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The Impact of Water Chlorination on Faecal Carriage Rates of Antimicrobial Resistant Bacteria in Bangladeshi Children

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Division of Water Resources Engineering
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Abbreviations

AMR	Antimicrobial resistance
ESBL	Extended-spectrum-beta-lactamase
E	*Enterbacteriaceae*
E.coli	*Escherichia coli*
EAWAG	Swiss Federal Institute of Aquatic Science and Technology
icddr,b	International Center for Diarrhoeal Disease Research
KESC	*Klebsiella, Enterobacter, Serratia, Citrobacte*r
LMICs	Low and middle income countries
WHO	World Health Organisation

Abstract

Antimicrobial resistance (AMR) is one of today's most pressing global health threats accounting for an estimated 700,000 deaths per year (WHO, 2019). Knowledge on how environmental interventions can effectively be used as barriers to reduce the spread of AMR is needed (Larsson *et al.*, 2018). This interdisciplinary study aims at assessing the impact of point-of-collection drinking water chlorination on faecal carriage rates of antimicrobial resistant bacteria in Bangladeshi children.
A culture-based method was used to detect and quantify the antimicrobial-resistant bacteria extended-spectrum-β-lactamase-producing *Enterobacteriaceae* (ESBL-*E*) in children's stool samples, which had been collected as part of a double-blind randomized clinical trial in the urban slums of Dhaka and Tongi (Pickering *et al.*, 2019). A preliminary analysis compared ESBL-*E* faecal carriage rates and concentrations in stool samples from children grouped as follows: The treatment group had consumed chlorinated drinking water (n=240). The control group had consumed non-chlorinated drinking water dosed with vitamin C (n=240). No significant difference was found between ESBL-*E* faecal carriage rates and concentrations of the groups. An observational analysis of supplementary data on the children was performed in order to identify risk factors associated with the prevalence and concentration of ESBL-*E* in stool samples. This analysis, in contrast, reveals a significant difference between ESBL-*Escherichia coli* (ESBL-*E. coli)* faecal carriage rates and concentrations in children from Dhaka and Tongi. Overall, a high prevalence of ESBL-*E* (69%) as well as ESBL-*E. coli* (64%) was detected in the stool samples.

The findings suggest that point-of-collection drinking water chlorination may not be an effective intervention to reduce the spread of AMR in highly contaminated environments. Future work will be undertaken to focus on identifying the major drivers influencing the difference in ESBL-*E. coli* faecal carriage rates between the study sites, in Dhaka and Tongi, with a view to develop and implement viable interventions which effectively reduce the spread of AMR.

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1. Introduction

Antimicrobial resistance (AMR) is one of today's most pressing global health threats (WHO, 2019). Treatment failure of infectious diseases caused by antimicrobial resistant bacteria have become more common, leading to longer hospital stays, higher mortality rates and increased financial costs for health care systems (Rottier *et al.*, 2012). In order to combat this global health problem the World Health Organization (WHO) has emphasized the need for cooperative multisectoral action and the adoption of a holistic approach referred to as "One Health" (WHO, 2015). This approach considers that human, animal and environmental health are inseparably connected and solutions to mitigate the risks of AMR will require interdisciplinary research on the transmission of AMR between human, animal and environmental reservoirs (Figure 1).

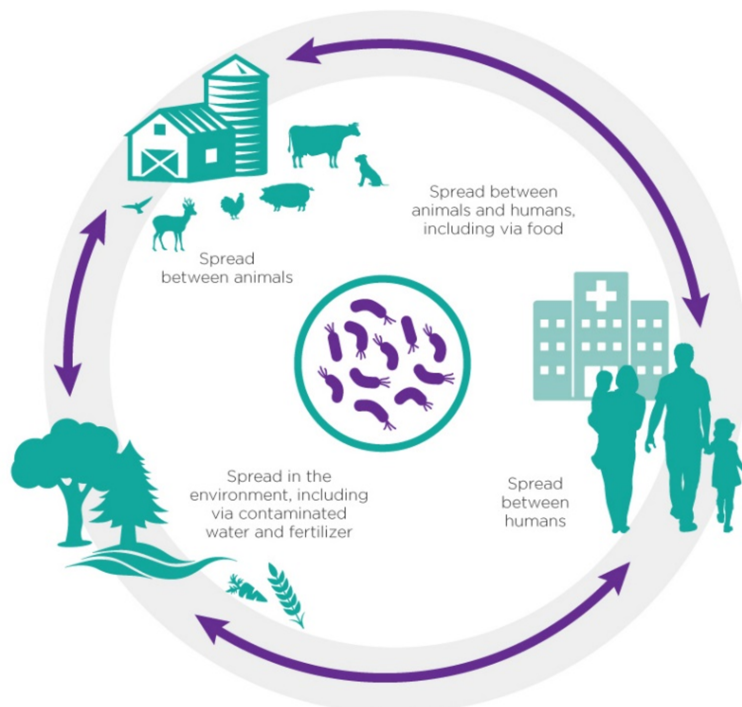

Figure 1: The continuous circle represents the connection between human, animal and environmental reservoirs of AMR. Credit: Public Health Agency of Canada

Increasing knowledge through interdisciplinary research on this multi-variable phenomenon is needed in order to develop viable environmental interventions and reduce the spread of AMR (Larsson *et al.*, 2018).

The aim of this study is to assess the effect of consuming chlorinated water treated at the point-of-collection with a passive dosing device on faecal carriage rates of antimicrobial resistant bacteria in Bangladeshi children. It seeks to contribute to interdisziplinary research by connecting expertise from the fields of water resources engineering, environmental microbiology and public health.

1.1. Interconnected drivers of AMR

Antimicrobials are agents which kill or inhibit the growth of microorganisms such as bacteria, viruses and fungi. There are multiple interconnected drivers of antimicrobial resistance arising not only from humans but also from animal and environmental reservoirs (Chatterjee *et al.*, 2018).

Antibiotic consumption has been associated with the development of AMR in humans (Bell *et al.*, 2014). There is evidence that over-prescription and misuse of antibiotics in healthcare and livestock production is related to an increase in antimicrobial resistant bacteria in the human and animal microflora (Laxminarayan *et al.*, 2013). As a result, large amounts of antimicrobial resistant bacteria are introduced into the environment via human and animal waste. High faecal carriage rates of antimicrobial resistant bacteria have been associated with densely populated urban areas in low and middle income countries (LMICs), where appropriate water and sanitation systems are lacking (Woerther *et al.*, 2013).

Figure 2: Overview of causes of antibiotic resistance according to the WHO

These areas often have limited access to water resources and electricity, which means that the delivery of water to residents can regularly be interrupted. In turn this leads to negative hydraulic pressure in the pipe network which, consequently, increases the risk of pathogenic faecal contamination of drinking water through cracks in the pipes (Lee and Schwab, 2005).

1.2. Water Chlorination

Water chlorination is a low cost and widely used treatment process for controlling levels of bacteria in drinking water and could be used as an effective control intervention to reduce the spread of AMR. However, it is common that the amount of chlorine used at centralized water treatment plants in LMICs is not enough to ensure sufficient chlorine residual to prevent recontamination in the drinking water supply network (Lee and Schwab, 2005). Decentralized drinking water chlorination methods have until recently mainly focused on point-of-use treatment. An example for point-of-use water chlorination is the manual addition of chlorine tablets to drinking water by household members. The disadvantages of this method are that it relies on users being motivated to change their behaviour over a long period of time and is effective only if used correctly and consistently. An alternative decentralized treatment method is community-based point-of-collection drinking water chlorination. This method is advantageous because it requires minimal behavioural change for users, provides consistent access to clean water to multiple households and is low cost (Pickering *et al.*, 2019).

