

## Unsealed tubewells lead to increased fecal contamination of drinking water

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### ABSTRACT

Bangladesh is underlain by shallow aquifers in which millions of drinking water wells are emplaced without annular seals. Fecal contamination has been widely detected in private tubewells. To evaluate the impact of well construction on microbial water quality 35 private tubewells (11 with intact cement platforms, 19 without) and 17 monitoring wells (11 with the annulus sealed with cement, six unsealed) were monitored for culturable *Escherichia coli* over 18 months. Additionally, two 'snapshot' sampling events were performed on a subset of wells during late-dry and early-wet seasons, wherein the fecal indicator bacteria (FIB) *E. coli*, Bacteroidales and the pathogenicity genes *eltA* (enterotoxigenic *E. coli*; ETEC), *ipaH* (*Shigella*) and 40/41 hexon (adenovirus) were detected using quantitative polymerase chain reaction (qPCR). No difference in *E. coli* detection frequency was found between tubewells with and without platforms. Unsealed private wells, however, contained culturable *E. coli* more frequently and higher concentrations of FIB than sealed monitoring wells ( $p < 0.05$ ), suggestive of rapid downward flow along unsealed annuli. As a group the pathogens ETEC, *Shigella* and adenovirus were detected more frequently (10/22) during the wet season than the dry season (2/20). This suggests proper sealing of private tubewell annuli may lead to substantial improvements in microbial drinking water quality.

**Key words** | adenovirus, Asia, Bacteroidales, *E. coli*, *Shigella*, tubewells

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## INTRODUCTION

Fecal bacteria and viruses have been detected in ground-water wells emplaced in aquifers of diverse geologic material (Rudolph *et al.* 1998; Abbaszadegan *et al.* 2003; Embrey & Runkle 2006; Borchardt *et al.* 2011; Johnson *et al.* 2011; Kozuskanich *et al.* 2011). In developed countries, such as the USA, wells are typically sealed with an expanding clay which fills the annulus between the well casing and the surrounding aquifer sediments from ground surface to approximately 1 m above the screened interval to prevent 'short circuiting' by downward flow of contaminated surface water or shallow groundwater. In developing countries, such as Bangladesh, the annulus of shallow drinking water wells, referred to as tubewells, is typically filled with soil or sediments obtained during drilling; a measure unlikely to prevent rapid annular flow. Recent programs have encouraged the construction of cement platforms and drainage channels around well-heads to drain wash water before it infiltrates down the tubewell annulus, but there is insufficient information to indicate whether this is successful (Luby *et al.* 2008; Leber *et al.* 2011; van Geen *et al.* 2011). Although tubewells are considered an 'improved' drinking water source by the World Health Organization (WHO 2000), these tubewells still do not have adequate protection from annular flow and are considered unsealed (personal communication, Peter Ravenscroft). The goal of this study was to evaluate the impact of tubewell construction (specifically annular seals and cement platforms) on the levels of human exposure to fecal contamination and pathogens.

Over 10 million of these tubewells provide drinking water for millions of inhabitants throughout rural Bangladesh. In this country, 11% of all deaths are estimated to be caused by diarrheal disease (Streatfield *et al.* 2001) with recent studies suggesting that sustained levels of diarrheal disease are caused in part by drinking untreated groundwater (Escamilla *et al.* 2011, 2013; Wu *et al.* 2011). The widespread fecal indicator bacteria (FIB) and bacterial and viral pathogens found in tubewells (Luby *et al.* 2008; van Geen *et al.* 2011; Leber *et al.* 2011; Ferguson *et al.* 2012) are known to be predictive of diarrheal disease in diverse populations around the world (Gundry *et al.* 2004).

Bangladesh is underlain by shallow aquifers consisting of unconsolidated sand and silt laid down by streams and rivers flowing through the Ganges-Brahmaputra delta (Goodbred & Kuehl 1998; Weinman *et al.* 2008). In addition to annular flow around the tubewell, it is possible that fecal contamination enters aquifers and tubewells through infiltration from latrines or seepage from the many ponds and canals found in rural villages (Knappett *et al.* 2011a). Recent field experiments in Bangladesh indicate efficient spatial removal of FIB in typical medium-grained aquifers, with 7-log<sub>10</sub> removal within 13 m from leaking latrine ponds (Knappett *et al.* 2012). This finding suggests that fecal contamination of unconfined aquifers from latrines, ponds and other sources should have relatively limited spatial extent. Contamination would be expected to be even more limited in areas where the aquifer is overlain by silt or clay layers. This is not consistent, however, with the widespread occurrence of FIB in tubewells located in both unconfined sandy aquifers and aquifers overlain by silt (Leber *et al.* 2011), suggesting the presence of rapid flow pathways, such as annular flow around the tubewell casing.

Recent programs in Bangladesh and other developing countries have encouraged the construction of 2–3 m<sup>2</sup> cement platforms with drainage channels around tubewells to remove standing water and reduce the likelihood of annular flow of contaminated water (Luby *et al.* 2008; WHO/UNICEF 2012). The utilization of cement platforms is supported by studies in rural Africa (Godfrey *et al.* 2006), which found links between the presence of standing water around the well-head and poor microbial water quality in the well.

Studies spanning multiple villages and seasons in Bangladesh, however, have shown that *E. coli* detection frequency in private tubewells is typically insensitive to both the presence and quality of a platform (Luby *et al.* 2008; van Geen *et al.* 2011; Leber *et al.* 2011) leading some to suggest that tubewells are not subject to annular flow (Luby *et al.* 2008). Only one study (Escamilla *et al.* 2013), considering a subset of approximately 90 wells from the study by van Geen *et al.* (2011), found that the presence of a well platform correlated to lower *E. coli* detection

frequency in private wells. This effect was only significant during the early monsoon period (April–June) and at no other time of year (Escamilla *et al.* 2013). Likely more critical than the presence of an intact platform in preventing annular flow is the presence of a seal between the borehole and the annulus of a well from surface to screened depth.

