank the Master students Ridwan Bin Rashid, Tasnimuzzaman Romel, Sabera Saima Rumi, Sumayia Mumu, Musharrat Jahan Prima for their continuous assistance in the laboratory work. Special thanks to Ridwan Bin Rashid, a bright, very eloquent, young researcher who joined as a first Master student of WP4 in the C5 project. Talking about him, it reminds me the discussions we often had in the laboratory which seemed very frivolous at that time but came out most outstanding ideas afterwards.

My heartiest gratitude and respect to Rebeca Sultana who helped me boundlessly until the end of my PhD. She played a key role in critical reviewing manuscript drafts, editing drafts and correcting citations. Her enormous support and guidance in scientific writing were very vital in successfully completing my dissertation. She helped me to find out the novelty of my research and to interpret the microbiological aspects of the study from the public health point of view.

I am very fortunate to have a very self-motivated and super talented PhD colleague like Zenat Zebin Hossain. Her persistent endeavours in research and commendable accomplishments have

always created a stimulating environment in the laboratory that encouraged and motivated me to conduct my laboratory work with success.

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I am incredibly thankful to my friends and family throughout the PhD process in numerous ways, including but not limited to, providing emotional support, listening to my complaints, frequently visiting me with homemade delicious foods. Words cannot express my gratitude to my parents who have always inspired me to pursue my higher studies, offered words of encouragement and laughter on the difficult days.

Last but not least, I am grateful to DANIDA due to the financial support provided for my PhD research through the project Combating Cholera Caused by Climate Change [grant number: 12-040KU].

Project summary

Bangladesh experiences a plethora of cholera and diarrhea outbreaks on a year-round basis, where contaminated drinking water plays an important role. While the earlier studies on drinking water microbiology emphasized the risk of contamination of water at the source, in recent years, studies have stated that controlling the microbial contamination of in-house water might be an important interim strategy until a safe, reliable piped-in water connection is provided to the household. Therefore, this thesis is primarily focused on addressing the effect of using a piped-to-plot improved communal source on point-of-drinking water during consumption in a low-income urban community of Bangladesh, from diverse microbiological points.

The study was conducted in the low-income urban community of East Arichpur, located in Tongi Township of Dhaka City, Bangladesh, as it has a history of outbreaks of waterborne diseases, including cholera. Water samples were collected from 430 households which were connected to 78 communal piped-to-plot sources. The research team collected samples from point-of-drinking water (i.e. in-house drinking water) and communal sources, simultaneously. Both point-of-drinking water and source water were collected during routine visits at 6-week intervals from September 2014 to December 2015.

To conduct microbiological investigation in a resource-limited laboratory setting, inexpensive, rapid and convenient molecular methodologies were required. For this purpose, a DNA extraction method was identified, and two qPCR methods were developed.

Three DNA extraction methods: a) boiled template, b) phenol: chloroform: isoamyl alcohol and c) the QiaAmp® DNA mini kit, were evaluated by quantitative Polymerase Chain Reaction (qPCR) and conventional end-point PCR for the detection of the *ctxA* gene of *V. cholerae* O1. The comparative evaluation showed that the boiled template extraction method was the most inexpensive and simple to apply of the three, and in a short period of time was able to extract DNA of a sufficient purity and yield to be detected by both qPCR and end-point PCR. Subsequently, a qPCR method was optimized for quantification of bacterial species to render results accurately (R^2 value). The detection and estimation of fecal *E. coli* was obtained by targeting the *uidA* gene and that of toxigenic *V. cholerae* was obtained by targeting the *ctxA*-gene for the qPCR method. A simple genomic DNA dilution provided a better R^2 value for both *V. cholerae* (0.99) and *E. coli* (0.99) than the cell suspension dilution method (0.96 and 0.93 for *V. cholerae* and *E. coli*, respectively). Our next attempt was to develop an assay that would detect

all species of *V. cholerae* in qPCR regardless of the epidemic-causing serotypes O1 and O139. The SYBR green-based qPCR primers were developed for the *ompW* gene as a target for species-specific detection, and quantification of *V. cholerae.* The suitability of the developed assay for detection of *V. cholerae* was examined in food and water samples. The developed assay could successfully detect *V. cholerae* in spiked food and water samples.

Fecal contamination assessment by a membrane filtration culture-based method showed that overall contamination of *E. coli* was higher in domestic domain (point-of-drinking) water than public domain (communal source) water. Most importantly, the same-day paired samples of connected communal source and point-of-drinking water showed that the level of fecal contamination increased from communal source to point-of-drinking water in 51% of samples, where 26% of samples had zero *E. coli* at the communal source. This implies that recontamination and post-contamination played a significant role in drinking water contamination. The recontamination/post-contamination pathways were dominant for *V. cholerae*, as significantly higher odds ($P < 0.05$) of *V. cholerae* presence in point-of-drinking water compared to communal source [OR = 17.24 (95% CI = 7.14–42.89)] water were found when samples were collected from connected communal source and point-of-drinking water within a seven-day interval (seven days before/after). The study found higher contamination of both *E. coli* and *V. cholerae* in wider mouth drinking vessels (mugs, glasses) than in narrow mouth drinking vessels (bottles). All these findings point to the fact that non-water routes (hands, flies, contaminated vessels) are prevailing in domestic domain contamination, specifically in point-of-drinking water.

The pathogenic identification of *E. coli* showed that ETEC was the most prevalent pathotype found in point-of-drinking water and communal source water. We found hybrid *E. coli* isolates (a combination of ETEC-EHEC and ETEC-EIEC) in the ‘intermediate risk’ group and ‘very high risk’ group from both point-of-drinking water and communal source water. Our study findings revealed that the ‘intermediate risk’ group should be equally prioritized with the high-risk groups as we found highly virulent and emerging ‘hybrid’ pathogens in both point-of-drinking water and source water after pathogenic characterization of *E. coli*.

Phylogenetic grouping of *E coli* isolates showed that communal sources of the study area were mostly contaminated by animal feces and, to a lesser extent, by human feces. Regarding the potential origin of fecal contamination of source water in the study community, non-human mammals (goats, cows) and birds (ducks and chickens) might have played an important role.

Contamination by human feces at the point-of-drinking water was higher compared to communal source water, suggesting that human fecal contamination might have prevailed in in-house point-of-drinking water contamination.

Future research should emphasize minimizing non-water routes of recontamination/post-contamination in the domestic domain, which might include regular cleaning of drinking vessels and the promotion of narrow necked drinking water vessels. Incorporating the investigation of pathogenic bacteria, and identification of their host of contamination using molecular methods, into the water quality monitoring guidelines may provide useful insights to reflect the unambiguous safety of drinking water, and information on the emergence of new pathogenic microbes.

Danish summary

Befolkningen i Bangladesh oplever et væld af kolera- og diarréudbrud året rundt, og forurenet drikkevand er en vigtig årsag bag. Mens de tidligste mikrobiologiske studier af drikkevand har haft fokus på drikkevandskilden, har forskningen i de senere år påpeget, at det kan være en effektiv midlertidig strategi at kontrollere mikrobiel forurening af drikkevand i selve husholdningen, indtil rent vand, som føres ind i hjemmet i vandrør, er tilgængeligt. Derfor har denne afhandling fokus på at undersøge, fra forskellige mikrobiologiske perspektiver, hvilken effekt det har på drikkevand i hjemmet, når vandet kommer med vandrør fra en kommunal vandboring.

Studiet er blevet udført i East Arichpur, et urbant område med lav gennemsnitsindkomst i Tongi Township i Dhaka, Bangladesh, som ofte rammes af udbrud af vandbårne sygdomme, inklusive kolera. Vandprøver blev indsamlet fra i alt 430 husholdninger, som var forbundet med rør til 78 forskellige kommunale vandboringer. Forskningsgruppen indsamlede, samtidigt, prøver fra den beholder (glas, flaske o. lign) man drak drikkevandet fra i hjemmene og fra den forsynende vandboring. Indsamlingen foregik ved rutinemæssige besøg med seks ugers mellemrum fra september 2014 til december 2015.

Da de mikrobiologiske studier fandt sted i et laboratorie med begrænsede ressourcer, var der behov for effektive molekylære metoder med lave omkostninger. I det henseende blev en metode til DNA-udtræk identificeret, og to qPCR-metoder blev udviklet.

Tre metoder til DNA-udtræk – a) *boiled template*, b) *phenol: chloroform: isoamyl alcohol*, og c) QiaAmp® DNA min kit – blev evalueret for deres evne til at detektere *ctxA*-genet i *V. cholerae* 01 ved hjælp af qPCR (quantitative Polymerase Chain Reaction) og konventionel *end-point* PCR. Denne comparative evaluering viste at *boiled template*-metoden både har de laveste omkostninger af de tre og er simple at anvende. Inden for et kort tidsrum kunne metoden udtrække DNA af en tilfredsstillende mængde og renhed, som kunne detekteres af både qPCR og *end-point* PCR. Endvidere blev en qPCR-metode optimeret til at kvantificere bakterier med et præcist resultat (R^2-værdi). Detektering og estimering af fækal *E. coli* muliggjordes, med qPCR-metoden, ved at spore *uidA*-genet, og for *V. cholerae* ved at spore *ctxA*-genet. En simpel *genomic DNA dilution* viste sig at give en bedre R^2-værdi for både *V. cholerae* (0,99) og *E. coli* (0,99) end en *cell suspension dilution*-metode (henholdsvis 0,96 og 0,93 for *V. cholerae* og *E. coli*). Vi forsøgte derefter at udvikle et *assay* til at spore alle *V. cholerae*-arter i qPCR uden at

tage hensyn til serotyperne O1 og O139. *SYBR green-based qPCR primers* blev udviklet med *ompW* som mål til art-specifik detektion og kvantificering af *V. cholerae*. Metodens egnethed blevet vurderet ved hjælp af mad- og vandeksempler, og viste sig med succes at kunne identificere *V. cholerae*.

Fækal kontaminering blev målt med en kulturbaseret membranfiltreringsmetode, som viste at *E. coli*-forurening var større i drikkevandet i hjemmet end i den kommunale vandboring. Endvidere viste prøverne fra de to miljøer, som var indsamlet samme dag, at forureningen steg fra vandboringen til drikkevandet i hjemmet i 51% af prøverne, mens 25% af prøverne fra den kommunale vandboring slet ikke indeholdte *E. coli*. Det indikerer at rekontaminering og postkontaminering har spillet en markant rolle i forureningen af drikkevandet. *V. cholerae* dominerede i højere grad re- og postkontamineringen, og der var markant større sandsynlighed for at finde *V. cholerae* i drikkevandet i hjemmet ($P < 0,05$) ift. den kommunale vandboring [OR = 17.24 (95% CI = 7.14–42.89)] når prøver blev indsamlet fra begge kilder inden for et interval på syv dage (syv dage før/efter). Yderligere fandtes en højere grad af forureningen af både *E. coli* og *V. cholerae* i drikkevandsbeholdere med bred åbning (kopper, glas) end dem med en smal åbning (fx flasker). Resultaterne tyder på at smitten i det hjemlige miljø ikke foregår gennem vand, men gennem andre veje såsom urene hænder, fluer og kontamineret service.

Den patogeniske identifikation af *E. coli* viste at ETEC var den hyppigst forekommende patotype i prøverne fra både det hjemlige drikkevand og vandboringen. Vi fandt *E. coli*-hybridisolater (en kombination af ETEC-EHEC og ETEC-EIEC) i '*intermediate risk*'-gruppen og i '*very high risk*'-gruppen i både det hjemlige drikkevand og i vandboringen.

Vores resultater har vist, at '*intermediate risk*'-gruppen bør prioriteres på linje med *'high risk'*-gruppen, da vi fandt højvirulente 'hybrid'-patogener både i vandboringer og i drikkevandet i hjemmene efter patogenisk karakterisering af *E. coli*.

En fylogenetisk gruppering af *E. coli*-isolater viste at de kommunale vandboringer primært var forurenet af afføring fra dyr, og kun sekundært afføring fra mennesker. Pattedyr (geder og køer) og fugle (ænder og høns) har formentlig spillet en vigtig rolle i forureningen af vandboringerne i studieområdet. I forhold til de kommunale vandboringer var drikkevandet i hjemmene i højere grad forurenet af afføring fra mennesker, hvilket tyder på at det her er den primære forureningskilde.

Fremtidig forskning bør fokusere på at minimere re- og postkontaminering fra andre smitteruter end vand i de hjemlige miljøer. Det kunne eksempelvis ske gennem rengøring af vandbeholdere og ved at arbejde for udbredelsen af snæverhalsede drikkevandbeholdere. Hvis molykulære undersøgelser efter patogeniske bakterier, samt en indsats for at identificere kilden til forureningen, inkorporeres i monitoreringsprogrammer for vandkvalitet kan det give nyttig indsigt i drikkevandets sikkerhed samt information om nye, opkommende patogeniske mikrober.

1. Introduction

1.1 Access to safe drinking water and burden of diarrhea

1.1.1 Global Scenario

Globally, 1.9 billion people lack reliable access to microbiologically safe drinking water sources, with the majority living in low- and middle-income countries (LMICs) [1]. In LMICs, 502,000 deaths were associated with unsafe or insufficient drinking water [2]. Recently, in 2015, the Global Burden of Disease (GBD) study ranked unsafe water as 14th among global health risks [3]. Among the LMICs, the population attributable fraction for diarrohea burden due to inadequate water was 0.32, with an estimated 207,774 deaths in South-East Asia [2]. Cholera, a life threatening diarrheal disease, annually affects an estimated 2.9 million people with an estimated 95,000 deaths in 69 cholera-endemic countries [4].

1.1.2 Scenario in Bangladesh

The United Nations International Children's Emergency Fund (UNICEF) report on Drinking Water, Sanitation and Hygiene in 2017 noted that access to safely managed water in Bangladesh is 56%, revealing that 71 million people in this country lack access to safely managed water [5]. Diarrhea and cholera are endemic to this country. A recent review by Ali et al. (2015) reported that, in Bangladesh, an estimated 66 million people are at risk for cholera, with an estimated incidence rate of 1.64 per 1,000 people, and 109,000 estimated annual cases with a three percent case fatality rate [4]. Authors claim that outbreaks of diarrheal illness, including cholera, predominantly occur due to contaminated drinking water in this country [6-9]. Although the national level data regarding incidence and prevalence rate of diarrheal diseases in Bangladesh is absent, there are some studies that provide location specific estimates of diarrheal illness [10]. A study conducted in the high risk diarrhea-prone urban areas of Dhaka City reported that prevalence of diarrhea among all ages was 16 per 1,000 and among young children it was 44 per 1,000 persons [10].

1.2 Drinking water: a primary route of diarrheal disease transmission

1.2.1 History of the role of water in the transmission of diarrheal diseases

The association of drinking water with waterborne pathogens came into context in 1854 when John Snow published an article attributing the devastating London cholera outbreaks to the drinking-water supply [11]. He identified the water pump on Broad Street in Soho, London as the source of the cholera outbreak, and the water company delivering water drawn from a

sewage-polluted section of the river Thames to the homes, as the leading cause of the high cholera incidence. In 1958, Wagner and colleagues first proposed the F-diagram to describe the fecal-oral transmission of diseases, including diarrheal diseases, through multiple pathways [12]. Later on, during the 1970s, White et al. [13] proposed the classification of water-related diseases based on their transmission routes in the environment. According to White, water-related diseases can be classified as water-borne, water-washed, water-based and water-related insect vector diseases. The strength of this classification was that it could easily indicate the type of intervention that might be useful in reducing the incidence of water-related diseases. Afterwards, Rechard Feachement, in 1977, [14] significantly improved White, Bradley and White's contribution by proposing a classification of transmission routes rather than diseases, since some diseases could be transmitted by more than one route. In the 1990s, Cairncross et al. [15] proposed another division of transmission routes by classifying the preceding categories into two major domains: the domestic domain and the public domain. 'Domestic domain' refers to the area under the control of a household and 'public domain' includes public places such as work, education and recreation sites, as well as streets and fields. This paradigm brought much greater clarity to scientists and practitioners in controlling diseases through environmental interventions. Intervention of the water supply from communal sources entails a public domain intervention. Intervention involving in-house drinking water storage and point-of-use water at the household level constitutes a domestic domain intervention.

1.2.2 Debate on the impact of the public vs domestic domain on health

Numerous disputes exist on the effectiveness of water source intervention versus in-house drinking water interventions. Conventional interventions to improve water supplies at the source were recognized as an effective strategy in preventing diarrhea [16, 17]. Vanderslice and Briscoe (1993) [18] stated that in-house contamination of water is not a serious risk for diarrhea, as family members might acquire some level of immunity to the pathogens to which they are repeatedly exposed in the household. The authors argued that even in the absence of such immunity, transmission of pathogens through stored water might be less effective as compared to other routes of transmission (i.e. person-to-person contact, food contamination) within the household. Thus, they concluded that improvement of the source water quality is more important since it may introduce new pathogens into the household. However, this argument fails to explain the fact that acquired immunity develops slowly and, as a result, in-house contamination may still affect the health of very young children, especially during weaning [19]. Secondly, the

argument also ignores that these recycled pathogens can act as opportunistic pathogens for elderly immunocompromised persons.

A meta-analysis led by Clasen et al. in 2006 [20] emphasized that interventions to improve microbial water quality at the household level are more effective than intervention at the source. Likewise, a similar study on blinded trial of household water treatment and safe storage (HWTS) reported that there is no additional benefit on the reduction of disease incidence from improvements in water supply [21]. In the recent Cochrane review, Clasen et al. (2015) stated that controlling microbial contamination of in-house water might be an important interim strategy until a safe, reliable piped-in water connection is provided to the household [22]. While the earlier studies on drinking water microbiology emphasized the risk of water contamination at the source, the later studies on this topic placed more emphasis on in-house water contamination.

Cairncross et al. (1996) [15] suggested that intervention should be carried out at the source, in transit, and on in-house drinking water to prevent transmission through contaminated water. The authors discussed that preventing in-house water contamination might be an important measure to avert endemic disease occurrence, while avoiding contamination at the source and in transit might be beneficial in preventing epidemics of severe diseases. The study by Jensen et al. in 2002 [23] showed that when the source water is highly contaminated (>100 *Escherichia coli* per 100 ml of water), intervention to prevent in-house water contamination would have a negligible impact on water quality. While there has been an ongoing debate regarding the relative importance of fecal contamination of water in the public vs the domestic domain and its corresponding health effects, these findings of Jensen et al. echoed the Cairncross et al. recommendation of 1996 that intervention should be both at the source and at in-house drinking water to prevent diarrheal diseases. The fecal-oral transmission pathway, that includes drinking water, can be demonstrated using the following diagram (Figure 1).

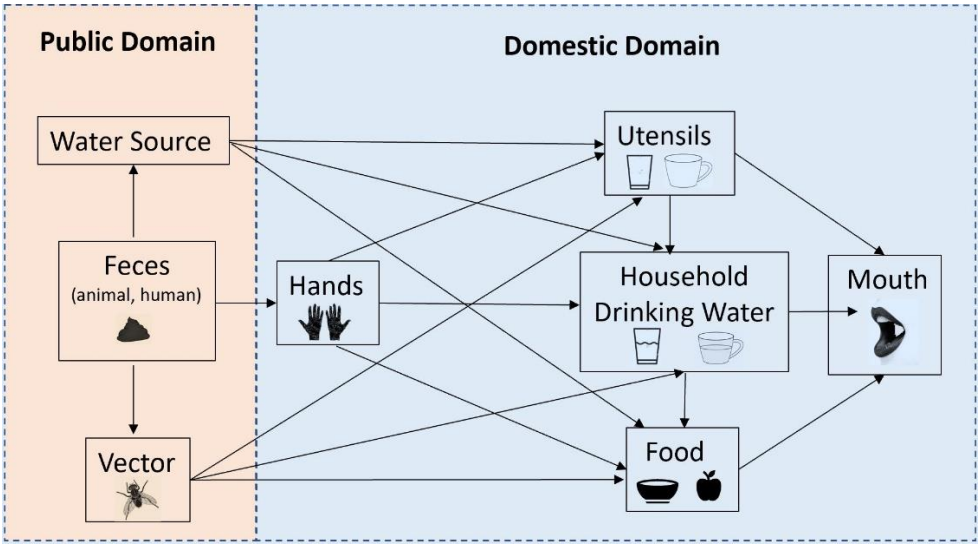

Figure 1. Schematic diagram of fecal-oral transmission routes. The diagram represents possible fecal-oral transmission routes that include drinking water

1.2.3 Water contamination: source to point-of-consumption

Water quality at the source might change over the course of collection, transport, and home storage [24-26]. A good quality water source does not always ensure safe drinking water and thus does not guarantee full health benefits if proper storage and sanitation are not maintained [27]. Hence, some researchers proposed to monitor the quality of water in the households [19, 28, 29]. Household drinking water includes both in-home water storage and point-of-consumption (Figure 2).

The extent of fecal contamination during the cascade from source to storage has been examined by many studies in rural Sierra Leone, rural Honduras, India, Pakistan, South Africa and Zimbabwe [23, 26, 28, 30, 31]. Bacterial counts in household stored water can be greater or lower than the source water [23, 31]. Human hands and household sanitary conditions were considered as predominant causes of increased in-house bacterial counts [19]. In contrast, decreased counts in the household stored water may result from bacterial die-off due to the time elapsed after collection and home-based water treatment [31, 32]. However, home-based water treatment during water storage is not always effective in maintaining drinking water quality. A study conducted in a community of Lima, Peru found that although 99% of households boiled their drinking water, 30% of water samples were contaminated with *E. coli* [33].

Ensuring the water quality of stored water in the household does not necessarily ensure safe drinking water for consumption. Point-of-consumption refers to the drinking vessels (i.e. mug, glass, bottle) that are used to serve water for drinking. Studies confirmed that recontamination occurs between in-home storage and point-of-consumption [32, 34]. Oswald [32] found that in

the peri-urban households of Lima, fecal contamination was higher in drinking cups compared to in-house storage containers and the source. Another study conducted in Bolivia found that the median concentration of *E. coli* was significantly higher at point-of-consumption compared to the source [34]. While most of the studies [23, 26, 28, 30, 31] investigated the influence of contamination from an 'unimproved' source to storage vessels and drinking cups in the households, only two studies [26, 34] exist that investigated the influence of contamination from an 'improved' source to the point-of-consumption. However, these two studies showed the effect of the water quality of 'improved' sources collectively (boreholes, standpipes, bowser trucks) on 'point-of-consumption' water and did not document the specific effect of the piped-to-plot (improved) source on point-of-consumption. Therefore, the quality of water at the source and at point-of-consumption among the communities with an in-house water connection from an 'improved' (piped-to-plot and tap water as defined by the WHO/UNICEF Joint Monitoring Programme for Water Supply and Sanitation in 2012) [35] source is still unexplored.

In this thesis, I endeavored to investigate the water quality of 'improved' (i.e. piped-to-plot) sources and of point-of-consumption in a low-income urban community with in-house water connection, using microbiological and molecular methods.

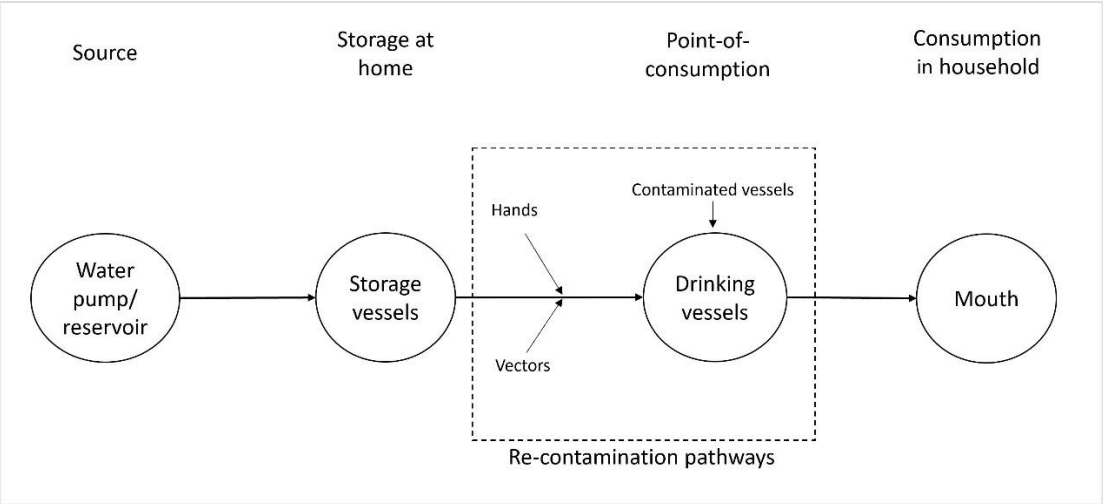

Figure 2. Schematic presentation of water contamination from source to point-of-consumption. The dashed box indicates potential pathways of contamination and/or recontamination of points-of-consumption in the household.

1.3 Waterborne diarrheal diseases and etiologies

Contaminated drinking water can lead to many types of waterborne diarrheal diseases such as cholera, typhoid fever, amebiasis, and dysentery [36]. Disease-causing organisms (pathogens) transmitted via ingestion of drinking water are predominantly of fecal origin and known as

enteric pathogens [37, 38]. The Global Burden of Disease (GBD) study in 2015 highlighted that 13 diarrheal infections were the leading cause of diarrheal deaths among all ages [39]. These infections include: cholera, *Salmonella* infections, shigellosis, enteropathogenic *Escherichia coli* (EPEC) infections, enterotoxigenic *Escherichia coli* (ETEC) infections, *Campylobacter* enteritis, amebiasis, cryptosporidiosis, rotaviral enteritis, *Aeromonas* infections, *Clostridium difficile* infections, norovirus infections, and adenovirus infections. A wide range of bacteria, viruses and parasites were responsible for these diarrheal diseases.

The type of 'pathogen' is crucial in predicting the health risk of re-contaminated drinking water. Several pathogen characteristics (persistence, virulence, infective dose and growth rate) are of particular relevance for transmission to a new host. These characteristics vary widely between pathogens and, in some cases, between pathogen strains. In this thesis, enteric pathogens, with a specific focus on *Escherichia coli* (*E. coli*) and *Vibrio cholerae* (*V. cholerae*), will be discussed and their manifestation of diseases, epidemiology, transmission and molecular detection will be highlighted.

1.3.1 Microbial water quality assessment: use of *E. coli* as an indicator

The greatest risk to public health from microbial aspects of water is associated with consumption of drinking water that is contaminated with human and animal excreta. Public health protection policies require an indicator of fecal pollution, as it is not yet feasible to analyze all pathogens in the aquatic system. The use of thermotolerant *E. coli* has been widely accepted since the 1990s as the indicator microorganism to assess fecal contamination from both human and animal sources in drinking water. The characteristics that facilitate the selection of thermotolerant *E. coli* as a suitable indicator organism over other fecal coliform group bacteria include: it is found only in feces; it has a longer survival time and does not survive well outside of the intestinal tract; it is easy, fast and inexpensive to detect; and a small volume of water is needed for its detection [40].

E. coli is found in all mammal feces at concentrations of 10^9 cells per gram, but it does not multiply appreciably in the environment [41]. *E. coli* is a Gram-negative, oxidase-negative, rod-shaped bacterium from the *Enterobacteriaceae* family and is found in the large intestine of warm-blooded animals [42]. Theodor Escherich first reported the isolation and characterization of this bacterium from infant stool, which he named *Bacterium coli commune*, in his 1885 publication. Later, it was renamed after him as *Escherichia coli.*

The World Health Organization (WHO) urges periodic testing, sanitary inspection and assessment of microbial quality for community drinking-water supplies. Along with the United States Environmental Protection Agency (USEPA) and Canadian Drinking Water Quality Guidelines, WHO recommends that the acceptable limit of *E. coli* is <1 per 100 mL of drinking water, meaning that there should be no fecal contamination in drinking water. However, in many developing and developed countries, a high proportion of household and small community drinking-water systems, in particular, fail to meet the requirements for water safety, including the absence of *E. coli*. In such circumstances, in order to implement a realistic goal for progressive improvement of water safety linked to priority action, a grading scheme for scoring risk was suggested for implementation by WHO. The scheme was stratified into four categories of risk based on the number of *E. coli* per 100 mL sample of water, and thereby prioritized remedial actions for household water systems. The categories include: low risk/safe: no action required (< 1 *E. coli*/100 mL), intermediate: low action priority (1–10 *E. coli*/100 mL), high: higher action priority (11–100 *E. coli*/100 mL), and very high risk: urgent action required (> 100 *E. coli*/100 mL) [43].

1.3.2 *E. coli* as a major diarrheal pathogen in developing countries

All waterborne pathogens exhibit persistence, the ability to survive outside the human host, to some extent. *E. coli* strains are generally considered as ubiquitous commensals of the gastro-intestinal tract of warm-blooded animals, including humans, with minimal survivability in the external environment. However, certain strains often possess particular virulence-associated genes that make them genetically distinct from commensal strains and enable them to cause intestinal infections such as diarrhea or hemolytic colitis, or extra-intestinal infections such as meningitis/septicemia, and urinary tract infections [44, 45].

Pathogenic *E. coli* strains can be classified as intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC) on the basis of their virulence factors and clinical symptoms [46, 47]. InPEC strains are commonly known as diarrheagenic *E. coli* (DEC) and are further classified into six well-characterized pathovars: enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) (e.g. enterohemorrhagic *E. coli* [EHEC]), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC) [44, 46, 48]. ExPEC strains carry different combinations of virulence genes than those of InPEC strains, and thus cause different clinical symptoms [47]. ExPEC are sometimes categorized into uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC). Recently, highly virulent hybrid pathotypes of *E. coli*

strains have been reported, which are believed to have evolved due to the acquisition of several toxin encoding genes through mobile genetic elements and subsequent genetic combinations within the previously defined pathotypes; however, their significance in drinking water contamination remains uncertain [49, 50].

InPEC strains are commonly known as diarrheagenic *E. coli* (DEC). In 2005, O'Ryan et al. [51] reported that DEC accounts for 30–40% of all the diarrheal episodes in developing countries. Among all DEC, ETEC and EPEC are the most important enteric pathogens that cause acute diarrhea in infants and young children in developing countries [52]. The widespread association of DEC in causing diarrheal illness has been documented in various studies, which were focused on DEC detection in stool samples of diarrhea patients in urban slums of Bangladesh [52-54]. Disease outbreaks and deaths linked with exposure to surface water, freshwater and recreational water contaminated with pathogenic strains of *E. coli* are well documented [55-57]. The importance of region-specific prevalence studies of various *E. coli* pathotypes has been witnessed by previous studies [57-59]. However, the occurrence of pathogenic *E. coli* strains harboring virulence genes in the drinking water of low-income urban settings has been scantily documented in Bangladesh [60, 61]. A study conducted by Talukdar et al. (2013) [60] investigated the pathogenic diversity of *E. coli* in tap water samples of collection points in the southwest part of Dhaka City, but they did not investigate the pathogenic diversity of *E. coli* at the point-of-consumption (drinking cups, mug, glass). Harada et al. (2018) [61] conducted a study in a rural area of Bangladesh and analyzed the occurrence of pathotypes of *E. coli* in sanitary wastewater and drinking water, but the study did not investigate source water. To bridge this gap, in this thesis I will compare the prevalence of pathotypes of *E. coli* strains, including ExPEC isolates, in household drinking water and communal source water in a low-income urban community.

1.3.3 Transmission of pathogenic *E. coli*

Pathogenic *E. coli* is transmitted from host to host via the fecal-oral route through contaminated food, drinking water, surfaces, weaning fluids, and human carriers (Figure 3) [62-64]. Food can be contaminated by infected food handlers, cross contamination, poor kitchen hygiene, [33, 65, 66], flies [67], asymptomatic carriers, contaminated domestic water, or contact with untreated irrigation water [68-70]. Contaminated runoff water, flooding, and irrigation water can also taint nearby water sources, rivers, lakes, and private drinking water wells. Common reservoirs of ETEC and EPEC include humans, humans, ruminants, pigs and other domesticated animals such as goats, dogs and cats [71, 72]. Exposure to ETEC is usually from contaminated food and drinking water (Figure 3).

Some examples of high-risk foods contaminated with ETEC include food that is left at room temperature, table-top sauces, certain fruits, and foods from street vendors [73, 74]. Additionally, these organisms have been found in surface water of low-income regions like Bangladesh and may serve as an important source of infection [75].

Shiga toxin-producing *E. coli* (STEC) which is also known as verocytotoxin producing *E. coli* (VTEC) encompasses a diverse pathotype that can cause mild to bloody diarrhea and hemolytic-uremic syndrome (HUS). Enterohemorrhagic *E. coli* (EHEC) is a subset of STEC and was originally demonstrated by its association with hemorrhagic colitis. EHEC disease primarily appears in developed countries, causing fewer disease outbreaks in LMICs [64, 76, 77]. A study conducted in 2006 in 13 districts of Bangladesh identified EHEC in 6% (160) of *E. coli* isolates of the aquatic environment (e.g. ponds, rivers and lakes) [78]. Cattle (both meat and dairy) are primarily known to be major reservoirs for pathogenic STEC, and their fecal matter acts as an important source of human pathogens [79, 80]. Additionally, asymptomatic shedders may also act as a source of person-to-person transmission, especially when food handlers or highly susceptible recipients are involved.

The principal reservoir for EIEC, EAEC and DAEC are humans [46, 71]. Host-to-host spread of EIEC is mediated via the fecal-oral route, mostly through contaminated water and food or direct person-to person transmission [63] (Figure 3). Transmission of traveler's diarrhea, which is often caused by EAEC, occurs mostly through contaminated water and food, such as salads [81] (Figure 3). In Mexico, contaminated desserts and salsa have been found to be sources of EAEC [82-84]. Additionally, food handlers have been implicated as carriers of EAEC [85, 86]. Although atypical EAEC has also been identified in calves, piglets, and horses, animals are not an important reservoir of typical human-pathogenic EAEC [87]. Transmission and reservoirs of pathogenic *E. coli* are shown in Figure 3.

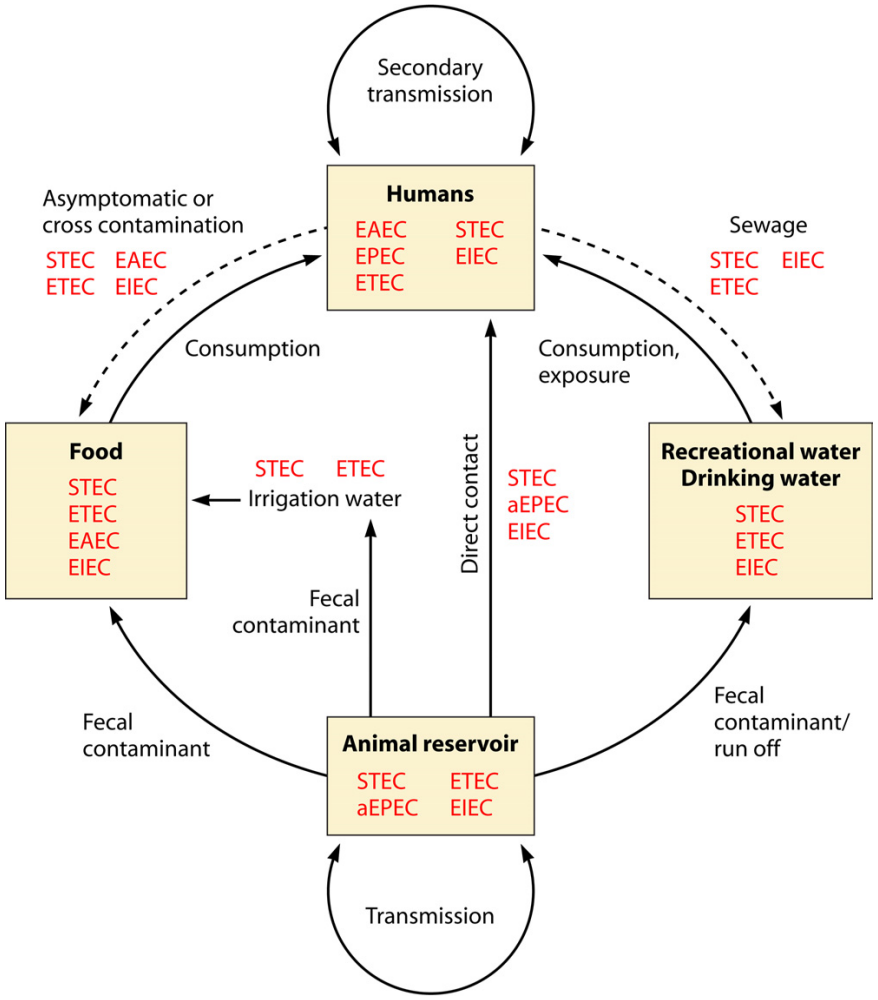

Figure 3. Transmission of pathogenic *Escherichia coli.* Potential reservoirs and modes of transmission of pathogenic *E. coli*. (Croxen et al. 2013 [88])

1.3.4 Source identification of *E. coli*

It is imperative to identify the major sources of fecal contamination (e.g. animal, human) for the effective management of water systems [89]. *E. coli* strains can be assigned to one of the main phylogenetic groups: A, B1, B2 or D [90-93]. These phylogroups apparently differ in their ecological niches, life-history, ability to utilize different sugar sources, antibiotic-resistance profiles and growth rate [94, 95]. The ExPEC strains usually belong to groups B2 and D, the commensal strains to groups A and B1, and the InPEC strains to groups A, B1 and D [45, 96-98]. Walk et al. (2007) [99] demonstrated that the majority of the *E. coli* strains that are able to persist in the environment belong to the B1 phylogenetic group.

Some authors have analyzed the distribution of the main phylogenetic groups among *E. coli* strains isolated from human and animal feces. Escobar-Páramo et al. (2006) [100] analyzed the fecal strains isolated from birds, non-human mammals and humans. They observed the prevalence of groups D and B1 in birds, A along with B1 in non-human mammals, and A as well

as B2 in humans. These authors concluded that one of the main forces shaping the genetic structure of *E. coli* populations among the hosts is domestication. Baldy- Chudzik et al. (2008) [101] analyzed feces from zoo animals and found a higher prevalence of group B1 in herbivorous animals and of group A in carnivorous and omnivorous animals. Not many studies have analyzed the distribution of phylogenetic groups and subgroups of *E. coli* strains isolated from drinking water, which is important in identifying the potential source of fecal contamination in drinking water. Hence, one of the aspects of this thesis is to examine the major sources of fecal contamination (e.g. human or animal) in drinking water.

1.3.5 Cholera and cholera like illnesses: *V. cholerae* in drinking water

Cholera is a diarrheal disease manifested by rapid dehydration due to severe water and electrolyte loss. Cholera results from ingestion of toxigenic *Vibrio cholerae*, which is a Gram-negative, curved-rod-shaped bacterium. *V. cholerae* is autochthonous to the aquatic environment. Ever since John Snow's investigation of the Broad Street, London water pump in the cholera outbreak, contaminated drinking water has primarily been linked as a risk factor for cholera [11, 102-104]. Cholera is endemic in Bangladesh, where it causes year-round cases, but peaks in cholera cases are generally witnessed during two points of the year (September-November, March-May) in urban Dhaka [105]. Dhaka, the capital of Bangladesh, has become one of the world's most densely populated cities with 36 percent of the country's 15,584,835 urban population living in Greater Dhaka [106]. Around 3.5 million people are slum dwellers [107] and close to one third do not have access to safe drinking water due to the lack of sanitation [108]. Water scarcity in Dhaka also contributes to cholera being present throughout the year [109]. There is, however, investigation of the presence of *V. cholerae* in point-of-consumption water (i.e. the quality of water in the drinking vessel immediately before consumption), in households of a low-income community within the diarrhea/cholera endemic region is under research. For this reason, I conducted a comparative assessment of the presence of *V. cholerae* and investigated the variability of the virulence profile between communal source water and household point-of-consumption water.