Figure 3: Drinking water point-of-collection in Dhaka, Bangladesh. Credit: Amy Pickering

The effect of water chlorination on antimicrobial resistant bacteria has not yet been well understood. Some studies have shown that chlorination has achieved an effective reduction of concentrations of antimicrobial resistant bacteria in

water (Lin *et al.*, 2016), (Samir *et al.*, 2019). However, it has also been shown that low dosages of chlorine induce a dormant state in bacteria, such as *E. coli.*, in which they become viable but non-culturable (VBNC). Bacteria in this VBNC state have been reported to be more resistant and persistent to various antibiotics (Lin *et al.*, 2017), (Murray *et al.*, 1984).

1.3. Extended-spectrum-ß-lactamase-producing *Enterobacteriaceae* (ESBL-*E*)

β-lactam antibiotics are the world's most commonly used class of antibiotics to treat infections caused by Gram-negative [1] pathogens. They are characterized by the β-lactam ring in their molecular structure and include the subgroups penicillin, cephalosporins, carbapenems and monobactams. One of the main mechanisms causing β-lactam resistance in Gram-negative bacteria is the production of β-lactamases. This enzyme hydrolyses the amide bonds in the β-lactam ring, inactivating the antibiotic. Extended-spectrum-β-lactamases (ESBLs) are enzymes which hydrolyse the β-lactam ring of penicillin and the first 3 generations of cephalosporin antibiotics (Bush and Fisher, 2011). Colonization of the human or animal intestinal tract with ESBL-producing *Enterobacteriaceae* [2] (ESBL-*E*) is mostly asymptomatic. However, the prevalence of ESBL-*E* in human faecal samples have been associated with infections by ESBL-*E.* This is partially due to the increased risk of contamination of normally sterile sites in the human body (Donskey, 2006). ESBLs were initially identified in hospital settings in the 1980s produced by species of *Klebsiella* [3] (Knothe et al., 1983). By the turn of the century,

[1] **Gram-negative bacteria** are one of two large classes of bacteria (the second being Gram-positive bacteria) which are differentiated using a staining method based on the chemical and physical properties of their cell wall. Gram-negative bacteria are generally characterized by a thin layer of peptidoglycan between two cell membranes.

[2] ***Enterobacteriaceae*** is a large family of Gram-negative bacteria including *Escherichia coli* and *Klebsiella, Enterobacter, Serratia* and *Citrobacter (KESC)*

[3] ***Klebsiella*** can be found in the natural environment as well as in the humans and animals. They are often associated with hospital acquired infections where they act as opportunistic human pathogens in immunocompromised patients (Ullmann, 1998).

patients' infections caused by ESBL-producing *Escherichia Coli* [4] were reported at community scale amongst people who had not been exposed to health care facilities (Arpin et al., 2007).

Over the past decades, a steady increase in faecal carriage rates of ESBL-*E* has been observed worldwide (Woerther *et al.*, 2013). The highest prevalence of ESBL-*E* was reported in South East Asia, where certain regions exceeded a 50% carriage rate in 2008 with an estimated 7.2% average yearly increase between 2002 and 2011 (Figure 4). The Eastern Mediterranean and Western Pacific also showed rapid yearly increases of 3.5% and 1.5%, respectively, followed by Africa with a 1.1% increase in ESBL-*E* carriage. Europe seems to lie further behind with carriage rates not exceeding 10% until 2008 and an average increase of 0.5% per year (Figure 4). Based on this data, associations have been made with the increase in ESBL-*E* carriage and faecal-oral transmitted diseases which are impacted by limited access to safe water and sanitation as well as high population densities (Woerther *et al.*, 2013).

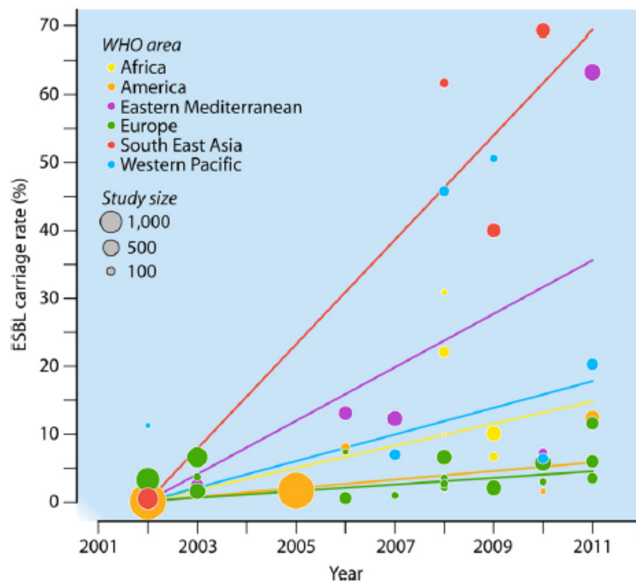

Figure 4: geographical and temporal distribution of ESBL-E community carriage rates. Different sized bubbles represent the size of different study populations and are colour coded based on the study site location. Lines represent the average increase in ESBL-E (Woerther *et al.*, 2013)

[4] ***Escherichia coli (E. coli)*** is found in most human and animal intestines and is the predominant aerobic organism in the gut. However, it only represents less than 1% of the total microbiota of a healthily developed human gut. *E.coli* may exist either as commensal bacteria in symbiosis with its host, preventing colonization of pathogens or it may carry a combination of virulence genes making it pathogenic (Delmas *et al.*, 2015).

1.4. AMR in Bangladesh

Bangladesh, located in South Asia, has one of the highest population densities in the world, with 1,115.62 people per square kilometre (World Population Review 2019). It has been identified as one of the countries that is most vulnerable to extreme weather events with health-related impacts. About 80% of the country falls under the flood plain and up to 34% is flooded for five to seven months every year. Water supplies and sanitation systems are often contaminated or damaged during floods increasing the spread of faecal contamination and waterborne diseases (Chanda Shimi, 2010). The highest ESBL-*E. coli* faecal carriage rate (74%) reported to date was recently detected in healthy infants living in rural areas of Bangladesh (Islam *et al.*, 2019). Multidrug resistance patterns in faecal samples from children in rural Bangladesh have been shown to vary during the year with the highest carriage rates measured during the post monsoon period (Mamun *et al.*, 1993).

1.5. Objectives

The hypothesis investigated in the scope of this project was that *point-of-collection chlorination of drinking water reduces child faecal carriage rates of ESBL-E.*

In order to test this hypothesis, the following objectives were defined:

1) to apply a culture-based method to determine the prevalence and amount of ESBL-*E* and ESBL-*E. coli* in Bangladeshi children's faecal samples (n=480) which were collected during a double-blind cluster-randomized controlled clinical trial.
2) To compare samples from children belonging to a treatment group (n=240), which had access to chlorinated water, to samples from children in a control group (n=240) which had access to non-chlorinated water dosed with vitamin C.
3) to identify, by means of an observational group analysis, other potential risk factors which may have an effect on ESBL-*E* faecal carriage rates and concentrations.

2. Project description

A double-blind cluster-randomized controlled clinical trial was carried out by Stephen P. Luby and Amy Pickering from Stanford and Tufts University in cooperation with Aminul Islam from the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b), between July 2015 and December 2016 (Pickering *et al.*, 2019). Faecal samples as well as supplementary data on the children who participated in the trial were sent to EAWAG to be analysed and are the basis of this study.