*E. coli* prevalence peaks in tubewells during the wet season (Leber *et al.* 2011), being closely associated with antecedent rainfall events (van Geen *et al.* 2011). Increases in allochthonous bacteria concentrations in aquifers following rainfall events are attributed to vertical flushing when bacteria are both introduced to the vadose zone and mobilized from grain surfaces due to increasing water content and shear velocity (De Novio *et al.* 2004; Pronk *et al.* 2007). Pit latrines and ponds are the primary repository of human feces in Bangladesh and the water table is very shallow, lying 1–5 m below the surface, giving ample opportunity for contamination of the water table from these numerous point sources in densely populated rural villages. Further, flooding is widespread during the late wet season, potentially widening the spatial extent of fecal contamination sources at the surface during the time of year when the vadose zone is thinnest (van Geen *et al.* 2011; Knappett *et al.* 2012).

The objective of this study is to quantify the impact of private well construction on the frequency and concentrations of FIB detected in tubewells within a sandy aquifer underlying a village in rural Bangladesh. A further objective is to assess the utility of molecular FIB markers in predicting the year-round risk of fecal contamination of a well emplaced within a shallow, reducing aquifer. Quantitative PCR (qPCR) was used to screen for the pathogenicity genes *elt A* (enterotoxigenic *E. coli*; ETEC), *ipaH* (*Shigella*) and 40/41 hexon (adenovirus) to assess the ability of FIBs to indicate the presence/absence of pathogens.

Some of the monthly cultured *E. coli* measurements reported in this study for private tubewells have been published previously in the context of a larger study finding a broad negative correlation between *E. coli* detection frequency and arsenic (van Geen *et al.* 2011). The previously published monthly *E. coli* detection frequencies for 35 private wells at Site K is compared here to synoptic measurements made on 17 additional monitoring wells. Additionally, the previously unpublished results of two

snapshot monitoring events for FIB DNA and pathogens on a subset of private and monitoring wells are presented here.

## METHODS

### Site description

The village of Char Para is referred to herein as Site K (Knappett *et al.* 2011a, b). Char Para overlies a sand bar deposit of the neighboring ‘Old Brahmaputra’ river, which flows throughout Arai hazar upazilla and transported far more water and sediment, in the past, than it does today (Weinman *et al.* 2008). Bangladesh has a dry season during which the region receives little rain from November through May. The dry season is followed by the monsoon, a period of 3–4 months during which Bangladesh receives the vast majority of its total rainfall for the year. In this study, rainfall was measured using a HOBO weather station (ONSET, Bourne, MA) in the region of Matlab, 50 km south of Site K. The unconfined water table at Site K fluctuates throughout the year from 4 m below the surface at the end of the dry season to within 1 m of the surface during the wet season (Knappett *et al.* 2012). Although there is an influence of the local river which bounds three sides of the village, lateral hydraulic gradients throughout Site K are small and affected by irrigation pumping in the surrounding rice fields. Approximately 1,500 people live in Site K. Roughly 50 ponds and 180 latrines are scattered throughout the site. Half of these latrines spill effluent onto the open ground (Knappett *et al.* 2011a), consistent with the country-wide improved sanitation coverage for rural Bangladesh in 2010 of only 43% (WHO/UNICEF 2012).

Private wells in Bangladesh, referred to as ‘tubewells’, are inexpensive PVC pipes with 1–1.5 m screened intervals equipped with iron hand pumps and are typically screened at depths ranging from 8 to 30 m below the ground surface (van Geen *et al.* 2003). As of 2009 our exhaustive survey of Char Para (Site K) indicated that it contained 144 private tubewells. Therefore 1 tubewell supplies drinking water for 10 people, as there are 1,500 inhabitants in the village (Knappett *et al.* 2011a). Drillers will typically drill no further than the depth necessary to ensure a year-round supply. In the absence of poorly conductive surface deposits, tubewells

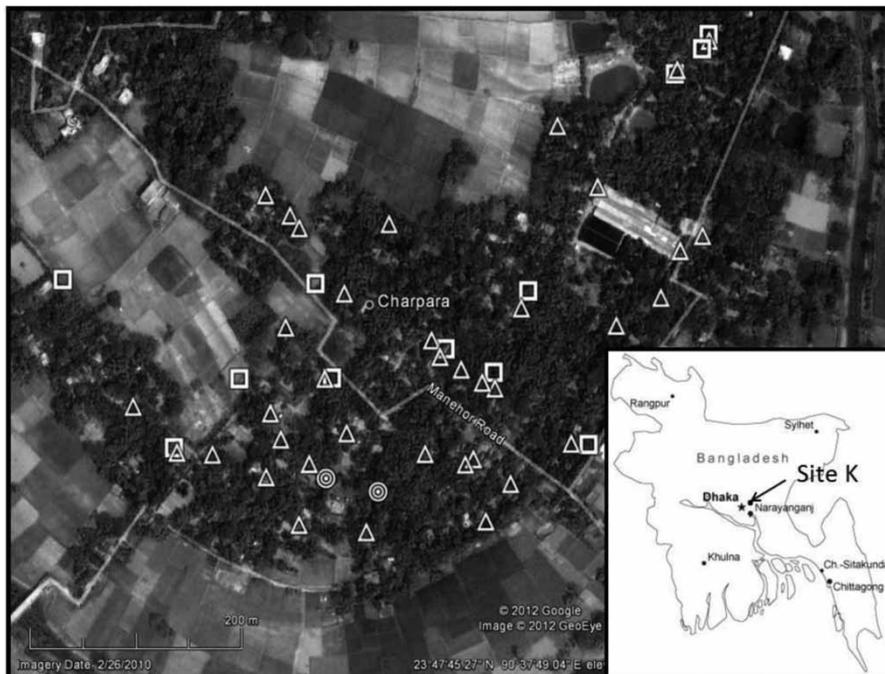
will be quite shallow as they are at Site K (Leber *et al.* 2011). The minimum and maximum depths of these private tubewells, reported by the owners, were 6.1 and 91.5 m, respectively, with a median depth of 9.1 m. The positions of all wells in this study were determined using high accuracy (sub-meter) GPS using a Trimble GeoXH receiver and Terrasync 2.4 software. GPS data were post-processed using Pathfinder Office 3.0 (Trimble Navigation Ltd, Sunnyvale, CA).