V. cholerae is an extremely diverse species, with more than 208 different identifiable serogroups [110]. The serogroups are classified based on the structure of the cell surface lipopolysaccharide O antigen of *V. cholerae* and it is known that only serogroup O1 and O139 are linked to epidemic cholera, worldwide [111]. The pathogenicity of serogroups O1 and O139 is associated with the expression of two major virulence factors: the cholera toxin (CT) and toxin co-regulated pilus (TCP), and are believed to be accountable for extended and large-scale outbreaks. However, there

are reports that CT and TCP are not always exclusive to serogroups O1 and O139, as many other non-O1/non-O139 strains have been found to carry one or both virulence factors [112, 113]. Sporadic outbreaks of non-O1/non-O139 have been quite common in Bangladesh, India, Brazil and USA [114-117]. Symptoms of infection due to pathogenic non-O1/non-O139 *V. cholerae* strains range from mild gastroenteritis to violent diarrhea, which resembles cholera caused by the pandemic O1 *V. cholerae* strains [117]. A minority of these *V. cholerae* strains are responsible for extra-intestinal infections such as ear infection, septicemia and meningitis [118, 119]. Similarly, nontoxigenic *V. cholerae* O1 has been associated with gastroenteritis and localized cholera outbreaks [120, 121]. Therefore, reports suggest that mild and moderate cases of diarrhea may go unnoticed if surveillance studies are largely dependent on the specific O1 and O139 serogroups of *V. cholerae* [122-125]. Hence in this thesis, to avoid inadvertently missing detection of serogroups other than O1 and O139, I emphasized the presence of *V. cholerae* regardless of their specific serotypes.

1.4 Bacterial survival in VBNC state and importance of quantification

Several human pathogenic bacterial species have been found to adopt a unique survival strategy, the viable but non-culturable (VBNC) state, which supports long-term survival under adverse environmental conditions. The existence of the VBNC state was first documented in the pioneering study by Xu et al. (1982) [126] who described that estimating survival and viable populations of indicator organisms, such as *E. coli*, and water-borne pathogens including *V. cholerae*, has severe limitations in the aquatic environment. Unlike normal cells, bacteria in the VBNC state fail to grow and develop into colonies on the routine bacteriological media, although they are alive [127]. Factors such as nutrient deprivation, oxidative stress, chlorination, light, extremes in temperature and salinity can induce VBNC formation in bacteria [128-132]. In nutrient-poor environments, Jubair et al. (2012) [133] found that *V. cholerae* can enter a starvation state for long time survival (>700 days) in a nutrient-poor filter-sterilized lake water microcosm. *V. cholerae* appear predominately as VBNC cells within the bacterioplankton and as culturable cells in biofilm consortia, either as aggregates or attached to biotic and abiotic surfaces [134].

E. coli, including pathogenic ETEC and EHEC, can also exist in the VBNC state in response to adverse environmental conditions [135, 136]. Aurass et al. (2011) [137] concluded that the EHEC/EAEC O104:H4 strain, which was linked with the 2011 German outbreak of HUS, was capable of entering into the VBNC state. Filip et al. (1987) [138] reported that *E. coli* survived in groundwater at 10°C for up to 100 days. One study showed that ETEC was able to survive for up to three months in freshwater [139] and was able to form biofilms in drinking water sources [140].

Despite the non-culturability of bacterial cells in the VBNC state, they are metabolically active, carry out respiration and, more importantly, can actively express virulence and colonization traits when resuscitation occurs under suitable conditions [141-143]. Therefore, the importance of the VBNC state in the epidemiology of infectious disease has been demonstrated by several studies.

Colwell et al. (1996) [144] showed that *V. cholerae* cells that had been in the VBNC state for seven weeks were capable of colonization in the human intestine and were excreted as culturable cells. Furthermore, biofilms of *V. cholerae* in the VBNC state have been implicated as an important environmental reservoir of *V. cholerae* during interepidemic periods of cholera [134].

The ability to enter the VBNC state may be beneficial for bacteria but poses a risk to human health. If VBNC cells are present in a sample, their non-culturability in conventional culture methods will lead to an underestimation of their population. For example, estimates of the infectious dose of STEC may become complicated since they have been shown to form VBNC cells on food, when stressed. The toxin-producing capability of STEC is independent of their culturability state since VBNC STEC have been shown to produce Shiga toxin [136]. The non-detection of viable cells in water quality assessment may pose serious risks to the public, as ‘zero’ CFU of fecal *E. coli* by culture method might not represent absence of fecal contamination, due to the formation of VBNC cells. Therefore, it is of the utmost importance to apply reliable detection methods to quantify the accurate population of viable cells, including both culturable and VBNC cells. In this thesis, a brief quantitative comparison of culturable and non-culturable state *E. coli* and *V. cholerae* has been shown.

2. Research hypothesis and objectives

As a diarrhea endemic country, Bangladesh experiences a plethora of cholera and diarrhea outbreaks on a year-round basis. The inhabitants of resource-poor, low-income urban areas are more prone to these diseases because of poor hygiene and sanitation infrastructure and practices. As discussed above, consumption of contaminated drinking water has been widely implicated as one of the primary reasons of gastrointestinal disease outbreaks worldwide. Although substantial improvement of drinking water and sanitation facilities has been achieved to date in this country, eradication of diarrheal diseases is yet to be achieved. Policy makers have been emphasizing the use of an 'improved' source to lessen the burden of diarrheal diseases attributable to unsafe drinking water (Millennium Development Goal: 7.c target). The term 'improved' source has been used to refer to the sources that have some measure of protection from outside fecal contamination, such as: piped supply, boreholes, protected dug wells, protected springs, and rainwater. Researchers have mostly investigated the outcome of using 'improved' sources on household storage for drinking (Cambodia, Peru, Bolivia) [34, 145, 146] water, with the 'improved' source including all the types of improved water sources mentioned above. To accurately assess the risk of the regional burden of diarrheal disease due to unsafe drinking water, region/country specific research is needed which will address the result of using not only improved but also a piped-to-plot improved communal source on drinking water during consumption, which is lacking in the existing research. Additionally, there is a need to add new considerations on measuring the health risk of unsafe drinking water from a diverse microbiological point of view. For instance, ongoing studies [23, 26, 28, 30, 31, 146, 147] evaluate the health risk of unsafe drinking water by simply measuring the coliform/fecal coliform test which may not adequately address the actual health risk from water since waterborne pathogens like *V. cholerae* can go unnoticed. In addition, the existence of VBNC state of *E. coli* and *V. cholerae* is neglected in the recent studies; this state may pose a risk for human health and requires a reliable detection and quantification method for accurate measurement of culturable and non-culturable cells. Added to all these points, there is no contemporary research, particularly in Bangladesh, addressing the issue from the communal water source to the point-of-consumption, which ultimately affects the consumer.

Therefore, the main objective of this thesis is to provide a comparative assessment of water contamination between communal source and point-of-consumption (i.e. drinking glass, mug,

bottle) water in a low-income urban area of Dhaka City, Bangladesh, with a specific focus on two microorganisms: *E. coli* and *V. cholerae.*

In order to achieve the main objective, microbiological approaches were employed by setting the following specific objectives:

2.1 Specific objectives

1) To optimize and validate molecular methodologies for accurate detection and quantification of bacterial pathogens (*E. coli* and *V. cholerae*) in a resource-strained laboratory (Manuscripts I, II, III).
2) To compare fecal contamination in communal source and point-of-consumption water by two analytical approaches (culture-based and qPCR) (Manuscript IV).
3) To characterize pathogenic *E. coli* isolates collected from water and to identify their probable fecal origin/host (Manuscript V).
4) To compare *V. cholerae* contamination in communal source and point-of-consumption water, and to investigate virulence patterns of *V. cholerae* (Manuscript VI).

2.2 Chapter outlines

In order to achieve the specific objectives, six manuscripts were produced and included in the thesis. The thesis includes four published manuscripts, and two draft manuscripts. To provide a transparent layout, the thesis is divided into the following three chapters. A precise description of each of the chapters is given below:

Chapter 3 provides a detailed description of the project area, selection of study households and sample collection procedures.

Chapter 4 contains the overall results and summaries of six manuscripts (manuscript I to VI). This chapter is divided into two major sections. The first section contains the results and discussion from manuscript I to III, which deals with the methodology set-up for the PhD project. The second section contains results and discussion from manuscript IV to VI, speaking of the findings of the investigation to achieve the overall objective of the thesis.

Chapter 5 includes the concluding remarks and further perspectives of the research based on the findings from Chapter 4. This chapter highlights the recommendations and interventions needed

in the public and domestic domains for reduction of the diarrheal burden attributed to unsafe drinking water in a low-income urban area.

The title of each of the manuscripts included in the thesis is as follows:

Manuscript I

Quantitative Analysis of Nucleic Acid Extraction Methods for *Vibrio cholerae* Using Real-time PCR and Conventional PCR. Hossain, Z. Z., Ferdous, J., Tulsiani, S. M., Jensen, P. M., & Begum, A. (2018). Mymensingh Medical Journal, **27**(2), 327-335.

Manuscript II

Optimization and Validation of Real Time PCR Assays for Absolute Quantification of Toxigenic *Vibrio cholerae* and *Escherichia coli*. Ferdous, J., Hossain, Z. Z., Tulsiani, S., Rashid, R. B., Jensen, P. K. M., & Begum, A. (2016). Tropical Biomedicine, **33**(4), 641-651.

Manuscript III

Development and Validation of a Novel Real-time Assay for the Detection and Quantification of *Vibrio cholerae*. Rashid, R. B., Ferdous, J., Tulsiani, S., Jensen, P. K. M., & Begum, A. (2017). Frontiers in Public Health, **5**, 109.

Manuscript IV

Comparative assessment of fecal contamination in 'improved' piped-to-plot communal source and point-of-drinking water. Ferdous, J., Sultana, R., Rashid, R. B., Begum, A., & Jensen, P. K. (2019) (Draft to be submitted).

Manuscript V

The fecal origin of pathogenic *E. coli* in 'improved' piped-to-plot communal source and point-of-drinking water of a low-income urban community, Bangladesh. Ferdous, J., Rashid, R. B., Begum, A., & Jensen, P. K. (2019) (Draft to be submitted).

Manuscript VI

A Comparative Analysis of *Vibrio cholerae* Contamination in Point-of-Drinking and Source Water in a Low-Income Urban Community, Bangladesh. Ferdous, J., Sultana, R., Rashid, R. B., Tasnimuzzaman, M., Nordland, A., Begum, A., & Jensen, P. K. (2018). Frontiers in Microbiology, **9**, 489.

3. Project description

In 2012, a multidisciplinary collaborative research project entitled “Combating Cholera Caused by Climate Change (C5)” was funded by the Danish International Development Agency (DANIDA) of the Ministry of Foreign Affairs (MFA) of Denmark. The project aimed at examining the relative risk of climate change-induced cholera and diarrhea influenced by environmental and household hygiene behavior in a water-stress environment of a low-income area (Arichpur) on the outskirts of Dhaka City, Bangladesh (**http://cope.ku.dk/research/cholera/**). This PhD project encapsulates a sub-study (Work Package 4) of the broader, comprehensive C5 study and the central role of this PhD project was to assess the water quality in the Arichpur area.

In this chapter, I provide a brief description of the project area (sections 3.1 and 3.2), data collection procedures for inclusion of households and sample collection (section 3.2), and microbiological procedures for sample collection (section 3.3).

3.1 Brief description of Arichpur area and population

The study took place in East Arichpur, located in Tongi Township of Dhaka City, Bangladesh (Figure 4). Arichpur is a low-income urban community encompassing 1.2 km^2 with a high population density (>100,000 residents per km^2) [148]. Approximately 129,000 residents are living in 29,000 households where many nuclear families share one room and up to 10-15 families may share a stove, toilet, and water source [148]. The study was conducted in East Arichpur as it has a history of outbreaks of waterborne diseases including cholera [148, 149]. On its southern edge, the community is bordered by one of the peripheral rivers of Dhaka City, Turag River, which is heavily polluted due to sewage dumping.

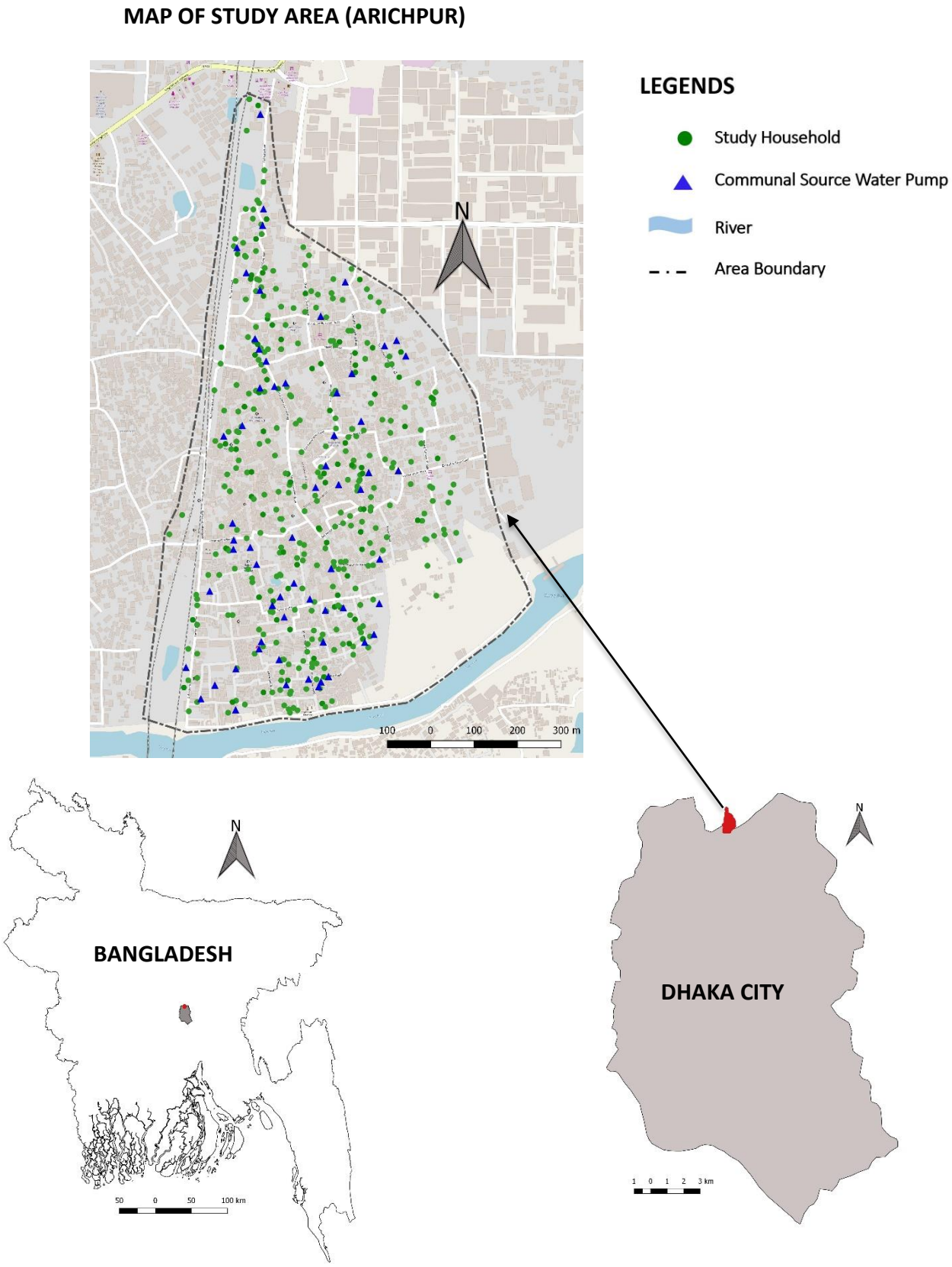

Figure 4. Map of the study area. Distribution of study households and communal source water pumps in the Arichpur area.

3.2 Infrastructure of water sources in Arichpur

The communal sources of Arichpur community were dependent on government provided public water supply and private water supply. These two types of 'improved' (piped-to-plot) ground water sources are: public-supply (locally known as 'WASA' as it stands for Water Supply and Sewerage Authority and installed by the municipal government), private supply (locally known as 'submersible pump' supply and installed by the individual owner/s). WASA water is supplied to the households through underground networks of pipes. Submersible water is distributed to the households using over ground networks of pipes. All the communal sources of our studied households abstracted groundwater and the depth was >85 meters. The area around the pumps is not usually protected with a wall and floor made of concrete (Figure 5).

(i) WASA pump

(ii) Submersible pump

(iii) Underground network pipes

(iv) Aboveground network pipe

Figure 5. Communal water sources and network system in Arichpur area. Types of 'improved' category water sources are (i) WASA pump and (ii) Submersible pump. Network system of water lines: (iii) Underground network pipes, and (iv) Aboveground network pipes.

3.3 Data collection procedures

In the C5 study, a total sample size of 400 households was calculated to ensure the power of the primary outcome of the study [150]. To avoid an insufficient sample due to losses to follow-up of migrating households, > 400 households (477 households) were enrolled in the study. The 477 households enrolled were connected to 81 communal sources.

A team of four field research assistants trained in water sample collection collected water samples from 430 households (47 households dropped out of the study) which were connected to 78 communal sources. The research team collected samples from point-of-drinking water (i.e. in-house drinking water) and communal sources simultaneously. We specified the term ‘point-of-drinking’ instead of the commonly used term ‘point-of-consumption’, since ‘point-of-consumption’ refers broadly to consuming water for various purposes such as bathing, cooking, hand washing and drinking. For point-of-drinking water, the team requested household members to provide drinking water samples using their preferred drinking vessels (i.e., a mug, glass, bottle, jug, or pitcher), as they normally would do to serve drinking water (Figure 6, Figure 7). For the communal source water, the team collected samples from the communal source point used by each study household. Both point-of-drinking water and source water were collected during routine visits at 6-week intervals from September 2014 to December 2015 as a part of the longitudinal study of diarrhea incidence and water use in a low-income urban community [151]. During water sample collection, any home-based water treatment (i.e. boiling, filtration, adding alum, etc.) of drinking water employed in the household was recorded. The coordinates of sample collection sites (households and communal sources) were obtained using a global positioning system (GPS). Q-GIS software was used to locate the sites on a Google map (Figure 4).

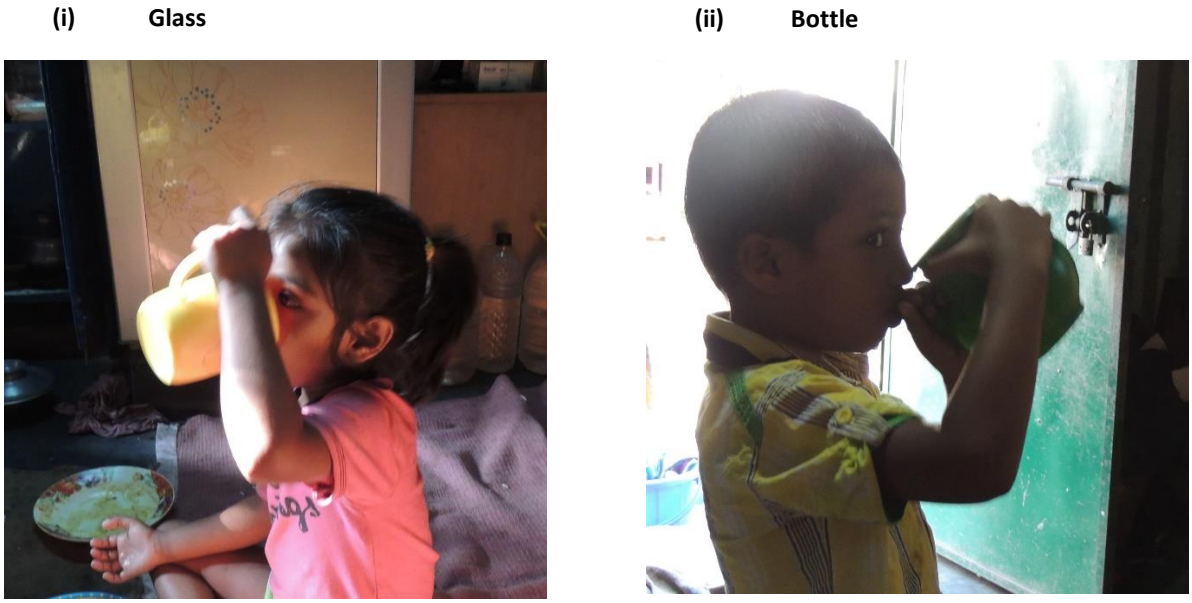

Figure 6. Point-of-drinking water vessels. Preferred drinking water vessels used in the household. (i) Glass, (ii) Bottle, (iii) Mug, (iv) Pitcher.

Figure 7. Collection of point-of-drinking water. (i) & (ii) Collection of point-of-drinking water directly from the tap on plot, (iii) Collection of point-of-drinking water from the intermediate storage in the household.

3.4 Microbiological procedures for sample collection and sample processing

A volume of 150-200 mL water sample was collected both at the communal source and point-of-drinking. Water samples were collected in pre-sterilized wide-mouth water sampling bottles (SPL Life Sciences, Korea) and transported in a cool box to the Environmental Microbiology Laboratory, University of Dhaka, within two to four hours of collection.

Aliquots of water samples were used for the following three basic purposes:

(1) Assessment of water quality by the membrane filtration (MF) culture method: Aliquots of 100 mL water samples were filtered through 0.45 µm size pores and 47 mm diameter white gridded S-Pak Filters (Merck Millipore, Germany) and the filters were placed on membrane Thermotolerant *E. coli* agar (m-TEC agar, Oxoid, UK) plates. Plates containing the filters were incubated at 44.5 +/- 0.5° C for 18-24 hours. Typical reddish-purple or magenta colonies of *E. coli* were enumerated and recorded as colony forming units (CFUs) per 100 ml of water [152]. This step was followed for all samples (a total of 4,008 water samples: 2,514 samples from point-of-drinking and 1,494 samples from communal source water) collected throughout the whole study period. Discrete *E. coli* colonies were isolated from a subset of samples (184 water samples:108 samples from point-of-drinking and 76 samples from source water samples) and preserved for further analysis.

(2) Assessment of water quality by qPCR: For a subset of samples (a total of 676 samples: 404 samples from point-of-drinking and 272 samples from communal source water), aliquots of 1 mL of water were taken aseptically and inoculated in vials containing 2 mL of nutrient broth (NB) enrichment medium. The vials were incubated at 37°C for 4 hours to recover the cells in lag phase or injured and stressed condition. After DNA extraction, *E. coli* was detected and quantified using quantitative Polymerase Chain Reaction (qPCR).

(3) Detection of *V. cholerae* by PCR: Aliquots of water were added to 10 mL of alkaline peptone water (APW), to enrich injured and stressed *V. cholerae* cells. After overnight incubation at 37° C for 18–24 hours [153], 1 mL culture suspension was taken, and DNA was extracted from the enriched water samples. The DNA templates were subsequently tested for the presence of species-specific *ompW* gene of *V. cholerae* by PCR. This step was also performed for a subset of samples (1,463 water samples: 1,082 samples from point-of-drinking and 381 from source water).

3.5 Data analysis

All data analyses were conducted using IBM SPSS software, version 23. $P < 0.05$ was considered to indicate statistical significance.

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

In a resource-poor laboratory setting of a low-income country like Bangladesh, selection and validation of the analytical procedures for microbiological investigation require considerations from several perspectives (cost, rapidity and convenience) that would be realistic for implementation and further scale up. Thus, in the following sections, this thesis will first rationalize the need for optimization and development of microbiological methods and will also describe the applicability and precision of the methods by trying them out on a variety of environmental samples and bacterial species.

The second section will represent the overall results of the study by providing comparative assessment of water contamination between the public domain and domestic domain targeting two microorganisms: *E. coli* and *V. cholerae,* which are the two common etiologies that pose a major public health concern for diarrheal diseases, particularly in Bangladesh. Fecal contamination in water samples will be assessed and compared using the traditional culture-based method and the developed molecular methods, with a subsequent discussion of the utility of both methods. Along with comparative assessment, genetic characterization of the pathogenic *E. coli* will point out the origin/host of fecal contamination (animals/humans) in both public and domestic domains. The uniqueness of this PhD is the use of a holistic approach (combining each of the approaches of *E. coli*, *V. cholerae* and pathogenic characterization) to identify the drinking water contamination in a low-income urban setting which had not been previously studied. The findings of the whole PhD work will answer some unresolved questions that will be useful to better inform future public health measures including target specific interventions to ensure safe/pathogen free drinking water, which is one of the SDGs to achieve by 2030 [154].

4.1 Selection, validation and optimization of microbiological methodologies for molecular investigation in drinking water

In the following sub-sections, the summaries of the manuscripts (Manuscripts I, II, and III) are discussed from methodological performance standpoints. Section 4.1.1 will present the summary result and discussion of a suitable DNA extraction method (Manuscript I), which was chosen from three DNA extraction methods based on ease of use, rapidity and cost-effectiveness. Section 4.1.2 will discuss a newly optimized qPCR method that showed desirable accuracy in

quantification of *E. coli* and toxigenic *V. cholerae* (Manuscript II). Section 4.1.3 will discuss a newly developed assay for detection and quantification of both toxigenic and non-toxigenic *V. cholerae* (Manuscript III). All these methods were employed in this PhD project for the molecular investigation of *E. coli* and *V. cholerae* in drinking water.

4.1.1 Manuscript I: Quantitative analysis of nucleic acid extraction methods for *Vibrio cholerae* using real-time PCR and conventional PCR

DNA extraction is a crucial step for further downstream processing of pathogens in a given sample; for example, molecular detection, amplification of targeted genes and sequencing. As this PhD project required the analysis of a large number of water samples routinely, a simple, inexpensive, time efficient, and easy to modify DNA extraction method was required to optimize for molecular detection of pathogenic genes. For this purpose, we compared the three DNA extraction methods and identified a suitable one.

We prepared four defined mock community controls- i) pure culture of *V. cholerae* in enrichment media, ii) spiked water, iii) spiked phosphate-buffered saline and iv) spiked suspension of rice samples. The optimization and spiking of the samples was carried out by using a known quantity of *V. cholerae* O1 biovar El Tor strain N16961 which possesses the *ctxA* gene. The pure cultures and spiked samples of *V. cholerae* were subjected to three DNA extraction methods: a) boiled template, b) phenol: chloroform: isoamyl alcohol, and c) the QiaAmp® DNA mini kit (Qiagen GmbH, Hilden, Germany). The concentration and purity of DNA yielded by each of the extraction methods from each of the samples were initially measured by the Colibri Microvolume Spectrometer (Titertek-Berthold, Berthold Detection Systems GmbH, Germany). The suitability of the DNA extraction methods, in terms of qualitative and quantitative analysis, was compared by downstream amplification using both quantitative Polymerase Chain Reaction (qPCR) and conventional end-point PCR. The methods were also compared with respect to sample processing time and cost when a high turnover of samples requiring molecular tests is expected.

The comparative evaluation of the three DNA extraction methods by qPCR was presented by C_T values (threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold in the amplification plot) and the copy number of bacteria per reaction. For all three of the extraction methods, the C_T values for all mock samples (spiked by *V. cholerae*) ranged from 14 to 34, which is within the desirable limit of detection by the Step One ABI real-time PCR machine [155]. The copy numbers were also detected for all the sample types by all three methods except for

negative control and no template control. The comparative evaluation of the three DNA extraction methods by end-point PCR was depicted by measurement of band intensity from a gel image. The three DNA extraction methods also resulted in measurable band intensity ranging from 7.36% to 28.26%. However, with respect to cost, the least expensive DNA extraction method was the 'boiled template' extraction method (0.16 USD/sample) whereas 'phenol: chloroform: isoamyl alcohol' cost 1.02 USD/sample and the 'QiaAmp® DNA mini kit' cost 5.04 USD/sample [155]. In terms of time from the start of sample processing to the end of DNA retention, the boiled template method was in the middle position, meaning that it took 90 minutes for overall DNA extraction, whereas the QiaAmp® DNA mini kit method took 70 minutes and the phenol: chloroform: isoamyl alcohol method took 270 minutes. In view of its being the most inexpensive and simplest method to apply, producing a purity and yield detectable by both qPCR and end-point PCR, the boiled template extraction method was chosen as a reliable method for subsequent routine DNA extraction from a large number of samples.

4.1.2 Manuscript II: Optimization and validation of real time PCR assays for absolute quantification of toxigenic *Vibrio cholerae* and *Escherichia coli*

The conventional approved methods for measuring fecal indicator bacteria to assess water quality are: membrane filtration, multiple-tube fermentation and defined-substrate technology (DST) [156]. The utility of these methods is limited by a lengthy incubation period with longer-lasting verification and confirmation steps ranging from 18 to 96 hours, and their inability to detect VBNCs [126], which has made them less reliable and outdated for protecting public health.

Real-time PCR is one of the recent advances in technology that can be used quantitatively (quantitative real-time PCR/qPCR) to measure bacterial load more efficiently with rapid, specific and sensitive detection compared to culture-based methods. Generally, typical qPCR methods for quantification of microorganisms are conducted on the genomic DNA extracted from a serial dilution of cell suspensions. A disadvantage of this method is that the standard curve attained from serial dilutions of the samples produces an R^2 value (a regression coefficient, a critical parameter to evaluate PCR efficiency) which is not a 'best fit' for the quantification of unknown samples. To identify a method that could provide a better R^2 value compared to the dilution of cell suspension, we implemented standard curves of *E. coli* and toxigenic *V. cholera*e from genomic DNA dilution.

The detection and estimation of fecal *E. coli* was obtained by targeting the *uidA* gene (encoding ß glucuronidase) and that of toxigenic *V. cholerae* was obtained by targeting the *ctxA*-gene for the qPCR method. DNA was extracted from culture suspension and the concentration of extracted bacterial DNA was measured. A range of calibration standards were prepared using 7-$\log_{10}$ serial dilution (1:10) of starting bacterial genomic DNA in triplicates. Using the culture method, quantification was performed by 7-$\log_{10}$ serial dilution (1:10) of the same culture suspension (which was used for genomic DNA extraction) and colony forming units (CFU) were recorded. The quantification of bacteria by the culture-based method was checked in parallel with the qPCR method. In order to identify the best approach for the quantification of bacteria in the original samples, the regression coefficients (R^2 values) were compared between C_T vs copy number by the genomic DNA dilution method and C_T vs CFU counts by the cell suspension dilution method.

Our genomic DNA dilution method showed a better R^2 value, and more accuracy in quantification of bacterial copy numbers compared to the cell suspension dilution method (Table 1). The PCR efficiency by genomic DNA dilution was more accurate than cell suspension dilution, as dilutions of genomic DNA gave an R^2 value closer to 1. The estimated copy numbers of genomic DNA of *V. cholerae* and *E. coli* were higher than the bacterial counts of the same sample attained from plate counts by cell suspension (Table 1). The higher bacterial counts of our genomic DNA dilution might have resulted due to detection of VBNC cells by our method which are undetectable in the culture-based method [126]. The C_T value of the lowest concentration of DNA (7 log 10-fold dilution) was 33.82 for genomic DNA whereas the C_T value (37.51) of the lowest concentration of cell suspension (7 log 10-fold dilution) exceeded the cut-off value (>8 to <35) for positive sample detection. As a high C_T value refers to a low amount of target DNA in the sample, the C_T value of the lowest concentration of cell suspension implies that loss of DNA might have occurred during DNA extraction in each dilution of cell suspension.

Table 1. Comparative evaluation of the genomic DNA dilution and cell suspension dilution methods for qPCR

	Genomic DNA dilution		**Cell suspension dilution**	
	V. cholerae	*E. coli*	*V. cholerae*	*E. coli*
R^2 value	0.99	0.99	0.96	0.93
Copy number of the starting sample	2.48×10^7	2.21×10^7	5.2×10^5	2.3×10^5
Copy number of the end sample (7 log 10-fold dilution)	248	221	5.2	2.3
Sensitivity (C_T value for lowest concentration of DNA)	33.82	29.39	37.51	30.68

The specificity for detection of fecal *E. coli* by targeting the *uidA* gene and for detection of toxigenic *V. cholerae* by targeting *ctxA* was 100% by the genomic DNA dilution method in qPCR. The PCR efficiency of the TaqMan assay for *V. cholerae* was 99.21% and that of the SYBR green assay for *E. coli* was 103.80%, which is within the acceptable limits (generally, an efficiency between 90 and 110% is considered acceptable). For both of the target organisms, our developed assay could detect organisms from a DNA concentration as low as 0.1 pg.

The results suggest that quantification by qPCR can provide both detection and quantification as an absolute number of copies or a relative amount when standardized to DNA input of specific nucleotide sequences. Moreover, this DNA dilution method is more time efficient compared to the culture dilution method. This qPCR method will allow more accurate detection and quantification within a short timeline, which could be useful for defining strategy quickly in large outbreaks. Therefore, in this PhD project, a standard curve input from dilution of genomic DNA was utilized to assess the water quality of a subset of water samples.

4.1.3 Manuscript III: Development and validation of a novel real-time assay for the detection and quantification of *Vibrio cholerae*

Along with *V. cholerae* O1/O139, *V. cholerae* non-O1/non-O139 have also been documented as incriminating in several outbreaks in developing countries [114-117]. A number of assays exist for the detection of *V. cholera*e [157-160], many of which lack empirical data for reproducibility and repeatability. Therefore, we aimed to develop and validate a new qPCR assay that would permit the detection and quantification of all species of *V. cholerae*, regardless of their serotypes, and would be reproducible and repeatable.

The outer membrane protein (ompW) sequence is highly conserved among *V. cholerae* species belonging to different biotypes and/or sero-groups [161]. Hence, the *ompW* gene was used as a target for species-specific detection, identification, and quantification of *V. cholerae. V. cholerae ompW* reference sequences including both O1 and non-O1/non-O139 genes were downloaded from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) GenBank®. A range of primers were attained when the reference sequences were run in the FastPCR 6.05 software (PrimerDigital, Helsinki, Finland). Primers that conformed to the ideal range of nucleotide length, melting temperature (Tm) range (°C), CG content (%), and linguistic complexity (LC%) as described by Kalendar et al. [162] were analyzed for their complementarity with the reference sequence using NCBI's Basic Local Alignment Search Tool (BLAST). The forward and reverse primer sequences were checked, and the pair that had the

highest identity with the Query Sequences (reference sequences) was selected for further analysis. The selected primer set included forward sequence (5′-acatcagytttgaagtcctcgc-3′) and reverse sequence (5′-gtggtgtaattcaaacccgc-3′).

To examine the suitability of the assay for detection of *V. cholerae* in food and environmental samples, four different types of samples were taken for experimentation: (i) drinking water, (ii) pond water, (iii) boiled rice, and (iv) shrimp. The samples were spiked with different concentrations of *V. cholerae* CT^+ O139, *V. cholerae* CT^+ O1, and *V. cholerae* CT^- non-O1/ non-O139, following standard procedures. The assay could successfully detect the presence of *V. cholerae* in spiked food and environmental samples while showing absence of *V. cholerae* in un-spiked food and water samples, and negative controls.

The specificity of the selected primer set in the assay was 100% in detection of the *ompW* gene of all *V. cholerae* (both CT^+ *V. cholerae* O1/O139 and CT^- *V. cholerae* O1/O139). The detection limit of the PCR assay ranged from 2,500 pg to 2.50 fg (10-fold serial dilution up to 6-$\log_{10}$) of purified genomic *V. cholerae* DNA, which is equivalent to 5.46×10^5 to 5.46×10^{-1} genomic copies. Thus, it shows that the assay can detect as low as a single copy in a sample.

The melting curve analysis was performed for four dilutions of two *V. cholerae* strains using the Step One ABI real-time PCR system. A single distinct peak was seen, indicating that all the PCR products had similar Tm values (78.46°C) and neither secondary non-specific products nor primer dimers were formed. The inter-assay and intra-assay precision were calculated by coefficient of variation (CV%) and was found to be within acceptable limits, suggesting that the assay is reproducible and repeatable. The developed assay had high specificity and sensitivity in detection and quantification of *V. cholerae* from food and environmental samples.

4.2 Public domain and domestic domain comparison

In the following sub-sections, the summaries of the manuscripts (Manuscripts IV, V and VI) are discussed from a comparative assessment standpoint between the public domain (communal source) and domestic domain (point-of-drinking) water. Section 4.2.1 will present the summary results and discussion of the comparative assessment of fecal contamination through *E. coli* in both domains and will compare the results using the culture-based method and qPCR method (Manuscript IV). Section 4.2.2 will explain the occurrence of highly virulent pathogenic strains of *E. coli* isolated from communal source and point-of-drinking water, and their probable origin of fecal contamination (Manuscript V). Section 4.2.3 will present a discussion of the

comparative assessment of *V. cholerae* between the communal source and point-of-drinking water (Manuscript VI). The detailed methods and more descriptive results will be found in each of the manuscripts.

4.2.1 Manuscript IV: Comparative assessment of fecal contamination in 'improved' piped-to-plot communal source and point-of-drinking water

World Health Organization guidelines recommend an acceptable limit of *E. coli* as <1 per 100 mL of drinking water. In many developing and developed countries, maintaining this acceptable limit was not feasible for a high proportion of household and small community drinking-water systems [43]. In such circumstances, WHO proposed a realistic practical solution and provided a classification of scoring risk linked to the progressive improvement of water safety for remedial actions of drinking water systems [43]. The WHO *E. coli* risk category for remedial actions includes four risk groups: low risk/safe: no action required (< 1 *E. coli*/100 mL), intermediate risk: low action priority (1–10 *E. coli*/100 mL), high risk: higher action priority (11–100 *E. coli*/100 mL), and very high risk: urgent action required (> 100 *E. coli*/100 mL) [41]. In our study, we used the WHO risk categories for risk assessment of communal source and point-of-drinking water quality.

The manuscript has two objectives: (i) to assess the water quality of 'improved' (i.e. piped-to-plot) communal source and point-of-drinking water, and (ii) to examine the variation of results by the conventional membrane filtration (MF) culture method and the quantitative PCR (qPCR) method using our developed assay. The MF culture method was used for all 4,008 samples and the qPCR method was used on 676 water samples for water quality assessment. Water quality was initially measured by a binary variable indicating presence or absence of *E. coli* in 100 mL samples of water. Quantification of *E. coli* was categorized into four risk groups following the WHO risk categories described above.

Twenty three percent (587/2,514) of point-of-drinking and 42% (625/1,494) of the communal source water samples had no detectable *E. coli* (Figure 8) by the MF culture method. Communal source water was less contaminated than point-of-drinking water in all the risk groups (Figure 8).

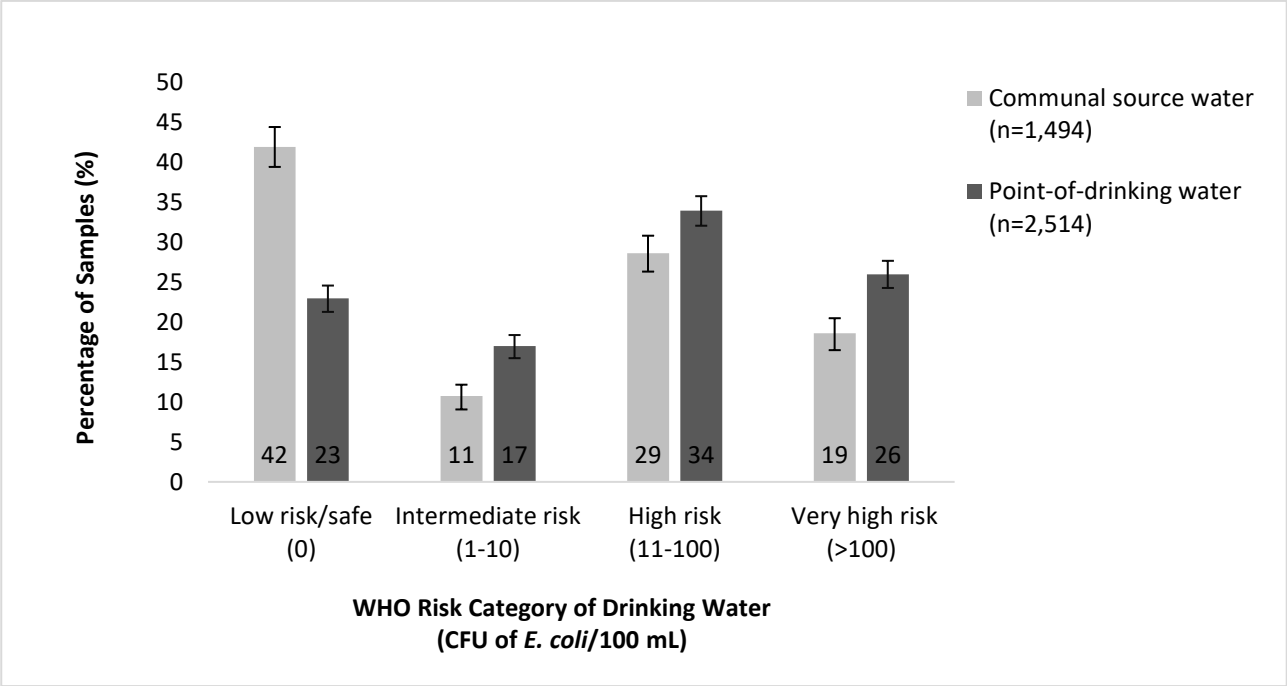

Figure 8. Fecal contamination of drinking water according to WHO risk category. The graph represents the WHO risk categories of communal source and point-of-drinking water. Error bars represent 95% confidence intervals.