2.1. Study sites

The clinical trial was carried out in the urban slums of Dhaka and Tongi, Bangladesh. The city of Dhaka is surrounded by five rivers; the Buriganga to the south, the Balu and Shitalakhya to the east, the Tongi to the north and the Turag to the west. Tongi lies just north of the Turag. These rivers frequently overflow during the monsoon and winter period (Alam, 2007). A study on Dhaka's faecal sludge management in 2014 reported that 98% of faecal waste entered the environment untreated (Peal *et al.*, 2014). Due to frequent flooding and insufficient waste management and sanitation the risk of drinking water contamination is very high in the city. Tap water samples collected from the municipal water supply in different parts of the city revealed a high prevalence (36%, n=84) of multidrug resistant bacteria (Talukdar *et al.*, 2013).

2.2. Field study design

The aim of the clinical trial was to evaluate the effect of passively chlorinated water at the point-of-collection on child diarrhoea prevalence. The subjects of the trial were children under the age of five living in households in the study areas whose primary drinking water source was a shared water point connected to a water storage tank. The trial was an open cohort, meaning that participants could enter or leave the trial after it had started. Children who no longer fulfilled participation criteria were unenrolled as soon as this occurred. Children who were enrolled from beginning to the end of the trial were referred to as "baseline children". Households were clustered into groups according to the water point they used and were randomized at a 1:1 ratio into treatment and control groups. Passive dosing devices manufactured by Medentech were installed at the inlet to the water storage tanks, treating water from the

municipal piped supply. The devices did not require electricity as they were designed to passively disinfect water by using gravity driven flow, which eroded the chlorine (trichloroisocyanurate) tablets housed within the device (Pickering, Amy J., et al.,2019). Chlorine tablets were supplied to the water points of the treatment groups. The dosing devices at the water collection points of the control groups were filled with vitamin C, chosen as an active control because it is beneficial to children's health without affecting diarrhoeal disease (ClinicalTrials, 2015). In order not to affect the taste and smell of the water, 0.2 – 0.5 ppm was targeted as a residual chlorine dose. Free and total chlorine, as well as *E. coli* measurements were taken from taps at point-of-collection and in stored water. The primary outcome of the study was based on caregiver reported infant's diarrhoea, defined as three or more loose watery stools within 24-hours. Data was collected during a 14 month follow-up period, within which households were visited every two months. The trial was blinded meaning that staff collecting non water related data and the members of enrolled households did not know which devices were dosing chlorine and which vitamin C (Pickering, Amy J., et al., 2019).

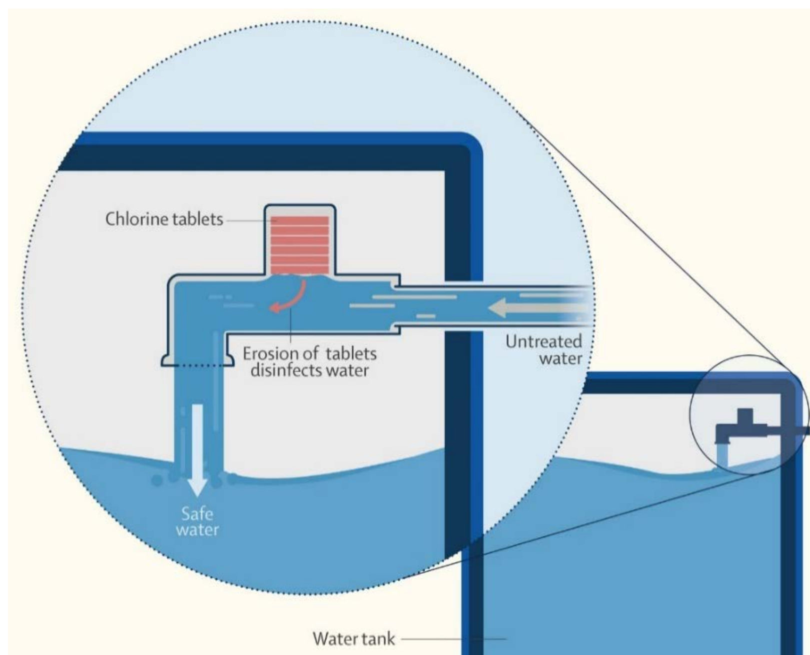

Figure 5: Passive point-of-collection chlorination device (Pickering *et al.*, 2019)

2.3. Faecal sample collection

Faecal samples were collected from the children approximately one year after the automatic dosing devices were installed. The collection was undertaken by care givers and the samples were stored in households for up to 24 hours before being collect by project staff. The faecal samples were frozen upon collection. 480 faecal samples were selected, balancing the amount from the treatment and the control group and sent to EAWAG in Switzerland where they were stored at -80 °C before being analysed.

2.4. Ethics statement

The trial's study protocol was approved by icddr,b scientific and ethical review committees as well as the Institutional Review Board (IRB) on Human Subjects Research at Stanford University (Pickering, Amy J., et al., 2019).

2.5. Outcome of the clinical trial

The data presented in Table 1 was collected and analysed by project partners at Stanford, Tufts University and the icddr,b (Pickering *et al.*, 2019) and were not part of the work carried out for this study.

Table 1: Overview of results of the clinical trial (Pickering et al., 2019)

Parameter	Treatment Group	Control Group
Care-giver reported (WHO-defined) diarrhoea	156 [7.5%] of 2073	216 [10%] of 2154
Total chlorine mean concentration at tap	0.37 mg/L	0 mg/L
Detection frequency of total chlorine at tap	83%	0%
Detection of total chlorine in stored water	45%	0%
Detection frequency of *E. coli* at tap	15%	64%
Detection of *E. coli* in stored water	36%	63%
Participants thought they were in treatment group	14%	15%

The results from the double-blind cluster-randomized controlled trial revealed a significant reduction in caregiver reported diarrhoea amongst children under the age of five in the treatment group compared to the control group (Pickering, Amy J., et al., 2019). Passive point-of-collection water chlorination was shown to effectively disinfect water at collection taps with total chlorine being detected 83% of the time at taps of the treatment group and not at all at taps from the control group. The average total chlorine concentration was of 0.37 mg/L measured at taps used by the treatment group. 45% of measurements taken from stored water at household level detected total chlorine. *E. coli* was detected 15% and 36% of the time in water from the collection tap and in stored water at household level, respectively. A higher prevalence of *E. coli* was observed in the water of the control group with 64% detection in tap water samples and 63% detection in stored water (Pickering, Amy J., et al., 2019).

3. Material and methods

3.1. Detection and quantification of ESBL-*E*

A culture-based method was used to determine the presence and quantify the amount of culturable ESBL-*E* in Bangladeshi children's faecal samples. The project group at EAWAG were blinded to which group was the treatment group and which the control group. The groups were known to them as group A and group B and will be referred to as such within this report. A total of 480 samples were processed by; 240 from group A and 240 from group B. The order in which the samples were processed was randomized making sure that samples from group A and B were processed at an equal rate.
An aliquot of 0.1-0.14 g of each thawed faecal sample was added to 0.9 ml of 0.9% NaCl solution, vortexed for 1 min with an oscillator speed of 2800 rpm and centrifuged for 30 s at 100 g/1000 rpm. Serial dilutions 10^{-1} and 10^{-2} were prepared with the supernatant of the faecal solution. Starting from the highest dilution each tube was vortexed at maximum speed before 0.1 ml of the sample was pipetted onto chromID ESBL media. ChromID ESBL is chromogenic selective agar supplemented with the third-generation cephalosporin antibiotic cefpodoxime, which relies on ß-glucuronidase substrates that release colour when they are hydrolysed (chromID ESBL, bioMerieux Inc, Marcy l'Etoile, France). Plastic L-shaped spreaders were used to distribute the faecal sample homogeneously on the media surface. Pipette tips and spreaders were renewed and disposed of after every step (Figure 6).