Drillers in Bangladesh do not typically use any material to seal the outside of the PVC pipe (personal communication, Peter Ravenscroft) from rapid annular flow or 'short-circuiting' of surface water or near-surface water to screened interval depth. In developed countries, bentonite, an expanding clay, will typically be used to seal wells. To control for the possibility of annular flow, 11 monitoring wells with cement seals (MS) were installed in January 2008 throughout Site K. Ten of these had 1.5 m screens at the same depth as a nearby private well, and one was installed at some distance from all the 35 monitored private wells (Figure 1) and was screened at a typical depth. In addition six monitoring wells were installed within two

multilevel nests, previous to 2008 without cement seals (M) (Table 1). These unsealed monitoring wells served as intermediates between private (P) and sealed monitoring (MS) wells to test for an effect of regular pumping only (as opposed to pumping absence and seal presence combined) on the frequency of *E. coli* detections in the well. The depths of the 35 class P wells varied between 5.8 and 30.5 m (5th and 95th percentile were 6.1 and 15.2 m, respectively) and the median depth was 7.6 m. Depths of the 11 MS wells varied between 7.2 and 15.4 m and the median depth was 7.7 m. All wells were monitored for *E. coli* monthly for the period from April 2008 through November 2009.

### Well sampling

Continuous pumping from both P and MS tubewells has been observed to dramatically decrease measured concentrations of *E. coli* over a 24-hour period (Knappett *et al.* 2010b), therefore prior to sampling all wells were purged for a consistent three well-bore volumes. Duplicate 100 mL water samples were taken from every well. Private tubewells



**Figure 1** | Locations of 35 unsealed private tubewells (triangles), six unsealed (circles) and 11 sealed monitoring wells (squares) within Char Para (Site K). The six unsealed monitoring wells are contained within two multilevel piezometer nests. Image produced in Google Earth®. The inset country map is from [www.mapresources.com](http://www.mapresources.com). The scale bar in the bottom left corner represents 200 m.

**Table 1** | Classification of tubewells at Site K

Well type (notation)	Seal (y/n)	Pumping frequency	Count
Private (P)	N	Daily	35
Monitoring (M)	N	Monthly	6
Monitoring (MS)	Y	Monthly	11
		<i>Total</i>	52

were sampled using the existing iron hand pump whereas monitoring wells were pumped using submersible electric pumps (Typhoon, Groundwater Essentials, LLC) at flow rates ranging from 2 to 8 L/min. In between monitoring wells, all tubing and electric pumps were flushed with a bleach and TWEEN solution diluted in water derived from the well just sampled, followed by rinsing once with well water and once with sodium thiosulfate as detailed in Knappett *et al.* (2011b).

Two 'snapshot' sampling events were performed, once during the dry season (March 16–18) and once during the wet season (July 3–7) in 2009 to analyze for a broad spectrum of FIBs and pathogens. Cultured *E. coli* was measured concurrently, only during the wet season sampling event.

### Microbial analyses

*E. coli* was quantified using the Most Probable Numbers (MPN) based Colilert™ test kit (IDEXX Laboratories, Inc.). Duplicate 100 mL groundwater samples were collected in sterile containers and measurements were carried out in a laboratory within 8 hours of sample collection. MPN of *E. coli* were determined by combining the numbers of discrete positive wells in both trays (Hurley & Roscoe 1983; Knappett *et al.* 2011b).

For enumeration of fecal bacteria genomes, 4–8 L of groundwater was filtered onto 0.22 µm nitrocellulose filters. The filters were removed from the plastic housing, placed in sterile Petri dishes, frozen and transported on dry ice to the University of Tennessee. DNA was extracted and purified from the filters using the FastDNA® SPIN for Soil Kit (MP Biomedicals, LLC, Solon, Ohio) following the manufacturer's protocols. DNA was measured using a nanodrop and the extracts were diluted to 5–10 ng/µL of total DNA to avoid inhibition, and this was further verified by

measuring the amount of a known plasmid spike added to each sample for each PCR run.

Quantitative PCR was performed to detect *E. coli* and Bacteroidales using the same assays and laboratory methods as described in our previous study (Knappett *et al.* 2011b). The gene targets for the *E. coli* (herein referred to as *mE. coli*) and Bacteroidales assays were the 23S rRNA gene and the 16S rRNA gene, respectively (Bernhard & Field 2000; Scott *et al.* 2002; Layton *et al.* 2006; Noble *et al.* 2006; Kildare *et al.* 2007) and the primer and probe sequences are provided in Table 2. Pathogen genes (*eltA*, *ipaH* and adenovirus 40/41) were assayed in duplicate or triplicate using the primers, probes and master mix types listed in Table 2 and following standard qPCR protocols described in previous studies (Knappett *et al.* 2011b). Standards for the quantitative PCR reactions were made from a relevant gene fragment cloned into PCR4-TOPO cloning vector (Layton *et al.* 2006). The method detection limit (MDL) was determined from the standard curve to be 20 gene copies per qPCR reaction. Data were calculated only for samples in which at least two PCR reactions had >1 gene copy and were quantified as copies/µL nucleic acid extract. Gene copies were adjusted to copies/100 mL for tubewell water based on the fraction of the filter extracted, multiplied by the volume of DNA extract and divided by the filtered sample volume. Due to differences in the volume of water filtered for each sample, the MDL varied somewhat with each sample (Knappett *et al.* 2011b) with a mean detection limit of four copies/100 mL for the groundwater samples (Ferguson *et al.* 2012).

### Experimental design and statistical analyses

The three well types (P, M and MS) were compared for *E. coli* prevalence using binned wet/dry season box plots and a monthly time series comparison between P and MS wells. A 6-month dry season was defined here from November 15 through May 15 with the wet season being the other half of the year. A total of 18 months were available (April 2008 through October 2009) for which all three classes of wells were sampled each month (class P wells were also sampled from January 2008 through April 2008). For statistical testing on binned wet/dry season data a minimum of five sampling events were required in each season for each well, causing