A set of stratified samples of point-of-drinking water (1,236 water samples) and their linked communal sources (1,206 samples) collected within the same day, were examined to assess the influence of fecal contamination of communal source on point-of-drinking water. Our results showed that level of fecal contamination decreased from communal source to point-of-drinking water in 33% of water samples which might have resulted from bacterial die-off caused by predation by other microorganisms, lack of nutrients, or other factors contributing to inhospitable conditions [163, 164].

Results also showed that the level of fecal contamination increased from communal source to point-of-drinking water in 51% of samples, where 26% of the samples had zero *E. coli* at the communal source. In this 26% of the samples, where the communal source had zero *E. coli* but point-of-drinking water had *E. coli*, we assume that recontamination by hands, flies, interim storage vessel contamination, contaminated drinking vessels were the contributing factors in the domestic domain [32, 67, 146]. However, for the other 25% of water samples, where we observed that the communal source had *E. coli* ≥ 1 and the level increased even more in point-of-drinking water, both bacterial re-growth and post contamination might have contributed. However, we anticipate that in-house contamination had more contribution than re-growth in the increased count of point-of-drinking since the communal sources of the study households abstracted groundwater and groundwater contains very low level of organic nutrients to support

the multiplication of bacteria. Furthermore, our findings revealed that 38% of point-of-drinking water samples had increased *E. coli* count, which were connected to the cleaner sources (*E. coli*: 0-10 CFU). Possibly compromised hygiene behavior of the household members was the key contributing factor for this scenario.

However, as a higher proportion of water samples showed increased bacterial contamination in point-of-drinking water samples compared with communal source water, it is anticipated that point-of-drinking water contamination is much more influenced by recontamination rather than source contamination, which is consistent with other studies [34, 146]. Hence, the provision of piped-to-plot improved water sources did not ensure safe drinking water at point-of-drinking. Although SDG targets to expand the access to a piped-to-plot water supply, [154] routine water purification measures need to be included along with the infrastructure changes, if real improvements in the incidence of water related diseases are to be seen.

When point-of-drinking water samples were stratified as treated and non-treated water, we found that 79% (260/333) of treated and 76% (1,662/2,175) of non-treated point-of-drinking water samples had *E. coli.* Among the treated water samples, *E. coli* was found in most of the 'boiled' (197/254) and 'filtered' (59/76) samples. The result indicates that no improvement of water quality was achieved by treating drinking water, which was also witnessed in studies conducted in Peru [32, 33]. The study conducted in peri-urban communities of Lima, Peru observed a significant increase of *E. coli* counts in drinking cups after boiling drinking water [32]. Similarly, a study conducted at the household level in rural areas of Peru reported that 69% of jars in which drinking water was stored had fecal coliforms, though the water was treated by boiling [33]. The authors explained that longer storage time of treated water provides ample opportunity for contamination, because hands and the handle or outer surface of collecting vessels frequently carry fecal pathogens. Additionally, fecal contamination circulated within kitchen environment of the domestic domain can contribute in contamination of drinking water and food [165, 166].

A comparison of drinking vessels indicated that the presence of *E. coli* was lower in bottles (67%, [232/344]) compared to mugs (78%, [1,036/1,336]) and glasses (78%, [568/726]). The logistic regression analysis reveals narrow mouth drinking vessels were less likely (p=0.000, OR=1.72, [1.34-2.20]) to be contaminated than wider mouth vessels. Thus, our study also corroborates that wider mouth glasses/mugs/jugs have a higher chance of contamination than narrow mouth bottles [23]. Moreover, it was observed in our study that when household

members used a bottle, they usually collected water directly from the tap and then drank from the bottle; however, when they used a glass or mug for drinking, they usually stored water in an intermediate storage vessel. Therefore, in such circumstances where the household members used mugs or glasses (wider mouth vessels), bacterial re-growth and post-contamination might have contributed to the fecal contamination of point-of-drinking water. Bacterial re-growth is defined as a process; i.e. when a small number of microorganisms (fecal coliforms) are provided with a conducive environment (i.e. optimum temperature, concentration of organic nutrients) they can act as seeds and multiply in the intermediate storage containers [167]. In addition to bacterial re-growth, post-contamination due to hand-to-water contact and/or contaminated drinking water vessels, and vector exposure, can eventually increase fecal contamination in the point-of-drinking water in the wider mouth drinking vessels [19].

From the above findings, we can conclude that fecal contamination remains commonplace in water quality deterioration within the domestic domain, particularly at point-of-drinking. Additionally, treatment of drinking water did not ensure absolute removal of microbes which might be due to compromised kitchen hygiene practices. In order to reduce domestic transmission of fecal-oral pathogens, hygiene improvement efforts should target repeated cleaning of drinking vessels and the promotion of narrow mouth drinking vessels. Additionally, education efforts should emphasize safe handling of drinking water after treatment; otherwise, treatment will not offer any benefit for a positive health outcome.

Of 676 samples, 272 were from communal source and 404 were from point-of-drinking samples tested for the presence of *E. coli* using both qPCR and MF culture methods. The qPCR method showed that 98% (266/272) of communal source and 90 % (363/404) of point-of-drinking water samples were positive for *E. coli*, while the MF culture method showed that 52% (141/272) of the same communal source and 73% (296/404) of point-of-drinking water samples were positive for *E. coli*. There was a higher detection of *E. coli* by qPCR as it measures genetic material from the target DNA of culturable cells, VBNC cells and dead or dying cells. Conversely, the culture-based method showed a lower detection of *E. coli* as it measures only viable cells and cannot detect VBNC or dead/dying cells. Hence, the 42% of communal source water samples and 23% of point-of-drinking water samples that showed safe levels (no *E. coli*) by the MF culture method (Figure 8) might not really be safe and might fall into the ‘intermediate’ or the successive risk groups. Therefore, the non-detectability by MF culture can undermine the results and pose a major health concern if the sample contains pathogenic bacteria. This phenomenon led us to investigate the occurrence of

pathogenic *E. coli* in the 'intermediate' to 'very high risk' groups, which is one of the aims of Manuscript V and presented in the following 4.2.2 section.

To further assess the credibility of *E. coli* as an indicator for the presence of pathogenic bacteria by MF culture and qPCR, we tested the association between the presence of *E. coli* and the presence of *V. cholerae* in water samples. The detection rate of *E. coli* as an indicator for the presence *V. cholerae* was 87% (chi-sq test p=0.000). However, for 13% (19/142) of water samples, MF culture was unable to forecast the presence of *V. cholerae.* This poses a serious health concern because this 13% of the sample which seems to be safe (no *E. coli*), is not truly safe, and might cause cholera or cholera like illness if it contains *V. cholerae* at an infectious dose level. In contrast, the detection rate of *E. coli* as an indicator for the presence *V. cholerae* was 94% (58/62) by qPCR. Thus, the results suggest that the qPCR method has the ability to supplement or replace the MF culture method as a means of assessing the levels of fecal contamination in drinking water. However, one of the limitations of qPCR is that it cannot distinguish between dead and live cells, since it measures target DNA from the organism. To selectively quantify viable *E. coli*, Taskin et al. (2011) developed a qPCR assay using propidium monoazide (PMA) [168]. As PMA is an expensive reagent and the PMA treatment steps are complicated and time consuming, we could not afford to use PMA treated qPCR for routine sample analysis. However, dead cell estimation still plays an important role for human health as these cells can produce endotoxins that can cause food poisoning [169].

Generally, in emergency outbreak settings, culture-based methods are used for detecting *E. coli* to monitor water quality. Our findings suggest that depending on a culture-based method alone can provide spurious results which might not reflect the actual fecal contamination. Moreover, several several species-specific target pathogenic bacteria should be regularly monitored for assuring water quality, as a single species-specific bacterium for assessing water quality can be misleading.

4.2.2 Manuscript V: The fecal origin of pathogenic *E. coli* in 'improved' piped-to-plot communal source and point-of-drinking water of a low-income urban community, Bangladesh

Characterization of pathogenic *E. coli* is needed to accurately assess the health risk associated with fecal contamination of drinking water, since certain strains of *E. coli* have virulence properties that may account for life threatening human infections. Additionally, detection of the host of virulent *E. coli* (e.g. animal, human) is crucial for portraying the transmission pathway of *E. coli* through drinking water. Therefore, the aim of this manuscript was to see the extent of

diverse pathotypes of *E. coli* isolates collected from point-of-drinking water and communal source water, and to identify their origin of fecal contamination (human or animal fecal origin), using molecular methods.

Traditional culture-based and molecular tests were performed for confirmation of the *E. coli* isolates. Pathogenic genes and phylogenetic groups of *E. coli* were examined by single-plex and multiplex PCR, using published primers. Details of the methodology will be found in the draft manuscript included with the thesis. A total of 108 water samples were randomly selected from 2,514 point-of-drinking water samples, and 76 water samples were randomly selected from 1,494 communal source water samples for isolation of *E. coli*.

Pathogenic *E. coli* was identified in 41%-71% of samples of both point-of-drinking water and communal source water, distributed across the risk groups (Figure 9). This is higher than the other study conducted in Dhaka, Bangladesh that found 7% of pathogenic *E. coli* in source water samples from Dhaka municipality [60]. This difference might be due to chlorine treatment of the municipal water of Dhaka City [170], which is not maintained in the Arichpur location. In addition, submersible pumps are the predominant communal water sources in the community and no treatment of water at the communal source was performed.

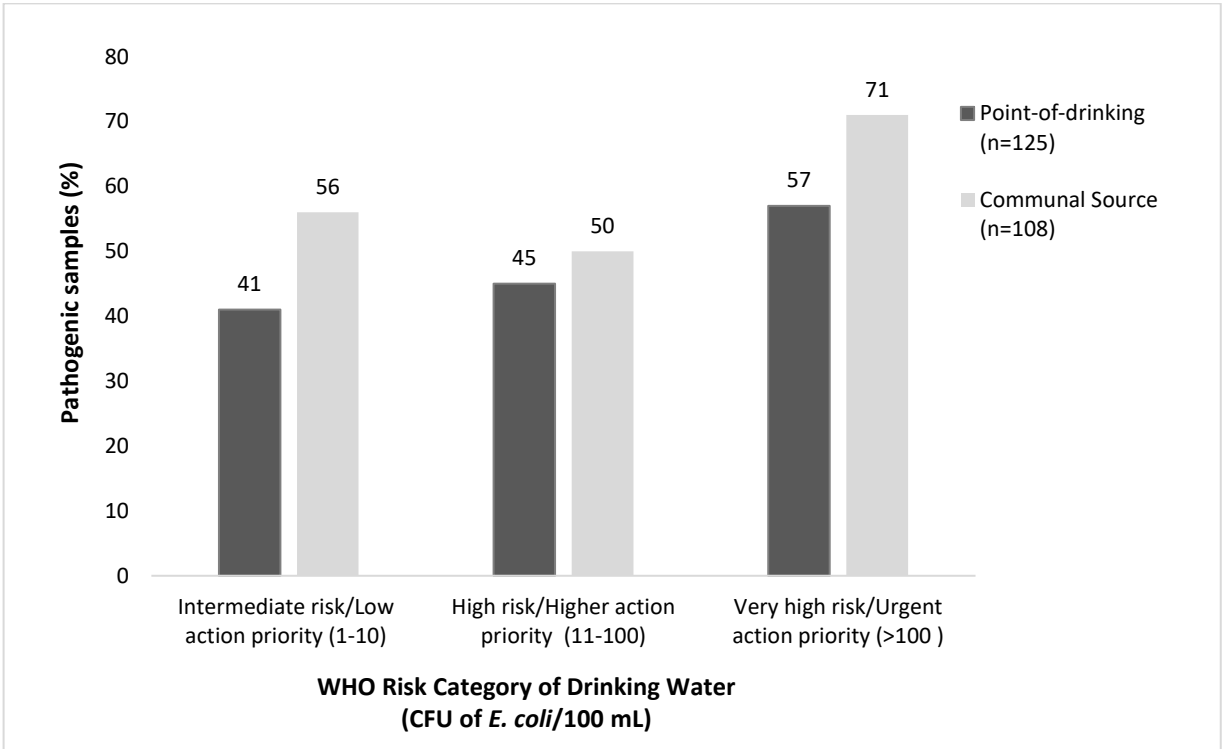

Figure 9: Presence of pathogenic *E. coli* in different risk groups. The graph represents the presence of pathogenic *E. coli* in point-of-drinking water and communal source water in 'intermediate', 'high' and 'very high' risk groups.

Of the pathogenic isolates, ETEC was the most prevalent pathotype found in point-of-drinking and communal source water (Table 2). Although Harada et al. (2018) [61] found the highest proportion (16.3%) of ETEC in sanitary wastewater, they did not find any ETEC in stored drinking water in Khulna, Bangladesh. Talukdar et al. (2013) [60] found a high percentage (69% [11/16]) of ETEC in Dhaka municipal tap water. The high detection of ETEC in drinking water in our study can be threatening, particularly to young children aged ≤ 2 years, since ETEC is the primary cause of diarrhea for young children living in a low-income urban community [171]. Moreover, ETEC also contributes to 11% of diarrheal cases among all age groups in the icddr,b hospital of Dhaka, Bangladesh [172].

Of the pathogenic *E. coli* isolates, ExPEC isolates were found in the 'high risk' and 'very high risk' groups from point-of-drinking water and in the 'very high risk' group from source water (Table 2). Although, the prevalence of ExPEC was low in this study, it should be noted that ExPEC is an opportunistic pathogen that has the potential to cause some fatal diseases when the isolates exit the gut and enter the sterile body [173, 174]. For instance, ExPEC is associated with neonatal meningitis, which is one of the most common infections accounting for high mortality and morbidity (10–30 %) in newborn children [46, 175, 176]. Furthermore, ExPEC is the primary cause of community-acquired urinary tract infections (UTIs), affecting an estimated 20% of women over the age of 18 years [177, 178].

Apart from the pre-existing pathotypes of *E. coli* isolates, we found hybrid *E. coli* isolates in the 'intermediate risk' and 'very high risk' groups from both point-of-drinking water and communal source water (Table 2). Our finding is not unique, as a number of past studies have shown hybrid strains carrying virulence marker genes of two different *E. coli* pathotypes [179], which were clinically linked to hybrid *E. coli* strains from India [180], Brazil [181, 182], Denmark[183], Switzerland [184], France [185], Germany [186] and Mexico [187]. An extremely pathogenic strain that possessed EAEC and STEC associated virulence genes emerged and caused a sprout-borne outbreak in Germany within a very short period of time [188]. A hybrid strain carrying genes from ETEC and EHEC genes was isolated from contaminated drinking water during an outbreak in Finland [189]. Although in our study a low number of hybrid strains were isolated, the widespread distribution and clinical relevance worldwide indicates their virulence potential to cause an outbreak. Thus, the presence of hybrid *E. coli* in drinking water can pose a great public health concern for the people of Bangladesh, especially when it is point-of-drinking water.

Table 2: Prevalence of pathotypes of *E. coli* in source and point-of-drinking water samples in different risk groups

	Point-of-drinking water			**Communal source water**		
	Intermediate risk n = 17 (%)	High risk n = 45 (%)	Very high risk n = 63 (%)	Intermediate risk n = 11 (%)	High risk n = 33 (%)	Very high risk n = 60 (%)
ETEC	5 (29)	13 (29)	28 (44)	4 (36)	12 (36)	32 (53)
EIEC	1 (6)	1 (2)	-	-	1 (3)	-
EAEC	-	1 (2)	-	-	-	-
EHEC	-	2 (4)	3 (5)	-	-	1 (2)
EPEC	-	1 (2)	-	-	-	3 (5)
ExPEC	-	2 (4)	3 (5)	-	-	1 (2)
Hybrid (ETEC+EHEC, ETEC+EIEC)	1 (6)	-	1 (2)	1 (9)	-	2 (3)
Commensal	10 (59)	25 (56)	28 (44)	6 (55)	20 (61)	21 (35)

For progressive improvement of water safety linked to remedial actions for contamination in drinking water systems, the WHO guideline emphasizes the ‘high risk’ (11–100 *E. coli*/100 mL) and ‘very high-risk’ groups (> 100 *E. coli*/100 mL) by giving them a higher action priority and urgent/immediate action priority, respectively, but gives less emphasis to the ‘intermediate risk’ group, stating that this group requires a low action priority [43]. Our study findings revealed that the ‘intermediate risk’ group should be equally prioritized with the high-risk groups, as we found highly virulent and emerging ‘hybrid’ pathogens in both point-of-drinking water and source water after pathogenic characterization of *E. coli*. We recommend that, in addition to the fecal contamination of water quality assessment criteria, the WHO guidelines should also include investigation of pathogenic bacteria to assess water quality safety.

Phylogenetic grouping of the 229 *E. coli* isolates showed that strains isolated from point-of-drinking water belonged to six subgroups (i.e. A1, B1, B2-2, B2-3, D1 and D2), and communal source water isolates belonged to four subgroups (B1, B2-2, B2-3, D2) of the four major phylogenetic groups (i.e. A, B1, B2 and D). As discussed in section 1.3.4, groups D and B1 were usually found in birds, A and B1 in non-human mammals, and A and B2 in humans. Carlos et al. [190] worked on the phylogenetic determination of *E. coli* isolates collected from a variety of hosts (i.e. humans, cows, chickens, pigs, goats, sheep, sewage) and found that subgroup B2-3 was present only in the human sample. In our study, we found the substantial presence of subgroup B1 in both point-of-drinking (50%, 91/181) and communal source (50%, 90/181) water isolates, followed by the presence of B2-3 in point-of-drinking (65%, 13/20) and communal source (35%, 7/20) water (Table 3).

Table 3. Assignment of phylogroups of the 229 *E. coli* isolates collected from point-of-drinking water and communal source water

Phylogenetic subgroup	**Total no. N=229 (%)**	**Point-of-drinking water**			**Communal source water**		
		n=125	%	95% CI	n=104	%	95% CI
A1	2 (1)	2	2	0-6	-	-	-
B1	181 (79)	91	73	64-80	90	87	78-92
B2-2	4 (2)	2	2	0-6	2	2	0-8
B2-3	20 (9)	13	10	6-17	7	7	3-14
D1	4 (2)	4	3	1-8	-	-	-
D2	18 (8)	13	10	6-17	5	5	2-11

Our findings suggest that communal sources of the study area are mostly contaminated by animal feces, and to a lesser extent by human feces. Regarding the potential origin of fecal contamination of source water in the study community, non-human mammals (goats, cows) and birds (ducks and chickens) might have played an important role, since Harris et al. (2016) [191] reported the significant contribution of domestic animals to the fecal contamination of urban household environments in Dhaka. Furthermore, point-of-drinking water was substantially contaminated by animal, bird and human feces, where contamination by human feces at the point-of-drinking was higher than at the communal source. Our finding is consistent with a study conducted in India, in which human fecal markers were detected much more frequently in the domestic domain (45% of stored water samples in households) than in public domain sources (8% of ponds; 4% of groundwater drinking sources) and animal fecal markers were widely detected in both domains (74% of ponds, 96% of households, 10% of groundwater drinking sources) [192] .

In 2014, a systematic review revealed that 69% of studies examining the relationship between domestic animal husbandry and diarrheal disease in humans showed a significant positive association worldwide, and this increased to 95% when considering only studies assessing specific diarrheal pathogens (i.e. excluding studies looking at non-pathogen specific diarrhea) [193]. In our study (see Figure 5 in section 3.2), the communal source water pumps were not well protected and were surrounded by soil, which might have contributed to the higher fecal contamination from animal sources than human sources [194]. In contrast, human and animal fecal contamination prevailed in point-of-drinking water contamination, which is indicative of the recontamination pathways which I described in the previous section (Manuscript IV).

To prevent diarrheal diseases, considerable attention has been paid to the contamination of drinking-water supplies, and the contribution of drinking-water quality, sanitation and hygiene.

In the recently adopted SDGs to be achieved by 2030, sanitation is primarily focused on the proper management of human fecal matter, to reduce the diarrheal burden [195]. Less attention has been given to the influence of animal feces on water contamination and diarrheal disease, and the proper management of fecal matter from domestic animals is largely ignored. Our results indicate that addressing human sanitation without consideration of fecal contamination from livestock sources will not be enough to prevent drinking-water contamination and thus it will persist as a great contributor of diarrheal pathogens.

4.2.3 Manuscript VI: A comparative analysis of *Vibrio cholerae* contamination in point-of-drinking and source water in a low-income urban community, Bangladesh

In parallel with water quality assessment based on the presence of *E. coli* in water samples, a subset of samples was tested for the presence of *V. cholerae* and their virulence associated genes. The objective was to conduct a comparative analysis of the presence of *V. cholerae* between point-of-drinking water and communal source water, and to investigate the variability of their virulence profile using the PCR method.

The methodology of sample collection, sample processing and enrichment was described in section 3.3. The DNA was extracted from the samples using the method confirmed in section 3.4.1 (Manuscript I, [155]). The *V. cholerae* species-specific *ompW* gene [161] was chosen for detection of *V. cholerae* in water samples by PCR. The serogroups and pathogenic determinants of *V. cholerae* in total DNA were determined by PCR using published primers. Direct PCR from enriched water samples was conducted to reliably detect all forms of *V. cholerae* (both VBNC and culturable), as VBNC cells are non-detectable by existing culture methods. All measures of associations were tested using a logistic regression analysis.

Overall, 10% (110/1,082) of point-of-drinking water samples and 9% (33/381) of communal source water samples tested positive for *V. cholerae* by *ompW* PCR. Although there was no association between the presence of *V. cholerae* in point-of-drinking water and communal source water, significantly higher odds ($P < 0.05$) of *V. cholerae* presence in point-of-drinking compared to communal source [OR = 17.24 (95% CI = 7.14–42.89)] water were found when samples were collected within a seven-day interval. During this seven-day interval, 53% (17/32) of communal source water samples were negative for *V. cholerae*, while linked point-of-drinking water samples were positive. The results are indicative of post-contamination/in-house contamination, as a higher proportion of communal source water was negative for *V. cholerae.* Hence, consistent with other studies, this finding provides evidence that pathogen-free water at

the source is not a guarantee of safe and pathogen-free drinking water at the point-of-consumption [23, 34, 196].

There was a higher probability of the presence of *V. cholerae* in non-treated water compared to treated water, though it was not statistically significant (P = 0.22). This finding suggests that water treatment might not have effectively changed the drinking water quality. However, this finding could also be limited by the respondent's self-reporting bias, as no other observation was conducted to crosscheck the reported information. Nevertheless, this study's findings were consistent with the findings from a study in Peru [33] which found that the effect of specific types of treatment (boiling or filtration) did not sufficiently change the water quality in drinking cups.

The odds of the presence of *V. cholerae* in bottle water was lower compared to glass and mug water, which was similar to Jensen et al.'s 2002 [23] findings, suggesting that narrow-necked vessels can prevent contamination. However, although the mouth width of mugs and glasses is almost the same, water from mugs showed lower odds of *V. cholerae* than water from glasses. The presence of a holding shaft on a mug might play a role in reducing direct hand contamination of drinking water to some extent, and thus contribute to the lower odds of *V. cholerae* in mug water than glass water.

Similar to the findings of Manuscript IV, all the findings of this manuscript (i.e. high contamination at point-of-drinking water, no difference in contamination between treated and non-treated water and less contamination in narrow mouth vessels) echoed the same conclusion that different routes (by hand, drinking vessel, flies) might have facilitated the contamination of drinking water within the domestic domain.

A higher prevalence of non-O1/non-O139 *V. cholerae* (85%) compared to toxigenic O1/O139 *V. cholerae* (15%) was found in both communal source and point-of-drinking water samples. Although most epidemic cholera cases are caused by toxigenic *V. cholerae* O1/O139, a large proportion of sporadic cases or outbreaks of cholera-like disease [115, 121, 197] and many extra-intestinal infections [118, 119] are caused by non-O1/non-O139 *V. cholerae*. Hence, non-O1/non-O139 *V. cholerae* have also gained public health importance.

Analysis of genotypic profiling is important since this can provide an understanding of the virulence traits present in the samples and monitor the evolution of pathogenic strains that can cause outbreaks. The genotypic profiles of *V. cholerae*-positive samples revealed that some of the samples

contained virulence genes (*hlyA, rtxC, rtxA, toxR*) that have the potential to cause diarrhea via non-O1/non-O139 *V. cholerae*.

Apart from the potentiality of the bacterial genes to induce disease in humans, the genotypic profiles revealed that the samples also contained some *V. cholerae* genes that have implications for the survival of the organism in the environment. For example, 39% of the samples possessed *chxA* genes that play a significant role in the survival of the organism in an aquatic environment [198]. The *mshA* gene, which has been implicated in attachment on abiotic (fomites) and biotic surfaces (cellulose), was present in 32% of the samples [199]. This might explain the higher frequency of *V. cholerae* detection in mugs, glasses and reservoir tanks.

5. Conclusions and perspectives for further research

During the expiration of the MDG monitoring period in 2015, the Joint Monitoring Program (JMP) for Water Supply and Sanitation proposed new targets and indicators for measuring the expansion of access to safe drinking water. One of the proposed indicators is “safely managed drinking”, which is defined as the use of an improved drinking water source that is located on the premises, available when needed and free from fecal contamination.

This thesis shows that access to an on-premises ‘improved source’ (one that by nature of its construction and design protects the source from outside fecal contamination; e.g. piped supply, boreholes, protected dug wells, protected springs, and rainwater) [43] has been accomplished in the low-income urban community of Arichpur, Bangladesh. However, access to “safely managed drinking” water in the community has yet to be achieved, as 58% of communal source water was found to be fecally contaminated. The water quality in the domestic domain worsened even more, specifically in the point-of-drinking water (with 77% contamination).

Our findings suggest that there is a need to take appropriate policy initiatives in controlling fecal contamination of water at the communal sources and in households in LMICs in order to achieve SDG targets for “safely managed drinking” water. For example, to minimize the frequent fecal contamination in the domestic domain, specifically at point-of-drinking, interim measures should be emphasized to prevent recontamination (contamination after collection from the source) and post-contamination (after treatment) pathways (via hands, contaminated vessels and vectors) in the households, for safe drinking water. Particularly for cholera or cholera-like illnesses, the prevention strategy of drinking water quality within the households (domestic domain) should be much more stringent, as the findings of this thesis suggest that probable recontamination/post-contamination pathways through non-water routes were dominant for the presence of *V. cholerae* species in point-of-drinking water.

We propose regular cleaning of drinking vessels and promotion of narrow-necked drinking water vessels that would prevent hand contamination and access of vectors. Additionally, treated drinking water should be handled with the precautions necessary to avoid its recontamination. Future research efforts should include the identification of appropriate methodological approaches for post-handling of treated water and the assessment of health outcomes linked to the use of clean, narrow-necked vessels.

It is important to note that fecal matter contaminating drinking water does not necessarily arise solely from human sources, and contamination can occur both at the source and within the home. In addition, close proximity between human and domestic livestock populations in many low-income settings substantially increases the risk of zoonotic diseases, including water-borne [200, 201] diseases. According to the current priorities in the SDG era, sanitation is primarily focused on the management of human fecal matter, and largely ignores the management of fecal matter from domestic animals. Thus, a broader approach may be necessary to provide safely managed drinking water in the households, as interventions to improve human sanitation will not be adequate to prevent fecal contamination of water. Promotion of specific husbandry methods such as housing larger herds or flocks at a greater distance from the household, and subsequently examining the impact on the source and household water quality may provide new knowledge to identify better approaches for reducing fecal contamination from animal sources.

Furthermore, this thesis provides some insights to re-think some of the commonly practiced guidelines. It is evident from this thesis that what was thought to be a low action priority, with regard to remedial action for the WHO 'intermediate risk' category, in fact requires the same level of attention as the 'high risk' or 'very high risk' categories, as highly pathogenic *E. coli* were also found in the 'intermediate risk' group. However, investigation of newly emerging pathogens in all the risk groups should be monitored with high importance as the emergence of a pathogen can be an important public health problem due to changes in virulence factors or host susceptibility that can cause outbreaks. In our study, we discerned some newly emerged 'hybrid *E. coli*' isolates and a variant virulence profile of *V. cholerae* in water samples. Future, in-depth studies such as genomics and proteomics of these strains may provide new insights on their potential to cause disease and help develop vaccines for combating such diseases.

Our study findings also highlight that *E. coli* is not sufficiently sensitive to forecast the presence of other pathogens in drinking water, as we observed *V. cholerae* in 13% of water samples where *E. coli* was absent. Thus, investigation of some common waterborne pathogens along with fecal contamination will be useful to reflect the unambiguous safety of drinking water. However, it was also verified from our study that the use of certain advanced methodologies (i.e. qPCR) might improve the sensitivity of using *E. coli* as an indicator organism for detection of both fecal contamination and *V. cholerae*.

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Original Contribution

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

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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.

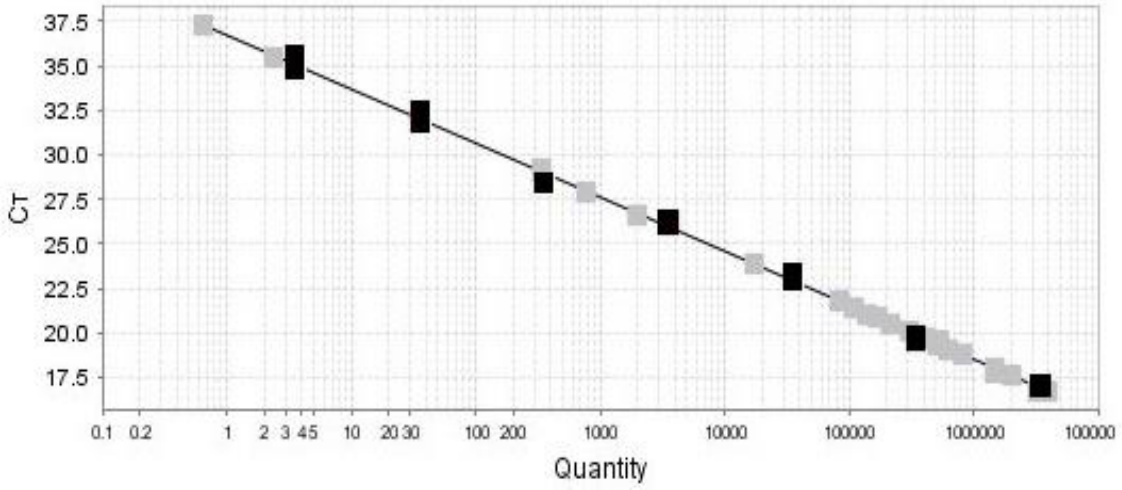

Figure 1: Standard curve for ctxA gene of *V. cholerae* O1 N16961 showing correlation between Cycle threshold (Ct) value with log value of quantity (copy no/reaction) calculated from concentration of serially diluted DNA. ■ Standard ■ Unknown

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

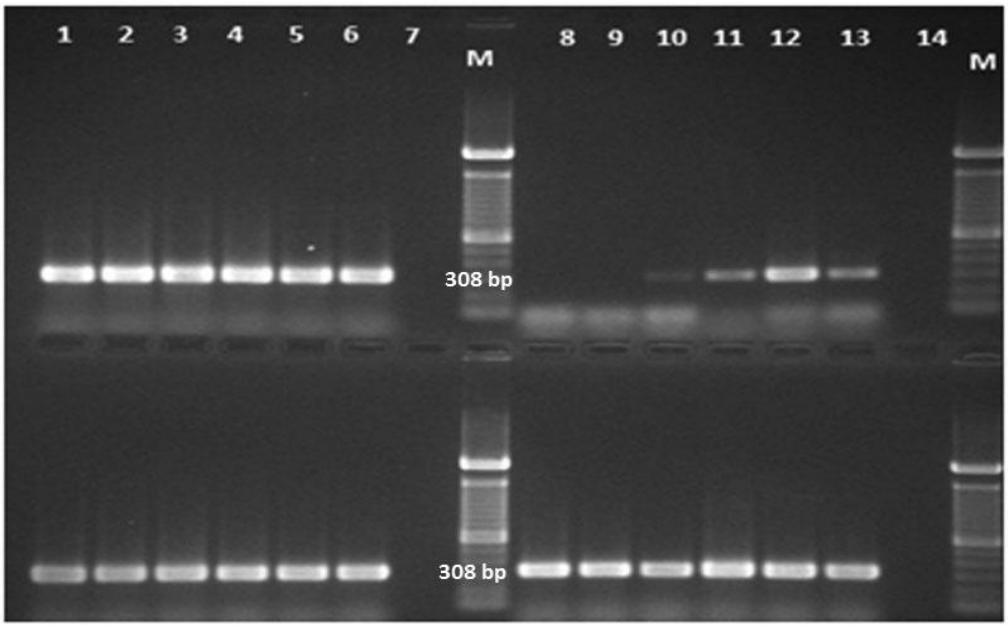

Figure 2: Detection of *V. cholerae ctxA* gene (308 bp) by conventional PCR. Upper gel lane 1-7 crude DNA templates extracted by three methods, lane 1, 2 - boiling, lane 3, 4 - Phenol method, lane 5, 6 kit method, lane 7 - NTC,100 bp Marker (Invitrogen, USA), lane 8-14 spiked water samples, lane 8, 9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

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Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for Escherichia coli and Vibrio cholerae

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	B
2. Planning of the experiments and methodology design, including selection of methods and method development	B
3. Involvement in the experimental work	B
4. Presentation, interpretation and discussion in a journal article format of obtained data	B

**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 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

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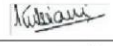
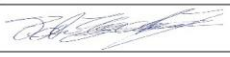
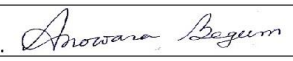
23/11/2018	Suhella Mohan Tulsiani	PhD	
7/01/2019	Peter Kjær Mackie Jensen	Associate Professor	

7/01/19	Anowara Begum	Professor	

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Optimization and Validation of Real Time PCR Assays for Absolute Quantification of toxigenic *Vibrio cholerae* and *Escherichia coli*

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Abstract. Quantitative real-time PCR (qPCR) is a dynamic and cogent assay for the detection and quantification of specified nucleic acid sequences and is more accurate compared to both traditional culture based techniques and 'end point' conventional PCR. Serial dilution of bacterial cell culture provides information on colony forming unit (CFU) counts. This is crucial for obtaining optimal standard curves representative of DNA concentration. This approach eliminates variation in the standard curves caused by loss of DNA by serial dilution of nucleic acid elute. In this study, an assay was developed to detect and quantify DNA by real-time PCR for two pathogenic species, *Escherichia coli* (*E. coli*) and *Vibrio cholerae* (*V. cholerae*). In order to generate a standard curve, total bacterial DNA was diluted in a 10-fold series and each sample was adjusted to an estimated cell count. The starting bacterial DNA concentration was 11ng/µL. An individual *E. coli* cell has approximately 5.16 femtograms of DNA. Therefore, 11 ng/µL of DNA would indicate 2.48×10^7cells. Both SYBR Green and TaqMan assays were validated for *uidA* region in *E. coli* and *ctxA* region in *V. cholerae*, respectively and was based on previously published assays for this standard curve experiment. PCR efficiency for *uidA* gene and *ctxA* gene were obtained 103.8% and 99.21%, respectively. Analysis of Variance (ANOVA) and coefficient of variation (CV %) indicated that standard curve generated by genomic DNA dilution had higher repeatability. Although not statistically significant, low F ratios indicated that there was some variation in C_T values when genomic DNA dilution was compared to dilution of cell suspension in media. Different water samples spiked with pure cultures of *E. coli* and *V. cholerae* were used as unknown samples. The standard curve constructed by the serial dilution of genomic DNA exhibited greater efficiency when compared to that of the standard curve obtained from serial dilution of cell suspension since in the former method DNA is not lost during extraction from culture dilutions.

INTRODUCTION

Diseases caused by consumption of water contaminated with bacteria such as *Escherichia coli* (*E. coli*) and *Vibrio cholerae* (*V. cholerae*) may result in serious illnesses like diarrhea, cholera, and may even lead to death (Hunter, 1997; Momba, Malakate & Theron, 2006; Swerdlow *et al.*, 1992). Studies suggest that adverse health effects associated with contaminated drinking-water are mainly due to the presence of human and animal fecal materials. Therefore, from a public health perspective, an indicator is necessary to confirm fecal pollution in water. Since monitoring the presence of all pathogens is not feasible. *E. coli*, a common inhabitant of the gastro intestinal (GI) tract of warm blooded animals (Hartl & Dykhuizen, 1984), is used as a surrogate for fecal contamination. In addition, *Vibrio cholerae*, needs to be

monitored for their presence in the environment due to their ability to cause epidemics of cholera.

Quantitative PCR (qPCR) allows the measuring of bacterial loads efficiently and rapidly with specific and sensitive detection compared to culture-based methods. It is designed to quantify microorganisms by directly targeting genomic DNA and can yield results within a few hours (Noble & Weisberg, 2005) by eliminating steps requiring lengthy incubation. In this study, estimation of fecal indicator bacteria (*E. coli*) was obtained by targeting *uidA* gene (encoding β-glucuronidase) and the assay was developed by using SYBR green qPCR assay. Likewise, for detection of *Vibrio cholerae*, *ctxA*-gene (encoding the A subunit of cholera toxin present in Serogroup O1 andO139) was targeted with TaqMan qPCR assay. This assay was found more specific, sensitive and rapid for detection of toxigenic *Vibrio cholerae* compared to conventional-PCR and culture-dependent methods (Chapela *et al.*, 2010).

Generally, typical qPCR methods for quantification of microorganisms, are performed on the genomic DNA isolated from serial dilution of cell suspensions. A disadvantage of this method is that the standard curve attained from serial dilutions of the samples produce R^2 value which is not a 'best fit' for the quantification of unknown samples. So, in this study, we assessed the relative performance of standard curves by direct 10-fold serial dilution of genomic DNA without the dilution of cell suspension. The objectives of this study were to generate a standard curve for *E. coli* thereby enabling one to quantify indicator microorganism in water, to implement a standard curve for the quantification of toxigenic *Vibrio cholera*e, and to compare the accuracy and efficiency of quantification of DNA templates by dilution of DNA and dilution of cell suspension. This study involved simultaneous application of qPCR analysis and culture based quantification. The two methods were compared to identify the best approach for the quantification of bacteria in the original samples.

METHODS AND MATERIALS

Study Design

The qPCR method involved 21 DNA samples consisting triplicates of each of seven variable amounts of DNA samples for standard curve calibration. This method was applied for both *Escherichia coli* B170 and *Vibrio cholerae* O1 biotype el Tor N16961 which produced two different standard curves with different efficiencies and R^2 values. The quantity of bacteria in the unknown samples was estimated from the standard curve. In the culture based method, 1mL bacterial culture of target organism was serially diluted 10-fold to yield 10^7 down to 1 CFU/mL and plated on to Nutrient Agar (NA) in triplicate. After overnight incubation, colony forming units (CFU) were recorded. The starting stock bacterial culture was the same for both, qPCR and culture based method.