In order to improve the detection of ESBL-*E* an additional enrichment step was performed (Jazmati et al. 2016). Pre-enrichments were made by adding 0.1 ml of the faecal solution supernatant to 0.9 ml of non-selective tryptic soy broth (TSB). These were incubated together with the inoculated chromID ESBL plates at 37 °C under aerobic conditions for 16-24 h. The pre-enrichment solutions of the faecal samples which did not produce growth on chromID ESBL plates were plated again onto the chromID ESBL selective media. These were then incubated at 37 °C under aerobic conditions for 16-24 h (Figure 7).

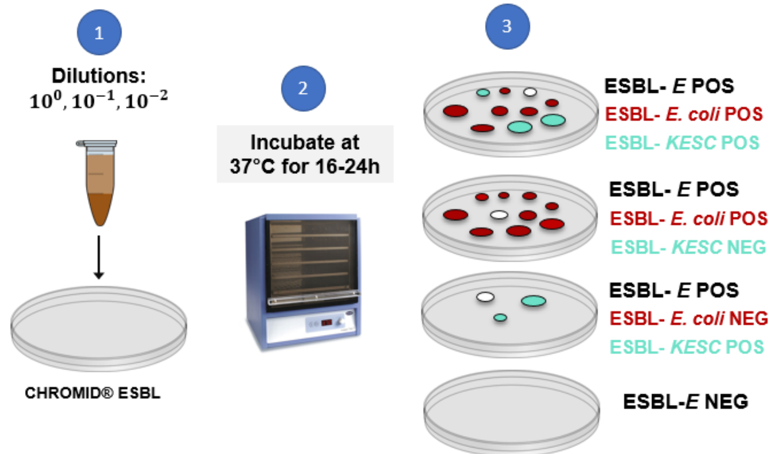

Figure 6: Culture based method: 1) Serial dilutions prepared with an aliquot of faecal sample were plated onto CHOMID ESBL media, 2) Plates were incubated overnight, 3) presumptive species of colony forming units on the ESBL-E positive plates were determined and counted

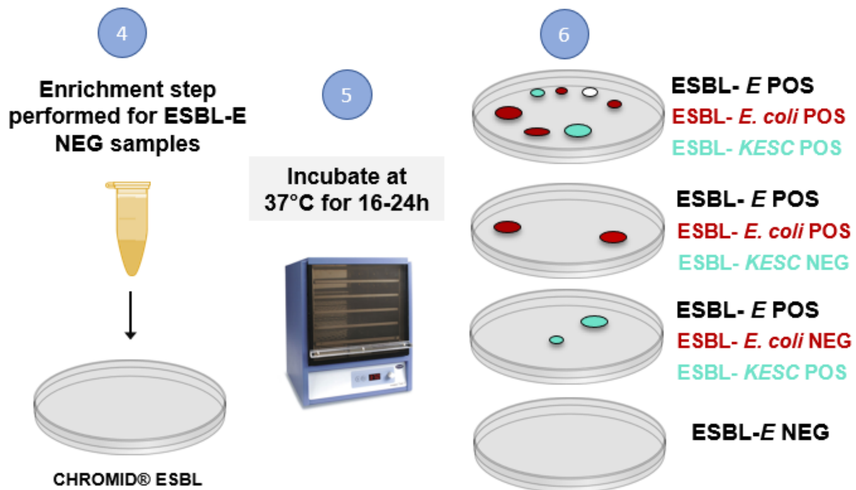

Figure 7: 4) pre-enrichment solutions of ESBL-E NEG samples were plated, 5) Plates were incubated overnight, 6) results were categorized as ESBL-E POS or NEG and ESBL-E. Coli POS or NEG

3.2. Archiving of isolate strains

Colonies were extracted from the chromID ESBL media and sub-cultured on CHROMagar ESBL media to obtain pure cultures. This was done primarily to allow future research to be carried out with these strains. However, this method also allowed a comparison to be made of phenotypic characteristics of single colonies on two different chromogenic media. For each sample two pink colonies (presumptive β-glucuronidase-positive *E. coli*), one turquoise (presumptive β-glucuronidase-positive *Klebsiella, Enterobacter, Serratia, Citrobacte*r, collectively referred to as *KESC*) and one white colony (presumptive β-glucuronidase-negative bacteria) were chosen from chromID ESBL plate which colonies had grown on and streaked onto CHROMagar ESBL agar. This was done using disposable plastic loops to obtain single colonies. The plates were incubated for 16-24 hours at 37 °C. The media surface was then wiped with cotton swabs and the collected colonies were mixed into tubes with 0.8 ml of TSB solution with 30% glycerol. The tubes were archived at -80 °C degrees.

3.3. Calculation and categorization of results

Colony forming units (CFU) present on each plate were differentiated according to the reference colour description provided by the manufacturer. The number of CFU per plate were manually counted and divided into six categories based on different ranges of CFU detected per plate (Figure 9). The categories were defined according to recommendations from the *United States Pharmacopeia* (USP) for determining the resistance of biological indicators (USP, 2011) as well as the USP informational chapter <1227> based on a review from Scott Sutton (Sutton, 2011). These consider the accepted range for countable colonies on a standard agar plate to lie between 25 and 250 CFU but not fewer than 6 CFU. The categorization of results was carried out in order to facilitate future analysis of results from certain ranges of countable colonies.

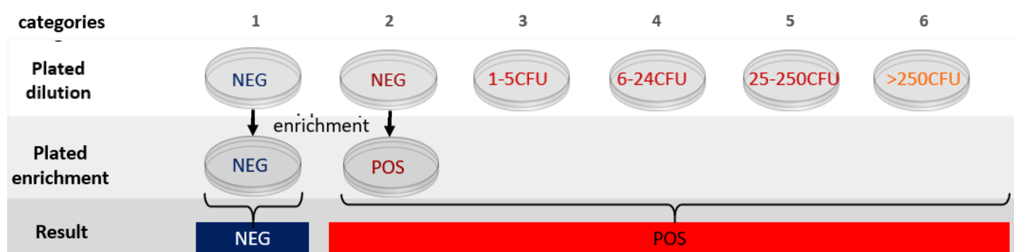

Figure 8: Categorization of results based on the rage of CFU per plate

The lower limit of detection (LLOD) was calculated by multiplying the lowest number of detectable CFUs on a plate (1 CFU) by the lowest dilution plated and dividing this by the lowest wet faecal weight used (0.1 g) to produce the dilution (equation 1).

Category 1 encompassed samples of which both the plated dilutions and the plated enrichments were negative for ESBL-*E*. These were given a value of the base 10 logarithm of half of the LLOD (equation 2). These results were used to calculate ESBL-*E* carriage rates. Samples for which the plated dilutions were ESBL-*E* negative and the plated enrichments were ESBL-*E* positive were assigned to category 2 and given the value of the LLOD (equation 1). Samples which displayed 1 to 5 CFU were placed in category 3. It was noted that these results did not fulfil the precision requirements for quantification as they lay below the range of countable colonies recommended by the United States Pharmacopeia. Samples displaying between 6-24 CFU were assigned to category 4 and those with 25-250 CFU to category 5. ESBL-*E. coli* concentrations were calculated for categories 3-5 with the wet weight of faecal sample applied to the ESBL agar plate (equation 3). No differentiation was made between categories 3-5 for the statistical analysis within the scope of this report. Plates with more than 250 CFU were placed into category 6 and assigned the value of the upper limit of detection (ULOD) (equation 4). The ULOD was calculated based on the highest number of countable colonies on one plate (250 CFUs), the highest dilution (10^{-2}) used and the largest amount of wet faeces used (0.14 g).