**Table 2** | Quantitative PCR primer and probes used to target specific genes and organisms in tubewell water samples

Target organism	Assay gene target (assay type and annealing temperature)	Oligonucleotide sequences <sup>a</sup>	Reference
<i>E. coli</i>	23S rRNA (fluorogenic probe, 55 °C)	EC23Sf, 5' GAG CCT GAA TCA GTG TGT GTG 3' EC23Sr, 5' ATT TTT GTG TAC GGG GCT GT 3' EC23Srv1Taq, 5' CGC CTT TCC AGA CGC TTC CAC	Knappett <i>et al.</i> (201b)
All <i>Bacteroides</i>	16S rRNA (fluorogenic probe, 60 °C)	AllBac296f, 5'-GAGAGGAAGGTCCCCAC-3' AllBac412r, 5'-CGCTACTTGGCTGGTTCAG-3' AllBac375Taq, 5'CCATTGACCAATATTCCTCACTGCTGCCT (BHQ-1)-3'	Layton <i>et al.</i> (2006)
<i>Shigella</i> and enteroinvasive <i>E. coli</i> (dysentery-type <i>E. coli</i> )	<i>ipaH</i> (fluorogenic probe, 60 °C)	<i>IpaH</i> U1f- 5' CCTTTCCGCGTTCCTTG A-3' <i>IpaH</i> L1r- 5'- CGGAATCCGGAGGTATTG C-3' <i>IpaH</i> Taq- 5'-CGCCTTTCCGATACCGTCTCTGCA-3'	von Seidlein <i>et al.</i> (2006)
Enterotoxigenic <i>E. coli</i> strains	<i>eltA</i> (heat labile toxin LT) (fluorogenic probe, 60 °C)	<i>Elt311f</i> - 5' TCTGAATATAGTCCGGCAGA-3' <i>Elt414r</i> -5' CAACCTTGTGGTGCATGATGA-3' <i>Elt383Taq</i> r -5' TTCTCTCCAAGCTTGGTGATCCGGT-3'	Modified from Persson <i>et al.</i> (2007)
Adenovirus 40/41	<i>Hexon</i> (fluorogenic probe, 60 °C)	AV40/41-117f, 5'- CAGCCTGGGGAACAAGTTCAG 3' AV40/41-258r, 5' -CAGCGTAAAGCGCACTTTGTAA 3' AV40/41-157Taq, 5' ACCCACGATGTAACCACAGACAGGTC 3'	Rajal <i>et al.</i> (2007)

<sup>a</sup>All probes synthesized with FAM (fluorescein) and black hole quencher 1 (BHQ1) from Biosearch Technologies.

the numbers of wells in each category to be reduced to 33, 6 and 11 for P, M and MS, respectively. Analysis of variance (ANOVA) was performed three times with well class as the 'treatment' and *E. coli* frequency during year round, wet and dry seasons as response variables, to determine differences between the classes of wells. Further, the non-parametric Kruskal-Wallis test was performed on the ranks to confirm statistical differences between paired classes of wells using the statistical software NCSS (version 07.1.14, NCSS, LLC, Kaysville, Utah). *E. coli* prevalence in a given well (across time) or during a given month (across space) was accompanied by the approximation  $\pm 2 [p(1-p)/n]^{1/2}$  used to estimate 95% CIs for the proportion of wells  $p$  with detectable *E. coli* where  $n$  is the total number of sampling events or wells respectively (Gelman & Hill 2007).

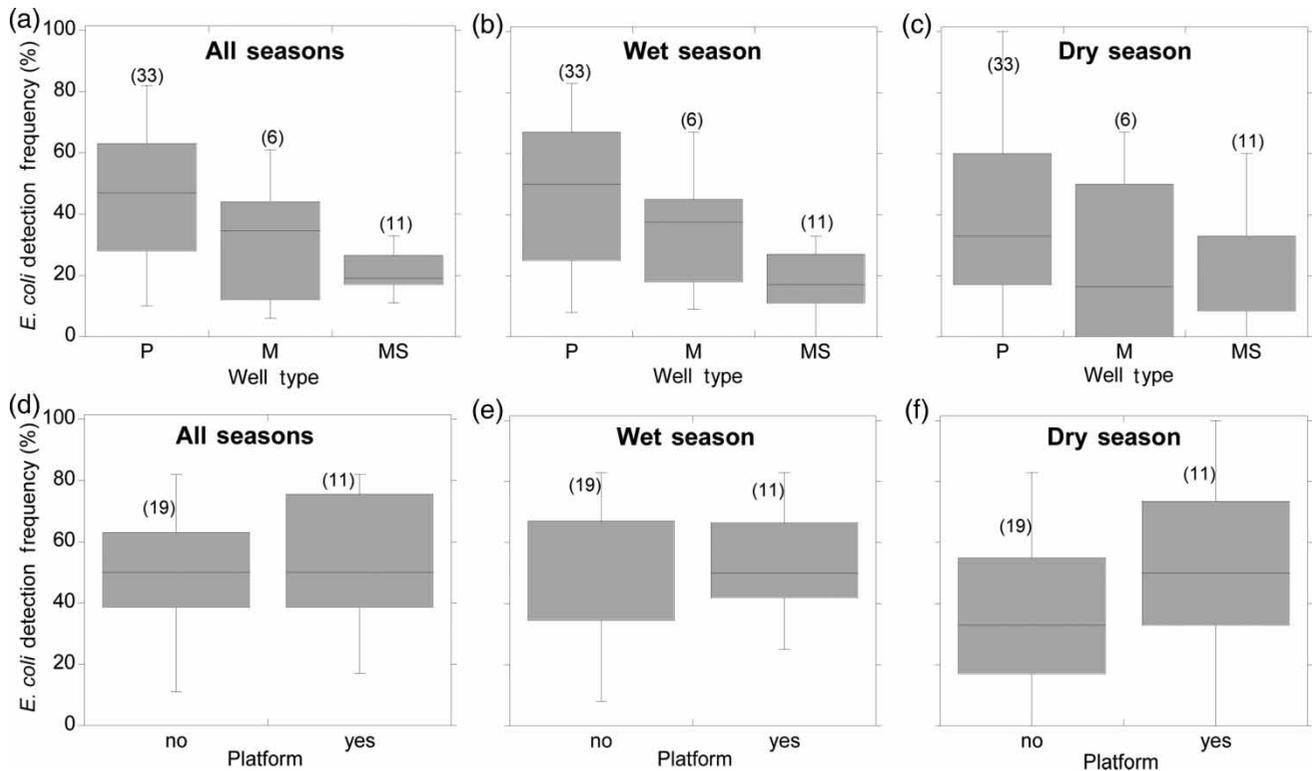
## RESULTS AND DISCUSSION

### Monthly *E. coli* detection frequency in sealed and unsealed wells

*E. coli* prevalence in tubewells was observed to be substantially higher in the monsoon than the dry season

(Figures 2(b) and 2(c)). An ANOVA on the *E. coli* prevalence data confirmed that well class (P, M, MS) significantly impacted the frequency of *E. coli* detected in a well, both year-round (Figure 2(a)) and during the wet season ( $p < 0.05$ ) (Figure 2(b)), but not during the dry season (Figure 2(c)). Non-parametric Kruskal-Wallis tests between pairs of well classes confirmed that P wells were more frequently contaminated than MS wells year-round and during the wet season ( $p < 0.05$ ) (Figures 2(a) and 2(b)). *E. coli* prevalence in M wells was intermediate and not significantly different from either P or MS wells. The lesser *E. coli* prevalence in M wells than P wells suggests that daily pumping from private tubewells plays a role in fecal contamination. Other possible causes of more frequent private tubewell contamination over monitoring wells (M and MS) include biofilm growth within the iron hand pump (Ferguson *et al.* 2011) and the introduction of *E. coli* into the well following pump priming of private wells (van Geen *et al.* 2011).