DNA extraction

Genomic DNA from overnight cultures of *Vibrio cholerae* and *E. coli* strains were extracted and purified according to the manufacturer's instructions by QIAamp DNA mini kit DNA mini kit (Qiagen, Hilden, Germany). The concentrations and purity of extracted DNA samples were measured at 260nm using Colibri Microvolume Spectrometer (Titertek-Berthold, Berthold Detection Systems GmbH, Bleichstrasse, Pforzheim, Germany). DNA templates were stored at -20°C until further use.

Calibration standards, controls, and standard curves

Prior to qPCR, a range of calibration standards and controls were prepared. Strains used for calibration standard were *E. coli* (ATCC B170) and *Vibrio cholerae* (N16961). Relative standards were prepared using 7-$\log_{10}$ serial dilution (1:10) of DNA isolated from these strains. The starting concentration of each stock DNA was measured by Colibri Microvolume Spectrometer. This value was then divided by the dilution factor of each consecutive DNA sample to find the concentrations of the remaining diluted DNA standards using the standard dilution formula, $C_1V_1=C_2V_2$.

To estimate the number of cells in a reaction, the mass of a single bacterial genomic DNA was calculated. Genome size of one *E. coli* and *Vibrio cholerae* were 4527247 bp (NCBI Genbank HG738867.1) and 4,033,460 bp (NCBI Genbank10952301), respectively. To calculate molecular mass, we used the following formula:

E. coli genome size = 4527247 bp

Average mass of a base pair = 675 Dalton

Mass of one *E. coli* genome
= (4527247 × 675) Dalton = 3055891725 Dalton
= $3055891725 \times 1.66 \times 10^{-24}$ gram
[*Note: 1 Dalton = 1.66×10^{-24} gram]
= $5072780263.5 \times 10^{-24}$ gram = 5.07×10^{-15} gram = 5.07 fg

Using this formula molecular mass of *Vibrio cholerae* was found 4.52 fg. Our measured concentration in the starting stock bacterial DNA was 11ng/µL which measured 0.1pg/µL in the final PCR reaction mixture. To find the number of cell number in reaction mixture, this 11ng/µL concentration was then divided by the molecular mass of the specific bacteria. After triplicate 7-log serial dilution (1:10) of the stock DNA, the equivalent cell numbers were calculated in the PCR reaction mixtures.

Reference genes, qPCR Assays and Reaction Conditions

Genome annotation report for *uidA* and *ctxA* were found only once on GenBank where *uidA* and *ctxA* genes were present as 1 copy number per genome (http://www.ncbi.nlm.nih.gov/nuccore/556503834?report=genbank). Sequences of primers and probes for *ctxA* and *uidA* genes were obtained from previous studies (Table 1). The probe for *ctxA* was validated by labelling FAM at the 52 end and a Black Hole Quencher 1 at the 32 end (Tag, Copenhagen-Oligo, Denmark).

In our experiment, we used existing TaqMan Universal Master Mix and SYBR Green Master Mix (Applied Biosystems, Life Technologies, Warrington, UK) for two of the target organisms. The qPCR was performed on Applied Biosystems StepOne™ (48-well) Real Time PCR systems.

The final *ctxA* reaction mixture (25µL) contained 12.5 µL 2XTaqMan® Universal Master Mix II (pre-mixed with passive reference dye ROX), 2.5µL of 100nM each

Table 1. List of primers, probes and their sequences

Target species and gene	qPCR Assay Mix	Sequences of Primers and Probe (52 to 32) and size	Amplicon length (bp)	References
Escherichia coli ATCCB170 (*uidA*)	*Power SYBR green® PCR master mix*	UAL1939b (terminal sense) 5'-ATGGAATTTCGCCGATTTTGC-3' (21-mer) UAL2105b (terminal antisense) 5'-ATTGTTTGCCTCCCTGCTGC-3' (20-mer)	187	(Heijnen & Medema, 2006
Vibrio cholera serotype O1 CT+ATCC N16961 (*ctxA*)	*TaqMan® Universal Master MixII with UNG*	ctxA (terminal sense) 5'-TTTGTTAGGCACGATGATGGAT-3' (22-mer) ctxA (terminal antisense) 5'-ACCAGACAATATAGTTTGACCCAC TAAG-3' (28-mer) ctxA (probe) FAM-TGT TTC CAC CTC AAT TAG TTT GAG AAG TGC CC- BHQ-1 (32-mer)	84	(Blackstone *et al.*, 2007)

sense and antisense primer, 2.5 µL 250nM probe and 5 µL of template DNA. PCR was performed under the following thermal conditions: UNG incubation at 50ºC for 2 min, polymerase activation at 95ºC for 10 min, followed by 40 cycles of 15 sec at 95ºC, and for extension, 1 min at 60ºC.

Similarly, the reaction mixture (25µL) for *uidA* contained 12.5 µL 2XPower SYBR green® PCR master mix (contains a propriety version of ROX dye), 2.5µL of 100nM each sense and antisense primer, 2.5 µL of Diethylpyrocarbonate treated H_2O and 5 µL of template DNA. The thermal conditions were maintained as following: polymerase activation at 95ºC for 5 min, followed by 40 cycles of 30 sec at 95ºC and extension for 1 min at 60ºC.

All primers and probes were purchased from Tag Copenhagen-Oligo, Denmark.

Analytical Sensitivity and Limits of Detection

The DNA sample was then serially diluted (10-fold) up to 7- $\log_{10}$ (10^7 CFU/mL down to 1 CFU/mL) in DEPC treated water. Five microliters from each dilution was used as template for detection. DNA from *E. coli* and distilled H_2O were used as negative control and no template control (NTC), respectively. For sensitivity, detection of *E. coli* possessing the *uidA* gene, the limit of detection was obtained by using the aforementioned procedure and DNA template from *Vibrio cholerae* O1 cells was used as negative control.

Analytical Specificity of the qPCR assay

In order to investigate the specificity of developed qPCR assay for detecting the chosen genes in presence of non-specific DNA, 27 DNA from isolates (as shown in Table 6) were used as templates.

Statistical analysis

In order to test the variation between the assays, Analysis of Variance (ANOVA) was performed using SPSS Version 16 (IBM, USA). The F ratio and corresponding P values were found to test the significance of variation.

RESULTS

Quantification of target organism in unknown samples

To verify the reliability of the qPCR assays used in this study, water sample spiked with 10µL of culture was used as unknown sample. Initially, copy number of each bacteria was determined by mass conversion of total DNA and the standard curve was obtained for 7-log 10-fold dilution of DNA. From this standard curve, a set of 10-fold dilution of bacterial DNA from the estimated copy number of bacteria from each dilution of pure culture of bacterial DNA was used as standard. Acquired number of copies for the unknown samples were 3.2×10^4 (*Vibrio cholerae*) and 7.6×10^3 (*E. coli*) from the developed standard curves.

Sensitivity and limit of detection

The limit of detection and sensitivity of the designed qPCR assays for both of the target organisms were determined. For both of the assays, lowest amount of DNA were restricted to approx. 0.1 pg in the diluted DNA from a starting amount of 11ng. For the successive increase of DNA concentration, C_T value increased by 3 cycles which allowed detection of 0.1pg of DNA to 11ng of DNA. The results obtained is summarized in Table 2 & Table 3.

Specificity of the two qPCR assays

Primers and probes employed in the TaqMan and SYBR green assay demonstrated PCR efficiencies of 99.21% and 103.80%, respectively (Table 2). Amplification was observed for *ctxA* harbouring *Vibrio cholerae* strains. The results have been summarized in Table 6. To observe the specificity of this assay, melt curve analysis was carried out for the 6-log 10-fold dilutions of *E. coli* DNA, which gave dissociation at the same temperature (82.80ºC). No other peaks were observed for *E. coli* which implies that neither non-specific products nor primer dimers were present. Multiple peaks would indicate that more than one product was formed.

Table 2. Variation of regression correlation, amplification efficiency, sensitivity of qPCR for two different organisms

Species	Correlation Co-efficient (R^2)	Amplification Efficiency (%)	Slope	Specificity	Limits of Detection
Vibrio cholerae	0.99	99.21	-3.34	100%	0.112 pg DNA
E. coli	0.99	103.80	-3.23	100%	0.114 pg DNA

Table 3. Comparison of sensitivity of detection from 10-fold dilution series of pure genomic DNA and 10-fold dilution of cell suspension DNA for *Vibrio cholerae* (A) and *E. coli* (B)

Vibrio cholerae

Genomic DNA (g-DNA)				Cell suspension		
Weight/25µl reaction mix	CT ± SD (n = 3)	Coefficient of variation (CV %)	Estimated copy number	CFU/ml	CT ± SD (n = 3)	Coefficient of variation (CV %)
11.2 ng	17.50±0.10	0.57	2.48×10^{7}	5.2×10^{5}	19.47±1.28	8.62
1.12 ng	20.29±0.09	0.45	2.48×10^{6}	5.2×10^{4}	20.01±0.72	3.6
112 pg	23.67±0.04	0.17	2.48×10^{5}	5.2×10^{3}	24.10±1.98	8.22
11.2 pg	27.09±0.05	0.18	2.48×10^{4}	5.2×10^{2}	30.15±1.73	5.73
1.12 pg	30.54±0.09	0.29	2.48×10^{3}	5.2×10^{1}	34.43±0.84	2.44
0.112 pg	33.82±.09	0.27	2.48×10^{2}	5.2×10^{0}	37.51±0.11	0.29

(A)

E. coli

Genomic DNA (g-DNA)				Cell suspension		
Weight/25µl reaction mix	CT ± SD (n = 3)	Coefficient of variation (CV %)	Estimated copy number	CFU/ml	CT ± SD (n = 3)	Coefficient of variation (CV %)
11.4 ng	14.71±0.08	0.54	2.21×10^{7}	2.3×10^{5}	12.11±1.32	10.9
1.14 ng	17.01±0.02	0.12	2.21×10^{6}	2.3×10^{4}	14.94±0.43	2.88
114 pg	20.17±0.05	0.24	2.21×10^{5}	2.3×10^{3}	20.77±3.32	15.98
11.4 pg	23.92±0.07	0.29	2.21×10^{4}	2.3×10^{2}	28.46±2.84	9.98
1.14 pg	26.55±0.08	0.3	2.21×10^{3}	2.3×10^{1}	29.47±1.85	6.28
0.114 pg	29.39±0.02	0.07	2.21×10^{2}	2.3×10^{0}	30.68±1.79	5.83

(B)

Table 4. Reproducibility of Real Time Assays

	Genomic DNA dilution	Cell suspension
Vibrio cholerae TaqMan Assay	F = 6.126E-4 P value = 0.999 Up to dilution 10E-7	F = 0.121 P value = 0.887 Up to dilution 10E-5
Escherichia coli SYBR Green assay	F = 1.681E-5 P value = 0.999 Up to dilution 10E-8	F = 0.026 P value = 0.974 Up to dilution 10E-8

Table 5. Comparison between Genomic DNA dilution and Cell Suspension in Real Time Assays

	Replicate 1	Replicate 2	Replicate 3
Vibrio cholerae Taqman assay	F = 0.561 P value = 0.475 Up to dilution 10E-5	F = 0.038 P value = 0.849 Up to dilution 10E-5	F = 0.107 P value = 0.752 Up to dilution 10E-5
Escherichia coli SYBR green assay	F = 0.354 P value = 0.561 Up to dilution 10E-8	F = 0.342 P value = 0.568 Up to dilution 10-8	F = 0.663 P value = 0.429 Up to dilution 10E-8

Table 6. Detection of *ctxA* gene and *uidA* gene for specificity test

Sr#.	Species	Collection or Isolation number	Origin	*ctxA* presence	*uidA* presence
1	*Escherichia coli* (*E. coli*)	[a]ATCC AN33859	Clinical	–	+
2	*E. coli* EPEC	ATCC B170	Clinical	–	+
3	*E. coli* EAEC	ATCC MG1214C2	Clinical	–	+
4	*E. coli* ETEC	ATCC MGL-IC1	Clinical	–	+
5	*E. coli* EPEC	AE3171	Clinical	–	+
6	*E. coli* EHEC	NF 9422	Clinical	–	+
7	*E. coli*	MMLA	Clinical	–	+
8	*E. coli* EIEC	2V	Clinical	–	+
9	*E. coli* EHEC	NF 9877	Clinical	–	+
10	*E. coli* ETEC	C600	Clinical	–	+
11	*Enterococcus faecium*	T7	Environmental	–	–
12	*Enterococcus faecium*	B10	Environmental	–	–
13	*Enterococcus faecium*	B4	Environmental	–	–
14	*Enterococcus faecalis*	T11	Environmental	–	–
15	*Salmonella* spp	29	Food	–	–
16	*Salmonella* spp	36	Soil	–	–
17	*Salmonella* spp	19 (b)	Food	–	–
18	*Salmonella enteritidis*	A	Environmental	–	–
19	*Salmonella typhimurium*	Ifo-3313	Environmental	–	–
20	*Vibrio parahaemolyticus*	1	Environmental	–	–
21	*Vibrio parahaemolyticus*	2	Environmental	–	–
22	*Vibrio parahaemolyticus*	3	Environmental	–	–
23	*Vibrio cholerae* serotype O1 CT^+	ATCC C6706	Clinical	+	–
24	*Vibrio cholerae* (*VC*) serotype O1 CT^+	ATCC N16961	Clinical	+	–
25	*VC* serotype nonO1 CT^-	ATCC 4460	Clinical	–	–
26	*VC* serotype O1 CT^-	ATCC SA 317	Clinical	–	–
27	*VC* serotype O1 CT^+ O139	ATCC NIHC0270	Clinical	+	–

[a]Reference strains: American Type Culture Collection, ATCC were collected from Laboratory of Molecular Genetics, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B). Other isolates were obtained from clinical laboratories of ICDDR, B and Environmental Microbiology Laboratory of University of Dhaka.

DISCUSSION

To assess the microbiological quality water, real-time quantitative PCR demonstrates quantification of gene targets with higher sensitivity, specificity, and is more time efficient compared to traditional end-point PCR or conventional culture based methods. One of the disadvantages of culture-based methods is that injured cells or cells that have

evolved into viable but non-culturable state are not detectable and therefore cannot be enumerated by culture based methods (Pommepuy *et al.*, 1996). Another limitation is that a lengthy incubation period renders the protocol cumbersome. In comparison, while traditional end-point PCR improves detectability, an additional step of gel electrophoresis is required and accurate quantification cannot be achieved.

Quantification of bacterial cells by current qPCR methods primarily depends on the correlation between CFU counts obtained from culture plates with the C_T values associated with the DNA from culture suspension. Our study investigated both the correlations of C_T versus CFU counts by plate method and C_T versus serial dilutions of genomic DNA. A critical parameter to evaluate PCR efficiency is measured by a regression coefficient (R^2 value) that defines the closeness of data to the fitted regression line. R^2 value close to 1 indicates good PCR efficiency. We found higher R^2 value of C_T versus serial dilutions of genomic DNA than the R^2 value (Figure 2, 3) the former one for both of the target bacteria. Moreover, the estimated bacterial counts we found from the g-DNA dilution were higher (Table 3) in several magnitudes (i.e copy number ranged from 2.48×10^7 to 248) than the plate counts (corresponding CFU ranged from 5.2×10^5 to 52) for each bacterium since stress-induced VBNC cells are undetectable in plate counts. This result indicates that dependence on traditional plate count might result in the underestimation of potentially infectious bacterial cells in food and water (Lyon, 2001). Higher sensitivity of detection from direct g-DNA dilution was observed when compared with dilution of cell suspension DNA. The C_T value of the lowest concentration of DNA (0.1 pg) was 33.82±0.09 for genomic DNA whereas the C_T value of the lowest concentration of cell suspension exceeded the cut-off value for positive sample detection

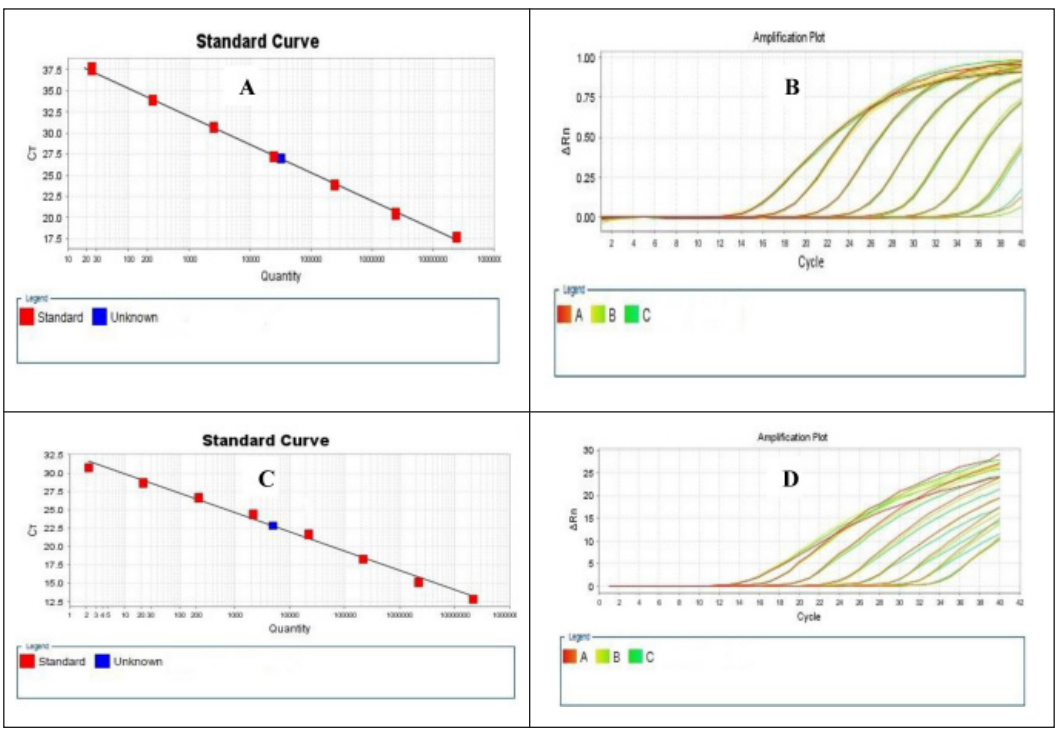

Figure 1. Standard curve and amplification plot of 10-fold series dilution for *Vibrio cholerae* and *E. coli*: Standard curves are plotted in C_T (cycle threshold) vs estimated copy number of each dilution. Amplification plots are in cycle vs "Rn. Standard curve and amplification plot of *uidA* - A, B and *ctxA*-C, D.

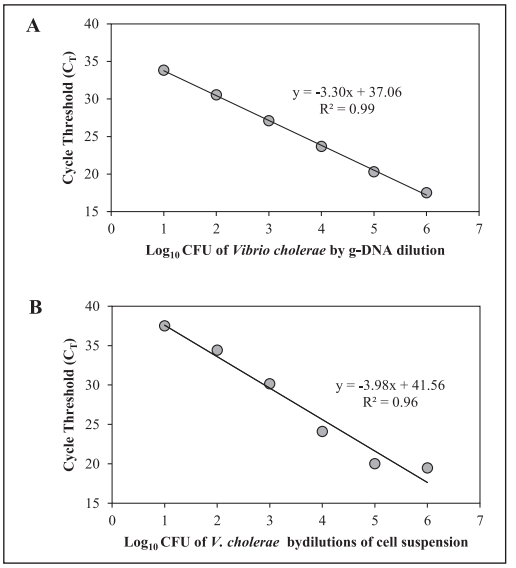

Figure 2. Analysis and comparison of two different types of dilutions for standard curve generation using target organism *Vibrio cholerae* N16961. (A) g-DNA of *Vibrio cholerae*. (B) Cell suspension of *Vibrio cholerae* Standard samples are represented by grey circles (A, B).

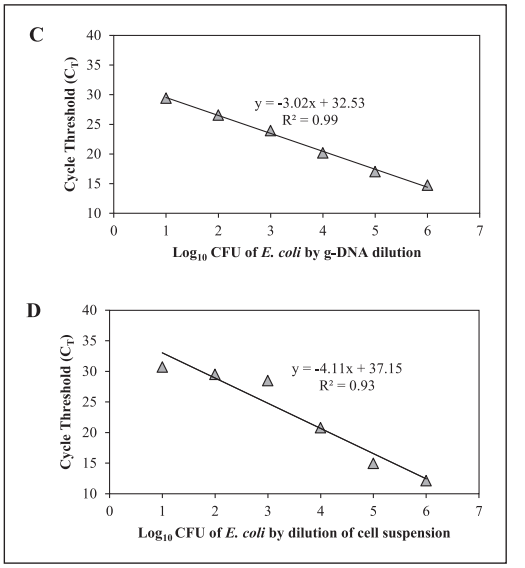

Figure 3. Regression analysis of standard curves generated by two different types of dilutions for *E. coli* B170. (C) g-DNA of *E. coli* (D) Cell suspension of *E. coli*. Standard samples are represented by grey triangles (C, D).

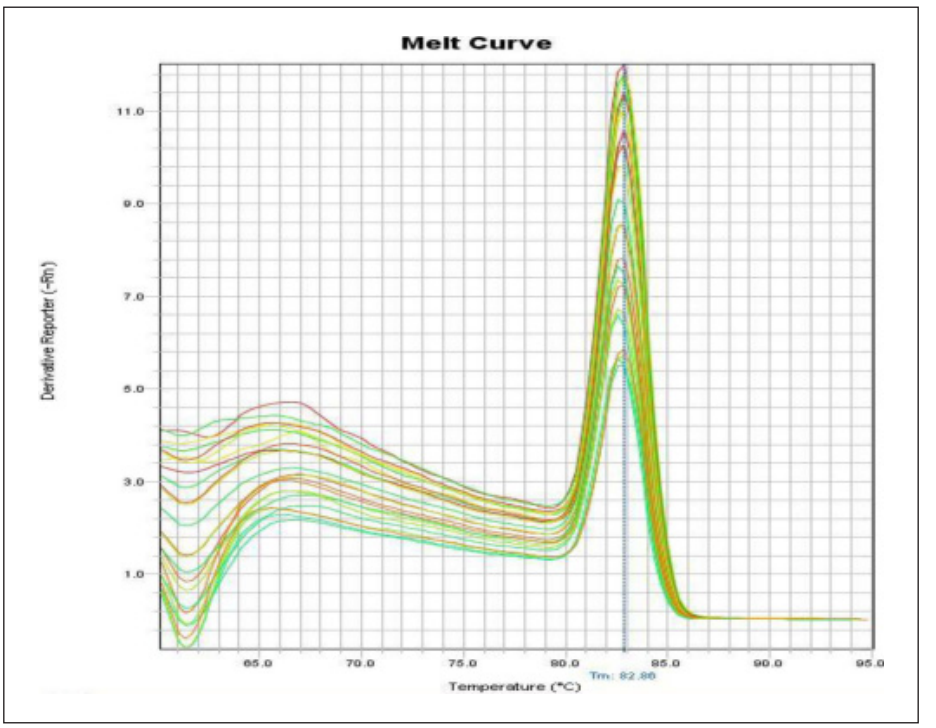

Figure 4. Melt curve analysis for *E. coli*. Dissociation was observed at 82.80°C for all dilutions of *E. coli* DNA.

(37.51±0.11>35). This difference may be due to the loss of DNA during sample processing for DNA extraction.

For analyzing the repeatability of assay, Analysis of Variance (ANOVA) was carried out to see if there were variations in the C_T values between the replicates. A lower F ratio indicates lower variation between the replicates and hence higher repeatability. In the case of the *ctxA* TaqMan assay, the F ratio observed for the genomic DNA dilution and cell suspension dilution was 6.126E-4 and 0.121, respectively. The coefficient of variation ranged from 0.17–0.57 for the genomic dilution method and for the cell suspension dilution method it ranged from 0.29–8.62. For the *uidA* SYBR Green assay the observed F ratio for the genomic DNA dilution and cell suspension dilution was 1.681E-5 and 0.026 respectively. The coefficient of variation ranged from 0.07–0.54 for the genomic dilution method and for the cell suspension dilution method it ranged from 2.88–15.98. In both cases it was seen that the F ratio and the CV% were lower for the genomic DNA dilution compared to the cell suspension dilution. The genomic DNA dilution method for the TaqMan assay exhibited higher repeatability at higher dilutions (10E-7) compared to the cell suspension dilution which was not reproducible beyond a dilution of 10E-5. This shows the former method can detect DNA at lower concentrations. However the consistency of detection for the SYBR green assay was same for *Escherichia coli* and *Vibrio cholerae*.

In case of variation between the genomic DNA dilution and cell suspension dilution, some variation was seen. Hence there is evidence to suggest that there will be no variation between replicates TaqMan and SYBR green assay. For the *ctxA* TaqMan assay, the F ratios that were obtained when comparing the genomic DNA dilution with the cell suspension dilution for replicates 1, 2, 3 were 0.561, 0.038 and 0.107 respectively. For the *uidA* SYBR green assay, the F ratios that were obtained when comparing the genomic DNA dilution with the cell suspension dilution for replicates 1, 2, and 3 were 0.354, 0.343 and 0.663 respectively.

For the health and well-being of individuals in a community, quantitative assessment of potentially hazardous pathogens is essential (Haas, Rose & Gerba, 1999). Our study sought an effective quantification technique of bacterial number which relied on only g-DNA dilution and excluded the necessity of quantitative approach by culture suspension. The calibrator control equivalents achieved by direct dilution of DNA for standard curve generation reduced the time of analysis since it only requires the preparation of stock DNA followed by serial dilution. In case of standard curve generated from cell suspension, each of the dilutions of culture suspension needs to be processed for DNA extraction. This study was also found to be suitable for analysis of wide range of samples for example rice, PBS, water and can be implemented when the number of samples is high (data not shown here).

The choice of the gene target is also an important factor for precise quantification of bacterial cells in samples. *Vibrio cholerae* is commonly present in many tropical and temperate regions of aquatic environments (R. Colwell, Kaper & Joseph, 1977; R. R. Colwell *et al.*, 1981; Islam *et al.*, 1994; Kaper, Lockman, Colwell, & Joseph, 1979) and strains harboring *ctxA* gene is a major public health concern. Some of the previous works on *Vibrio cholerae* by qPCR involved detection of multiple genes, for instance sequences encoding repeat in toxin, extracellular secretory protein, mannose-sensitive pili and the toxin coregulated pilus (Gubala, 2006) while our study targeted single gene *ctxA*, since it discriminates between toxigenic and non-toxigenic strains. Moreover, database searches demonstrated the presence of single copy per genome of *ctxA* gene and has high specificity for the toxigenic strains (Blackstone *et al.*, 2007). Similarly, for detection of *E. coli* by qPCR, many of the studies targeted 16S rDNA gene (Nadkarni, Martin, Jacques & Hunter, 2002) the internal transcribed spacer region and the 23S rRNA gene (16S-ITS-23S gene region; (Khan *et al.*, 2007). In all known human bacterial pathogens, 16S rRNA gene is present in multiple copies (Brosius, Dull,

Sleeter & Noller, 1981) and thus species-specific discrimination and quantification remains questionable in a heterogeneous DNA sample. Another study, revealed that the internal transcribed region (ITS), flanking the conserved regions of 16S rRNA gene and 23S rRNA gene having poor specificity of 85.9% (Maheux *et al.*, 2009). In the present study, our gene of interest for detection and quantification of *E. coli* was *uidA* which was found single copy per genome by database search of previously published study (Taskin, Gozen & Duran, 2011) and exhibited 100% specificity (Maheux *et al.*, 2009). In order to calculate the exact number of bacterial cell, single copy gene was chosen for each of the target organisms.

The use of TaqMan assay for detection of pathogen was more sensitive compared to SYBR Green assays. This is because additional probe is used in the reaction system. Furthermore, non-specific amplified products can increase the fluorescence due to the non-specific incorporation of the SYBR Green dye into double-stranded DNA (Bel, Ferré & Escriche, 2011). The purpose of our study was to quickly screen samples *E. coli* and *Vibrio cholerae* and estimate cell numbers, thereby allowing rapid analysis before consumption of food and water samples. Hence, optimization of protocols for the generation of the standard curve was imperative to quantify the bacterial load in samples.

Our investigation suggests the need for a standard curve generated from dilution of genomic DNA over the standard curve generated from cell suspension dilution as this method is capable of accurate and rapid quantification of bacterial pathogens in a range of environmental samples.

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GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
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DECLARATION OF CO-AUTHORSHIP

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Principal supervisor	Peter Kjær Mackie Jensen

Title of PhD thesis:
Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for Escherichia coli and Vibrio cholerae

This declaration concerns the following article:
Optimization and Validation of Real Time PCR Assays for Absolute Quantification of toxigenic Vibrio cholerae and Escherichia coli

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	C
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 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

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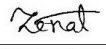
02/12/18	Zenat Zebin Hossain	PhD student	
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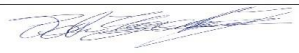
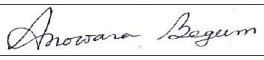
07/01/19	Peter Kjær Mackie Jensen	Associate Professor	
07/01/19	Anowara Begum	Professor	

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Development and Validation of a Novel Real-time Assay for the Detection and Quantification of *Vibrio cholerae*

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Vibrio cholerae O1 and O139 has been known for its ability to cause epidemics. These strains produce cholera toxin which is the main cause of secretory diarrhea. *V. cholerae* non-O1 and non-O139 strains are also capable of causing gastroenteritis as well as septicemia and peritonitis. It has been proven that virulence factors such as T6SS, *hapA*, *rtxA*, and *hlyA* are present in almost all *V. cholerae* strains. It is imperative that viable but non-culturable cells of *V. cholerae* are also detected since they are also known to cause diarrhea. Thus, the aim of this study was to develop an assay that detects all *V. cholerae* regardless of their serotype, culturable state, and virulence genes present, by targeting the species specific conserved *ompW* sequence. The developed assay meets these goals with 100% specificity and is capable of detecting as low as 5.46 copy number of *V. cholerae*. Detection is rapid since neither lengthy incubation period nor electrophoresis is required. The assay had excellent repeatability (CV%: 0.24–1.32) and remarkable reproducibility (CV%: 1.08–3.7). Amplification efficiencies in the 89–100% range were observed. The assay is more economical than Taqman-based multiplex real-time PCR assays. Compared to other real-time assays, the *ompW* assay is specific and sensitive, has better repeatability and reproducibility, and is more economical.

Keywords: *Vibrio cholerae*, OmpW, C_T value, sensitivity and specificity, gene copy number, real-time PCR

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INTRODUCTION

Vibrio cholerae is a Gram-negative, comma shaped facultative pathogen responsible for causing cholera. The global incidence of cholera was about 2.8 million cases per year, with 91,000 deaths (1). *V. cholerae* O1 has been the etiological agent for several cholera epidemics. The serogroup O139 was responsible for cholera outbreaks in India and other countries in Asia during 1992 (2) and was also isolated during the outbreak in November 2000 in India (3) and March–April 2002 in Bangladesh (4).

Vibrio cholerae O1 and O139 serogroups express toxin coregulated pilus which confers the bacteria the ability to colonize the intestine while the cholera toxin is associated with secretory diarrhea (5). Depending on severity, the infectious dose for *V. cholerae* varies from 10^6 to 10^{11} cells (6).

Toxigenic and non-toxigenic non O1, non-O139 have been documented as incriminating in several outbreaks in developing countries (7–10). In non-CT-producing vibrios, virulence factors such as type 3 secretion systems, hemolysin (HlyA), repeat in toxin (RTX), and heat-stable enterotoxin have

major roles in causing infections (11). Hasan et al. (10) reported 98% *V. cholerae* strains carried hemagglutinin protease *hap* (98%) irrespective of their source, i.e., clinical or environmental. Other virulence factors present are T6SS (94–99%), *rtxA* (96%), *toxR* (87%), and *hlyA* (83%), and all these virulence factors might be responsible for diarrhea caused by non-toxigenic non-O1/non-O139 variants.

Vibrio cholerae in the viable but non-culturable (VBNC) state can express virulence factors required to produce infection (12). The VBNC cells have the capacity to revert to the culturable state and colonize the intestine (13) the mechanism of which is largely unknown (14). These organisms may go undetected if conventional culture based methods are used (15). Conventional identification of *V. cholera*, which may be done by biochemical tests, is time consuming and laborious. Available commercial biochemical identification systems, such as dipstick test used for the detection of O1 and O139 strains, are not always accurate (16). *V. cholerae* has been shown to possess similar biochemical properties with other species in the Genus *Vibrio* and *Aeromonas*, hence complicating an accurate identification (17).

Compared to conventional PCR, real-time PCR is less labor intensive, more safe, and rapid due to the elimination of gel electrophoresis. It has greater sensitivity and can detect minute amounts of target amplicons that might be missed by the conventional PCR. Real-time PCR can directly target genomic DNA and thus eliminate extensive incubation periods (18). Furthermore, VBNC cells can be detected which might be missed by culture-based methods. The *ompW* sequence is highly conserved among *V. cholerae* species belonging to different biotypes and/or serogroups (17). Hence, the *ompW* gene could be used as a target for species-specific detection, identification, and quantification.

A number of assays exist for the detection of *V. cholerae* (19–24) but many of these assays lack empirical data for reproducibility and repeatability. Some of these assays have not been validated in terms of detecting non-specific products that might accompany the amplification reaction. Furthermore, a number of assays are based on virulence factors that might not be present in certain strains and might yield false negative results.

The aim of this study was to develop an assay that detects and quantifies both O1/O139 and non O1/O139 disease causing strains of *Vibrio* spp. In addition, the assay would be able to quantify VBNC cells that cannot be detected or quantified by conventional methods.

MATERIALS AND METHODS

Assay Controls and Growth Conditions

A total of 28 bacterial strains were used as assay controls. *V. cholerae* strains were grown in alkaline peptone water for enrichment, and all other strains were grown in nutrient broth for 24 h in 37°C. Genomic DNA from overnight cultures controls were extracted and purified according to the manufacturer's instructions by QIAamp DNA mini kit (Qiagen, Hilden, Germany).

Sample Preparation and Spiking

Four different types of samples were taken for experiment: (i) drinking water, (iii) pond water, (ii) boiled rice, and (iii) shrimp. Rice sample were prepared by homogenization of 25 g of boiled rice with 225 µL of phosphate-buffered saline (1 L distilled H_2O, 10 g L^{-1} NaCl, 0.25 g L^{-1} KCl g L^{-1}, 1.8 g L^{-1} Na_2HPO_4, 0.3 g L^{-1} KH_2PO_4; pH 7.4) in a Stomacher Lab Blender (Seward Stomacher® 80, Lab Biomaster, UK). Shrimp sample was also prepared by following the same procedure for rice. All of the samples were spiked with different concentrations of *V. cholerae* CT^+ O139, *V. cholerae* CT^+ O1, and *V. cholerae* CT^- non-O1/non-O139. Prior to the evaluation of this assay for these environmental samples, absence of *V. cholerae* was confirmed by qPCR. DNA extraction was conducted using QiaAmp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction.

PCR Primer Design

The Outer Membrane Protein W-OMPW Sequence of eight reference strains (**Table 1**) was downloaded from the NCBI database. The primer design was accomplished by FastPCR 6.05 (PrimerDigital, Helsinki, Finland). Primers that conformed

TABLE 1 | *Vibrio cholerae ompW* sequences with their GenBANK accession numbers used for primer designing.

Strain	Accession number
Vibrio cholerae strain 08-5735 *ompW*gene, partial cds	FJ462446
V. cholerae strain 08-5739 *ompW* gene, partial cds	FJ462447
V. cholerae strain 08-5738 *ompW* gene, partial cds	FJ462448
V. cholerae strain 08-5737 *ompW* gene, partial cds	FJ462449
V. cholerae strain ATCC 27070 *ompW* gene, partial cds	FJ462450
V. cholerae strain ATCC 55056 *ompW* gene, partial cds	FJ462451
V. cholerae strain 08-5742 *ompW* gene, partial cds	FJ462453
V. cholerae O1 strain N16961 *ompW* gene, complete cds	KJ722608

TABLE 2 | *ompW* gene primers used for real-time PCR along with their properties.

	Sequence(5′–3′)	Length (nt)	Tm (°C)	PCR_ Fragment_Size (bp)	T_{opt} (°C)
Forward	Acatcagytttgaagtcctcgc	22	56.8	191	61
Reverse	Gtggtgtaattcaaacccgc	20	55.8		

TABLE 3 | Primer parameters obtained for the designed primers together with the default and ideal range as stated by Kalendar et al. (25).

Criteria	Default	Ideal	Obtained
Length (nt)	20–24	>21	Forward (22 nt) Reverse (20 nt)
T_M range (°C)	52–68	60–68	Forward (56.8) Reverse (55.8)
T_M 12 bases at 3′ end	30–50	41–47	Forward (42.9) Reverse (41.3)
CG (%)	45–65	50	Forward (47.7) Reverse (50.0)
Linguistic complexity (LC%)	>75	>90	Forward (95) Reverse (89)
Sequence quality (PQ%)	>70	>90	Forward (93) Reverse (87)

TABLE 4 | Comparison of sensitivity of detection and precision of two replicate runs.