ESBL-*E. coli* concentrations were calculated for categories 3-5 with the wet weight of faecal sample applied to the ESBL agar plate (equation 3). In the case that more than one plated dilution fell within category 5 the results were selected from the plate which had between 60-100 CFU prioritizing the plate with the higher number of CFU. When both dilutions did not fall within this range then priority was given to the plate with the higher number of CFU.

Samples from category 3 with results below the LLOD were given a value of $2\log 10\left(\frac{CFU}{g}\right)$.

$$LLOD = log10\left(\frac{1\,colony*10}{0.1g}\right) = 2\log 10\,(\frac{CFU}{g})\quad \textit{equation1}$$

$$log10\left(\frac{1\,colony*10}{0.1g*2}\right) = 1.7\;log10(\frac{CFU}{g})\quad \textit{equation 2}$$

$$\frac{CFU}{g} = log10(number\ of\ colonies * \frac{dilution}{(weight\ of\ fecal\ aliquote)})\ \textit{equation 3}$$

$$ULOD = log10\left(\frac{250\,colony*1000}{0.14g}\right) = 6.25\;log10(\frac{CFU}{g})\quad \textit{equation 4}$$

3.4. Statistical analysis

3.4.1. Distribution of ESBL- *E. coli* faecal concentrations

The software package R (power analysis package pwr) and Microsoft Excel were used for data cleaning and the statistical analysis of the laboratory culture-method results. The distribution of ESBL-*E. coli* faecal concentrations within the total sample set were analysed and graphically presented using histograms with overlying weighted kernel density plots and boxplots with points jittered for improved visualization.

3.4.2. Analysis of ESBL-*E* and ESBL-*E. coli* faecal carriage rates

ESBL-*E* and ESBL-*E. coli* faecal carriage rates were compared between groups A and B. Additionally, associations were observed between different environmental factors such as 1) the study site location, 2) previous antibiotic consumption and 3) the period the children were enrolled in the trial. For this ESBL-*E* and ESBL-*E. coli* faecal carriage rates were compared between 1) the study sites Dhaka and Tongi, 2) children who had used antibiotics 2 months prior to sampling and children who had not used antibiotics as well as 3) children who had been enrolled at baseline and children who were not enrolled at baseline (Figure 9).

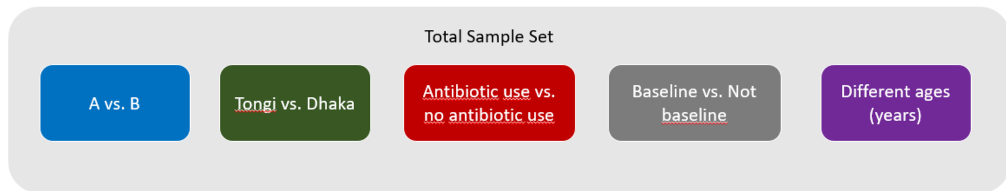

Figure 9: Groups analysed observing the effect of water chlorination, study site location, previous antibiotic consumption and the period the children were enrolled in the trial

Woerther, Paul-Louis, et al. reported a 7% yearly increase of ESBL-*E* carriage rates in South East Asia between 2002 and 2011. Based on this value the assumption was made that a 10% difference in ESBL-*E* or ESBL-*E. coli* carriage rates between two groups would be meaningful. An appropriate effect size was estimated to be 0.27 using the following equation:

$$h = 2 * \sin^{-1}(p1) - 2 * \sin^{-1}(p1 - 0.1)$$ *equation 5* (Cohen, J.,1988)

h represents the effect size and p1 is an estimated value of ESBL-*E* carriage rates in the study population. 0.74 was used for p1 based on a recent study which detected 74% ESBL-*E. coli* carriage rates in infants in rural Bangladesh (Islam *et al.*, 2019). The power analysis package in R was used to estimate the significance level needed to observe the desired effect considering the sample sizes of the groups. For this the function *two proportion testing for unequal sample sizes between groups* (pwr.2p2n.test) was applied with 80% power. The observed effect size was calculated with the following equation and used as a comparative parameter:

$$h = 2 * \sin^{-1}(p1) - 2 * \sin^{-1}(p2)$$ *equation 6* (Cohen, J.,1988)

In this case p1 and p2 were the proportions of positive samples from group 1 and group 2, respectively. Tests of proportions were carried out comparing ESBL-*E* and ESBL-*E. coli* carriage rates of independent groups with a significance level of 5%.

3.4.3. Analysis of ESBL-*E. coli* faecal concentrations

ESBL-*E. coli* faecal concentrations were compared between the groups A and B. Additionally, ESBL-*E. coli* faecal concentrations were compared between 1) Dhaka and Tongi, 2) children who had used antibiotics 2 months prior to sampling and children who had not used antibiotics as well as 3) children who had been enrolled at baseline and children who were not enrolled at baseline. Unpaired two-sided independent T-tests with a significance level (α) of 0.05 were used to determine if there was a statistically significant association between ESBL-*E. coli* faecal concentrations in the different groups mentioned.

4. Results

4.1. Faecal carriage rates and concentrations of ESBL-*E* and ESBL-*E. coli*

4.1.1. Total sample set

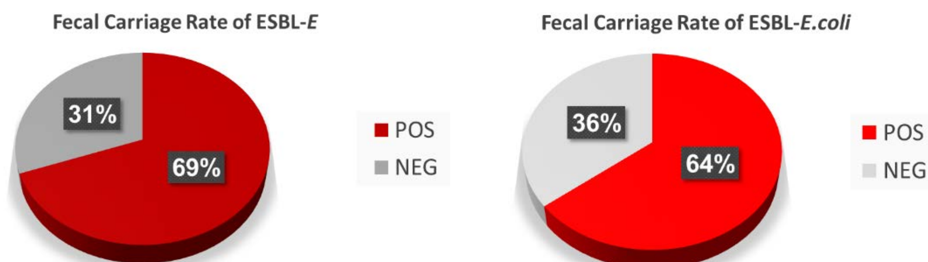

Figure 10: ESBL-E faecal carriage rate (n=480)

Figure 11: ESBL-E. coli faecal carriage rate (n=480)

The culture-based analysis of the 480 faecal samples revealed a 69% carriage rate of ESBL-*E* (Figure 10) and a 64% carriage rate of presumptive ESBL-*E. coli* (Figure 11). 5% (n=24) of ESBL-*E* positive samples were negative for ESBL-*E. coli*. These samples did not show pink colonies when plated on chromID ESBL media but rather white and turquoise colonies. The turquoise colonies were identified as presumptive ß-glucuronidase-producing *KESC* (n=8) according to the manufacturer's description. White colonies were not described in the chromID ESBL manufacturers user manual and were presumed to be non β-glucuronidase-producing bacteria. Multiple white colonies from chromID ESBL media appeared dark pink when they were streaked to single cell colonies onto CHROMagar ESBL media during the archiving process. Dark pink colonies characterize *E. coli* on CHROMagar ESBL media according to the manufacturer. Information was not found on the chromogens used in CHROMagar ESBL or the specific enzyme which influence the dark pink coloration of the colonies. Based on the phenotypical differences between the same cell colonies on these two different types of media it was assumed that a large proportion of the white colonies present on the chromID ESBL media were non ß-glucuronidase-producing ESBL-*E. coli*. Calculations of ESBL-*E. coli* faecal concentrations only included colonies which were dark pink on both chromID ESBL and CHROMagar ESBL media.