Although annular sealing appeared to substantially reduce *E. coli* detection frequency, the presence of an intact cement platform had no impact on the microbial drinking water quality from a private tubewell in either the wet or dry seasons (Figures 2(e) and 2(f)). The WHO/



**Figure 2** | Detection frequencies of *E. coli* in monthly monitored private (P), unsealed monitoring (M), and sealed monitoring (MS) wells from April 2008 through November 2009. For the well type plots (a)–(c) the number of wells with at least 5 months of monthly data in each season were 33, six and 11 for P, M and MS respectively. There were a total of 12 possible wet season sampling events and 6 dry season months. In the platform presence plots (d)–(f), only private wells are presented here since no monitoring wells had cement platforms. A reduced number of private wells ( $n = 30$ ) was available due to missing information.

UNICEF (2012) well construction guidelines emphasize the importance of an adequate drainage channel to allow the spilled water to leave the well-head area; however, strictly speaking this is not required for the classification of a tubewell as an ‘improved’ drinking water source (WHO/UNICEF 2012). As of 2010, 80% of the rural population in Bangladesh was recorded drinking from ‘improved’ water sources (WHO/UNICEF 2012). In our exhaustive survey of the 144 private tubewells at Site K in 2009, however, only 42% (61/144) of private tubewells had intact platforms, and many of these did not have drainage channels. In a related study combining 32 wells from Site K with 93 wells in Matlab upazilla, 50 km south of Site K, only 51% (64/125) of all tubewells had intact platforms (van Geen *et al.* 2011). In the present study, we did not differentiate between intact platforms with and without good drainage channels. The sample size in the present study for which monthly *E. coli* measurements were available is small, with 11 tubewells having good platforms and 19 that did

not have platforms or had broken platforms. Other studies, however, have reported the insensitivity of platform quality and presence for FIB detection frequency in private tubewells in Bangladesh (Luby *et al.* 2008; Leber *et al.* 2011; van Geen *et al.* 2011). This finding has led some to conclude that annular flow is not important in Bangladesh where wells are drilled and not dug (Luby *et al.* 2008). Based on the information presented in this study, annular flow seems to degrade microbial water quality, but the presence of an intact platform has little protective effect. This may be due to the general absence of good drainage channels, or the flat terrain which leads to ponding around well-heads, rendering intact platforms and long drainage channels irrelevant following large rainfall events.

It was hypothesized that *E. coli* detection frequency would decrease with depth. Well depth was not found to correlate to *E. coli* detection frequency for any class (P, M, MS) of wells in this study for any month or binned season. This is consistent with other studies measuring

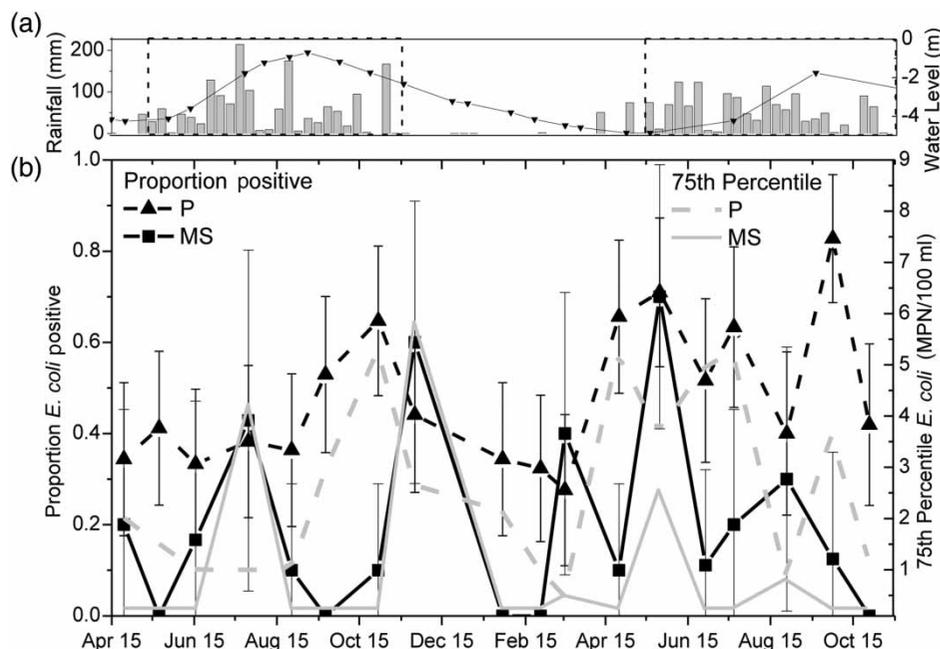
FIB contamination risk factors in private tubewells in Bangladesh (Luby *et al.* 2008; Leber *et al.* 2011; van Geen *et al.* 2011). One study showed consistent decreases in FIB concentration with depth in paired MS wells installed only 3 m apart vertically in highly contaminated aquifers in the vicinity of latrine ponds (Knappett *et al.* 2012). It is likely that sediment heterogeneity and well-specific processes such as biofilm growth (Ferguson *et al.* 2011) and rapid flow down unsealed annuli confound a simple relationship between depth and *E. coli* detection frequency in studies where comparatively shallow tubewells (<36 m) are compared across village(s) (Leber *et al.* 2011; van Geen *et al.* 2011).