	Replicate run 1			Replicate run 2			
	Efficiency = 89.161%			Efficiency = 97.374%			
	Slope = −3.612			Slope = −3.386			
	R^2 = 0.975			R^2 = 0.982			
Copy number	**SD (n = 4)**	**Mean (n = 4)**	**Coefficient of variation (CV%)**	**SD (n = 4)**	**Mean (n = 4)**	**Coefficient of variation (CV%)**	**Inter-assay CV%**
$5.46E10^{5}$	0.222951	18.806	1.185533	0.196337	17.90175	1.096748	2.838649
$5.46E10^{4}$	0.04455	18.91	0.235588	0.089388	18.55025	0.481871	1.084382
$5.46E10^{3}$	0.099654	22.7365	0.438301	0.382781	22.2215	1.722573	1.68125
$5.46E10^{2}$	0.347915	27.26575	1.276015	0.190516	26.42575	0.720947	1.932086
$5.46E10^{1}$	0.175279	31.034	0.564796	0.196538	30.3475	0.647623	1.321016
$5.46E10^{0}$	0.28061	34.67725	0.809206	0.382505	33.864	1.129533	1.558894
$5.46E10^{-1}$	0.517502	39.26467	1.317984	0.332131	36.731	0.904225	3.792876

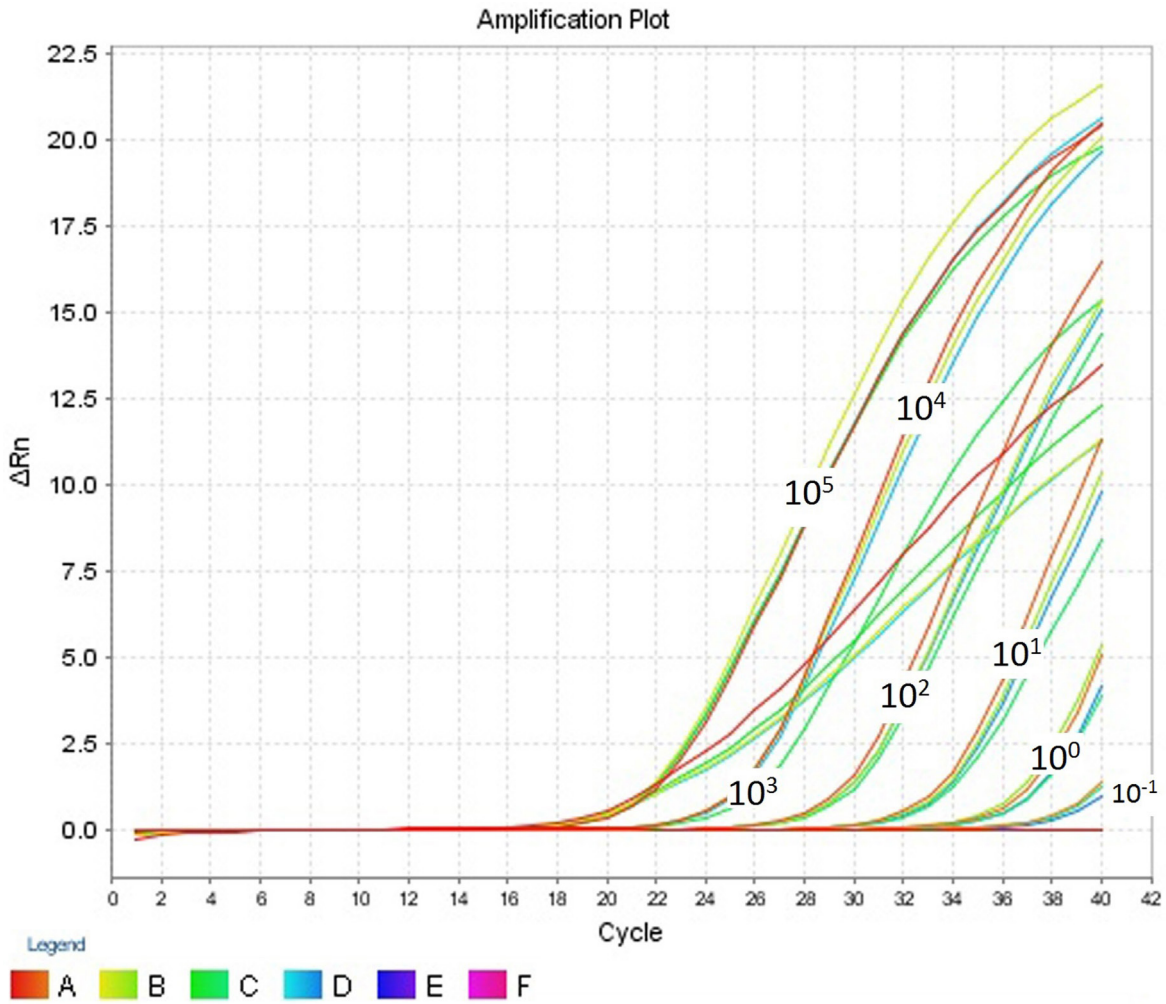

FIGURE 1 | Amplification plot (ΔRn vs Cycle) for testing the sensitivity and precision of the first replicate run.

to the criteria and summarized in **Table 3** were analyzed for their complementarity with the reference sequence by Basic Local Alignment Search Tool (NCBI, Bethesda, MD, USA). The forward and reverse primer sequences were checked, and the pair that had the highest identity with the Query Sequences (reference sequences) was selected for further analysis (**Table 2**).

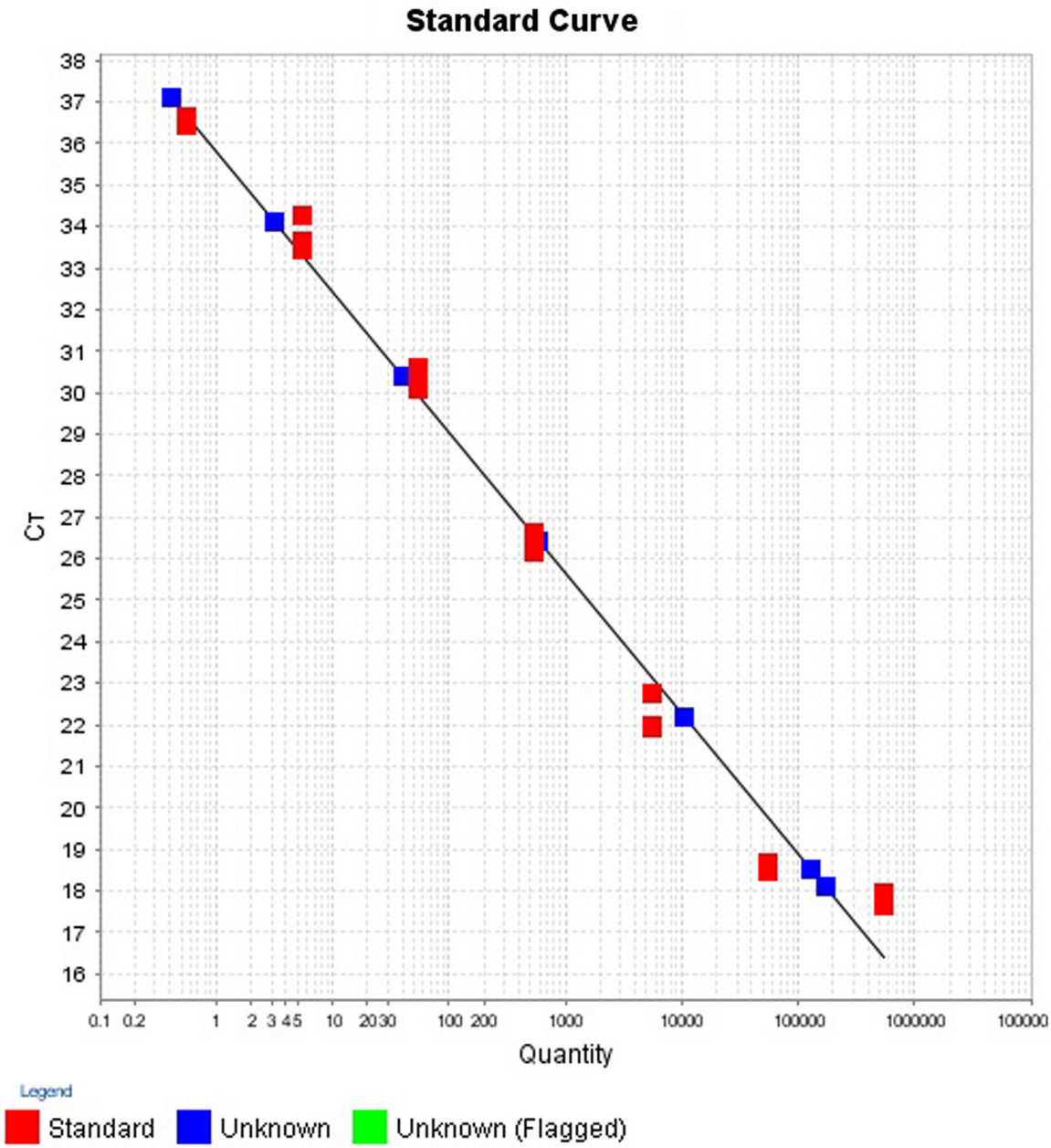

FIGURE 2 | Standard curve for the quantification of samples in first replicate run.

Calculation of the Physical Parameters of Primers

Primer quality was calculated by the consecutive summation of the points according to the parameters: total sequence and purine–pyrimidine sequence complexity, the melting temperatures of the whole primer, and of the 12 bases from each of the terminal 3′ and 5′. The melting temperature of the 12 bases at the 3′ terminus is calculated by nearest neighbor thermodynamic parameters (26). Linguistic complexity measurements (Eqs 1–3) were performed using the alphabet-capacity *L*-gram method (27, 28). The Tm was calculated by the nearest neighbor thermodynamic parameters (26, 29). The optimal annealing temperature (*T*a) was calculated by the Eq. 4 (30).

Real-time PCR Conditions

A Mastermix consisted of 12.5 µL 2× Power SYBR green® PCR master mix containing passive reference of ROX dye (Applied Biosystems, Life Technologies, Warrington, UK), 2.5 µL of 100 nM each sense and antisense primer, 2.5 µL of DEPC treated H_2O, and 5 µL of template DNA. The thermal conditions were maintained under the following conditions: polymerase activation at 95°C for 5 min, followed by 40 cycles of 30 s at 95°C for

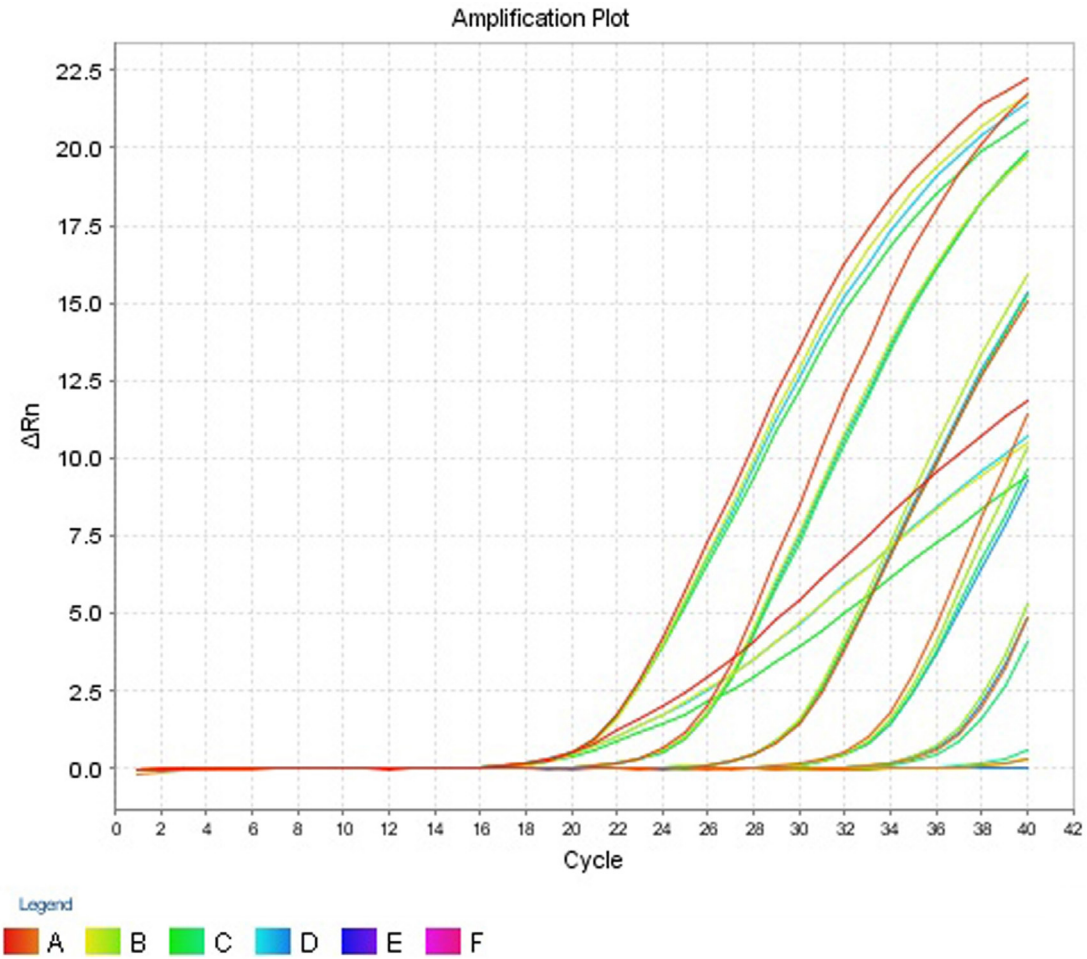

FIGURE 3 | Amplification plot (ΔRn vs Cycle) for testing the sensitivity and precision of the second replicate run.

and 1 min at 60°C. The real-time PCR was performed using the machine Applied Biosystems StepOne™ (48-well).

Specificity of the qPCR Assay

In order to investigate the capability of the assay to distinguish between target and non-target, DNA from 10 isolates of *E. coli*, 5 isolates of *Enterococcus* spp., 6 isolates of *Salmonella* spp., 3 isolates from *Vibrio* spp., and 7 isolates of *V. cholerae* were used as templates. The concentration of all DNA samples from the isolates was kept almost same (approximately 10 ng/μL) by diluting with DEPC-treated water or concentrating by DNA concentrator (Eppendorf Concentrator 5301).

Melt Curve Analysis and Detection of Non-Specific Products

Four dilutions of two *V. cholerae* strains were subjected to qPCR as stated above, and the reaction mixtures containing the SYBR Green PCR products were gradually warmed to 95°C at a ramp rate of 0.3°C/s with continuous fluorescence acquisition. The melting curves were created by plotting the derivative reporter vs the temperature. The melting curve analysis was performed with duplicates of four serial dilutions of template DNA ranging from 10^6 to 10^3 gene copies per reaction using the ABI software version 2.2.2. The SYBR green PCR products were also resolved for identity in 1.5% agarose gel by electrophoresis.

Sensitivity and Limits of Detection (LOD)

The DNA sample of *V. cholerae* was then serially diluted (10-fold) upto 7-$\log_{10}$ (5.46×10^5 copy numbers down to 5.46×10^{-1}) in DEPC-treated water. Five microliters from each dilution were used as template for detection. Distilled water was used as no template control.

Calibration Standards for Standard Curves

To estimate the number of cells in a reaction, the mass of a single bacterial genomic DNA was calculated. The genome size of one *V. cholerae* was 4,033,460 bp (NCBI Genbank10952301). The molecular mass of the genome was found by multiplying the genome size with the mass of base pair. The molecular mass

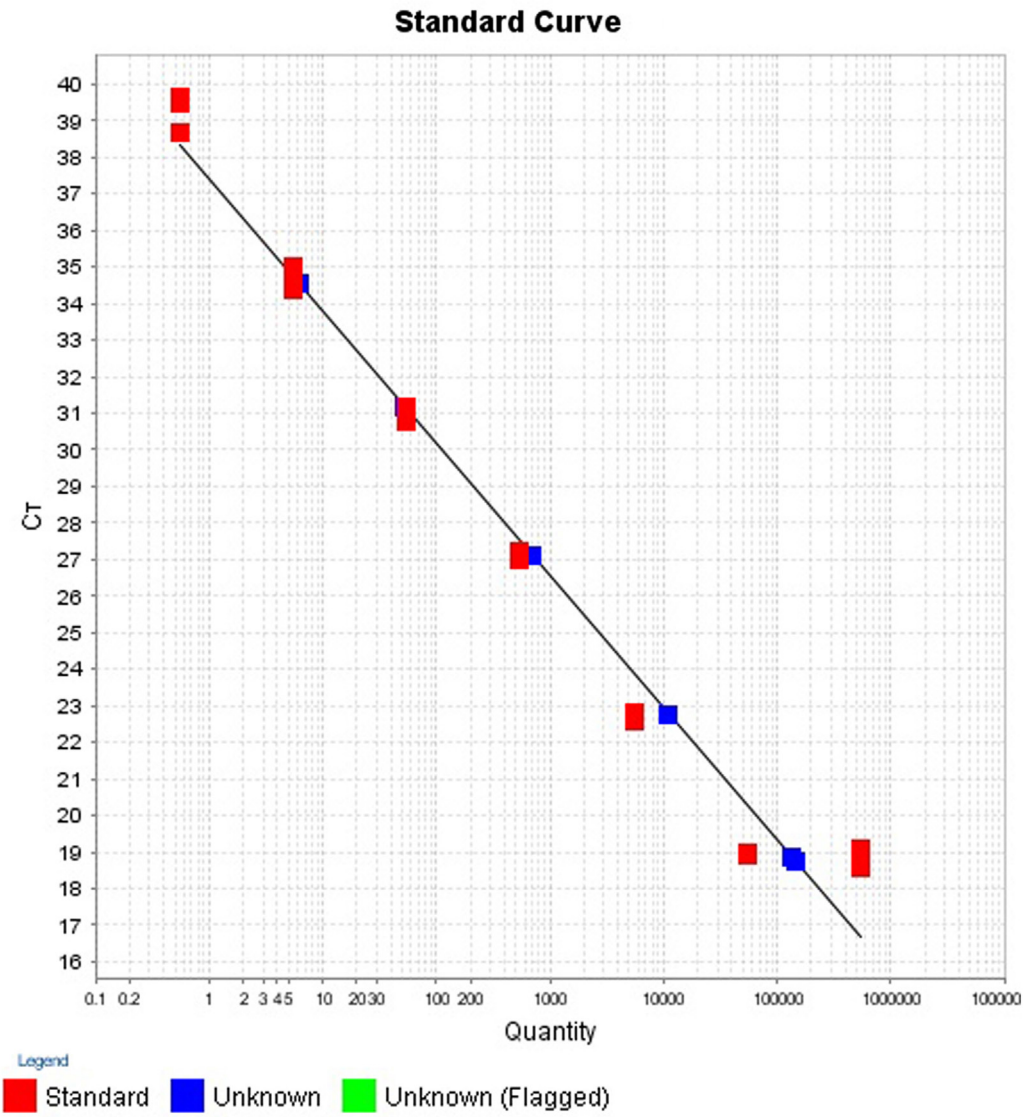

FIGURE 4 | **Standard curve for the quantification of samples in second replicate run**.

of *V. cholerae* was found to be 4.52 fg. The starting concentration of each stock DNA was measured by ColibriMicrovolume Spectrometer (Titertek-Berthold, Berthold Detection Systems GmbH, Bleichstrasse, Pforzheim, Germany) at absorbance 260 nm. To establish the number of cells in final reaction mixture, the stock concentration was divided by the molecular mass of the specific bacteria. The 7-log serial dilution (1:10) of the stock DNA was prepared in triplicate and the corresponding cell numbers were calculated in the final PCR reaction mixture.

Repeatability and Reproducibility

The precision of the PCR assays was evaluated for dilutions ranging from 5.46×10^5 gene copies per reaction down to 5.46×10^{-1} copy numbers. The dilutions were tested in four replicates in two separate PCR runs. The SD of the C_T values of each concentration was then calculated by using Eqs 1 and 2.

$$\mathrm{SD} = \sqrt{\sum \frac{(C_T - \overline{C_T})^2}{n}} \quad (1)$$

where $\overline{C_T}$ is the mean C_T value and n is the number of observations. The value obtained was used to calculate the coefficient of variation, CV, with Eq. 2.

$$CV = \frac{SD}{\overline{C_T}} \quad (2)$$

The intra-assay precision (repeatability) was assessed by calculating the coefficient of variation (CV%) for individual runs. The inter-assay precision (reproducibility) was calculated

by determining the coefficient of variation (CV%) of both runs combined.

Ethical Clearance

The study did not involve any human or animal related issues. Therefore, we did not seek any ethical clearance in this study. Besides, the lab is facilitated with biosafety level II functions. The test and control strains of this study fall under the BSL II category.

RESULTS

Physical Parameters of Primers

The physical parameters of the primers obtained are summarized in **Table 3**. Sequence quality and T_M 12 bases at 3′ end of both forward and reverse primers, LC and length of forward primer, and CG% of reverse primer were all in the ideal range (see **Table 3**). All the others parameters were within the default range.

Repeatability and Reproducibility

The intra- and inter-run precision obtained has been summarized in **Table 4**. The coefficient of variation for the first replicate varied from 0.24 to 1.32 and for the second replicate the CV% ranged from 0.48 to 1.1. The CV% for the inter-run reproducibility varied from 1.08 to 3.79. The amplification plot and standard curve have been shown (**Figures 1**–**4**).

Sensitivity and LOD

The LOD or analytical sensitivity was found to be 5.46 copies since among 8 replicates. The 5.46 was the lowest gene copies that were consistently detected. For higher dilution, i.e., 0.546 copy number, the assay failed to register a C_T value in 2 of the 8 replicates.

Specificity

The assay registered C_T values which ranged from 18.778 to 19.697 for the 4 *V. cholerae* strains and was detectable in the amplification plot (**Figure 5**). Two *E. coli* strains, EHEC and EIEC, had C_T values of 35.073 and 38.439, respectively. The C_T values for all other strains were undetermined. Strains which had C_T values of less than 35 were considered as *ompW* positive. Hence, the assay was able to correctly detect *V. cholerae* and gave a negative result for all other strains, thus proving the assay was *V. cholerae* specific. The results have been summarized in **Table 5**.

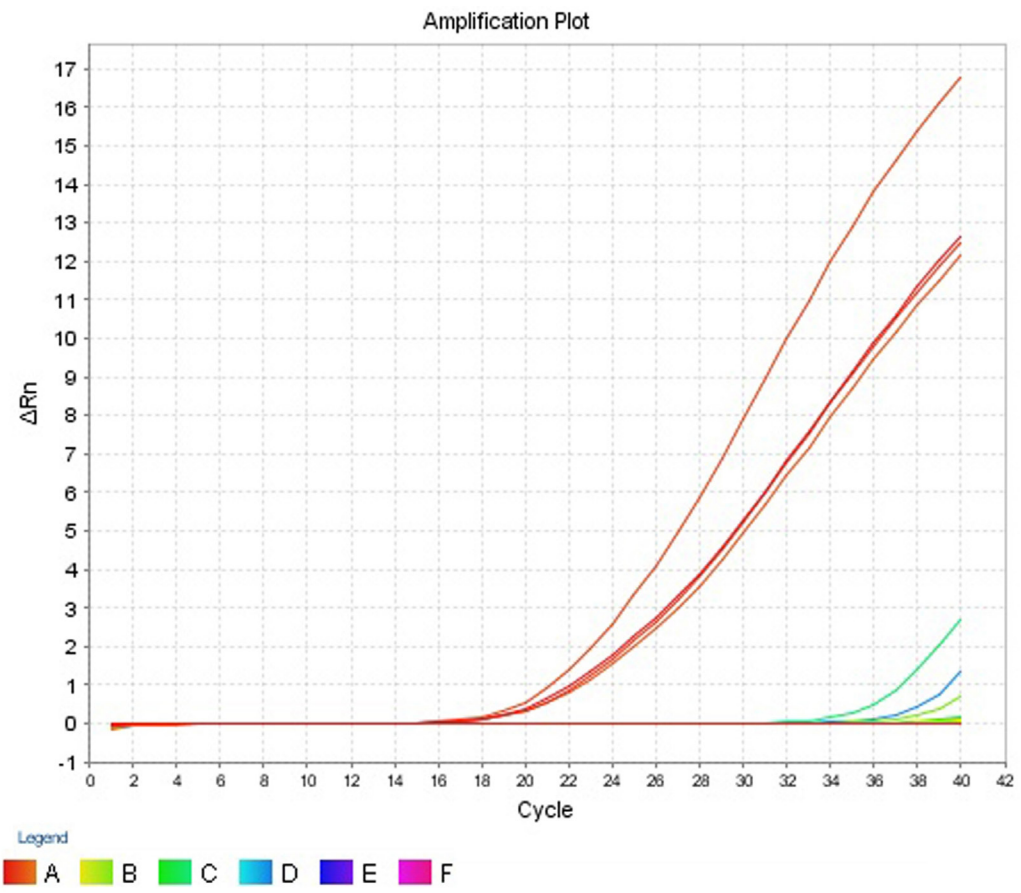

FIGURE 5 | Amplification plot (ΔRn vs Cycle) obtained for specificity test.

Melt Curve Analysis and Detection of Non-Specific Products

In the melt curve (**Figure 6**), a single distinct peak was seen, indicating that all the PCR products had similar Tm values which was approximately 78.46°C. Agarose gel electrophoresis of SYBR green PCR products gave a single distinct band of about 191 bp (**Figure 7**). It could be concluded that neither secondary non-specific products nor primer dimers were formed.

DISCUSSION

We have developed a real-time assay with designed primers for the detection and quantification of *V. cholerae*. The assay was based on SYBR Green PCR Mastermix and targeted the *ompW* gene, which is present in all species of *V. cholerae*. Initially, the physical properties of primers were assessed, followed by validation of sensitivity, precision, specificity, and melt curve analysis.

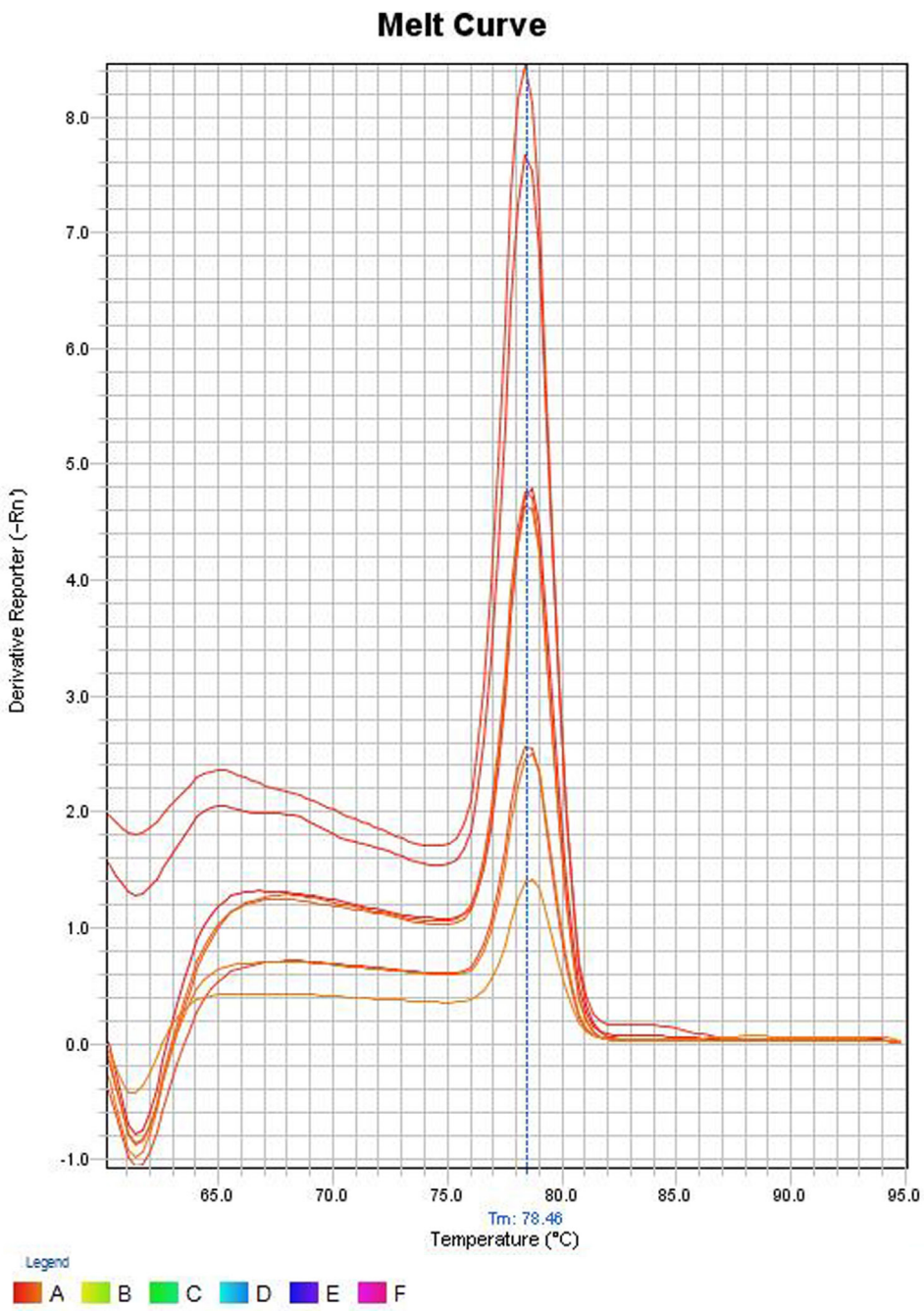

FIGURE 6 | Melt curve of SYBR green PCR products. The *Y*-axis represents the derivative reporter (–Rn) while *x*-axis represents the temperature (°C). The figure shows a melting temperature (31) of human *ompW* PCR products as 78.46°C.

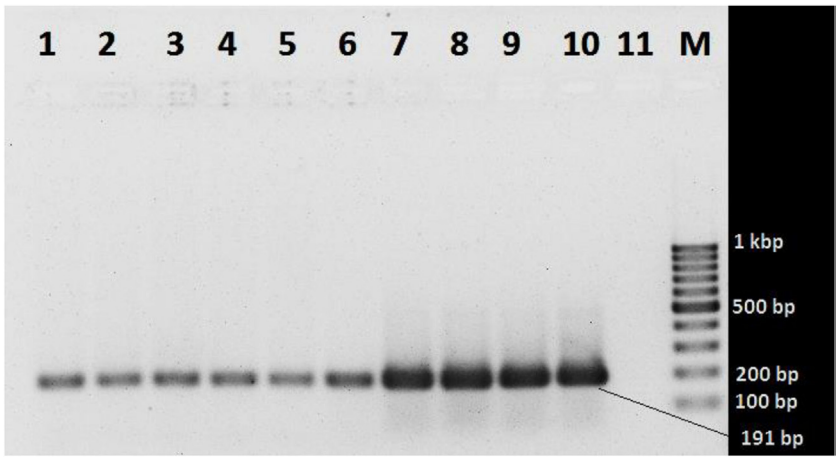

FIGURE 7 | **Agarose gel electrophoresis of SYBR green PCR products**. Lanes 1 and 2 (10^3 gene copy): *Vibrio cholerae* O1 ATCC N16961 and *V. cholerae* O139 ATCC NIHC0270, respectively; lanes 3 and 4 (10^4 copies): *V. cholerae* O1 ATCC N16961 and *V. cholerae* O139 NIHC0270 ATCC, respectively; lanes 5 and 6 (10^5 copies): *V. cholerae* O1 ATCC N16961 and *V. cholerae* O139NIHC0270ATCC, respectively; lanes 7 and 8 (10^6 copies): *V. cholerae* O1 ATCC N16961 and *V. cholerae* O139NIHC0270ATCC, respectively; lanes 9 and 10 (10^7 copies): *V. cholerae* O1 ATCC N16961 and *V. cholerae* O139NIHC0270ATCC, respectively; Lane 10 (M): molecular weight marker (100 bp DNA Ladder, Karl Roth, Germany), 11 no template control.

TABLE 5 | Detection of *ompW* gene for specificity test.

Sr#.	Species	Collection or isolation number	Function of the strains	Origin	C_T value	*ompW* presence
1	*Escherichia coli* (*E. coli*)	[a]ATCC AN33859	Test strain	Clinical	U	–
2	*E. coli* EPEC	ATCC B170	Test strain	Clinical	U	–
3	*E. coli* EAEC	ATCC MG1214C2	Test strain	Clinical	U	–
4	*E. coli* ETEC	ATCC MGL-IC1	Test strain	Clinical	U	–
5	*E. coli* EHEC	NF 9422	Test strain	Clinical	U	–
6	*E. coli*	MMLA	Test strain	Clinical	U	–
7	*E. coli* EIEC	2 V	Test strain	Clinical	38.439	–
8	*E. coli* ETEC	C600	Test strain	Clinical	U	–
9	*E. coli* EIEC	H2	Test strain	Clinical	U	–
10	*E. coli* EHEC	BH29	Test strain	Clinical	35.073	–
11	*Enterococcus faecium*	T7	Test strain	Environmental	U	–
12	*E. faecium*	B10	Test strain	Environmental	U	–
13	*E. faecium*	B4	Test strain	Environmental	U	–
14	*Enterococcus faecalis*	T11	Test strain	Environmental	U	–
15	*E. faecalis*	B4PE	Test strain	Environmental	U	–
16	*Salmonella* spp.	29	Test strain	Food	U	–
17	*Salmonella* spp.	36	Test strain	Soil	U	–
18	*Salmonella* spp.	19 (b)	Test strain	Food	U	–
19	*Salmonella enteritidis*	A	Test strain	Environmental	U	–
20	*Salmonella typhimurium*	Ifo-3313	Test strain	Environmental	U	–
21	*S. typhimurium*	S1	Test strain	Environmental	U	–
22	*Vibrio parahaemolyticus*	1	Test strain	Environmental	U	–
23	*V. parahaemolyticus*	3	Test strain	Environmental	U	–
24	*Vibrio mimicus*	1	Test strain	Environmental	U	–
25	*V. cholerae* serotype O1 CT^+	ATCC C6706	Control strain	Clinical	19.624	+
26	*V. cholerae* (*VC*) serotype O1 CT^+	ATCC N16961	Control strain	Clinical	19.324	+
27	*VC* serotype O1 CT^-	ATCC SA 317	Control strain	Clinical	19.697	+
28	*VC* serotype CT^+ O139	ATCC NIHC0270	Control strain	Clinical	18.778	+
29	*V. cholerae* non-O1 CT^-	Lab isolate-2P-16	Test strain	Environmental	22.201	+
30	*V. cholerae* non-O1 CT^-	Lab isolate-2P-203	Test strain	Environmental	21.329	+
31	*V. cholerae* non-O1 CT^-	Lab isolate-M-299	Test strain	Environmental	23.706	+

[a]*Reference strains: American Type Culture Collection, ATCC were collected from Laboratory of Molecular Genetics, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). Other isolates were obtained from clinical laboratories of ICDDR,B and Environmental Microbiology Laboratory of University of Dhaka.*
U, undetermined.

The LC describes nucleotide arrangement and composition of a sequence and the likelihood of PCR success of each primer. LC values of 80 and higher serve as excellent candidate primers. The primers developed had LC values of 89 and 95 for reverse and forward primers, respectively. Low-complexity regions such as Simple Sequence Repeats, imperfect direct or inverted repeats, triple-stranded DNA structures, and G/C quadruplexes (32) were unlikely to be formed if primers with high LC values are used. The parameter "Primer Quality" determines the possibility of primer dimer formation since dimers reduces the PQ value. The designed primers had PQ values of 87 and 93 for reverse and forward primers, respectively. Thus, these high values suggest that self-complementarity was not apparent. Two terminal C/G bases, recommended for increased PCR efficiency (33) were present in the designed primers.

The efficiency of a PCR assay is the amount of DNA that is amplified in each cycle. An efficiency of 100% indicates the target DNA has been doubled. The efficiencies obtained for the replicates 1 and 2 were 89.16 and 97.37%, respectively. Generally, efficiencies ranging from 90 to 100% are considered to be satisfactory. Inadequate primer design, production of non-specific amplicons and primer dimers may be responsible for reduced efficiencies (34). This is, however, only an estimate of the PCR efficiency and a real test sample, such as food, may contain inhibitory substances that decrease the PCR efficiency (35).

TABLE 6 | Evaluation of the assay using direct environmental samples.

#SL	Strain	Dilution	C_T	Sample type
1	*Vibrio cholerae* CT+ O139	10^5	16.88	Spiked drinking water
2	*V. cholerae* CT+ O1	10^5	16.87	Spiked drinking water
3	*V. cholerae* CT+ O1	10^4	20.18	Spiked drinking water
4	*V. cholerae* CT+ O1	10^3	24.67	Spiked drinking water
5	*V. cholerae* CT− non-O1/non-O139	10^4	20.15	Spiked drinking water
6	*V. cholerae* CT− non-O1/non-O139	10^3	23.97	Spiked drinking water
7	–	–	U	Unspiked drinking water
8	–	–	U	Unspiked drinking water
9	*V. cholerae* CT+ O139	10^5	16.84	Spiked pond water
10	*V. cholerae* CT+ O1	10^5	16.84	Spiked pond water
11	*V. cholerae* CT+ O1	10^4	20.85	Spiked pond water
12	*V. cholerae* CT+ O1	10^3	26.81	Spiked pond water
13	*V. cholerae* CT− non-O1/non-O139	10^4	20.29	Spiked pond water
14	*V. cholerae* CT− non-O1/non-O139	10^3	24.83	Spiked pond water
15	–	–	U	Unspiked pond water
16	–	–	U	Unspiked pond water
17	*V. cholerae* CT+ O139	10^5	16.75	Spiked boiled rice
18	*V. cholerae* CT+ O1	10^5	16.37	Spiked boiled rice
19	*V. cholerae* CT+ O1	10^4	20.67	Spiked boiled rice
20	*V. cholerae* CT+ O1	10^3	24.19	Spiked boiled rice
21	*V. cholerae* CT− non-O1/non-O139	10^4	20.34	Spiked boiled rice
22	*V. cholerae* CT− non-O1/non-O139	10^3	26.78	Spiked boiled rice
23	–	–	U	Unspiked boiled rice
24	–	–	U	Unspiked boiled rice
25	*V. cholerae* CT+ O139	10^5	16.75	Spiked shrimp
26	*V. cholerae* CT+ O1	10^5	16.33	Spiked shrimp
27	*V. cholerae* CT+ O1	10^4	21.00	Spiked shrimp
28	*V. cholerae* CT+ O1	10^3	23.97	Spiked shrimp
29	*V. cholerae* CT− non-O1/non-O139	10^4	20.37	Spiked shrimp
30	*V. cholerae* CT− non-O1/non-O139	10^3	25.36	Spiked shrimp
31	–	–	U	Unspiked shrimp
32	–	–	U	Unspiked shrimp
33	–	–	U	No template control
34	*V. cholerae* CT+ O1	10^6	10.13	Positive control

The precision of the assay was assessed by calculating both repeatability (intra-assay precision) and reproducibility (inter-assay precision). The coefficient of variation (CV%) for the repeatability ranged from 0.24 to 1.32 for both replicates. The CV% for the reproducibility varied from 1.08 to 3.79. The reproducibility is an important parameter since changed conditions such as different equipment and operators might affect the outcome. Pipetting and other human errors might account for poor precision. The precision usually increases with decreasing gene copy concentration (34) but this pattern was not observed for the developed assay. Retesting is required if the % CV of the PCR replicates exceeded 30% (36). All the CV% values for the assay were acceptable.

Specificity is ability to detecting chosen gene in the presence of non-specific DNA (34). The specificity is an important parameter since, in clinical and food samples, DNA from a wide range of organisms might be present. The developed assay was able to correctly detect the 7 *V. cholerae* and gave C_T values that ranged from 18.778 to 23.706. Though the assay did not give any C_T values for the 22 non-*V. cholerae* strains (**Table 5**), two *E. coli* strains—*E. coli* EIEC 2V, *E. coli* EHEC BH29 showed C_T values of 38.439 and 35.073 respectively. Since the cut point C_T value for ABI StepOne real-time machine is between >8 and <35, these C_T values of *E. coli* strains can be considered as negative results.

The LOD is the lowest gene copy number that the assay is able to consistently detect (37). A satisfactory LOD is 10 gene copies per reaction, and the assay was able to meet this requirement by consistently detecting 5.46 copies of the gene. The LOD sheds light on how sensitive the assay is.

The assay was evaluated for its ability to detect *V. cholerae* O1/O139 and non-O1/non-O139 in food and environmental samples over different dilutions. It was observed that drinking water, pond water, shrimp, and boiled rice spiked with these strains registered C_T values that ranged from 16.33 to 26.78 (**Table 6**).

To assess if the assay is affected by interference from non-target DNA, unspiked drinking water, pond water, shrimp, and boiled rice were examined by qPCR. Before this assessment, absence of *V. cholerae* was confirmed. Results showed that no C_T values were obtained for these unspiked food and water samples. Thus, this assay is suitable for detecting both *V. cholerae* O1/O139 and non-O1/non-O139 in food and environmental samples since non-specific amplification was not seen in negative controls.

Melt curve analysis was done to assess whether secondary products such as primer dimers or non-specific products were formed. The melt curve gave a single peak with a Tm value of about 78.46°C. Agarose gel electrophoresis of SYBR Green PCR products gave a single band at 191 bp. These results suggest that

the amplification was specific and only one type of amplicon was produced. Non-specific products hamper the efficiency of the assay and affect precision. Non-specific products were absent suggests that the primer design was adequate. The primers were specific and intended amplicons were produced. We can conclude the primers were not complementary to one another since primer dimers were not produced.

Many assays have been developed for detection and quantification of *V. cholerae* (19–24). Though impressive none of these presented any statistical figures (such as coefficient of variation) which would inform us about the reproducibility and repeatability. Many of these assays did not undergo melt curve analysis or the PCR products were not subjected to agarose gel electrophoresis and hence we do not know their status regard the formation of non-specific products. Since they are multiplex in nature, they add to the cost and hence are not suitable for purposes. For instance, during quality control testing of seafood where only quantification is required to see if the levels in food is acceptable to the standards set by the governing bodies.

An extremely impressive multiplex real-time assay has been developed by Bliem and colleagues (38). The assay is multiplex in nature, and hence the use of multiple primers might add to the cost. The assay developed by Bliem and colleagues had inter-assay variance of 2–28% for *ompW*. But our assay, which utilizes a primer for *ompW* gene with different sequence, was more precise with inter-assay variance of 1.08–3.79.