The distribution of ESBL-*E. coli* faecal concentrations of the total set of processed samples was right skewed with a mean concentration of 3.1 $\log_{10}$(CFU/g) (Figure 12). The most commonly observed concentrations were below the LLOD (n=223) (2 $\log_{10}$(CFU/g)).

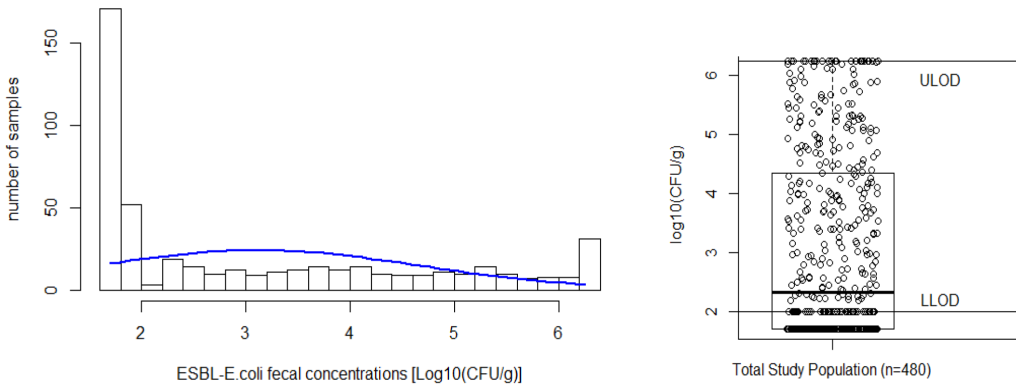

Figure 12: Distribution of ESBL-E. coli faecal concentrations

4.1.2. Faecal carriage rates in groups A and B

ESBL-*Enterobacteriaceae* faecal carriage rates in groups A and B

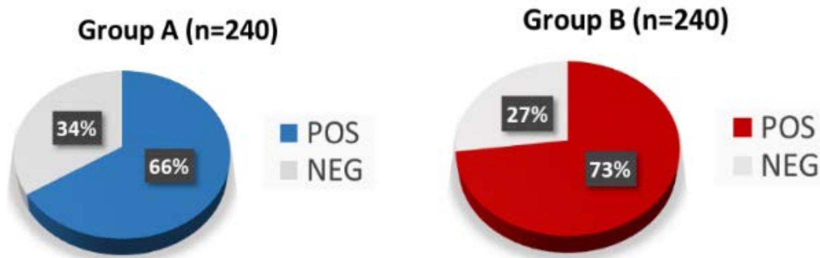

Figure 13: ESBL-Enterobacteriaceae faecal carriage rates in groups A and B

ESBL-*E. coli* faecal carriage rates in groups A and B

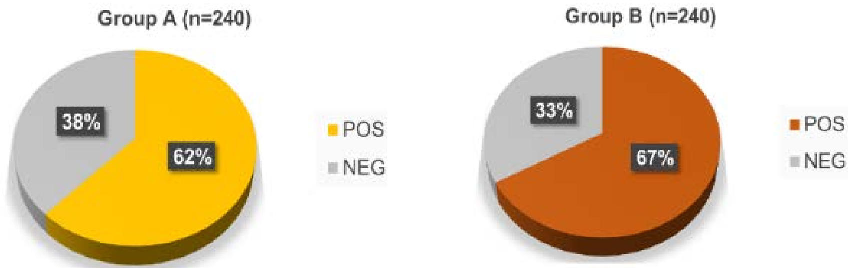

Figure 14: ESBL-E. coli faecal carriage rates in groups A and B

The preliminary comparative data analysis revealed a 66% ESBL-*E* faecal carriage rate in group A (n=240) and 73% in group B (n=240) (Figure 13). ESBL-*E. coli* carriage rates lay at 62% in group A and 67% in group B (Figure 14). The result of the statistical analysis showed that the resulting 7% size difference in ESBL-*E* faecal carriage between A and B had an estimated effect size of 0.2 and was not statistically significant (p=0.11). Similarly, the 5% size difference in ESBL-*E. coli* faecal carriage between groups A and B was not statistically significant with an estimated effect size of 0.13 (p=0.34). The average ESBL-*E. coli* faecal concentrations in groups A and B also did not differ significantly in the two-sided t.test performed (p=0.72) (Figure 15). Based on these results no association could be made between the use of point-of-collection chlorinated drinking water and the presence or concentration of ESBL-*E* in children's faecal samples.

Concentrations of ESBL-E.coli in group A and B (p=0.72)

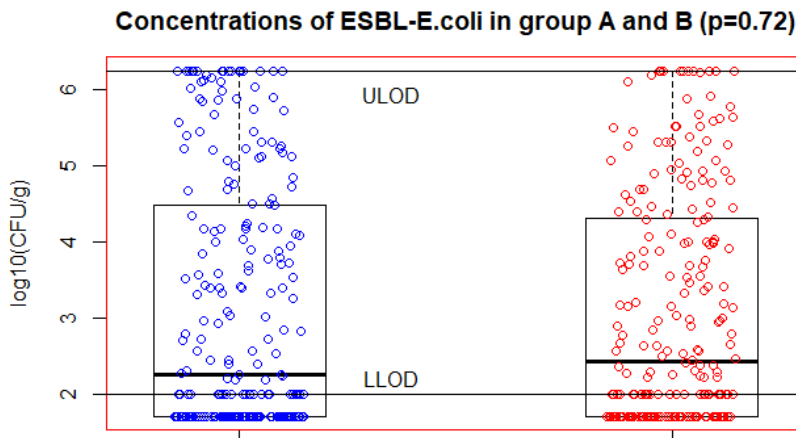

Figure 15: Distribution of ESBL-E.coli concentrations in groups A and B (p values calculated using two-sided t.test)

4.1.3. Risk factor analysis

No significant associations were observed between ESBL-*E* faecal carriage rates and 1) the study sites the children lived at (p=0.09), 2) their previous antibiotic consumption (p=47) or 3) the length that they took part in the trial (p=0.31) (Table 2).

Table 2: Group analysis of ESBL-E faecal carriage rates using two proportion testing for unequal sample sizes between groups

Factor	Groups	ESBL-E carriage rate	Δ ESBL-*E* carriage rate	Observed effect size	p-value
Location	Dhaka (n=196)	65%	8%	0.21	0.09
	Tongi (n=284)	73%			
Antibiotic use (2 months before)	Antibiotics used (n=183)	72%	4%	0.1	0.47
	Antibiotics not used (n=297)	68%			
Trial enrolment	Baseline (n=247)	72%	5%	0.13	0.31
	Not Baseline (n=233)	67%			

In contrast to ESBL-*E* faecal carriage rates, a statistical difference in ESBL-*E. coli* faecal carriage rates was observed between the study sites the children lived at. Significantly higher ESBL-*E. coli* faecal carriage rates were observed in samples collected in Tongi than in Dhaka (p=0.04). Associations between ESBL-*E. coli* faecal carriage rates and the children's previous antibiotic consumption (p=1) as well as the length that they took part in the trial were not statistically significant (p=0.1) (Table 3).