Throughout the 18-month monitoring period MS wells were typically less frequently contaminated on a month-to-month basis (Figure 3(b)). During the early monsoon season *E. coli* was just as prevalent in MS wells as P wells; however, *E. coli* prevalence tended to decrease later in the monsoon in MS wells but continued to increase in P wells (Figure 3(b)). Two exceptions to this pattern were when *E. coli* prevalence in MS wells exceeded P wells, following major rainfall events in the 2008 wet season.

These findings can be explained the following way. Sealed monitoring wells act to sample aquifer water that becomes contaminated via vertical infiltration through the vadose zone in a 'first flush' event when the water table is still >4 m below the surface (Figure 3(a)). Since 90% of all wells in this study have total depths that vary between 6 and 15 m, this 'first flush' water reaches the saturated water table only 0.5–9.5 m above the screened interval (1.5 m). As the water table rises the impetus for downward movement of infiltrating rainwater through sediment decreases with the thinning of the vadose zone. Similarly, *E. coli* prevalence increases in P wells at the start of monsoonal rains, but in contrast to the MS wells, as the water table rises unsealed P wells become more frequently contaminated (Figure 3(b)). This can be explained by vertical flow along the annulus of the private wells enhanced by regular pumping.

#### Dry and wet season snapshots of molecular FIBs and pathogens

During the wet season, snapshot sampling event concentrations of molecular *E. coli* and Bacteroidales in MS

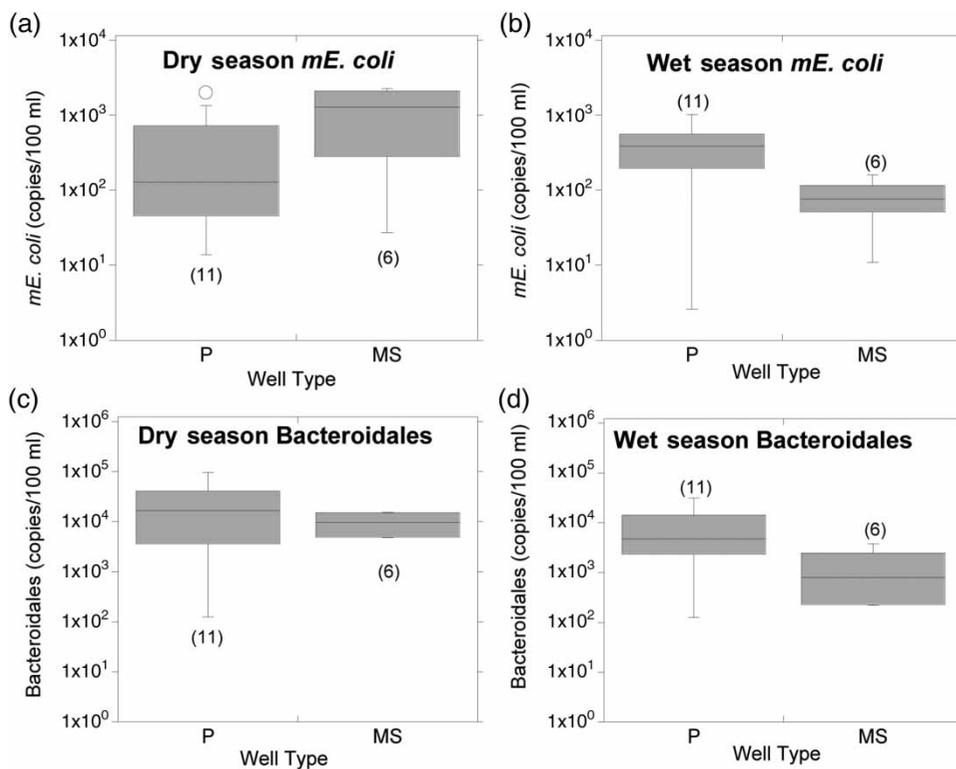


**Figure 3** | Comparing culturable *E. coli* prevalence in wells with rainfall and water table levels. (a) Weekly precipitation (vertical grey bars) for Matlab located 50 km south of Site K (left-axis). Manual groundwater levels are displayed at Site K (black line with grad symbols) from April 1 2008 through November 1 2009 (right-axis). Months assigned to the wet season are indicated by boxes outlined by dashed lines. (b) Monthly proportion of private (P) ( $n = 35$ ) and sealed monitoring (MS) ( $n = 11$ ) wells testing positive for cultured *E. coli* (left-axis). 75th percentile cultured *E. coli* concentrations (MPN/100 mL) for both P (dashed grey line) and MS (solid grey line) wells (right-axis).

wells were significantly lower than in P wells as assessed by the Kruskal-Wallis test ( $p < 0.05$ ). Although FIB DNA concentrations were high during the dry season, no significant difference in concentration was observed between MS and P wells (Figures 4(a) and 4(c)). This contrasts with the cultured *E. coli* data, which shows all three classes of tubewells have lower frequency of *E. coli* detected during the dry season than the wet (Figures 2(b) and 2(c)). The similar concentrations of molecular FIBs in MS and P wells during the dry season suggests that short circuiting is not active during the dry season, and rather these concentrations of FIB DNA represent background levels in the aquifer sustained throughout much of the year.

This finding suggests that FIB DNA persistence in these shallow anaerobic aquifers is longer than culturable *E. coli*. This agrees with findings in Knappett *et al.* (2012) where *mE. coli* and Bacteroidales were detected well above detection limit in fine sediments adjacent to latrine ponds in the absence of culturable *E. coli*. Even in sediments containing abundant culturable *E. coli*, Bacteroidales and *E. coli*

DNA was observed to be transported further laterally and especially vertically (Knappett *et al.* 2012). Another study found widespread high concentrations of FIB DNA in private wells across several villages and seasons (Ferguson *et al.* 2012). It is likely that the reduced metabolism and size of starving fecal bacteria in oligotrophic aquifers allows them to pass much further than readily culturable *E. coli*. This was found in a study by Jansen *et al.* (2010) in sand columns with *Pseudomonas fluorescens* where the starved, coccoid bacteria were less efficiently removed than metabolically active rod-shaped bacteria. This phenomenon has important implications for understanding the perceived threat of FIB and pathogen DNA in groundwater. Traditionally culturing of *E. coli* has been relied upon to indicate the relative health risk from drinking water (WHO 2008). Fecal bacteria DNA, contained in dead or starving cells, however, may be transported further and persist longer in an aquifer than the more metabolically active culturable cells. Therefore, the relative health risk represented by different concentrations of FIB and