Future objectives of our study might include the optimization of this assay to detect and quantify *V. cholerae* in food, water, and clinical samples. Some samples might contain inhibitory substances that decrease PCR efficiency (35) and hence optimization of the methods involving sample processing, DNA extraction, and assay itself might be required.

AUTHOR CONTRIBUTIONS

PJ and AB are the principal investigators of the project and contributed to the manuscript revision and final version approval to be published. RR conducted the study in the laboratory, performed statistical analysis, and wrote the first draft of the manuscript. ST contributed to revising the manuscript critically for important intellectual content. JF contributed to the study designing, implementation, manuscript reviewing, and revising it critically. The authors have agreed to be accountable for answering questions related to the accuracy and integrity of the work appropriately done.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DECLARATION OF CO-AUTHORSHIP

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This declaration concerns the following article:
Development and Validation of a Novel Real-time Assay for the Detection and Quantification of Vibrio cholerae

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	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	B

**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 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

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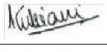
23/11/18	Suhella Mohan Tulsiani	PhD	
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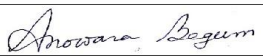
07/01/19	Anowara Begum	Professor	

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Comparative assessment of fecal contamination in 'improved' piped-to-plot communal source and point-of-drinking water

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Abstract

The aim of this study was to assess the water quality piped-to-plot communal source and point-of-drinking water in household in a low-income urban area of Bangladesh. Drinking water samples were taken directly from household drinking water i.e. point-of-drinking (n=2,514) water and from their linked communal source water (n=1,926) for basic water quality analysis for *Escherichia coli* (*E. coli*) using membrane filtration (MF) culture method. A subsample of the collected water samples was tested for the presence of *Vibrio cholerae* (*V. cholerae*) and for the presence of *E. coli* by the quantitative Polymerase Chain Reaction (qPCR) to compare the detection rate of qPCR with MF culture method. In the same-day paired data of connected communal source and point-of-drinking water showed that the level of fecal contamination increased from communal source to point-of-drinking water of the linked households in 51% (626/1,236) of samples. Most importantly, for a total of 38% of

point-of-drinking water samples, communal source had *E. coli:* 0-10 CFU/100 mL and this level had subsequently increased in the point-of-drinking water samples Results also showed that 78% of (260/333) treated and 76% (1,662/2,175) of non-treated point-of-drinking water were found to be contaminated with *E. coli.* Comparison between bottle vs other wide mouth vessels (i.e. glasses, mugs, jugs) showed significant lower odds (p=0.007, OR=0.68, [0.51-0.90]) of fecal contamination for >100 *E. coli*/100 mL compared to other drinking vessels. The detection rate of *E. coli* in drinking water samples was 93% by qPCR and 65% by MF culture method in the same water samples. Our study reveals that recontamination and post contamination at point-of-drinking plays significant role for water contamination at domestic domain. To reduce domestic transmission of fecal-oral pathogens, hygiene education efforts should target to improve kitchen hygiene practices including safe handling of drinking water after treatment and promotion of narrow mouth drinking vessels can be encouraged. Additionally, certain methodological approaches can be included for accurate assessment of water quality i.e. qPCR as a complement of MF method and detection of other pathogens.

Introduction

In 2015, Global Burden of Disease (GBD) study placed unsafe water as 14th among global health risks [1]. Globally, 1.8 billion people lack microbiologically safe drinking water sources, with the majority living in low- and middle-income countries (LMICs) [2]. Prüss-Ustün et al. (2014) [3] estimated the burden of diarrheal disease and reported that 502,000 deaths were associated with unsafe or inadequate drinking water. The United Nations International Children's Emergency Fund (UNICEF) reported in 2017 that 71 million people in Bangladesh lack access to safely managed water [4]. Inadequate drinking-water, sanitation and hygiene is not only responsible for diarrhoeal disease but also has complex impacts on undernutrition, growth stunting and environmental enteropathy, with subsequent life-long consequences [5].

Drinking water plays an important role in transmission of diarrheal diseases including cholera. Cairncross et al. (1996) classified transmission routes of water related diseases into two major domains: the domestic domain and the public domain [6]. ‘Domestic domain’ refers to the

area within a household premises and ‘public domain’ includes public places such as work, education and recreation sites, as well as streets and fields. This paradigm greatly helped to clarify scientists and practitioner to control diseases by environmental interventions. However, numerous disputes exist on the relative importance of the effectiveness of water domestic domain versus public domain interventions on health effects. In the Cochrane review, Clasen et al. (2015) stated that controlling microbial contamination of in-house drinking water might be an important interim strategy until a safe, reliable piped-in water connection is provided to the household [7].

The World Health Organization/United Nations Children’s Fund (WHO/UNICEF) Joint Monitoring Programme (JMP) for Water Supply and Sanitation tracked global coverage of safe drinking since 1990, by classifying water sources into ‘improved’ and ‘unimproved’ sources. The ‘improved’ source was defined to the sources those had some measures of protection from outside fecal contamination (i.e. piped supply, boreholes, protected dug wells, protected springs, and rainwater) and presumed to pose no health risk [8]. This definition did not include any measure of the consistency of access, the microbiological or chemical quality of water. During the end of MDG monitoring period in 2015, JMP noted that the use of an ‘improved’ source is not an adequate proxy for ‘safe drinking water’ as there was a growing body of evidence of fecal contamination in several countries using ‘improved’ drinking water supplies [9-12] . Therefore, as part of Sustainable Development Goals (SDGs), in 2015 JMP proposed to include a new indicator, ‘safely managed drinking water’ which is defined as the use of an ‘improved’ drinking water source that is located on the premises, available when needed and free from fecal contamination [13] with an expectation of better health outcome.

Researchers have repeatedly observed that the microbiological quality of water change over the course of collection, transport, home storage and consumption [14-16]. Consequently, in-house contamination may reverse the health benefits that are gained by improvements in community water supply. However, there have been very few attempts globally to measure the effect of piped-to-plot improved water sources on in-house drinking water immediate before consumption, specifically in Bangladesh there is no studies to date that showed the effect of on-premises improved water sources on point-of-consumption. The health risk of

unsafe drinking water was mostly investigated by simply measuring the coliform/fecal coliform test by culture-based method [16-22] which may not adequately address the actual health risk from water since several human pathogenic bacterial species have been found to adopt a unique survival strategy, the viable but non-culturable (VBNC) state, which supports long-term survival under adverse environmental conditions. Unlike culturable cells, bacteria in the VBNC state fail to grow and develop into colonies on the routine bacteriological media, although they are alive [23]. The advent of quantitative Polymerase Chain Reaction (qPCR) technology has facilitated these limitations to overcome, by its ability to detect and quantify bacterial load of culturable and non-culturable cells. However, the use of qPCR for assessment of water quality was limited to the recreational water and surface water quality [24-26] ; hence the water quality of household drinking water remains unexplored. Therefore, the primary objective of this study was to assess the water quality of 'improved' (i.e. piped-to-plot) communal source and point-of-drinking water (i.e. in-house drinking water immediate before consumption using preferred drinking vessels) in a low-income urban area of Bangladesh. This study also aimed to compare the drinking water quality between qPCR and culture-based methods to identify more accurate and useful method for detection of pathogens as a secondary objective.

Methods and Materials

Study site and population

We conducted this study in East Arichpur, located in Tongi Township of Dhaka city of Bangladesh (Figure 1). The population density of Arichpur is high (>100,000 residents per km^2) and it is a low-income urban community encompassing 1.2 km^2 [27]. East Arichpur has a history of outbreak of waterborne diseases including cholera [27, 28]. Many nuclear families share one room and up to 10-15 families may share a stove, toilet, and water source [27]. On its southern edge, the community is bordered by one of the peripheral rivers of Dhaka city, Turag River which is heavily polluted due to sewage dump (Figure 1).

Types of water sources in Arichpur

The communal sources of Arichpur community were dependent on government provided public water supply and private water supply. These two types of 'improved' (piped-to-plot) ground water sources are: public-supply (locally known as 'WASA' as it stands for Water Supply and Sewerage Authority and installed by the municipal government), private supply (locally known as 'submersible pump' supply and installed by the individual owner/s). WASA water is supplied to the households through underground networks of pipes. Submersible water is distributed to the households using over ground networks of pipes. All the communal sources of our studied households abstracted groundwater and the depth was >85 meters. The area around the pumps is not usually protected with a wall and floor made of concrete.

Data collection

In this study, a total of 430 households were enrolled which were connected to 78 communal sources. A research team collected water samples from communal source and point-of-drinking (i.e. in-house drinking water) water simultaneously from September 2014 to December 2015 during routine visits at 6-week intervals as a part of a longitudinal study of diarrhea incidence and water use [29]. The term 'point-of-drinking' was specified for the in-house drinking water instead of the commonly used term 'point-of-consumption' which broadly refers to the use of water for various purposes such as bathing, cooking, hand washing and drinking. Point-of-drinking water samples were taken from the household members' preferred drinking utensils (i.e. a mug, glass, bottle, jug, or pitcher), as they normally use to drink water. The water samples from communal sources were taken directly from taps attached to the communal pumps. In the absence of a direct tap, samples were collected from taps attached to the nearest over-ground reservoir connected to the pump. Information on home-based water treatment (i.e. boiling, filtration, adding alum etc.) were collected from the participant during water sample collection from point-of-drinking. The coordinates of sample collection sites (households and communal sources) were obtained using a global positioning system (GPS). Q-GIS software was used to locate the sites on a Google map (Figure 1).

Microbiological procedures for sample collection and sample processing

The team collected a total of 2,514 point-of-drinking water samples and 1,494 communal source water samples. A volume of 150-200 mL water samples was collected both at source and point-of-drinking. Water samples were collected using pre-sterilized wide-mouth sampling bottles (SPL Life Sciences, Korea) and transported to the Environmental Microbiology Laboratory, University of Dhaka within 2-4 hours of collection maintaining temperature in cool box.

To achieve our primary objective, basic water quality analysis was assessed by conducting membrane filtration (MF) culture method for detection of *Escherichia coli* (*E. coli*) in all the collected water samples. To achieve our secondary objective a comparative evaluation of qPCR and MF culture method for detection of *E. coli,* and the reliability of *E. coli* for detection of another pathogen i.e. *Vibrio cholerae* (*V. cholerae*) was tested in a subset of samples. Briefly the microbiological methods are described below:

Assessment of water quality using membrane filtration (MF) culture method: Laboratory technicians filtered aliquots of 100 mL water samples were through 0.45 µm porous 47 mm diameter white gridded membrane filters (S-Pak, Merck Millipore, Germany), placed the membrane filters on modified Thermotolerant *E. coli* agar (m-TEC agar, Oxoid, UK) plates and incubated the plates at 44.5 +/- 0.5° C for 18-24 hours. Typical reddish-purple or magenta colonies of *E. coli* were enumerated and recorded as colony forming units (CFUs) per 100 ml of water [30]. All the water samples (a total of 4,008 samples: 2,514 samples from point-of-drinking and 1,494 samples from communal source water) were examined for the presence of fecal contamination by MF culture method.

***Assessment of water quality by quantitative Polymerase Chain Reaction (qPCR)*:** Aliquots of 1 mL of water were taken aseptically from a subset of samples (a total of 676 samples: 404 samples from point-of-drinking and 272 samples from communal source water), inoculated in vials containing 2 mL of nutrient broth (NB) enrichment medium and incubated at 37°C for 4 hours to recover the cells in lag phase or injured and stressed condition. DNA extraction was carried out from 1 mL of enriched culture suspension. *E. coli* was detected using quantitative Polymerase Chain Reaction (qPCR). *E. coli*-specific housekeeping gene *uidA* was the target

gene, and qPCR procedures were performed using the described method in Ferdous et al. (2016) [31].

***Assessment of water quality by detection of V. cholerae and E. coli*:** From a subset of samples (1,463 water samples: 1,082 samples from point-of-drinking and 381 from communal source water) aliquots of water samples were added to 10 mL of alkaline peptone water (APW), incubated at 37° C for 18–24 hours [32]. After overnight incubation, 1 mL culture suspension was taken, total DNA was extracted, and DNA templates were subsequently tested for the presence of species-specific *ompW* gene of *V. cholerae* by PCR [33]. A total of 143 samples (110 samples from point-of-drinking and 33 from communal source water) were found positive for *V. cholerae* [34] which were also tested for the presence of *E. coli* by using qPCR and MF culture method.

Data analysis

Water quality was considered as uncontaminated if *E. coli*/100 mL of water sample was zero and contaminated if *E. coli*/100 mL of water sample was ≥ 1. Descriptive statistics used for calculating proportion of *E. coli*. The distribution of water samples were categorized into four risk groups following the WHO risk categories: low risk/safe (< 1 *E. coli*/100 mL), intermediate risk (1–10 *E. coli*/100 mL), high risk (11–100 *E. coli*/100 mL), and very high risk (> 100 *E. coli*/100 mL) for human consumption [35]. For continuous variables within the 'intermediate' 'high' and 'very high' risk groups, means and medians were calculated. Logistic regression tests were conducted to estimate the odds ratios (ORs) for the association of household characteristics (treatment and drinking vessels types) with each of the WHO risk groups. For estimating the odds ratio between treated vs non-treated drinking water, non-treated water was used as the referent group. For comparing the odds ratios within each of the drinking vessels (mug, glass, bottle, jug), bottle water was used as referent group. The difference of contamination from communal source to the linked households' point-of-drinking water were calculated for those samples that were collected on the same day by deducting the number of communal sources' *E. coli* CFU/100 mL from the point-of-drinking water *E. coli* CFU/100 mL counts. The difference of positive integer values was denoted as 'in-house contamination', the difference of zero value

was indicated as ‘no-change’ and the difference of negative integer values was denoted as ‘die-off’.

Results

E. coli was detected in 77% (1,926/2,514) of point-of-drinking water samples and 58% (866/1,494) of source water samples. A total of 13% (333/2,514) treated water samples were collected throughout the study period from 27% (115/430) of the study households. The study households used mug, glass, bottle, jug, pitcher and *bodna* (almost similar to pitcher) as point-of-drinking water vessels. From the drinking vessels samples of point-of-drinking, 53% (1,335/2,514) was mug, 29% (726/2,514) was glass, 14% (344/2,514) was bottle and 3% (74/2,514) was jugs. Point-of-drinking water contaminated with *E. coli*, stratified by various characteristics were represented in Table 1.

Of the 78 communal sources, 3 sources were WASA pumps and 75 sources were submersible pumps. Communal source water contaminated with *E. coli*, stratified by types of communal source and collection points were represented in Table 1.

Water quality assessment by WHO risk categories

Point-of-drinking water was more contaminated than communal source water in all the risk groups and a high percentage of communal source water samples belonged to low risk group (Figure 2). The mean and median of the ‘high’ and ‘very high risk’ group for point-of-drinking water was comparatively greater than the communal source water (Table 2). In point-of-drinking water samples contamination level predominantly belonged to the ‘high risk’ group for both treated, non-treated and vessels types (Figure 3 and Table 3). *E. coli* was found in the treated point-of-drinking water (Figure 4). There was not a noticeable difference in the mean and median range of *E. coli* in the treated and non-treated water (Table 2).

The logistic regression analysis of treated vs non-treated showed significant higher odds of *E. coli* contamination in the ‘intermediate’ risk group (p=0.000, OR=1.68, = [1.28-2.21]) and within the treatment types ‘boiling’ showed significant higher odds (p=0.047, OR= 1.94 [1.01-3.733]) ‘intermediate’ level of contamination than ‘filtration’. When the point-of-drinking water samples were categorized according to drinking vessel significant higher odds for bottle compared to

other vessels was found in the 'low risk' group (p=0.000, OR=1.72, [1.34-2.20]) and similarly significant lower odds for bottle compared to other vessels in the 'very high risk' group (p=0.007, OR=0.68, [0.51-0.90]) was found.

Water quality assessment by same-day paired data

Same day paired data of communal source water and point-of-drinking water of the connected households showed that the level of fecal contamination increased from communal source to point-of-drinking water in 51% (626/1,236) of samples which is included as in-house contamination in Table 4. Of these 51% in-house contaminated samples, 26% (314/1,236) had 'zero' *E. coli* at the communal source. In this 26% (314/1,236) samples that had 'zero' *E. coli* CFU at communal source, CFU range increased to 1-10 in 21% (66/314), CFU range increased to 11-100 in 50% (156/314) and >100 CFU increased in 29% (92/314) of point-of-drinking water samples. Furthermore, for 6% (69/1,236) point-of-drinking water samples, communal source had *E. coli*: 1-10 CFU which increased to *E. coli*>10 CFU in point-of-drinking water and in additional 6% (76/1,236) of point-of-drinking water samples had *E. coli*> communal source having *E. coli* 1-10 CFU.

The comparison between the treated and non-treated point-of-drinking water among the same-day paired data showed that in-house contamination was more frequent (56%) in treated water (Table 5). The comparison within the drinking vessels, among the same-day paired data showed that in-house contamination was less frequent than other drinking vessels (Table 5).

Detection rate comparison by qPCR and MF culture

In the communal source water samples, qPCR method showed 98 % (266/272) positive and MF culture showed 52% (141/272) positive for *E. coli* in the same samples. In point-of-drinking water samples, qPCR method showed 90 % (363/404) positive and MF culture method showed 73% (296/404) positive for *E. coli* in the same samples. The detection rate of *E. coli* in drinking water samples was 93% by qPCR and 65% by MF culture method (Table 6).

Among *V. cholerae* positive communal source water samples, qPCR method showed no detectable *E. coli* in 5 % (1/19) samples and MF culture showed 16% (3/32) non-detectable *E. coli*. In point-of-drinking *V. cholerae* positive water samples, qPCR method showed 8% sample

(3/40) negative for *E. coli* and MF culture method showed 15% (94/110) negative for *E. coli*. The sensitivity of using *E. coli* as an indicator for the presence *V. cholerae* was also higher (94%) by qPCR than MF culture method (87%) (Table 6).

Discussion

Detection and quantification of high percentage of *E. coli* in point-of-drinking water compared to communal source water suggest that fecal contamination is highly prevalent at point-of-drinking water in our study households of Arichpur. Similarly, increased *E. coli* at point-of-drinking compared to communal sources in the same-day paired data reveals that recontamination and post contamination at domestic domain might have occurred. Particularly absence of *E. coli* in the communal source and presence of *E. coli* within the connected household in the same day water sample explicated that communal sources water might not have played any role in contamination of drinking water at households. Furthermore, the findings of high detection rate of qPCR method suggest that using qPCR to measure water contamination might be more reliable molecular method than membrane filtration culture method.

Our study finding reflected the similar finding of other studies conducted by Wright et al. (2004) and Rufener et al. (2010) [36] that reported higher contamination of water in the domestic domain (i.e. point-of-drinking) compared to public domain (i.e. communal source). Our study showed a total of 51% increase of fecal count in point-of-drinking water from the linked communal source on the same day. Specifically, zero *E. coli* in the communal source water but presence of *E. coli* in the connected point-of-drinking water on the same day implied that in these situations, fecal contamination presumably solely originated from in-house contamination. Studies identified that in-house water contamination of domestic domain can occur through several pathways i.e. dirty hands [6, 37], dirty drinking vessels [22, 38] and flies [39-41]. Additionally, fecal contamination circulated within kitchen environment of the domestic domain can contribute in contamination of drinking water and food [42, 43]. However, for the samples where there was *E. coli* in the communal source and the level of contamination had subsequently increased in the point-of-drinking water on the same day, implies that in-house contamination/recontamination together with bacterial re-growth were

possibly responsible for increased count in point-of-drinking water. Bacterial re-growth is defined as a process; i.e. when a small number of microorganisms (fecal coliforms) are provided with a conducive environment (i.e. optimum temperature, concentration of organic nutrients) they can act as seeds and multiply in the elapsed time of water sample collection from the source [44]. However, since all the communal sources of our studied households abstracted groundwater and as groundwater contains very low level of organic nutrients to support the multiplication of bacteria, we anticipate that in-house contamination had more impact than re-growth in the increased count of point-of-drinking. Most importantly, our study findings revealed that for a total of 38% of point-of-drinking water samples were found to be contaminated with increased count of *E. coli* which were connected to the cleaner sources (*E. coli*: 0-10 CFU). Perhaps compromised hygiene behavior of the household members was the key factor for this scenario. These findings can be linked to the findings of Ferdous et al. (2019) [45] that showed human fecal source originated pathogenic *E. coli* prevailed more in point-of-drinking compared to communal source water.

Moreover, our findings of no improvement on drinking water quality after treatment at home, particularly presence of *E. coli* after boiling also signifies that post contamination within household might be an important attributing factor of in-house water contamination at domestic domain. Ideally proper treatment of water would kill all the microbes that arrive from the source and thus should be free of contamination before drinking; nonetheless we have found majority (78%) of treated drinking water to be contaminated. Additionally, the significant higher odds of treated vs non-treated drinking water ($p = 0.000$, OR=1.68, 95% CI= [1.28-2.21]) in the 'intermediate risk group' implies that though the post-contamination occurs after treatment, the contamination level is low (within 1-10 CFU/100 mL). These results indicate that post-contamination occurred in the households, perhaps due to the poorly maintained kitchen hygiene (i.e. poor hand hygiene, uncovering kitchen pans, dirty vessels) that contributed to the treated drinking water contamination, which were also observed in other studies [40, 46, 47]. Moreover, the lower level contamination (1-10 CFU/100 mL) should not be neglected as we have discerned in the Ferdous et al. (2019) [45] that the fecal *E. coli* isolated from these water samples can be pathogenic and it is well documented that certain pathogenic *E. coli* have infectious dose as low as one organism.

Our study found that among the vessels, mug, glass and bottle were widely used by the households. For bottles, significant higher odds (p=0.000, OR=1.72, [1.34-2.20]) of fecal contamination in the 'low risk' group and significant lower odds (p=0.007, OR=0.68, [0.51-0.90]) of fecal contamination in the 'very high risk' group compared to other drinking vessels implies that fecal contamination occurs less frequently in bottles (narrow mouth vessels) and thus safer to use than other mugs and glasses (wide mouth vessels). This analysis was conducted for all the samples without deducting the communal source contamination which might seem biasness for ‘very high risk’ group. However, for the same day paired data where we deducted the communal source contamination from the point-of-drinking water and compared the prevalence of in-house contamination according to vessel types, we found bottle water was the least prevalent for in-house contamination. Our study finding was avowed by Jensen et al. (2002) [20] who provided a 5-week intervention using narrow-necked water pitcher to avoid water recontamination (i.e. through utensils or hands from retrieving water) within the households and found that in-house water improved significantly. In our study it was observed that when household members used a bottle, they usually collected water directly from the tap and then drank from the bottle; however, when they used a glass or mug for drinking, they usually stored water in an intermediate storage vessel. These practices of the household members for using bottles have provided less opportunity for fecal contamination in bottle water at point-of-drinking. The aforementioned findings of our study reflect that point-of-drinking water contamination is much more influenced by recontamination within domestic domain rather than communal source contamination and the provision of piped-to-plot improved water sources did not ensure safe drinking water at point-of-drinking.

On a different note, our study assessed a comparative evaluation of qPCR and MF culture method and found higher detection rate of qPCR than MF culture method for detection of *E. coli* and *V. cholerae*. The higher detection of *E. coli* by qPCR can be demonstrated by qPCR’s ability to detect the target DNA from both culturable cells, viable but non-culturable (VBNC) cells and dead or dying cells [24, 48, 49]. Conversely, the MF culture method showed a lower detection of *E. coli* as it measures only culturable cells and cannot detect VBNC cells. Hence, the proportion of point-of-drinking and communal source water samples that showed zero *E.*

coli by the MF culture method and fell into the safe/low risk group (Figure 2) might not presumably safe and fall into the 'intermediate/high/very high risk' group. Therefore, the non-detectability of the VBNC cells by MF culture can undermine the results and pose a major health concern if the sample contains pathogenic bacteria. Furthermore, we assessed the credibility of *E. coli* as an indicator for the presence of *V. cholerae* by MF culture and qPCR, and found lower (chi-sq test p=0.000) detection rate by MF culture. In addition, we found a number of positive *V. cholerae* water samples where *E. coli* was absent both by MF culture and qPCR. These findings suggest that *E. coli* as an indicator may not be adequate to accurately determine water quality. Furthermore, our findings of higher detecting ability of qPCR methods indicate that MF culture may not be sufficient for absolute determination of water quality. Therefore, testing of several species-specific pathogenic bacteria by qPCR could be useful for regular monitoring of water quality.

One of the limitations of our study is that we found the majority (>70%) of treated drinking water was found contaminated which might have resulted from the respondent's self-reporting bias as our study did not cross check the reported finding with observation. However, our study findings were consistent with the findings from a study in Peru which found that the effect of specific types of treatment (boiling or filtration) did not sufficiently change the water quality in drinking cups.

Another limitation is the result on significant higher possibilities of bottle water for *E. coli* zero CFU contamination should be interpreted with caution as analysis was conducted for all the samples without deducting the communal source contamination. However, for the same day paired data where we deducted the communal source contamination from the point-of-drinking water and compared the prevalence of in-house contamination according to vessel types, we found bottle water was the least prevalent for in-house contamination. Findings of other studies also reported that narrow neck vessel can help to reduce contamination as this produce less opportunity for hand contamination or contamination through spigot [20].

Limitations also include the inability of qPCR to distinguish between dead and viable cells, since qPCR measures target DNA from the organism. However, dead cell estimation still

plays an important role for human health as these cells can produce endotoxins that can cause food poisoning [50]. Nevertheless, to selectively quantify viable cells, a qPCR assay using propidium monoazide (PMA) developed by Taskin et al. (2011) could be used in future research [51].

Conclusion

From our study we can conclude that, provision of piped-to-plot improved water sources did not ensure safe drinking water at point-of-drinking in the household, although it is one of the targets of SDGs to expand the access to piped-to-plot water supply [52]. Fecal contamination remains commonplace in water quality deterioration within the domestic domain, particularly at point-of-drinking. Additionally, treatment of drinking water proved ineffective might be due to compromised kitchen hygiene practices. To reduce domestic transmission of fecal-oral pathogens, hygiene education efforts should target to improve kitchen hygiene practices including repeated cleaning of drinking vessels, safe handling of drinking water after treatment and promotion of narrow mouth drinking vessels can be encouraged. Another conclusion of our study is that depending on a culture-based method and detection of only *E. coli* for assessing water quality can provide spurious results which might not reflect the actual fecal contamination. Application of qPCR method as a supplement of culture-based method and targeting several species-specific pathogenic bacteria can be included for assessing water quality rigorously for providing actual health benefit.

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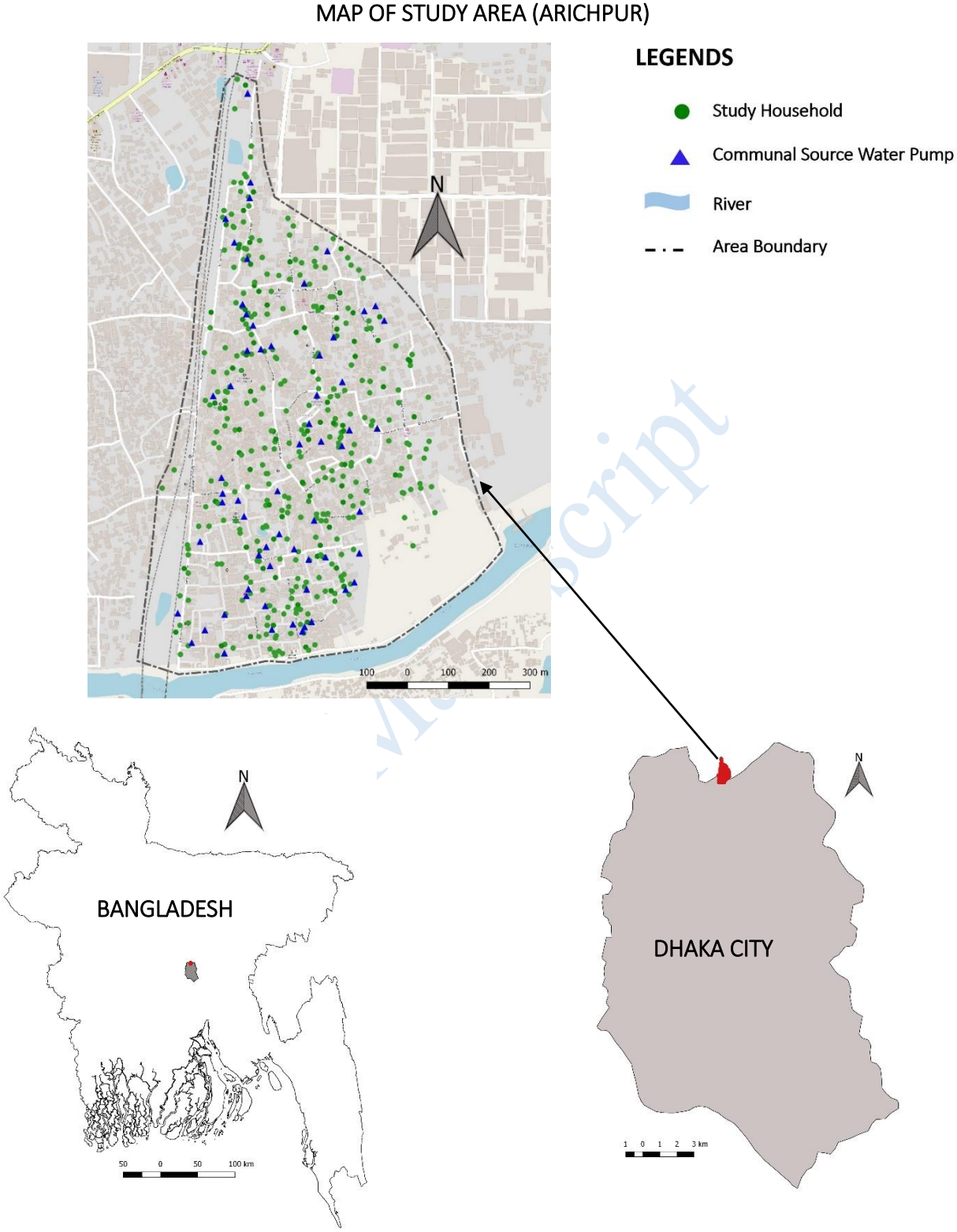

Figure 1. Map of the study area. Distribution of study households and communal source water pumps in the Arichpur area.

Table 1: Presence of *E. coli* in point-of-drinking water and communal source water of the study households, stratified by various characteristics

Characteristics	No. of sample	Contaminated with *E. coli*, n (%)
Point-of-drinking water		
Water treatment		
Yes	333	260 (78)
No	2,175	1,662 (76)
*Modes of water treatment**		
Boiling	254	197 (78)
Filtration	76	59 (78)
*Types of drinking vessels**		
Mug	1,337	1,036 (77)
Glass	726	569 (78)
Bottle	344	232 (67)
Jug	74	62 (84)
Communal source water		
Types of communal water sources		
'WASA' pump	122	73 (60)
'Submersible' pump	1,372	793 (58)
Collection points		
Taps attached to the communal pumps	440	208 (47)
Taps attached to the reservoir connected to the pumps	1,054	658 (62)

*Only 1% of the samples from treated water was from 'boiled and filtration' and 1% of the sample of drinking vessels consist of pitcher and *bodna*, and thus was not included.

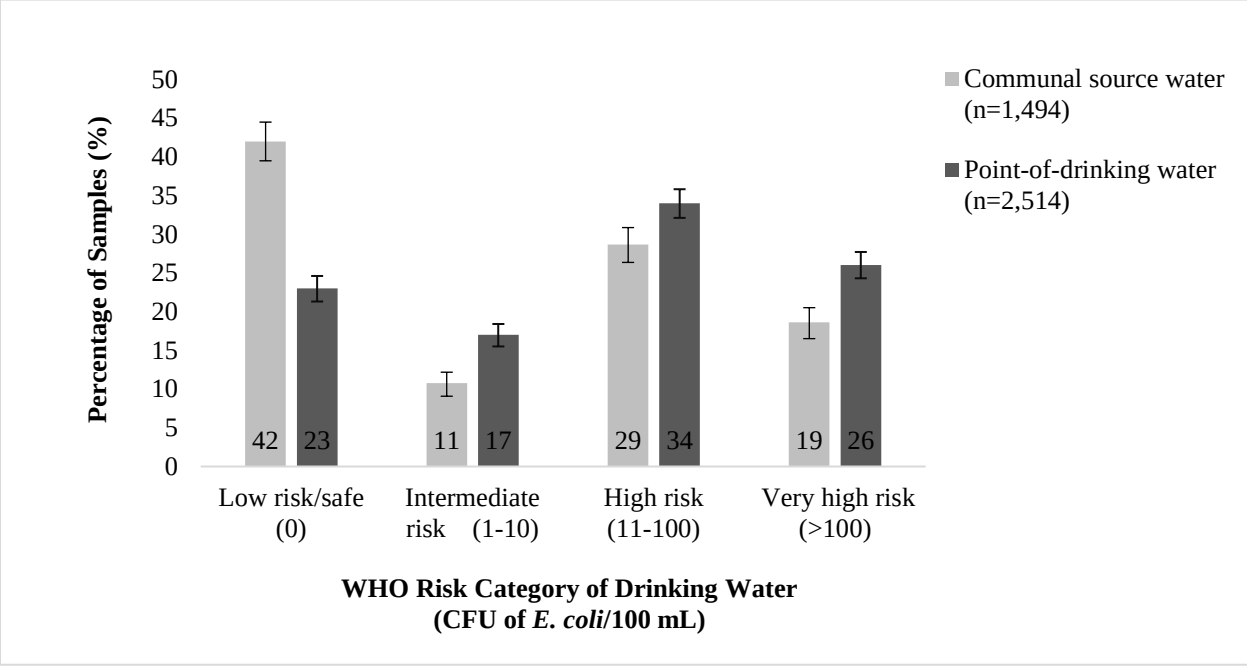

Figure 2. Fecal contamination of drinking water according to WHO risk category. The graph represents the WHO risk categories of communal source and point-of-drinking water. Error bars represent 95% confidence intervals.

Table 2: Median, geometric-mean, mean comparison between communal source and point-of-drinking water, and treated and non-treated point-of-drinking water

	Intermediate risk (1-10 CFU/100 mL)	High risk (11-100 CFU/100 mL)	Very high risk (>100 CFU/100 mL)
Comparison between communal source and point-of-drinking water			
Median (IQR)			
Communal source	4 (4, 8)	32 (20, 56)	196 (136, 313)
Point-of-drinking	4 (3, 8)	36 (20, 62)	272 (152, 428)
Mean (95% CI)			
Communal source	6 (5, 6)	40 (38, 43)	250 (231, 269)
Point-of-drinking	5 (5, 5)	42 (41, 44)	306 (293, 319)
Comparison between treated and non-treated point-of-drinking water			
Median (IQR)			
Treated	4 (2, 6)	37 (22, 60)	264 (175, 428)
Non-treated	4 (3, 8)	36 (20, 64)	276 (152, 428)
Mean (95%CI)			
Treated	4 (4, 5)	42 (37, 46)	299 (331, 267)
Non-treated	5 (5, 5)	43 (41, 44)	307 (321, 293)

IQR: Interquartile range indicates 1st and 3rd quartile in parenthesis. 95% CI: 95% confidence interval indicates upper and lower limits in parenthesis.

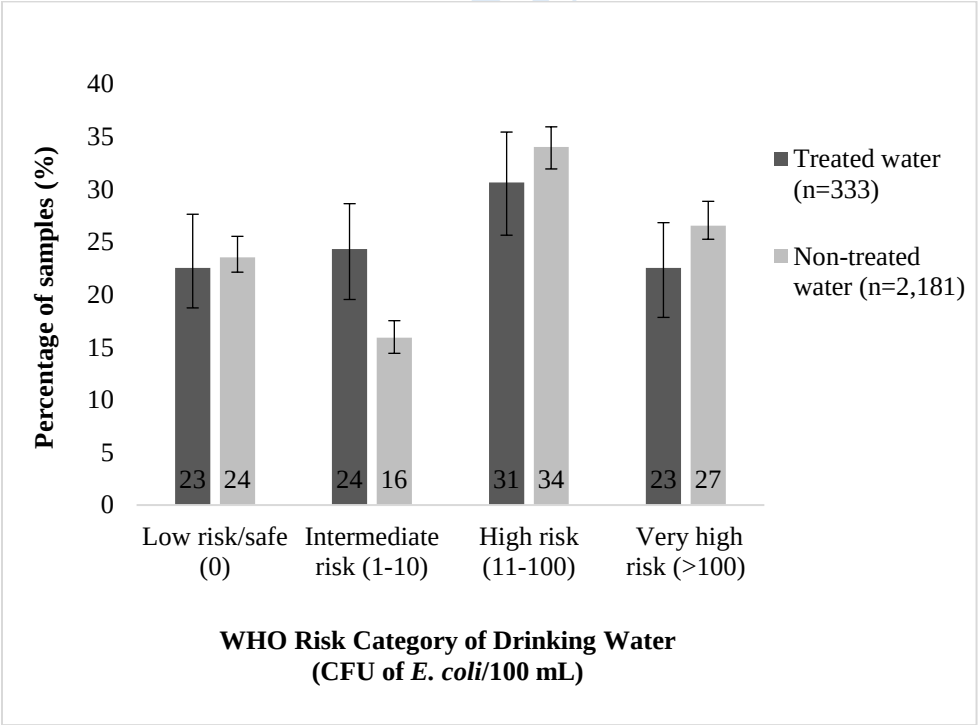

Figure 3. Fecal contamination of treated and non-treated point-of-drinking water according to WHO risk category. Error bars represent 95% confidence intervals.