Table 3: Group analysis of ESBL-E. coli faecal carriage rates using two proportion testing for unequal sample sizes between groups

Factor	Groups	ESBL-*E. coli* carriage rate	Δ ESBL-*E. coli* carriage rate	Observed effect size	p-value
Location	Dhaka (n=196)	59%	9%	0.23	0.04
	Tongi (n=284)	68%			
Antibiotic use (2 months before)	Antibiotics used (n=183)	64%	0%	0.00	1.00
	Antibiotics not used (n=297)	64%			
Trial enrolment	Baseline (n=247)	61%	7%	0.18	0.10
	Not Baseline (n=233)	68%			

ESBL-*E. coli* faecal concentrations were found to be significantly higher in Tongi than in Dhaka, based on the results of the two-sided t.test (p=0.01) (Figure 16). The impact of other factors such as antibiotic consumption or the period of enrolment in the trail showed no significance on ESBL-*E. coli* faecal concentrations (Table 4).

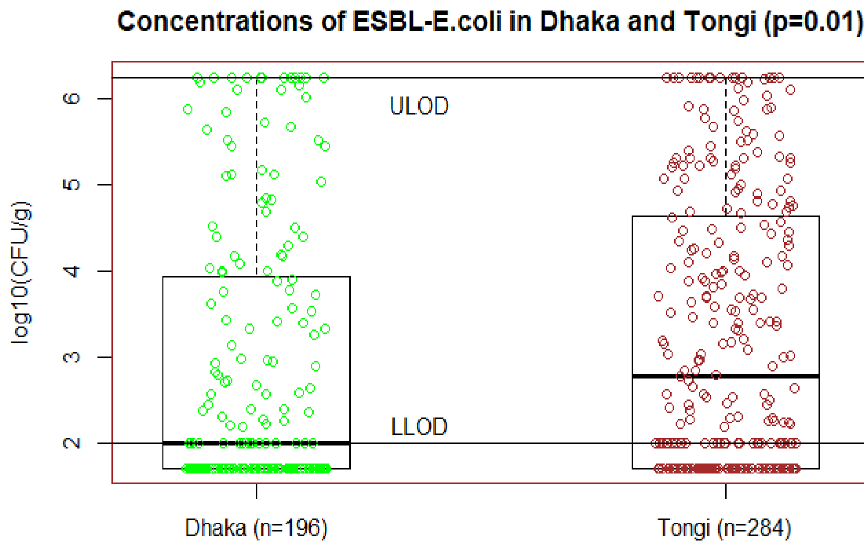

Figure 16: Distribution of ESBL-E. coli concentrations in Tongi and Dhaka (p values calculated using two-sided t.test)

Table 4: Groups analysis of ESBL-E. coli faecal concentrations using two-sided t.test

Factor	Group	Frequency (n)	mean ESBL-*E.coli* faecal concentration [log_{10}CFU/g]	p-value
Location	Dhaka	196	2.88	0.01
	Tongi	284	3.26	
Antibiotic use (2 months before)	Used	183	3.05	0.35
	Not used	297	3.2	
Trial enrolment	At Baseline	247	3.07	0.6
	Not at Baseline	233	3.15	

4.2. Sensitivity of method and ranges of countable colonies per plate

The application of a pre-enrichment stage increased the sensitivity of the method. 7.5% (n=25) of all ESBL-*E* positive samples were only detected after the pre-enrichment stage was performed (category 2 in Figure 17). 15% (n=74) of the total number of samples displayed less than 6 CFU per plate (*category 3*), 8% (n=38) displayed 6 to 24 CFU per plate (*category 4*), 34% (n=165) displayed between 25 and 250 CFU per plate (*category 5*) and 6% (n=31) displayed more than 250 CFU per plate (*category 6*) (Figure 17).

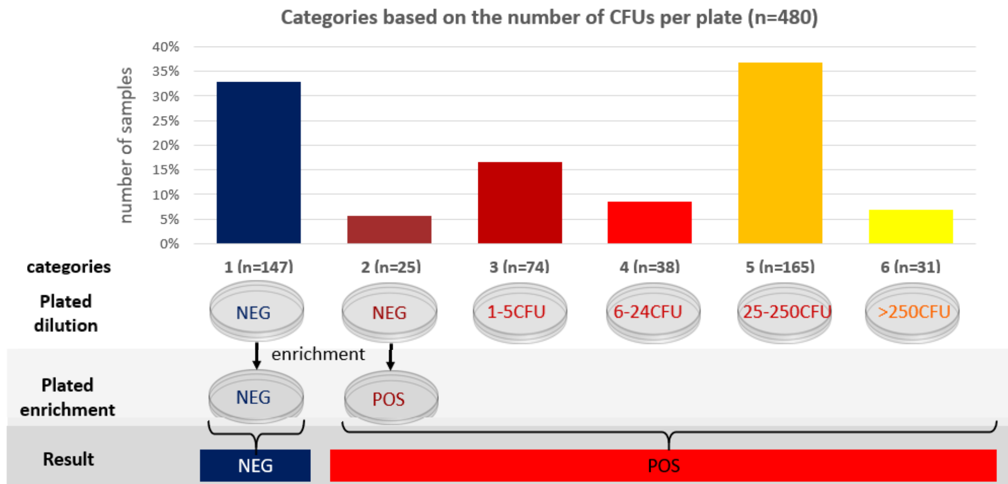

Figure 17: Categorization of results based on the number of CFU per plate

5. Discussion of main findings

Drinking water chlorination did not show a significant effect on ESBL-*E* faecal carriage rates in Bangladeshi children.

The results of the preliminary data analysis did not support the research hypothesis that *point-of-collection drinking water chlorination reduces ESBL-E faecal carriage rates in Bangladeshi children*. The observed 7 % size difference between ESBL-*E* faecal carriage rates in the treatment and the control group was not significant for the current sample size. Even with a larger sample size a statistical difference in ESBL-*E* faecal carriage rates may not be observed. There are multiple factors which influence the spread of AMR which may have a larger effect on the prevalence of ESBL-*E* than drinking water chlorination. The fact that the children in Dhaka and in Tongi were exposed to a highly contaminated environment, without proper sanitation and urban drainage systems, increases the risk of transmission of antimicrobial resistant bacteria via multiple faecal-oral routes. Monsoons and the flooding of the rivers surrounding the city are likely to worsen the situation by increasing the exposure of humans not only to faecal waste but also to untreated effluent from hospitals, pharmaceutical companies and other industrial wastewaters (Kamruzzaman *et al.*, 2013). Children under the age of five are commonly known to be more susceptible to faecal-oral transmission of pathogens as they often do not have preventive hygiene behaviour. For this reason, their contaminated living environments may have had a larger impact on their gut flora than their drinking water intervention.

In order to effectively evaluate the potential of drinking water chlorination as a barrier to the spread of AMR independently of other environmental drivers, it would have been necessary to measure levels of ESBL-*E. coli* in chlorinated and non-chlorinated water samples from taps and stored water. Based on the samples taken during the clinical trial, *E. coli* was detected in 15% of the samples measured at the taps with chlorinated water (treatment group) and in 36% of the water samples stored in households from the treatment group (Pickering *et al.*, 2019). This is a relatively large increase in culturable bacteria compared to water measurements from drinking water dosed with vitamin C (control group), where tap water and stored water samples showed a 64% and 63% *E. coli* positivity rate, respectively (Table 1) (Pickering *et al.*, 2019). One explanation for this difference in *E. coli* prevalence is that the low dosage of

chlorine residual induced the culturable bacteria into a viable but non culturable (VBNC) state. It has been reported that bacteria that enter a VBNC state have been observed to be more resistant than culturable ones to multiple antibiotics (Lin *et al.*, 2017). Water samples would have allowed the relative abundance of ESBL-*E. coli* in the 36% of *E. coli* positive samples, collected in households from the treatment group to be investigated.