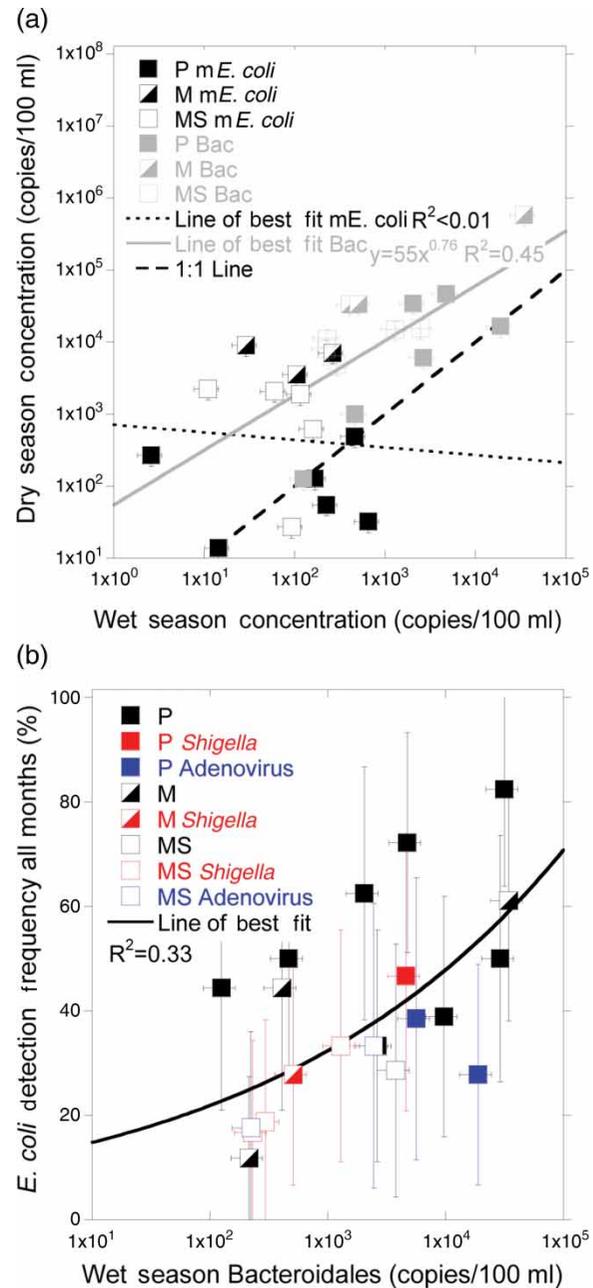


**Figure 4** | Concentrations of *mE. coli* and Bacteroidales DNA in private (P) and sealed monitoring wells (MS) during dry season and wet season snap shot sampling events. Sample size for each group is indicated in parentheses. Unsealed monitoring wells (M) were not included in this analysis due to low sample numbers ( $n < 5$ ).

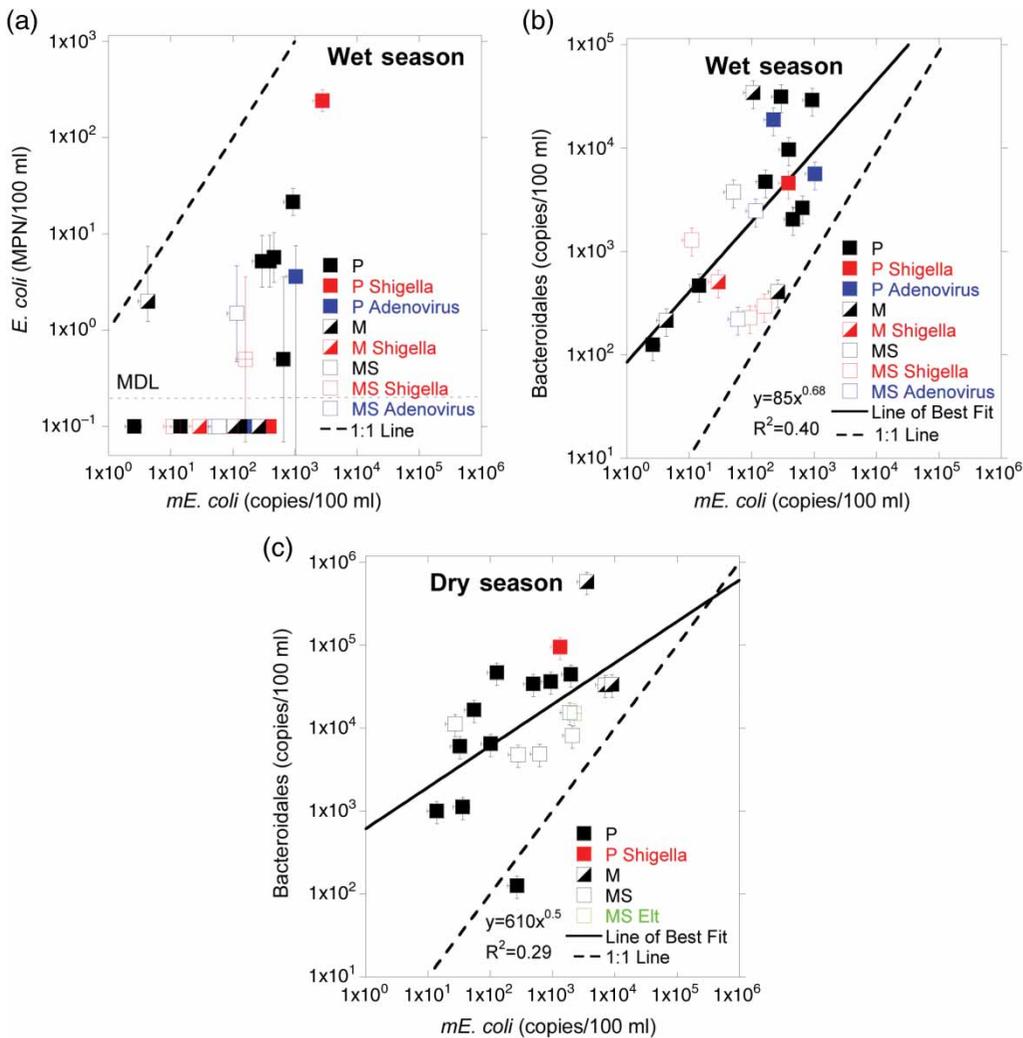
pathogen DNA occurring in groundwater (Ferguson *et al.* 2012) is currently unknown and needs to be assessed as it has been for recreational water exposure (Wade *et al.* 2008).