Table 3: Risk categories of WHO risk groups according to point-of-drinking water vessel types

Characteristics	**Low risk/safe**	**Intermediate risk**	**High risk**	**Very high risk**
E. coli CFU/100 mL	(<1)	(1-10)	(11-100)	(>100)
	n (%)	**n (%)**	**n (%)**	**n (%)**
Mug (n=1,335)	300 (22)	214 (16)	459 (34)	362 (27)
Glass (n=726)	158 (22)	129 (18)	247 (34)	192 (26)
Bottle (n=344)	112 (33) *	56 (16)	107 (31)	69 (20) *
Jug (n=74)	12 (16)	18 (24)	21 (28)	23 (31)

*indicates significance, P <0.01 for odds ratios in risk groups

Table 4: Difference of *E. coli* CFU/100 mL of water within the paired samples of communal source water and point-of-drinking water collected in the same day

Difference of CFU/100 mL between communal source and point-of-drinking water	**Total no. of sample (N=1,236)**
	n (%)
No net change (point-of-drinking = communal source)	204 (16)
In-house contamination (point-of-drinking > communal source)	626 (51)
Die-off (point-of-drinking < communal source)	406 (33)

Table 5: Difference of contamination from communal source to point-of-drinking water according to treatment and drinking vessels

	No. of sample	No-change, n (%)	In-house contamination, n (%)	Die-off, n (%)
Water treatment				
Treated	91	7 (8)	51 (56)	33 (36)
Non-treated	1,145	197 (17)	575 (50)	373 (33)
Point-of-drinking vessels				
Mug	707	111 (16)	372 (53)	224 (32)
Glass	290	48 (17)	135 (47)	107 (37)
Bottle	193	41 (21)	88 (46)	64 (33)
Jug	36	3 (8)	25 (69)	8 (22)

Table 6: Comparison between the detection rate of MF culture and qPCR method

Criteria	**Point-of-drinking**	**Communal source**	**Total**	**Detection rate**
Presence of E. coli in culture and qPCR method	*n=272*	*n=404*	*N=676*	
MF Culture method	141	296	437	65%

qPCR method	266	363	629	93%
Presence of E. coli in V. cholerae positive sample using MF culture method	*n=110*	*n=32*	*N=142*	
E. coli positive	94	29	123	87%
Presence of E. coli in V. cholerae positive sample using qPCR method	*n=43*	*n=19*	*N=62*	
E. coli positive	40	18	58	94%

DECLARATION OF CO-AUTHORSHIP

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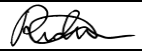
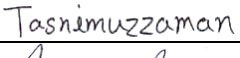
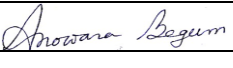
Principal supervisor	Peter Kjaer Mackie Jensen

Title of PhD thesis:
Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for Escherichia coli and Vibrio cholerae

This declaration concerns the following article:
Comparative assessment of fecal contamination in 'improved' piped-to-plot communal source and point-of-drinking water

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	C
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 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

Signature of the co-authors:			
Date:	**Name:**	**Title:**	**Signature:**
07/01/19	Rebeca Sultana	PhD Student	
23/11/18	Ridwan Bin Rashid	Ms Student	
27/11/18	Md. Tasnimuzzaman	Ms Student	
07/01/19	Anowara Begum	Professor	

07/01/19	Peter Kjær Mackie Jensen	Associate Professor	

Signature of the PhD student and the principal supervisor:	
Date: 07/01/19 **PhD student:** 	**Date:** 07/01/19 **Principal supervisor**: 

The fecal origin of pathogenic *E. coli* in 'improved' piped-to-plot communal source and point-of-drinking water of a low-income urban community, Bangladesh

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Abstract

The occurrence of pathogenic bacteria in drinking water is a global health concern. The aim of this study was to investigate the presence of diverse pathotypes of *E. coli* isolates in piped-to-plot 'improved' communal source water and in the point-of-consumption of drinking water and to identify their origin of fecal contamination in a low-income urban community, Bangladesh. multiplex and single-plex polymerase chain reaction (PCR) were performed for characterization of pathogenic *E. coli* and phylogenetic grouping, to identify the probable origin of fecal sources. Diverse pathotypes were identified in point-of-drinking water where ETEC was the most prevalent pathotype found in point-of-drinking water (37%, 46/125) and communal source water (46%, 48/104). Substantial presence of subgroup B1 (most prevalent in animals feces) in both of point-of-drinking (50%, 91/181) and source water (50%, 90/181) isolates followed by the presence of B2-3 (human feces) in (65%, 13/20) point-of-drinking and (35%, 7/20) source water was observed. Our findings suggest that both communal sources and point-of-drinking water of the study area are mostly contaminated by the feces from animals (181/229) and to a lesser extent by human feces (20/229). The presence of highly virulent pathogenic *E. coli* (hybrid *E. coli* isolates) in the 'intermediate risk' group (1–10 *E. coli*/100 mL) indicates that this group should get an urgent/immediate action priority as the 'high/very high-risk group' for remedial action which is included as 'low priority action' according to WHO. The non-human mammals

and birds played vital role in fecal contamination of the water and requires priority attention in future intervention effort of water quality improvement. Our results indicate that addressing human sanitation without consideration of fecal contamination from livestock sources will not be enough to prevent drinking-water contamination and thus will persist as a greater contributor of diarrheal pathogens.

Introduction

The occurrence of pathogenic bacteria in drinking water is a global health concern [1-3]. In the low- and middle-income countries, 502,000 deaths were associated with unsafe or insufficient drinking water [4]. Poor sanitation and contaminated drinking water are linked to outbreaks and transmission of waterborne diseases like diarrhea, cholera, typhoid, hepatitis, amoebiasis, and dysentery in developing countries specifically in South-central and Southeast Asia [5-9].

Escherichia coli is an inhabitant of the mammalian colon and is considered as commensal or harmless in nature. However, there are pathogenic variants of *E. coli* which are capable of causing diarrheal diseases with significant morbidity and mortality [10]. The Global Burden of Disease study in 2015, included *E. coli* as one of the 13 etiological agents of diarrheal infection [11]. The widespread association of diarrheagenic *E. coli* (DEC) in causing diarrheal illness has been documented in various studies in Bangladesh, which were focused on DEC detection in stool samples of diarrhea patients in urban slums [12-14].The DEC strains can be classified as six groups, namely enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC) and diffusely adhering *E. coli* (DAEC) [15-18]. The diseases caused by diarrhea causing pathogenic *E. coli* include traveler's diarrhea, hemorrhagic colitis, bloody diarrhea.

Detection of the host of virulent *E. coli* (e.g. animal, human) is crucial for displaying the transmission pathway of *E. coli* through drinking water. The identification of the source of fecal contamination is highly important for the effective management of water systems [2]. Clermont et al. [19] have developed a PCR based method to characterize the phylo-groups using the genetic markers *chuA, yjaA* and the DNA fragment TspE4.C2. Based on these genetic markers *E. coli* strains can be assigned to one of the main phylogenetic groups: A, B1, B2 or D [6-8]. These

phylo-groups apparently differ in their ecological niches, life-history [10] and some characteristics, such as their ability to exploit different sugar sources. their antibiotic-resistance profiles and their growth rate [11]. Escobar-Páramo et al. (2006) [19] isolated the fecal strains from birds, non-human mammals and humans and observed the prevalence of groups D and B1 in birds, A along with B1 in non-human mammals, and A as well as B2 in humans.

During the expiration of the Millennium Development Goal (MDG) monitoring period in 2015, the Joint Monitoring Program (JMP) for Water Supply and Sanitation proposed new targets and indicators for measuring the expansion of access to safe drinking water. One of the proposed indicators is "safely managed drinking", which is defined as the use of an improved drinking water source that is located on the premises, available when needed and free from fecal contamination. The JMP defined 'improved source' is characterized by nature of its construction and design, has some measures of protection against fecal contamination i.e. piped supply, boreholes, protected dug wells, protected springs, and rainwater) [20]. There are many studies that investigated the effect of 'improved' source water at various stages like from source to collection, storage condition and point of consumption [21-27] by simply enumerating coliform/fecal coliform bacteria.

Disease outbreaks and deaths linked with exposure to surface water, freshwater and recreational water contaminated with pathogenic strains of *E. coli* are well documented [28-30]. However, the occurrence of pathogenic *E. coli* strains harboring virulence genes in the drinking water of low-income urban settings has been scantily documented in Bangladesh [31, 32]. To the best of our knowledge there is no study that investigated the presence of pathogenic *E. coli* in the 'improved' source and in the household drinking water. An in-depth understanding on genetic characterization and phylogenetic grouping/analysis will provide an insight to the public health researchers on which domain to intervene to improve the drinking water quality for prevention of diarrhea. Therefore, the aim of this manuscript was to investigate the presence of diverse pathotypes of *E. coli* isolates in piped-to-plot 'improved' communal source water and in the point-of-consumption of drinking water in a low-income urban area of Bangladesh and to identify their origin of fecal contamination (fecal origin human or animal).

Materials and Methods

Study design

The study was conducted in Arichpur, located in Tongi Township of Dhaka, Bangladesh. Arichpur is an urban community with an area of 1.2 Km^2 and a population density over 100,000 per km^2; approximately 129,000 residents living in 29,000 households [33]. Water samples were collected both from 'point-of-drinking' and ‘communal source' of study household as part of routine visit at six weeks of interval from September 2014 to October 2015 [34]. Residents of this area use water from two types of communal pumps that collected ground water (>85-meter depth): 'WASA (Water Supply and Sewerage Authority) pump' installed by government municipality which is connected to households through underground networked pipe and/or 'submersible pump' installed by individuals or group of residents which is connected to households through over-ground networked pipe. Samples from ‘point-of-drinking’ was taken from the drinking vessels (i.e., mug, glass, bottle, jug and pitcher) that household members used for drinking water.

Sample collection and culture of bacterial strains

Water samples were collected in pre-sterilized wide-mouth water sampling bottles (SPL Life Sciences, Korea) and transported in a cool box to the Environmental Microbiology Laboratory, University of Dhaka within 2-4 hours of collection. Aliquots of 100 mL water samples were filtered through 0.45 µm 47 mm white gridded S-Pak Filters (Merck Millipore, Germany) and the filters were placed on membrane Thermotolerant *E. coli* agar (m-TEC agar, Oxoid, UK) plates. Plates containing the filters were incubated at 44.5 +/- 0.5° C for 18-24 hours. After overnight incubation, typical reddish-purple or magenta colonies on m-TEC were presumptively considered as *E. coli* colonies and enumerated. Isolation was carried out from randomly chosen m-TEC positive plates of water samples. Typical *E. coli* colonies were streaked on Eosin methylene blue EMB agar (Merck) and followed IMViC tests: indole, methyl-red, Voges-Proskauer and citrate tests.

Extraction of Bacterial DNA

Isolates of *E. coli* were routinely grown on nutrient broth (NB) at 37° C. Genomic DNA from overnight cultures of *E. coli* strains from NB were extracted using the boiled template method described by the method [35]

Detection of virulence genes and phylogenetic groups

All presumptive *E. coli* isolates were confirmed as *E. coli* by real-time PCR detection of the *E. coli*-specific housekeeping gene *uidA* [36] and underwent a more extensive virulence gene screen, as described below.

***DEC*:** Multiplex PCRs with previously published primers (S1 Table) were carried to detect the virulence markers of diarrhea causing *E. coli* (DEC). The criteria for determining the pathotypes of DEC were described by Nguyen et al. 2011 [37]. Briefly, the presence of *eltB* and/or *estA* genes for ETEC, the presence of *vt1* and/or *vt2* for EHEC (the additional presence of *eaeA* confirms the detection of a typical EHEC isolate), the presence of *bfpA* and *eaeA* for typical EPEC (but the presence of only *eaeA* for atypical EPEC), the presence of *ipaH* for EIEC and Shigella, and the presence of pCVD for EAEC.

***ExPEC*:** For detection of extra-intestinal pathogenic *E. coli* (ExPEC), all isolates were screened by multiplex PCRs for the presence of five virulence markers (S1 Table), i.e., *papA* and/or *papC* P fimbriae: counted as 1), *sfa/foc* (S and F1C fimbriae), *afa/dra* (Dr-binding adhesins), *kpsM* II (group 2 capsule), and *iutA* (aerobactin system). ExPEC isolates were categorized based on the presence of two or more of these five virulence markers [38].

***Extended virulence genes*:** All isolates were also examined for the presence of virulence genes other than DEC and ExPEC presented in the S1 Table. Amplifications of the target genes were carried out using multiplex and singleplex PCR assays, as described previously [39, 40]. Targeted genes and primer sequences are given in S1 Table.

***Phylogenetic group determination*:** The phylogenetic group of each isolate was determined according to Clermont et al. 2000 [41], by multiplex PCR of the genes *chuA* and *yjaA* and the DNA fragment TspE4.C2 (S1 Table). The isolates were assigned to the phylogenetic groups as follows: B2 (*chuA*+, *yjaA*+), D (*chuA*+, *yjaA*-), B1 (*chuA*-, TspE4.C2+) or A (*chuA*-, TspE4.C2-

). Subgroups within the phylogroups were determined to increase the distinction among the isolates according to the method described by Escobar-Páramo et al. 2006 [19]. The subgroups are as follows: subgroup A_0 (group A), *chu* A-, *yja* A-, TspE4.C2-; subgroup A_1 (group A), *chu* A-, *yja*A+ TspE4.C2-; group B1, *chu* A-, *yja* A-, TspE4.C2+; subgroup $B2_2$ (group B2), *chu* A+, *yja*A+, TspE4.C2-; subgroup $B2_3$ (group B2), *chu* A+, *yja* A+, TspE4.C2+; subgroup D_1 (group D), *chu* A+, *yja* A-, TspE4.C2- and subgroup D_2 (group D), *chu* A+, yjA-, TspE4.C2+ [19].

***PCR product visualization*:** Amplified products were resolved in 1.5% agarose (Carl Roth, Germany) gel using power pack (Bio-Rad, USA), at 80 volts for 45 min. For visualization of the PCR product, the gel was stained with 1% solution of ethidium bromide (AppliChem Panreac, 10mg per mL) and photographed under UV transilluminator ChemiDoc MP system (Bio-Rad).

Data analysis

The distribution of point-of-drinking and communal source water samples were stratified by the WHO *E. coli* risk categories: low risk/safe (< 1 *E. coli*/100 mL), intermediate risk (1–10 *E. coli*/100 mL), high risk (11–100 *E. coli*/100 mL), and very high risk (> 100 *E. coli*/100 mL) for human consumption [20]. Descriptive statistics were used to analyze the proportions of pathotypes, and phylogenetic groups of *E. coli* isolates collected from point-of-drinking water and source water. All analyses were conducted using IBM SPSS software, version 23. P, <0.05 was considered to be statistically significant

Results

Altogether 229 *E. coli* isolates were obtained where 125 were obtained from 108 water samples of point-of-drinking and 104 isolates were obtained from 76 communal source water samples. Most 93% (100/108) of the household drinking water samples were found non-treated water. Only 7% (8/108) samples were found treated where 7 samples were treated by boiling and 1 was treated by filtration. However, we found *E. coli* in treated water as well. ETEC isolates were found in 2 out of 8 treated water samples.

Molecular identification of the collected isolates revealed that pathogenic *E. coli* was identified in 41%-71% of samples of both point-of-drinking water and communal source water, distributed across the 'intermediate risk', 'high risk' and 'very high-risk' groups (Figure 1).

In our study 50 % (62/125, CI 95%: 41-58) of the isolates were found pathogenic from point-of-drinking water and 55% (57/104, CI 95%: 45-64) of the isolates were found pathogenic from communal source water. Of the DEC isolates, ETEC was found in the highest proportion both in point-of-drinking (84%) and in communal source water (91%) (Table 1). Of those ETEC isolates, *estA* gene containing ETEC was the most dominant pathotype which was the most frequently found both in point-of-drinking water and communal source water (Table 2). Other pathotypes of *E. coli* i.e. EPEC, EHEC, EIEC, EAEC have accounted for 16% (9/55) of DEC isolates in point-of-drinking and 9% (5/53) in source water (except EAEC, as no EAEC was found in source water).

ExPEC isolates were found in higher percentage from point-of-drinking water compared to communal source water, though some ExPEC-associated genes, were detected at relatively high frequencies i.e. *iutA* in 26% (31/119) and *sfa/foc* 28% (33/119) among the total pathogenic isolates of point-of-drinking and source water isolates (Table 2).

Among the extended virulence genes c*nf2*, *papG*, *papC* were not found in any of the *E. coli* isolates. All the isolates were *crl* gene positive. Majority of the isolates (≥ 98%) were *csg* and *fimA* gene positive (Table 2).

Phylogenetic grouping of the 125 *E. coli* isolated from point-of-drinking water belonged to six subgroups i.e. A1 (2%), B1 (73%), B2-2 (2%), B2-3 (10%), D1 (3%), D2 (10%), and 104 *E. coli* isolates from communal source water isolates belonged to four subgroups B1 (87%), B2-2 (2%), B2-3 (7%), D2 (5%). Subgroup A0 was not found in any of the isolates. Subgroups A1, D1 were absent in communal source water isolates. In our study, we found 50% of both point-of-drinking (91/181) and communal source (90/181) water isolates were in B1 subgroup. The presence of B2-3 in point-of-drinking was 65% (13/20) and 35% (7/20) in communal source water.

ETEC is predominant in B1 group, both point-of-drinking and source water (Table 4). The ETEC strains were distributed in four phylogenetic groups: most of them fell into group B1 (animals: birds/non-human mammals feces), followed by subgroup D2 (birds feces), D1 (birds feces) and B2-3 (human feces) in point-of-drinking. Similarly, in communal source water ETEC strains mostly fell into group B1 (animals: birds/non-human mammals) with a lesser presence (only one strain) of D2 (birds feces) and B2-3 (human feces) compared to point-of-drinking.

Discussion

A high percentage of pathogenic *E. coli* was distributed across the risk groups in both point-of-drinking water and in improved piped-to-plot communal source water samples indicating 'intermediate risk' group to 'very high-risk' group requires urgent attention for remedial action of contamination to provide safe drinking water in low-income urban community. Substantial presence of subgroup B1 in both point-of-drinking and communal source water isolates, followed by the presence of B2-3 suggest that fecal contamination originated from non-human mammals (goats, cows) and birds (ducks and chickens), and to a lesser extent from human feces. Among the pathogenic *E. coli*, ETEC was the most prevalent pathotype which is a major *E. coli* pathotype causing asymptomatic and symptomatic diarrhoea in low- and medium-income countries, including Bangladesh [10, 14]

In our study, pathogenic *E. coli* was identified in above 40% of point-of-drinking and communal source water samples which is higher than the other study conducted in Dhaka, Bangladesh that found 7% of pathogenic *E. coli* in source water samples from Dhaka municipality [31]. This difference might be due to chlorine treatment of the municipal water of Dhaka City by the authority [42], which is not maintained in our study area. In addition, privately owned submersible pumps were the predominant communal water sources in Arichpur and usually chlorination treatment of water at the communal source was not performed.

Similar to other studies conducted in Bangladesh [30-32, 43], our study also found higher presence of ETEC pathotype among pathogenic *E. coli* isolates [30-32, 43]. A study conducted in an urban slum area of Dhaka showed that ETEC form biofilms in household water tanks/reservoirs throughout the year [44]. Previously ETEC has been found in environmental

water in Dhaka and viable after long-term water incubation [43, 45]. In our study, *estA*-positive ETEC strains were the dominant pathotype among the ETEC isolates which is alarming as *estA*-positive ETEC are commonly associated with symptomatic cases of diarrhea [10]. The infectious dose of laboratory-grown cultures of ETEC is relatively high (i.e. 10^6 to 10^8 organisms) compared to that of other *E. coli* pathotypes [46]. However, the actual dose from natural transmission differed in an outbreak investigation in a Japanese prison that estimated the infectious dose in contaminated pickles was between 25 and 1,000 organisms [47]. In our study, we found ETEC across the 'intermediate', 'high' and 'very high' risk groups and might pose health risk since a person usually drinks water >1 liter per day.

Next to ETEC we found greater number of EHEC in point-of-drinking water than communal source water which is alarming since the infectious dose of EHEC can be as low as one organism to cause illness [48, 49]. Although in low abundance, we found other pathotypes of *E. coli* i.e. EPEC, EIEC, EAEC which have implications in causing severe diarrhea in humans [16-18]. Altogether, the presence of diverse pathotypes of DEC was higher in point-of-drinking water compared to source water.

In this study, a small number of isolates were found to be hybrid in 'intermediate risk' group and 'very high risk' group from both point-of-drinking water and from communal source water. A number of past studies have shown hybrid strains carrying virulence marker genes of two different *E. coli* pathotypes [50] which had clinical relevance to hybrid *E. coli* strains from India [51], Brazil [52, 53], Denmark[54], Switzerland [55], France [56], Germany [57] and Mexico [58]. An extremely pathogenic strain that possessed EAEC and STEC associated virulence genes emerged and caused a sprout-borne outbreak in Germany within a very short period of time [59]. In Bangladesh occurrence of hybrid (STEC–ETEC) strains in in domesticated animals have been documented but have not been described in drinking water. The hybrid *E. coli* strains carrying genes from ETEC and EHEC were isolated in our study, which was documented in an outbreak from contaminated drinking water in Finland [60]. Since hybrid is a newly emerged mixed pathovar of *E. coli* showed a high virulence potential in several regions of the world [52, 61-63], the presence of hybrid *E. coli* in drinking water can pose a great public health concern for the people of Bangladesh, especially when it is point-of-drinking water.

Presence of ExPEC isolates in point-of-drinking water could be a public health concern as infections at extraintestinal sites can develop due to colonization of ExPEC through consumption of ExPEC-contaminated water [64, 65]. ExPEC isolates have the potential to cause disease such as meningitis/septicaemia, urinary tract infections [16, 39, 66]. ExPEC is associated with neonatal meningitis, accounting for high mortality and morbidity (10–30 %) in newborn children [17, 67, 68]. The primary cause of community-acquired urinary tract infections (UTIs) is ExPEC that affect an an estimated 20% of women over the age of 18 years [69, 70].

WHO guideline emphasizes the 'high risk' (11–100 *E. coli*/100 mL) should get a higher priority action, 'very high-risk´ groups (> 100 *E. coli*/100 mL) should get an urgent/immediate action priority and the 'intermediate risk' group requires a low action priority for the remedial actions for contamination in drinking water [20]. Our study findings showed that pathotypes of *E. coli* i.e. ETEC, EIEC and most importantly newly emerged highly virulent hybrid strains of *E. coli* was detected in the 'intermediate risk' group both point-of-drinking water and communal source water, which poses public health concern as several outbreaks are linked to these pathotypes worldwide. Thus, our study findings revealed that the 'intermediate risk' group should be equally prioritized with the high-risk groups and inclusion of pathogenic bacterial investigation can provide important insight to assess water quality safety.

In point-of-drinking water *E. coli* isolates six subgroups were identified whereas in communal source water isolates belonged to four subgroups, suggesting that a diversified fecal origin was responsible for the contamination of point-of-drinking water compared to communal sources. Previous studies reported higher prevalence of group B1 and A in herbivorous animals/non-human mammals, groups D and B1 in birds [19, 71] and subgroup B2-3 was present only in the human sample [72]. Our finding is consistent with a study conducted in India, which reported animal fecal markers were widely detected in both public and domestic domains, and human fecal markers were detected much more frequently in the domestic domain than in public domain sources [73]. A significant positive association between domestic animal husbandry and diarrheal disease in humans was reported by a systematic review in 2014 [74]. In Bangladesh, poultry roaming within the household premises including living room is a common scenario [75, 76]. Most importantly, slaughtering, defeathering, or scavenging of poultry for cooking inside households specially in the kitchen area are common practice in Bangladesh [77] and are the

potential risk factors for fecal contamination. Therefore, the ubiquitous presence of animal feces should be given high attention, since it intensifies the risk of exposure to zoonotic pathogens as well.

To our best knowledge, this is the first study that showed association between the DEC strains collected from drinking water and their phylogenetic groups. Previous studies examined the phylogenetic groups of DEC strains collected from neonatal gut samples from India and for children stool samples from Libya, Colombia [78-80], and found majority (> 70%) of the isolated DEC strains as phylogenetic groups A and B1. In accordance with their findings we identified the majority (88%, 99/113) of the isolated DEC strains as phylogenetic B1 (non-human mammals and birds) from drinking water. All of the EPEC, EIEC, EAEC fell into group B1. EHEC mostly fell into group B1 but one strain of EHEC in point-of-drinking water fell into subgroup B2-3 (human origin) whereas in source water EHEC was only in B1 group. Similarly, the greatest number of strains carrying virulence genes were phylogroup B1 strains, reported in South Korea [81] and France [82]. Additionally, the ETEC isolates in our study possessed different virulence traits (hybrid isolates of combination from ETEC-EHEC and ETEC-EIEC) belonged to different phylogenetic groups (B1, B2-3, D1, D2) indicating their heterogeneity whereas the EAEC strains isolated from the weaned children with diarrhea of Nigeria [83] showed heterogeneity by exhibiting multiple pathogenic lineages and diverse phylogenetic groups (A, B1, D and B2). These findings suggest that ETEC strains of this study might have a genetic background that allows the acquisition of virulence factor coding genes of other pathotypes and their adaptability in different ecological niches. More studies are needed to understand the genomic and phylogenetic structure among the diverse lineages of ETEC.

Our study had some limitations. The study relied on *E. coli* isolation using m-TEC agar that contains a chromogen (5-Bromo-6-Chloro-3-Indoyl-beta-D-Glucuronide) which is converted into glucuronic acid in presence of β-glucuronidase-positive *E. coli* isolates [84]. Thus, the genotypic characteristics of the investigated *E. coli* isolates were ones those were β-glucuronidase-positive *E. coli*. Therefore, the β-glucuronidase-negative *E. coli* isolates were not studied in this study. Although future researches may consider using methods capable of isolating β-glucuronidase-negative *E. coli* isolates, our study relied on m-TEC as a screening media due to its widespread use in field studies. Another limitation of the study is that the

studied isolates of point-of-drinking and communal source water were randomly picked while disregarding the linked point-of-drinking and communal source water. Therefore, the influence of the pathogenic *E. coli* found in communal source water cannot be interpreted on the point-of-drinking water. However, the abundance of pathogenic *E. coli* in both domains provides a comparative understating of the prevalence of pathogenic *E. coli* and will serve as a baseline for future studies.

Conclusion

Our study findings revealed that the analysis of the *E. coli* population structure by phylogenetic grouping and subgrouping can be useful as an effective bacterial source tracking tool. More specifically, phylogenetic subgroups can be used as a first screening for pollution source identification (subgroups B2-3 for human contamination, B1 for domesticated animal contamination). In the recently adopted Sustainable Development Goals (SDGs) to be achieved by 2030, sanitation is primarily focused on the proper management of human fecal matter, to reduce the diarrheal burden [85]. In the SDGs goals, the proper management of fecal matter from domestic animals is largely ignored and less attention has been given to the influence of animal feces on water contamination and diarrheal disease. Whereas our study findings suggest that the non-human mammals (goats, cows) and birds (ducks and chickens) played a vital role in fecal contamination of the water and requires priority attention in future intervention effort of water quality improvement. The presence of highly virulent pathogenic *E. coli* in the 'intermediate risk' group (1–10 *E. coli*/100 mL) where WHO recommends low action priority for remedial action, appears to be dubious (needs to re-think) from our study. Moreover, fecal contamination from livestock sources should also be part of water and sanitation intervention to prevent drinking-water contamination and diarrheal pathogens. Our study showed that highly virulent diverse pathogenic *E. coli* were responsible for contamination of drinking water despite the provision of improved piped-to-plot communal sources. Thus, future research of water safety guideline should consider investigation of pathogenic bacteria to assess water quality safety as part of fecal contamination of water quality assessment criteria.

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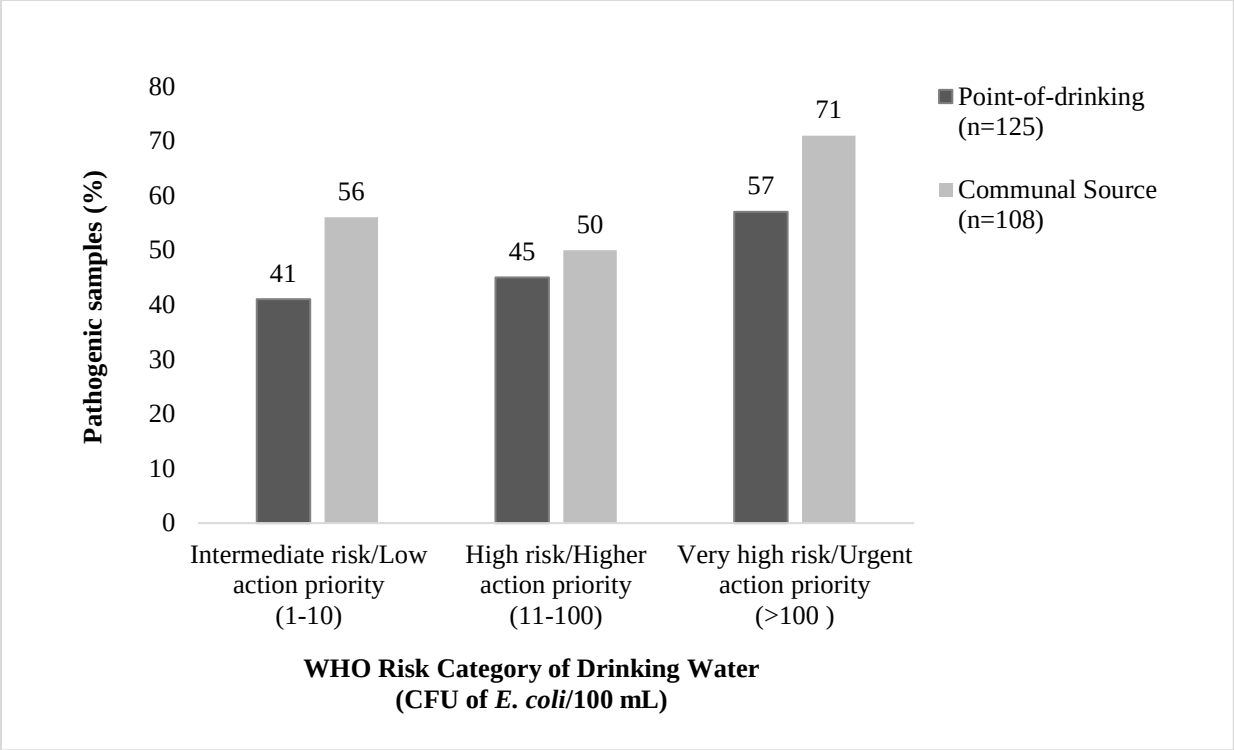

Figure 1: Presence of pathogenic *E. coli* in different risk groups. The graph represents the presence of pathogenic *E. coli* in point-of-drinking water and communal source water in 'intermediate', 'high' and 'very high' risk groups.

Table 1: Prevalence of pathotypes of *E. coli* in communal source and point-of-drinking water samples in different risk groups

Categories	**Point-of-drinking water**				**Communal source water**			
	Intermediate risk (1–10 *E. coli*/100 mL) n = 17 (%)	High risk (11–100 *E. coli*/100 mL) n = 45 (%)	Very high-risk (>100 *E. coli*/100 mL) n = 63 (%)	**Total, n=125 (%)**	Intermediate risk (1–10 *E. coli*/100 mL) n = 11 (%)	High risk (11–100 *E. coli*/100 mL) n = 33 (%)	Very high risk (>100 *E. coli*/100 mL) n = 60 (%)	**Total, n=104 (%)**
ETEC	5 (29)	13 (29)	28 (44)	**46 (37)**	4 (36)	12 (36)	32 (53)	**48 (46)**
EIEC	1 (6)	1 (2)	-	**2 (2)**	-	1 (3)	-	**1 (1)**
EAEC	-	1 (2)	-	**1 (1)**	-	-	-	**-**
EHEC	-	2 (4)	3 (5)	**5 (4)**	-	-	1 (2)	**1 (1)**
EPEC	-	1 (2)	-	**1 (1)**	-	-	3 (5)	**3 (3)**
ExPEC	-	2 (4)	3 (5)	**5 (4)**	-	-	1 (2)	**1 (1)**

Hybrid (ETEC+EHEC, ETEC+EIEC)	1 (6)	-	1 (2)	**2 (2)**	1 (9)	-	2 (3)	**3 (3)**
Total pathotypes	7 (41)	20 (44)	35 (56)	**62 (50)**	5 (45)	13 (39)	39 (65)	**57 (55)**
Commensal	10 (59)	25 (56)	28 (44)	**63 (50)**	6 (55)	20 (61)	21 (35)	**47 (45)**

Table 2. Assignment of pathotypes based on virulence gene content and distribution of other extended virulence genes among 229 isolates of point-of-drinking and communal source water, Arichpur, Dhaka

Virulence genes and pathotypes assignment	**Total no. with trait (% of 229)**	**No. of strains (%)**	
		Point-of-drinking water (n = 125)	**Communal source water (n=104)**
Pathotype assignment (DEC)			
eltB	27 (12)	14 (11)	13 (13)
estA	46 (20)	22 (18)	24 (23)
eltB+estA	21 (9)	10 (8)	11 (11)
ETEC	**94 (41)**	**46 (37)**	**48 (46)**
vt1	2 (1)	1 (1)	1 (1)
vt2	3 (1)	3 (2)	0
vt1+eae	1 (0.4)	1 (1)	0
vt2+eae	0	0	0
EHEC	**6 (3)**	**5 (4)**	**1 (1)**
Eae	4 (2)	1 (1)	3 (3)
eae+bfp	0	0	0
EPEC	**4 (2)**	**1 (1)**	**3 (3)**
ipaH	3 (1)	2 (2)	1 (1)
EIEC	**3 (1)**	**2 (2)**	**1 (1)**
pCVD	1 (0.4)	1 (1)	0
EAEC	**1 (0.4)**	**1 (1)**	**0**
eltB+estA+vt1	1 (0.4)	1 (1)	0
eltB+estA+vt2	3 (1)	1 (1)	2 (2)
estA +ipaH	1 (0.4)	0	1 (1)
Hybrid *E. coli*	**5 (2)**	**2 (2)**	3 (3)
Pathotype assignment (ExPEC)			
afa/dra	0	0	0
kpsMT II	9 (4)	5 (4)	4 (4)
iutA	31 (14)	20 (16)	11 (11)
papA	0	0	0
papC	0	0	0
sfa/foc	33 (14)	26 (21)	7 (7)
ExPEC*	**6 (3)**	**5 (4)**	**1 (1)**
Extended virulence genes			
csg	224 (98)	120 (96)	104 (100)
fimA	181 (79)	97 (78)	84 (81)
crl	229 (100)	125 (100)	104 (100)
fyuA	12 (5)	8 (6)	4 (4)
cnf1	30 (13)	24 (19)	6 (6)
cnf2	0	0	0
papG	0	0	0

cvaC	13 (6)	9 (7)	4 (4)
iss	32 (14)	27 (22)	5 (5)
traT	76 (33)	39 (31)	37 (36)
focG	58 (25)	45 (36)	13 (13)
hlyA	1 (0.4)	1 (1)	0
malX	5 (2)	4 (3)	1 (1)
ompT	18 (8)	8 (6)	10 (10)
iroN	29 (13)	11 (9)	18 (17)
ibe10	66 (29)	49 (39)	17 (16)
k1	4 (2)	1 (1)	1 (1)

* ≥ 2 markers present: *papA* and/or *papC*, *afa/dra*, *sfa/foc*, *iutA*, *kpsMT II*.

Table 3. Distribution of phylogenetic groups of 229 *E. coli* isolates isolated from point-of-drinking water and communal source water, Arichpur, Dhaka

Phylogroups	**No. of isolates**	**Point-of-drinking water (n)**	**Communal source water (n)**
A1	2	2	0
B1	181	91	90
B2-2	4	2	2
B2-3	20	13	7
D1	4	4	0
D2	18	13	5

Table 4. Phylogenetic distribution of pathotypes and commensal strains *E. coli* strains isolated from point-of-drinking water and communal source water, Arichpur, Dhaka

Categories	**Prevalence of pathotypes by phylogenetic group, no. (%)**									
	Point-of-drinking water (n=125)						**Communal source water (n=104)**			
	A1 (n=2)	B1 (n=91)	B2-2 (n=2)	B2-3 (n=13)	D1 (n=4)	D2 (n=13)	B1 (n=90)	B2-2 (n=2)	B2-3 (n=7)	D2 (n=5)
ETEC (n=94)	0	36 (38)	0	2 (2)	3 (3)	5 (5)	46 (49)	0	1 (1)	1 (1)
EHEC (n=6)	0	4 (67)	0	1 (17)	0	0	1 (17)	0	0	0
EPEC (n=4)	0	1 (25)	0	0	0	0	3 (75)	0	0	0
EIEC (n=3)	0	2 (67)	0	0	0	0	1 (33)	0	0	0
EAEC (n=1)	0	1 (100)	0	0	0	0	0	0	0	0
Hybrid *E. coli* (n=5)	0	2 (40)	0	0	0	0	2 (40)	0	0	1 (20)
ExPEC (n=6)	1 (17)	1 (17)	0	1 (17)	0	2 (33)	0	0	1 (17)	0
Commensal (n=110)	1 (1)	45 (41)	2 (2)	9 (8)	1 (1)	6 (5)	36 (33)	2 (2)	5 (5)	3 (3)

Manuscript entitled "**The fecal origin of pathogenic *E. coli* in 'improved' piped-to-plot communal source and point-of-drinking water of a low-income urban community, Bangladesh**"

S1 Table: Primers used in this study for PCR amplification

Genes	Primer name	Primer sequences (5′→ 3′)	Amplicon size (bp)	References
Fecal Detection				
uidA (real-time PCR)	UAL1939b	atggaatttcgccgattttgc	187	[1]
	UAL2105b	attgtttgcctccctgctgc		
DEC genes				
eltB	LT-F	tctctatgtgcatacggagc	322	[2]
	LT-R	ccatactgattgccgcaat		
estA	ST-F	gctaaaccagta$^{g}_{a}$ggtcttcaaaa	147	[2]
	ST-F	cccggtaca$^{g}_{a}$gcaggattacaaca		
vt1	VT1-F	gaagagtccgtgggattacg	130	[3]
	VT1-R	agcgatgcagctattaataa		
vt2	VT2-F	accgtttttcagatttt$^{g}_{a}$cacata	298	[2]
	VT2-R	tacacaggagcagtttcagacagt		
eaeA	eae-F	cacacgaataaactgactaaaatg	376	[2]
	eae-R	aaaaacgctgacccgcacctaaat		
bfpA	bfpA-F	ttcttggtgcttgcgtgtctttt	367	[2]
	bfpA-R	ttttgtttgttgtatctttgtaa		
ipaH	ipaH-F	gctggaaaaactcagtgcct	424	[4]
	ipaH-R	ccagtccgtaaattcattct		
pCVD	EA-F	ctggcgaaagactgtatcat	630	[5]
	EA-R	caatgtatagaaatccgctgtt		
Adhesin				
fimA	fimA-F	cgacgcatcttcctcattcttct	721	[6]
	fimA-R	attggttccgttattcagggttgtt		
sfa/foc	sfa-F	ctccggagaactgggtgcatcttac	410	[7]
	sfa-R	cggaggagtaattacaaacctggca		
papC	papC-F	gacggctgtactgcagggtgtggcg	328	[7]
	papC-R	atatcctttctgcagggatgcaata		
afa	afa-F	gctgggcagcaaactgataactctc	750	[7]
	afa-R	catcaagctgtlttgttcgtccgccg		
csg	csg-F	actctgacttgactattacc	200	[8]
	csg-R	agatgcagtctggtcaac		
crl	crl-F	tttcgattgtctggctgtat	250	[8]
	crl-R	cttcagattcagcgtcgtc		
Extra-intestinal				

iutA	iutA-F	ggctggacatcatgggaactgg	302	[9]
	iutA-R	cgtcgggaacgggtagaatcg		
fyuA	fyuA-F	tgattaaccccgcgacgggaa	880	[10]
	fyuA-R	cgcagtaggcacgatgttgta		
cnf1	cnf1-F	aagatggagtttcctatgcaggag	498	[11]
	cnf1-R	cattcagagtcctgccctcattatt		
cnf2	cnf2-F	aatctaattaaagagaac	543	[12]
	cnf2-R	catgctttgtatatcta		
papG	papG-F	ctgtaattacggaagtgatttctg	1070	[13]
	papG-R	actatccggctccggataaaccat		
kpsMT II	kpsMT-F	gcgcatttgctgatactgttg	272	[10]
	kpsMT-R	catccagacgataagcatgagca		
cvaC	cvaC-F	cacacacaaacgggagctgtt	680	[10]
	cvaC-R	cttcccgcagcatagttccat		
iss	Iss-F	cagcaacccgaaccacttgatg	323	[14]
	Iss-R	agcattgccagagcggcagaa		
traT	Trat-F	ggtgtggtgcgatgagcacag	290	[10]
	Trat-R	cacggttcagccatccctgag		
focG	focG-F	cagcacaggcagtggatacga	360	[10]
	focG-R	gaatgtcgcctgcccattgct		
hlyA	hlyA-F	aacaaggataagcactgttctggct	1177	[11]
	hlyA-R	accatataagcggtcattcccgtca		
malX	malX-F	ggacatcctgttacagcgcgca	930	[10]
	malX-R	tcgccaccaatcacagccgaac		
ompT	ompT-F	tcatcccggaagcctccctcactactat	496	[15]
	ompT-R	tagcgtttgctgcactggcttctgatac		
iroN	iroN-F	aatccggcaaagagacgaaccgcct	553	[10]
	iroN-R	gttcgggcaacccctgctttgacttt		
ibe10	Ib10-F	aggcaggtgtgcgccgcgtac	170	[10]
	Ib10-R	tggtgctccggcaaaccatgc		
kpsMT K1	kpsMT K1-F	tagcaaacgttctattggtgc	153	[10]
	kpsMT II-R	catccagacgataagcatgagca		
Phylogenetic				
chuA	chuA-F	gacgaaccaacggtcaggat	279	[16]
	chuA-R	tgccgccagtaccaaagaca		
yjaA	yjaA-F	tgaagtgtcaggagacgctg	211	[16]
	yjaA-R	atggagaatgcgttcctcaac		
tspE4C2	tspE4C2-F	gagtaatgtcggggcattca	152	[16]
	tspE4C2-R	cgcgccaacaaagtattacg		

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GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
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This declaration concerns the following article:
The fecal origin of pathogenic E. coli in 'improved' piped-to-plot communal source and point-of-drinking water of a low-income urban community, 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
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

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A Comparative Analysis of *Vibrio cholerae* Contamination in Point-of-Drinking and Source Water in a Low-Income Urban Community, Bangladesh

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Bangladesh is a cholera endemic country with a population at high risk of cholera. Toxigenic and non-toxigenic *Vibrio cholerae* (*V. cholerae*) can cause cholera and cholera-like diarrheal illness and outbreaks. Drinking water is one of the primary routes of cholera transmission in Bangladesh. The aim of this study was to conduct a comparative assessment of the presence of *V. cholerae* between point-of-drinking water and source water, and to investigate the variability of virulence profile using molecular methods of a densely populated low-income settlement of Dhaka, Bangladesh. Water samples were collected and tested for *V. cholerae* from "point-of-drinking" and "source" in 477 study households in routine visits at 6 week intervals over a period of 14 months. We studied the virulence profiles of *V. cholerae* positive water samples using 22 different virulence gene markers present in toxigenic O1/O139 and non-O1/O139 *V. cholerae* using polymerase chain reaction (PCR). A total of 1,463 water samples were collected, with 1,082 samples from point-of-drinking water in 388 households and 381 samples from 66 water sources. *V. cholerae* was detected in 10% of point-of-drinking water samples and in 9% of source water samples. Twenty-three percent of households and 38% of the sources were positive for *V. cholerae* in at least one visit. Samples collected from point-of-drinking and linked sources in a 7 day interval showed significantly higher odds ($P < 0.05$) of *V. cholerae* presence in point-of-drinking compared to source [OR = 17.24 (95% CI = 7.14–42.89)] water. Based on the 7 day interval data, 53% (17/32) of source water samples were negative for *V. cholerae* while linked point-of-drinking water samples were positive. There were significantly higher odds ($p < 0.05$) of the presence of *V. cholerae* O1 [OR = 9.13 (95% CI = 2.85–29.26)] and *V. cholerae* O139 [OR = 4.73 (95% CI = 1.19–18.79)] in source water samples than in point-of-drinking water samples. Contamination

of water at the point-of-drinking is less likely to depend on the contamination at the water source. Hygiene education interventions and programs should focus and emphasize on water at the point-of-drinking, including repeated cleaning of drinking vessels, which is of paramount importance in preventing cholera.