A high prevalence of ESBL-*E* and ESBL-*E. coli* was detected in the study population.

The preliminary analysis of faecal carriage rates in the 480 children's stool samples revealed a very high prevalence of ESBL-*E* (69%) as well as of ESBL-*E. coli* (64%). It can be assumed that children's ESBL-*E* faecal carriage rates in the local community at the study sites, Dhaka and Tongi, lay within a similar range in the year 2016, when the samples were collected. Even though the detected prevalence of ESBL-*E* exceeded most reported carriage rates worldwide, they were representative for South East Asia (Woerther *et al.*, 2013) and were comparable to the 74% ESBL-*E. coli* carriage rate reported in young infants living in rural areas of Bangladesh in 2017 (Islam *et al.*, 2019). The difference between the faecal carriage rate detected in this study and the one reported by Islam *et al.* could partially be explained by the rapid annual increase in the prevalence of ESBL-*E* in South East Asia (Woerther *et al.*, 2013). However, there are numerous other external factors which differ between urban and rural living environments, such as water and sanitation systems, hygiene behaviour, diet and access to medical care, which would need to be investigated in order to explain the size difference.

A significant difference was found between amounts of ESBL-*E. coli* detected in samples from Dhaka and Tongi.

ESBL-*E. coli* carriage rates as well as ESBL-*E. coli* faecal concentrations were significantly higher in children's stool samples from Tongi than from Dhaka. There are several environmental, socio-economical and geographical factors which may contribute to the difference in ESBL-*E. coli* faecal prevalence and concentrations in Dhaka and Tongi.

Drinking water contamination was reported to be higher at taps in Dhaka than in Tongi with *E. coli* detected in tap water samples 87% and 50% of the time, respectively. Awareness of water contamination was also higher in Dhaka, where people were more likely to treat their own water at home by boiling it meaning the population in Dhaka might have been drinking microbially safer water than in Tongi (Pickering *et al.*, 2019).

It was reported that there was a larger number of animals present in the settlements in Tongi than in Dhaka (Amy Pickering, personal correspondence). A study in low-income households in Dhaka showed evidence of ruminant-associated faecal contamination on child hands and household floors (Harris *et al.*, 2016). In order to access the risk of vector-transmission of antimicrobial resistant bacteria via animals living in close proximity to communities, faecal samples from animals would need to be taken and analysed for ESBL- *E. coli.* It would also be useful to analyse the different species and amounts of antimicrobial resistant bacteria in other environmental samples, such as soil and surface swabs, and compare these to the resistant bacteria in children's stool samples in order to gain a better understanding for how AMR spreads between the human, animal and environmental reservoir.

Differences in topography around the children's homes and the water collection points as well as their proximity to contaminated water bodies are additional factors that may be associated to ESBL-*E. coli* faecal carriage rates. Even though the area around Dhaka and Tongi is mostly flat, differences in elevation and proximity to river catchment areas could be an indicator for how severely homes are affected by flood events and therefore also by associated faecal contamination, that may correlate with contamination of antimicrobial resistant bacteria.

Finally, food availability may have resulted in differences in diet in different areas. Associations have been made between malnutrition and susceptibility to ESBL-*E. coli* carriage (Kayser et al., 2015). Further analysis of additional meta-data from the study sites would be needed in order to establish the relation between nutrition-status an ESBL-*E* faecal carriage rates.

In order to broaden our understanding of the multifaceted drivers of ESBL-*E. coli* carriage in both study sites, future work will include an additional analysis accounting for clustering according to the water points to which the children had access. Furthermore, the GPS coordinates of the water points from the treatment and control group could be used to investigate other environmental risk factors related to the household's location at the study sites. Additionally, satellite images and climate data could be used to evaluate the vulnerability of the study sites to sever flood events.

Several factors were identified limiting the reliability of ESBL-*E. coli* quantification.

Reports of ESBL-*E. coli* concentrations are limited which made it difficult to validate and compare the detected average ESBL-*E. coli* concentration of 3.1 $\log_{10}$(CFU/g). One recent study on healthy infants in rural Bangladesh reported higher average faecal concentrations of 6.86 $\log_{10}$(CFU/g) *E. coli* resistant to third generation cephalosporins of which approximately 90% were ESBL-producing (Islam *et al.*, 2019). The size difference between our results and the ones reported by Islam et al. is plausible as the faecal carriage rates observed by Islam et al. were also considerably higher than ours. However, several limitations were identified which reduce the reliability of our ESBL-*E. coli* concentrations.

The sample collection and culture-based method used in this project for processing the faecal samples was limited in various ways. Samples were collected in households by caregivers and stored for up to 24 hours before they were frozen. The length of time the faecal samples were stored at ambient temperature may have had a significant effect on the number of CFUs present in each sample due to bacterial growth. Unfortunately, no information was provided on the specific length of time each sample had been stored at ambient temperatures, making it difficult to assess the reliability of the results. 29% of the samples processed revealed colony counts outside of the countable range

recommended by the *United States Pharmacopeia,* thus, reducing their overall accuracy. Furthermore, the quantification of ESBL-*E. coli* did not include uncoloured colony counts, which means that non ß-glucuronidase producing ESBL-*E. coli* were not included in the calculated faecal concentrations. In order to estimate the proportion of non ß-glucuronidase producing ESBL-*E. coli* present in the samples, additional conformational tests would need to be performed, identifying the proportion of non ß-glucuronidase producing ESBL-*E. coli* on a representative number of plates.

6. Conclusion and future work

The preliminary analysis of results obtained from the culture-based method revealed that the consumption of chlorinated drinking did not have a significant effect on ESBL-*Enterobacteriaceae* (ESBL-*E*) faecal carriage rates in Bangladeshi children. A high prevalence of ESBL-*E* (69%) as well as ESBL-*E. coli* (64%) were detected in the study population. The results of an observational analysis based on supplementary data on the children suggested that environmental factors associated with differences between the study sites, Dhaka and Tongi, had a significant effect on ESBL-*E. coli* prevalence and faecal concentrations. It was assumed that other environmental factors which differed according to location, such as lack of sanitation, additional water treatment in households and vector transmission by animals, may have had a larger effect on ESBL-*E* faecal carriage rates than the water chlorination intervention.

Limitations were identified, reducing the reliability of the quantification of ESBL-*E. coli* in faecal samples of children. These were associated with longer periods of time that faecal samples may have been stored in households at ambient temperatures before being collected and frozen. Furthermore, additional conformational tests are needed in order to estimate the proportion of non-ß-glucuronidase producing ESBL-*E. coli* present in the samples in order to improve the accuracy of ESBL-*E. coli* quantification using ChromID ESBL.

Future work will focus on improving current understanding of major drivers of AMR which influenced the difference in ESBL-*E. coli* faecal carriage rates between the study sites, Dhaka and Tongi. This will include an additional subgroup analysis, accounting for clustering according to drinking water points. The insights gained through this study can serve as a basis for future development of viable environmental interventions and their effective implementation in highly contaminated environments.

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