One advantage of enumerating FIB DNA over culturing *E. coli* is that it is present in high concentrations year round at multiple field sites (Figure 4) (Ferguson *et al.* 2012). When concentrations of *mE. coli* and Bacteroidales from the dry and wet season snapshot sampling events are plotted against each other for 14 overlapping wells, Bacteroidales emerges as more consistent year round, whereas *mE. coli* concentrations are uncorrelated in the same wells in different seasons (Figure 5(a)). Furthermore, wet season Bacteroidales were the only FIB (and the only season) found to be somewhat predictive ( $R^2 = 0.33$ ,  $p < 0.05$ ) of annual *E. coli* detection frequency (Figure 5(b)). Bacteroidales may be a more seasonally unbiased estimate of year round susceptibility of a groundwater well to fecal contamination than either *mE. coli* or the sporadically detected cultured *E. coli*. Sampling at one or two points in time, rather than frequent monitoring over years, may save substantial labor.

Although correlated with each other during the wet season snapshot, no combination of FIBs was found to be predictive of pathogen presence/absence (Figures 6(a) and 6(b)), with 10/22 samples found to be positive for at least one pathogen. There were several false negatives with respect to the indicators, with pathogens being found in water samples containing low concentrations of *E. coli*, *mE. coli* and Bacteroidales. Annual *E. coli* detection frequency did not predict the wet season presence of pathogens (Figure 5(b)), either. In contrast, although only 2/20 dry season snapshot wells were positive for pathogens, both of these wells were highly contaminated with *mE. coli* and Bacteroidales (cultured *E. coli* was not measured simultaneously during the dry season snapshot) (Figure 6(c)). It is noteworthy that pathogens were detected in approximately equal numbers of both unsealed (P) and sealed (MS) wells, reinforcing the idea that although well construction effects lead to increased contamination of the tubewells themselves, fecal bacteria and pathogen DNA are clearly infiltrating the broader aquifer beyond the near-well environment (Knappett *et al.* 2012).



**Figure 5** | Inter-seasonal comparisons of FIB and pathogens detected in unsealed private wells (P), unsealed monitoring wells (M) and sealed monitoring wells (MS). (a) Measured FIB marker gene concentrations in wells from during the wet and dry season snapshot sampling events ( $n = 14$ ). Only the equation for the line of best fit for Bacteroidales is displayed since the fit was very poor for *mE. coli*. (b) Wet season concentrations of Bacteroidales 16S genes and pathogen presence/absence plotted against cultured *E. coli* detection frequency for all months ( $n = 22$ ). Samples where no pathogens were detected are indicated by black symbols. Samples positive for *Shigella* and Adenovirus are indicated by red and blue, respectively.



**Figure 6** | Comparison of FIB concentrations and pathogen presence/absence during wet ( $n = 22$ ) and dry season ( $n = 20$ ) snapshot sampling events. (a) Comparison of synoptic measurements of cultured *E. coli* (MPN/100 ml) and *mE. coli* (copies/100 ml) from the wet season snapshot sampling event. The culturable *E. coli* method detection limit (MDL) was 0.5 MPN/100 mL. (b) Comparison of Bacteroidales and *mE. coli* concentrations during wet season snapshot. (c) Comparison of Bacteroidales and *mE. coli* during dry season snapshot. Culturable *E. coli* was not measured synoptically during the dry season snapshot sampling event. Samples where no pathogens were detected are indicated by black symbols. Samples positive for *Shigella*, *EitA* (ETEC *E. coli*) and Adenovirus are indicated by red, green and blue, respectively.

## CONCLUSION

Multiple differences, including annular sealing and usage frequency, between private wells and monitoring wells led to more frequent detections of *E. coli* in unsealed tubewells. Clearly some simple well construction improvements can be made that will lead to decreases in FIB detections and cases of diarrheal disease obtained by drinking untreated groundwater (Gundry et al. 2004). In a related study, hand pumps themselves were found to harbor *E. coli* long after being exposed to high levels of *E. coli* (Ferguson et al. 2011). This

effect could have accounted for some of the more frequent contamination seen here in private wells. Amongst unsealed private wells, however, platform presence/absence had no impact on microbial water quality. This finding could possibly be due to the lack of adequate drainage channels accompanying intact platforms; however, this lack of sensitivity to platform presence and quality has been reported previously in Bangladesh (Luby et al. 2008; Leber et al. 2011; van Geen et al. 2011). Together these findings suggest that annular sealing or another private well construction factor may be much more important in determining

microbial drinking water quality than platforms and drainage channels in Bangladesh.

The molecular FIB Bacteroidales sampled in 22 wells during the wet season was found to be predictive of year-round fecal contamination in both unsealed and sealed wells, as assessed by annual *E. coli* detection frequency and dry season Bacteroidales. Pathogens were more frequently detected in the wet season, but their presence was uncorrelated to any FIB or well type. The high prevalence of *E. coli*, and high concentrations of FIB DNA, in sealed monitoring wells, especially during the early monsoon season, indicate that fecal contamination is indeed infiltrating and spreading over broad volumes of the aquifer. Infiltration pathways likely include both leaky tubewell annuli and infiltration from ponds (Knappett *et al.* 2012). Other factors potentially contributing to *E. coli* prevalence in tubewells in Bangladesh include the size distribution (Leber *et al.* 2011; Knappett *et al.* 2012) and mineralogy (Ryan *et al.* 1999; Flynn *et al.* 2004) of the overlying sediment, pore-water ionic strength and chemical composition (Fontes *et al.* 1991; Loveland *et al.* 1996), and the local spatial density of contamination sources (Knappett *et al.* 2011a; van Geen *et al.* 2011). Multi-season FIB monitoring could be conducted on both unsealed and sealed private wells with similar usage frequency to isolate for an effect of well sealing only apart from regular pumping and well-head effects (Ferguson *et al.* 2011). Further, private wells could be tested for leaks in the PVC pipe to determine how prevalent this is and whether leaking well pipes can explain the fecal contamination patterns in the unsealed private wells.

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