Keywords: *Vibrio cholerae*, drinking water, O1/O139, non-O1/non-O139, household, point-of-drinking, source water

INTRODUCTION

Cholera is a life-threatening disease with an estimated 2.9 million cases annually in 69 cholera-endemic countries, including Bangladesh (Ali et al., 2015). A recent review indicated that, in Bangladesh, around 66 million people are at risk for cholera, with an estimated incidence of 1.64 per thousand persons (Ali et al., 2015). In Bangladesh alone, the estimated annual number of cases is 109,000, with a three percent case fatality rate (Ali et al., 2015).Toxigenic and non-toxigenic *V. cholerae* can cause cholera and cholera-like diarrheal illness and outbreaks. *V. cholerae* has more than 200 serogroups based on variations in the "O" antigenic lipopolysaccharide (LPS). Cholera toxin-producing serogroups O1 and O139 have been shown to be the etiological agents of epidemic cholera (Kaper et al., 1995). Non-O1/non-O139 and non-toxigenic *V. cholerae* O1 strains, harboring a range of accessory virulence factors, can cause diarrheal diseases (Morris et al., 1984) and sporadic localized cholera outbreaks (Saha et al., 1996; Faruque et al., 2004; Pang et al., 2007) hence emphasizing the importance of research on both toxigenic and non-toxigenic *V. cholerae*. Accessory factors that can cause diarrheal diseases are repeats-in-toxin (*rtxA*) (Lin et al., 1999; Chow et al., 2001), non-O1 (NAG-ST) and O1 (O1-ST) heat-stable enterotoxins encoded by the *stn* and *sto* genes, respectively (Ogawa et al., 1990; Dalsgaard et al., 1995; Theophilo et al., 2006), hemolysins encoded by the *hlyA* gene (Zhang and Austin, 2005; Karlsson et al., 2013), transcriptional activator (*toxR*) (Waldor and Mekalanos, 1994), hemagglutinin protease encoded by *hap* (Silva et al., 2006; Mohapatra et al., 2009), ADP ribosylating exotoxin (*chxA*) (Awasthi et al., 2013), the type VI secretion system (T6SS) (Unterweger et al., 2012), a novel type III secretion system (T3SS) (Dziejman et al., 2005; Shin et al., 2011), and mannose-sensitive hemagglutinin subunit A encoded by *mshA* (Watnick et al., 1999).

V. cholerae can survive in nutrient limited drinking water for long periods of time in a viable but non-culturable state (VBNC) (Colwell, 2009) and can actively exert its infectious capability when in the human intestine (Colwell et al., 1996). This phenomenon poses serious risks to human health due to its non-detectability of VBNC cells by existing culture methods resulting underestimation of colony forming units (CFU) count of viable cells. *V. cholerae* can adapt to and persist in unfavorable environments, such as in conditions of nutrient deprivation and fluctuations in salinity and temperature, and can resist predation by heterotrophic protists and bacteriophages by adopting this unique survival strategy of the VBNC state (Ravel et al., 1995; Colwell et al., 1996; Carroll et al., 2001; González-Escalona et al., 2006; Thomas et al., 2006; Jubair et al., 2012; Mishra et al., 2012). Bacteria remain alive, metabolically active and can express virulence factors in this VBNC state; for example, *V. cholerae* can express *tcp* encoding a toxin co-regulated pilus (Krebs and Taylor, 2011) and the cholera toxin gene (*ctxA*) (Mishra et al., 2012). *V. cholerae* can exert its infectious properties when resuscitation occurs in human and animal digestive tracts (Colwell et al., 1996; Asakura et al., 2007; Senoh et al., 2010). In nutrient limited environments, *V. cholerae* can enter a starvation state in which cells are non-growing but culturable (Colwell et al., 1996; Thomas et al., 2006) and can survive for prolonged period of time (i.e., >700 days) (Jubair et al., 2012). Furthermore, both pathogenic and non-pathogenic *V. cholerae* can attach to abiotic surfaces, i.e., borosilicate glass (Watnick et al., 1999) and can survive in fomites in a VBNC state for more than 7 days (Farhana et al., 2016).

Cholera is endemic in Dhaka city (Patel et al., 2012), and low-income urban communities are particularly vulnerable to cholera and diarrheal diseases due to lack of hygiene and access to clean drinking water (Rafique et al., 2016). Drinking water is considered as one of the primary routes of cholera transmission in Bangladesh (Colwell et al., 2003; Huq et al., 2005; Akanda et al., 2009; Jutla et al., 2011). A recent study in Dhaka city established the association of cholera pathogen and its virulence in drinking water from households with confirmed or suspected cholera case patients (Rafique et al., 2016). There is, however, no known comprehensive evaluation of the burden of *V. cholerae* in source and point-of-drinking water in households in a cholera endemic community. Point of use or household water treatment can be an effective intervention in the prevention of diarrhea (Fewtrell et al., 2005). The World Health Organization has recognized that household water treatment and safe storage can provide rapid and significant health impacts (http://www.who.int/water_sanitation_health/publications/2011/9789241548151_toc.pdf). Therefore, investigating the contamination of drinking water in a population at risk for cholera will be useful to developing specific interventions to protect high risk populations from cholera and cholera-like illnesses. Studies that have investigated water quality at the point of use have focused primarily on water treatment, i.e., filtration, chlorination, flocculation, and solar disinfection of water stored in households (Clasen, 2015; Taylor et al., 2015). Few studies have investigated the microbiological water quality at the point of consumption/drinking (i.e., the quality of water in a drinking vessel immediately before consumption) (Rufener et al., 2010). The aim of this study is to conduct a comparative assessment of the presence of *V. cholerae* between point-of-drinking water and source water and to investigate the variability of virulence profile using molecular methods of a densely populated low-income settlement of Dhaka, Bangladesh.

METHODS AND MATERIALS

Study Design

The study was conducted in Arichpur, located in Tongi Township of Dhaka, Bangladesh. Arichpur is an urban community with an area of 1.2 km^2, population density of more than 100,000 residents per km^2, and approximately 129,000 residents living in 29,000 households (Azman et al., 2015). Residents of this area use water from two types of communal pumps: "WASA (Water Supply and Sewerage Authority) pump" installed by the municipal government and connected to households through underground networks of pipes, and/or "submersible pump" installed by individuals or groups of residents and connected to households through over ground networks of pipes. The area around the pumps is not usually protected with a wall and floor made of concrete. These pumps extract water at a depth of approximately 75–140 m.

Data Collection

A total of 477 households were enrolled in this study. Water samples were collected both at the point-of-drinking and at the source in each study household during routine visits at 6 week intervals from September 2014 to October 2015. Depending on the availability of the caretaker (i.e., the female or male family member who spent the most time in the house), point-of-drinking samples were taken from the drinking vessels (i.e., a mug, glass, bottle, jug, or pitcher) that household members used to drink water. Samples from sources were taken from the communal water source point used by each study household. On average, 20 samples were collected at each weekly visit from point-of-drinking and sources. Caretakers were asked if they treated the water (i.e., boiled, filtered, added alum, etc.) prior to consuming the drinking water. The water samples from sources were taken directly from taps attached to the communal pumps. In the absence of such a tap, samples were collected from taps attached to the nearest closed over-ground reservoir that was connected to the pump. The coordinates of sample collection sites (households and communal sources) were obtained using a global positioning system (GPS). Q-GIS software was used to locate the sites on a Google map.

Sample Collection and Enrichment

Each sample contained 100 mL of water that was collected in sterile bottles and transported in a cool box to the Environmental Microbiology Laboratory, University of Dhaka, within 2–4 h of collection. Aliquots of water were added to 10 mL of alkaline peptone water (APW), enrichment medium (1 L distilled H_2O, 10 gL^{-1} peptone, 10 gL^{-1} sodium chloride; pH 8.5) followed by incubation at 37°C for 18–24 h (Alam et al., 2014).

Extraction of Total DNA and Confirmation of *V. cholerae*

After overnight incubation, DNA was extracted from 1 mL of each enriched culture using the method described by De Medici et al. (2003). The presence of *V. cholerae* in water samples was confirmed by detection of the *V. cholerae* species-specific gene *ompW* (Nandi et al., 2000) by PCR. Due to the non-detectability of VBNC cells by existing culture methods, PCR was chosen to reliably detect all forms of *V. cholerae* (both VBNC and culturable).

PCR Reaction Mix and Primer Sequences

V. cholerae virulence genes were detected in 143 samples found positive for the *V. cholerae* species-specific gene *ompW* using PCR. A total of 22 *V. cholerae* virulence genes were selected for detection. PCR was performed using an MJ Research PTC-200 Peltier Thermal Cycler (Mexico). The 25-μL reaction mixture contained 2 μL of 10× PCR buffer, 20 mM $MgCl_2$, 0.4 μL of 10 mM deoxynucleoside triphosphates (dNTP) mix (Thermo Scientific, USA), 0.1 μL of 5 U Dream Taq DNA Polymerase (Thermo Scientific, USA) per μL, and 1.25 μL of each 25 μM primer (Tag Copenhagen A/S, Denmark). Sequences of the primers and target genes and their amplicon sizes are presented in S1 Table.

Real-time PCR was performed to detect the *V. cholerae ctxA* and *rtxA* genes using an Applied Biosystems StepOne (48-well) Real-Time PCR system. Real-time PCR was used as it provides higher sensitivity and specificity compared to conventional PCR. The fluorogenic probe and primer set (Tag Copenhagen A/S, Denmark) targeting the *ctxA* and *rtxA* genes are described in S2 Table. The formula of reaction mixture and cycling conditions for detection of *ctxA* gene were maintained as per supplier's instruction. The 25-μL reaction mixture containing 12.5 μL 2× TaqMan Universal Master Mix II with UNG (Applied Biosystems USA, with AmpliTaq Gold DNA Polymerase, dNTPs, ROX passive reference, Uracil-N glycosylase), 2.5 μL of each 100 nM of primer, 2.5 μL of 250 nM probe, and 5 μL of template. To detect the *rtxA* gene, a reaction mixture (25 μL) containing 12.5 μL 2× Power SYBR green PCR master mix (with a propriety version of ROX dye), 2.5 μL of each 100 nM sense and antisense primer, 2.5 μL of DEPC-treated H_2O, and 5 μL of template DNA was used. *V. cholerae* O1 N16961 genomic DNA was used as a positive control, and PCR grade water was used as a no template control for PCR screening.

Data Analysis

The proportions of samples positive for *V. cholerae* in point-of-drinking and source water were calculated. Logistic regression test was employed to examine the association of *V. cholerae* (and virulence genes) between point-of-drinking and sources, treated and non-treated water, drinking vessel type and all the virulence genes. We also examined the association of *V. cholerae* by logistic regression analysis of a set of stratified samples of point-of-drinking water and their linked sources that were collected within 7 days (before/after 7 days) of interval from each other.

Ethics Statement

The Ethical Review Committee (ERC) of icddr,b, Bangladesh reviewed and approved the study protocol. Informed written consent for collecting samples was obtained from caretaker of each household for "point of use" and from pump operator for "source" water.

RESULTS

A total of 1,463 water samples were collected: 1,082 from the point-of-drinking and 381 from the 66 sources for the 388 enrolled households. Most of the households used mugs (249/388), and/or glasses (195/388), and/or small bottles (75/388) to drink water. Drinking water was treated in 24% (93/388) of the households, and the majority of these households reported boiling (77/93) as the mode of treatment. Twelve households out of 93 reported filtration and three households reported both "boiling and filtration" as the mode of water treatment. Among the 66 water sources for these households, there were three communal "WASA pumps" installed by the government and 63 "submersible pumps" installed by individuals or groups. Of the 66 sources, 31 had direct taps attached to the communal pumps, and 51 had taps attached to the reservoir connected to the pumps.

TABLE 1 | Presence of *V. cholerae* in point-of-drinking and source water samples from the study households in Arichpur, Bangladesh, September 2014–October 2015.

Characteristics of point-of-drinking water	Sample	
	N (%) [*N* = 1082]	*V. cholerae* positive n (%) [*n* = 110]
Treated water	165 (15)	20 (12)
Non-treated water	917 (85)	90(10)
Types of treatment carried out	**[*n* = 165]**	
Boiling	125 (76)	14 (13)
Filtration	31 (19)	4 (4)
Boiling and filtration	4 (2)	2 (2)
Types of drinking vessels used at the point-of-drinking	**[*n* = 1069]***	
Mug	575 (54)	52 (47)
Glass	334 (31)	41 (37)
Bottle	125 (12)	8 (7)
Jug	30 (3)	6 (6)
Pitcher	5 (1)	1 (1)
Characteristics of source water	**N (%) [*N* = 381]**	**n (%) [*n* = 33]**
By types of collection points		
Taps attached to the communal pumps	146 (38)	6 (15)
Taps attached to the reservoir connected to the pumps	235 (62)	27 (82)
By types of pumps		
WASA pump	36 (9)	4 (12)
Submersible pump	345 (91)	29 (88)

**For some samples, the types of vessels used were not known.*

V. cholerae in "Point-of-Drinking" and "Source" Water

V. cholerae was detected in 10% (110/1082) of point-of-drinking water samples and in 9% (33/381) of source water samples (**Table 1**). Point-of-drinking water from 23% of households (88/388) and source water for 38% (25/66) of households were positive for *V. cholerae* at least once in the visits conducted at 6 week intervals. Most (76%, 67/88) households with point-of-drinking water samples positive for *V. cholerae* were also connected to 19 of 25 *V. cholerae* positive sources, irrespective of timing of collection. However, from the stratified data in 7 day intervals, 53% (17/32, [(95% CI = 0.360–0.70)]) of the sources were negative for *V. cholerae*, while point-of-drinking water samples linked to these sources were positive. The percentage of samples positive for *V. cholerae* was higher in the point-of-drinking water (11% [32/299], $P = 0.000$) compared to the sources water (9%, 28/299) in the 7 day-interval stratified data. The *V. cholerae* positive households were distributed throughout the study area, whereas the *V. cholerae* positive sources were mainly clustered in the southern part of the study area, which is adjacent to a water body (**Figure 1**).

In point-of-drinking water, *V. cholerae* was detected twice in 15% (13/88) of households, three times in 2% (2/88) of households, and six times in 1% (1/88) of households. The probability of the presence of *V. cholerae* was higher in glasses than mugs and bottles (**Table 2**). There was a higher probability of the presence of *V. cholerae* in non-treated water compared to treated water ($P = 0.22$; **Table 2**). *V. cholerae* was detected twice in 8% (2/25) of sources, three times in 4% (1/25) of sources, and five times in 4% (1/25) of sources. Samples from all three WASA pumps' were positive for *V. cholerae* at least once (**Figure 1**). Most (82%, 29/33) of the *V. cholerae* detected in source water came from taps attached to the reservoir connected to the pumps ($P = 0.008$), rather the taps directly attached to the communal pumps (**Table 2**).

Distribution of Virulence Genes

A total of 143 *V. cholerae* positive samples were identified, and virulence genes other than *ompW* were detected in these positive samples. In total, 11% (15/143) of *V. cholerae* samples were positive for the *rfb* O1 gene and 6% (9/143) of *V. cholerae* samples were positive for the *rfb* O139 gene (**Table 3**). The percentages of serogroups O1 and O139 were higher in source water compared to point-of-drinking water. There was a higher probability of having *V. cholerae* O1 [OR = 9.13 (95% CI = 2.85–29.26)] and *V. cholerae* O139 [OR = 4.73 (95% CI = 1.19–18.79)] in source water compared to point-of-drinking water (**Table 3**). Of the samples with non-O1/non-O139 serogroups, the *ctxA* gene was found in three of the point-of-drinking water samples and two of the source samples. The percentage of samples in which the *hlyA* gene was detected was higher in point-of-drinking water compared to source water, and this difference was statistically significant (**Table 3**).

Two of the *V. cholerae* positive point-of-drinking water samples carried virulence genes- *ctxA*, as well as, *rtxA, rtxC, toxR, hlyA, hap, msh1, chxA*, T6SS but lacked *tcpI, ompU, ace, nag-st. One* point-of-drinking water sample was found positive for *hlyA, rtxA, toxR, hap, ompU, cep, chxA,* and T6SS, but negative for *ctxA, rtxC, tcp,* and *ace*. One of the source water samples exhibited *hlyA, tcp*, *hap, cep, mshA, chxA,* T3SS, and T6SS, but

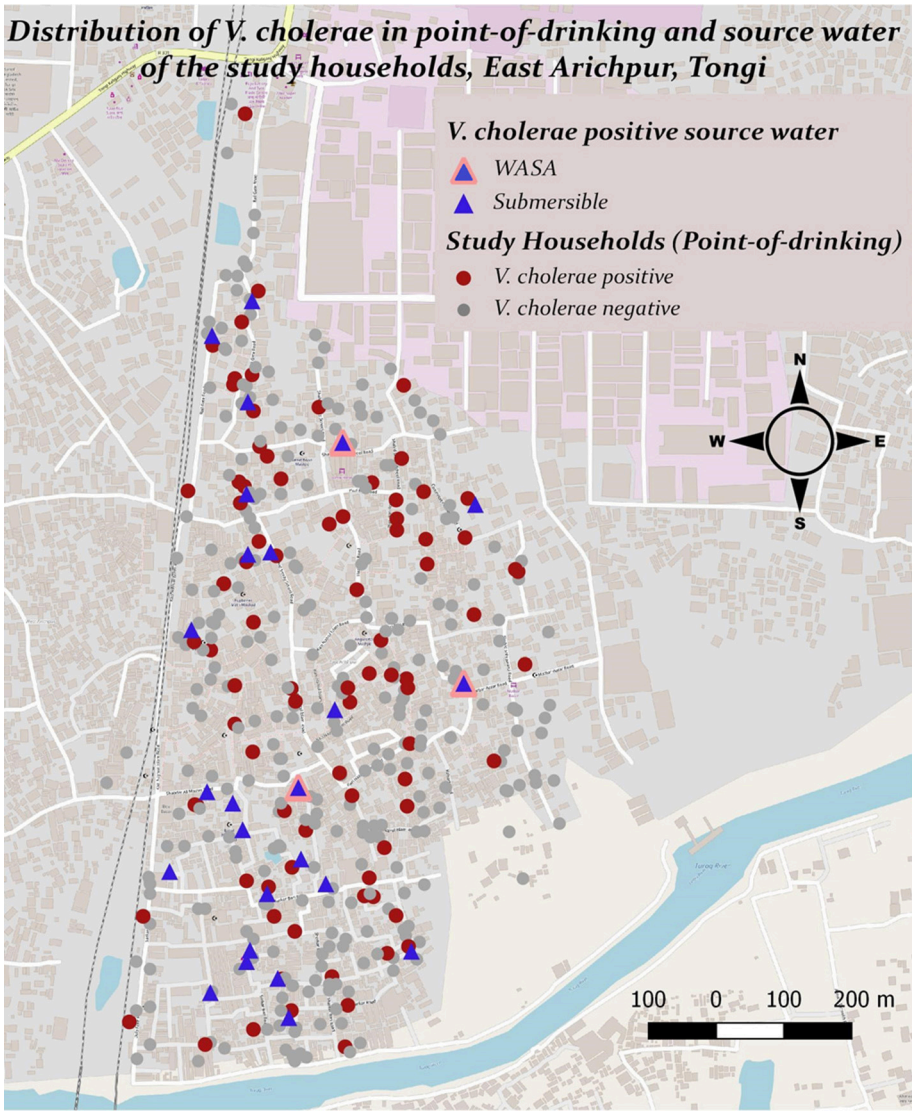

FIGURE 1 | Presence and distribution of *V. cholerae* in point-of-drinking and source water samples from the study households in Arichpur, Bangladesh, September 2014–October 2015.

not *ctxA*, *rtxA*, or *ompU*. However, most of the 121 non-O1/O139 *V. cholerae* positive samples carried *hlyA*, *rtxA*, *hap*, and *toxR*, as well as genes encoding T6SS.

DISCUSSION

Toxigenic and non-toxigenic *V. cholerae* were widely distributed in point-of-drinking and source waters throughout the low-income urban community of Arichpur. The estimated probability of the presence of *V. cholerae* in point-of-drinking water when absent in linked sources was 0.53 (95% CI = 0.36–0.70) within 7 day intervals, which suggests that post-contamination of point-of-drinking water might have occurred. The probability of the presence of *V. cholerae* O1 [OR = 9.13 (95% CI = 2.85–29.26)] and O139 [OR = 4.73 (95% CI = 1.19–18.79)] in source water was significantly higher than that in the point-of-drinking water, suggesting that the quality of point-of-drinking water might not be affected by the quality of sources.

Similar to other studies (Wright et al., 2004; Rufener et al., 2010), our study showed that the contamination of water was higher at the point-of-drinking compared to the source. In an observational study in Pakistan, Jensen et al. (2002) showed that water stored inside the household was more often contaminated than the source water when the source water contained < 100 *E. coli* per 100 mL (Jensen et al., 2002). In this same study, the researchers performed a 5 week intervention using narrow-necked water pitcher (that prevent utensils or hands from retrieving water) to prevent water contamination and found a significant improvement in in-house water quality (Jensen et al., 2002). A systematic review indicated that water quality

TABLE 2 | Logistic regression of factors associated with the presence of *V. cholerae* in water samples from Arichpur, Bangladesh, September 2014–October 2015.

Factors	OR (95% CI)	P
PRESENCE OF *V. cholerae*		
Point-of-drinking vs. source (irrespective of the timing of sample collection)	1.19 (0.79–1.79)	0.230
Point-of-drinking vs. source (samples collected within 7 day interval)	17.24 (7.14–42.89)	0.000*
Taps attached to the reservoir connected to the pumps vs. taps attached to the communal pumps	3.03 (1.22–7.53)	0.008*
Non-treated vs. treated point-of-drinking water	1.27 (0.76–2.12)	0.220
Point-of-drinking water in glass vs. mug	1.41 (0.91–2.17)	0.076
Glass vs. bottle	2.05 (0.93–4.50)	0.046
Mug vs. bottle	1.45 (0.67–3.14)	0.221
PRESENCE OF TOXIGENIC *V. cholerae* O1/O139		
Source vs. point-of-drinking	6.22 (2.54–15.25)	0.000*
Taps attached to the reservoir connected to the pumps vs. taps attached to the communal pumps	1.74 (0.55–5.58)	0.254

*OR, odds ratio; CI, confidence interval; *significance at a level of $P \leq 0.05$.*

improvement at sources were ineffective, because water from a good quality source was often contaminated at the point of use through poor hygiene practices in households (Taylor et al., 2015). Another study conducted in Bolivia showed that pathogen-free water at the source is not a guarantee for safe and pathogen-free drinking water at the point-of-consumption (Rufener et al., 2010), supporting our findings that the quality of water at the point-of-drinking did not depend on the presence or absence of *V. cholerae* in the source water.

Although there are reports that treatment type (boiling, chlorination) (Momba and Notshe, 2003; Levy et al., 2008), have significant impact on drinking water quality, our study did not evidence any significant association of *V. cholerae* with specific treatment type (boiling or filtration). A study conducted at the household level in rural areas of Peru reported that 69% of jars in which drinking water was stored had fecal coliforms though the water was treated by boiling (Gil et al., 2014). The absence of a holding shaft on a glass might play role in reducing direct hand contamination of drinking water to some extent and might explain the higher probabilities of *V. cholerae* contamination of water in drinking glasses compared to mugs. Compared to glasses and mugs, bottles were less frequently contaminated with *V. cholerae*, suggesting that narrow-necked vessels can prevent contamination, as shown by Jensen et al. (2002).

The higher prevalence of toxigenic *V. cholerae* O1/O139 [OR = 6.22 (2.54–15.25)] in sources compared to point-of-drinking water in this study matched findings of other studies conducted in Dhaka (Rafique et al., 2016) and in northern coastal Ecuador (Levy et al., 2008). In the source water, the number of *V. cholerae* was significantly higher in the water samples collected from the taps attached to the reservoir connected to the pumps compared to taps attached to the communal pumps which was also in agreement with a study conducted in Ecuador (Chalchisa et al., 2017). Larger storage tanks allowing longer storage times without regular cleaning (Schafer and Mihelcic, 2012) may potentially increase the risk of contamination and allow the persistence of bacteria by inducing the VBNC state (Colwell et al., 1996; Thomas et al., 2006).

V. cholerae lacking the *tcpI* gene was found in 5% *ctxA* positive samples. This is consistent with results of a study in Bangladesh (Hasan et al., 2013), where environmental O1 toxigenic strains were found to lack the *tcpA* and *tcpI* genes. Furthermore, we obtained O1 positive samples that did not carry *ctxA, tcpI* but carried *hlyA*, *hap*, *rtxA* genes. A research showed that variant virulence profile can be observed, since environmental strains are more heterogeneous than clinical strains (Hasan et al., 2013).

We found that non-O1/non-O139 *V. cholerae* was widely distributed throughout both source and point-of-drinking water samples. These strains are recognized to be of public health relevance, because they have been associated with sporadic cases or outbreaks of cholera-like disease (Crump et al., 2003; Dutta, 2013) and many extra-intestinal infections (Akoachere and Mbuntcha, 2014). While it is true that most epidemic cholera cases are caused by toxigenic *V. cholerae* O1/O139, a large proportion of diarrheal cases do not have a defined etiology where surveys take place (Islam et al., 2013).

After analyzing the genetic profiles of *V. cholerae* in samples, 85% of the *V. cholerae* in positive samples possessed *hlyA*, a gene whose product is an exotoxin related to CT, and *rtxA*, a heat-stable enterotoxin, both of which can be found in non-O1 strains isolated from patients with cholera (Saka et al., 2008) and from environmental strains from endemic areas (Faruque et al., 2004; Kumar et al., 2008; Mohapatra et al., 2009). These samples also possessed toxR, a 32-kDa transmembrane protein that acts as a master regulator of the *ctxAB* gene (DiRita et al., 1991). Finally, 10% of *V. cholerae* positive samples possessed *ompU*, whose product has been implicated in colonization and can also be found in some environmental isolates from endemic regions (Karunasagar et al., 2003). A gene *mshA*, also implicated in colonization, encoding a type IV pilus and biofilm formation on abiotic (borosilicate glass) and biotic surfaces (cellulose) (Watnick et al., 1999), was present in approximately half of the *V. cholerae* positive samples. This might explain the higher frequency of *V. cholerae* detection in the reservoir tanks.

Recently, fatal diarrheal disease caused by non-O1/O139 strains of *V. cholerae* has been shown to be associated with T3SS (Tam et al., 2010; Shin et al., 2011), a system absent in common pandemic O1 strains. Six percent of *V. cholerae* drinking water samples were positive for the presence of T3SS, implying the potential to cause fatal diarrhea via drinking water. Two other genes, *chxA* (encoding cholix toxin) was present in 39% and hap was present in 62% of the samples, which are also known to be associated with virulence in non-pandemic strains (Islam et al., 2013).

Our study had some limitations. Data presented here did not consider the inclusion of isolates. However, PCR performed directly on DNA samples allowed us to detect toxigenic genes

TABLE 3 | Presence and logistic regression of *V. cholerae* virulence genes in source and point-of-drinking water samples from Arichpur, September 2014–October 2015.

Genes	Total samples positive for *V. cholerae* n = 143, n (%)	Point-of-drinking water samples positive for *V. cholerae* n = 110, n (%)	Source water samples positive for *V. cholerae* n = 33, n (%)	Odds ratio of source vs. point-of-drinking water samples (95% CI)	*P*-value
ctxA	8 (6)	7 (5)	4 (9)	2.10 (0.47–9.30)	0.271
rfbO1	15 (11)	5 (5)	10 (31)	9.13 (2.85–29.26)	0.000*
rfbO139	9 (6)	4 (4)	5 (16)	4.73 (1.19–18.79)	0.031*
cep	62 (44)	50 (46)	12 (38)	0.67 (0.31–1.53)	0.235
ace	7 (5)	5 (5)	2 (6)	1.36 (0.25–7.32)	0.510
msh1	45 (32)	34 (31)	11 (34)	1.12 (0.49–2.56)	0.475
stn/sto	9 (6)	3 (3)	6 (19)	7.93 (1.86–33.75)	0.005*
rtxA	36 (25)	24 (22)	12 (38)	2.05 (0.88–4.75)	0.075
toxR	97 (68)	77 (70)	20 (63)	0.66 (0.29–1.48)	0.210
tcpI	3 (2)	2 (2)	1 (3)	1.69 (0.15–19.22)	0.548
hlyA	121 (85)	99 (90)	22 (69)	0.22 (0.09–0.58)	0.002*
ompU	10 (7)	7 (6)	3 (9)	1.47 (0.36–6.04)	0.417
nag-st	3 (2)	3 (3)	0 (0)	–	–
rtxC	61 (43)	47 (43)	14 (44)	0.98 (0.45–2.17)	0.569
hap	88 (62)	70 (64)	18 (56)	0.69 (0.31–1.51)	0.229
chxA	56 (39)	39 (36)	17 (53)	1.93 (0.88–4.24)	0.074
vcsC2	8 (6)	6 (5)	2 (6)	1.11 (0.22–5.82)	0.589
vcsN2	8 (6)	6 (5)	2 (6)	1.11 (0.22–5.82)	0.589
vopF	12 (8)	9 (8)	3 (9)	1.12 (0.29–4.41)	0.554
vasK	141 (99)	109 (99)	32 (100)	–	–
vasA	140 (99)	109 (99)	31 (97)	0.14 (0.01–1.62)	0.133
vasH	140 (99)	109 (99)	31 (97)	0.14 (0.01–1.62)	0.133

*$P \leq 0.05$.

of *V. cholerae* in both the culturable and non-culturable state, the latter of which can explain cholera or cholera-like diarrheal illness resulting from drinking water. In addition, PCR provides rapid detection with reduced cost compared to the culture method, and so might be useful for identifying the pathogen in outbreak settings. Although we found significantly higher presence of toxigenic *V. cholerae* O1/O139 in source water compared to point-of-drinking water, this assumption should be interpreted carefully, since the number of samples is low in this study.

CONCLUSION

Our study findings showed that contamination of water at point-of-drinking was less likely to depend on the contamination at sources and presence of *V. cholerae* in point-of-drinking water possibly did not depend on home-based water treatment suggesting that different routes (by hand, drinking vessel, flies) might have facilitated the contamination of drinking water at point-of-drinking. Hygiene education intervention and program should focus and emphasize on point-of-drinking including repeated cleaning of drinking vessels (such as mug, glass, bottle), which is of paramount importance in the prevention of cholera and cholera-like diarrheal illness. Data obtained in our study will serve as the baseline for the future investigations of *V. cholerae* in the environment, particularly in water.

AUTHOR CONTRIBUTIONS

JF designed the study concept, conducted the study in the laboratory, performed statistical analysis, and wrote the manuscript. RS contributed to framing the manuscript, data analysis, and writing and critical revision of the manuscript. RR contributed to the study concept, laboratory work, and data acquisition. MT contributed to the laboratory work and data acquisition. AN performed part of the statistical analysis. AB was the principal investigator of the project, contributed reagents, and approved the final version of the manuscript to be submitted. PJ was the functional principal investigator of the project and contributed to manuscript development, critical revision, and approval of the final version to be published.

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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.2018.00489/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DECLARATION OF CO-AUTHORSHIP

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Title of PhD thesis:
Assessment of microbiological quality of drinking water in an urban low-income settlement of Bangladesh: investigation for Escherichia coli and Vibrio cholerae

This declaration concerns the following article:
A Comparative Analysis of Vibrio cholerae Contamination in Point-of-Drinking and Source Water in a Low-Income Urban Community, 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
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
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 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

Signature of the co-authors:			
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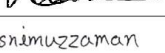
07/01/19	Rebeca Sultana	PhD student	
23/11/18	Ridwan Bin Rashid	MS student	
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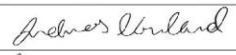
14-01-19	Andreas Nordland	PhD student	
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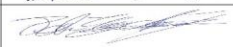
07/01/19	Peter Kjær Mackie Jensen	Associate Professor	

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Standard curve quantification for bacterial DNA using a real-time PCR assay

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Real-time PCR or quantitative PCR (qPCR) is a dynamic and cogent assay for the detection and quantification of specified nucleic acid sequences compared to both, traditional culture techniques and 'end point' conventional PCR for environmental samples. Serial dilutions of cell culture provide information on colony forming unit (cfu) counts which are crucial for optimal and standard curves representative of DNA concentration. This approach eliminates variation in standard curves caused by loss of DNA by serial dilution of nucleic acid elute .In this study, an assay was developed to detect and quantify DNA by real-time PCR on varying amounts of starting template of bacterial DNA for two pathogenic species, *Escherichia coli* and *Vibrio cholerae*. To generate a standard curve, total bacterial DNA was diluted in a 10-fold series and each sample was correlated with an estimated cell count. The starting bacterial DNA concentration was 96.12ng/µL and an individual *E. coli* cell consists of 5.16 femtograms DNA therefore, 96.12 ng/µL of DNA would consist of $1.86x10^7$cells. Both SYBR Green assay and TaqMan assay were validated for *uidA* region in *E. coli* and *ctxA* region in *V. cholerae* based on previously published assays for this standard curve experiment. PCR efficiency for *uidA* gene and *ctxA* gene were obtained 102.366% and 98.08% respectively. Different water samples spiked with pure culture of *E. coli* and *V. cholera*e were used as unknown samples to observe the bacterial cell count from this standard curve experiment. The standard curve established by this qPCR method demonstrates an efficient method to quantify bacterial cell counts since it excludes the possibility of DNA loss during DNA extraction from culture dilution and also yields quantity of DNA mass from non culturable cells.

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Title: High Prevalence of Virulent *Escherichia coli* Belonging B1 Phylogroup in Municipal Water Supply in Dhaka, Bangladesh

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Abstract:

Escherichia coli is a commensal organism of the digestive tracts of many vertebrates, including humans. Contamination of drinking water with pathogenic *E. coli* is a serious public health concern.This study focused on the distribution of phylogenetic groups and virulence gene profile of *E. coli* isolated from drinking water in Arichpur, a low income area of Dhaka, Bangladesh.The distribution of the phylogroups and virulence genes were investigated in 200 isolates among them 110 isolates were from municipal water supply system and 90 were from household drinking water. Gene profile of virulence factors was done based on the presence of *eltB, estA, vt1, vt2, eaeA,* pCVD432*, bfpA, ial, ipaH* by PCR. The classification of the isolates into 4/5 major groups (A, B1, B2, D2, D3) was done based on the distribution of *chuA, yjaA* and DNA fragment tspE4.C2 genes.Results demonstrated predominance of phylogroup B1 78.5 % (157/200) followed by B2 phylogroup 13% (26/200) and phylogroup D 8.5% (17/200).The genes *eltB, estA* and *eaeA* was present in 27.39% (43/157), 37% (74/157), and 10.5% (21/157) of B1 phylogroup isolates, respectively. The calculated chi-square value and P-value were 10.23 and 0.001. Therefore, it can be inferred municipal water supply was a greater contributor of pathogenic *E. coli* from the B1 phylogroup.Usually commensals fall in the Phylogroups A and B1. The presence of greater number of virulent B1 phylogroup isolates originating from municipal water supply indicates that the supply system might be contaminated with virulent *E. coli* such as enterotoxigenic *E. coli* carrying mobile genetic elements such as plasmids which might be transferred to the commensal strains. Greater proportion of commensal strains in household water demonstrates low level of contamination of virulent *E.coli* from asymptomatic shedders.

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Title: A Comparative Study of Cholera Transmission via Source of Supply and Point of Drinking Water at the Households

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Abstract:

Vibrio cholerae is the causative agent of the devastating diarrheal disease cholera, and is endemic to Bangladesh. This is an environmental bacterium that can survive up to two weeks in fresh water and can infect humans through ingestion of contaminated drinking water. Previous studies have established the association of cholera pathogen and its virulence in household drinking water of confirmed or suspected cholera case patients. There is however, no known comprehensive evaluation of the burden of *V. cholerae* in point of drinking in all households and corresponding source of supply within a cholera endemic community. The aim of this study is to investigate the presence and burden of *V. cholerae* in point of drinking water and in the source of supply water of the households in a densely populated low income settlement of Dhaka, Bangladesh using molecular methods. We collected 1463 water samples comprising of 1081 samples from point of drinking (the glass or mug used for drinking at household) and 381 samples from corresponding source of supply (the communal source that distributed water to households through network pipe) of randomly selected households within the study community from September, 2014 to October, 2015. We studied the virulence profile of all the *V. cholerae* positive water samples using 24 different virulence gene markers present in toxigenic O1/O139 and non-O1/O139. Logistic regression analysis was performed to measure the association of *V. cholerae* in source of supply and point-of-drinking of the study households. A total 143 water samples consisting of 110 (10.16%) water samples from point of drinking and 32 (8.26%) samples from corresponding source of supply were found to be positive for *V. cholerae* specific *ompW* gene by PCR. Significant association ($p<0.05$) of *V. cholerae* O1 [OR=9.54 (95% CI =2.96-30.68)] and *V. cholerae* O139 [OR=4.90 (95% CI=1.23-19.52)] in water from point of drinking was observed when compared with water from corresponding source of supply. Hence, the chances of having *V. cholerae* O1 and *V. cholerae* O139 was at 9.54 times and 4.9 times higher in point of drinking water compared to corresponding source of supply water. These findings highlight that the transmission of *Vibrio cholerae* within households may be of significant importance likely due to compromised hygiene related behavior rather than microbiological quality of the source water supply.

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