

## ARTICLE OPEN



# Waterborne pathogen monitoring in Jaipur, India reveals potential microbial risks of urban groundwater supply

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The Sustainable Development Goals require that 100 mL water samples contain no culturable *E. coli* to classify a water supply as “safely managed” from a microbial perspective. But small volume sampling is often insufficient for detecting microbial risks. We used culture-based measures of total coliforms and *E. coli* along with dead-end ultrafiltration (DEUF) and droplet digital PCR (ddPCR) to assess the microbial water quality of an urban water supply in Jaipur, India. Despite the absence of culturable *E. coli* in 90% of the 100 mL grab samples ( $n = 20$ ) during the 10-day sampling period, we detected genes associated with protozoan and bacterial pathogens (*Giardia*, *Cryptosporidium*, and enterotoxigenic *E. coli*) in 3 DEUF samples of groundwater ( $n = 9$ ; volume 59 to 122.4 liters). Of the three groundwater samples positive for waterborne pathogens, two were associated with 100 mL grab samples that were negative for culturable *E. coli*. Methods with improved analytical sensitivity, such as DEUF and ddPCR, can detect evidence of pathogens in drinking water supplies and supplement conventional culture-based methods to better inform pathogen-specific risk assessment and management.

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## INTRODUCTION

Fecal indicator bacteria have been used to assess the quality of drinking water and the risk of waterborne disease associated with its consumption for more than 100 years<sup>1</sup>. Despite its limitations, *Escherichia coli* or *E. coli*, a thermotolerant member of the coliform group, has emerged as the most widely used indicator of fecal contamination in drinking water<sup>2</sup>. The World Health Organization (WHO) Guidelines for Drinking-Water Quality (GDWQ) state that the detection of *E. coli*, which is less resistant to environmental degradation and disinfection than viruses or protozoan pathogens, in drinking water indicates recent fecal contamination<sup>3</sup>. Further, the Sustainable Development Goals (SDGs) define access to “safely managed” drinking water as an improved source on premise, available when needed, and free of priority chemical and fecal contamination with *E. coli* as the recommended fecal indicator<sup>4</sup>. Approximately 1.8 billion people use a fecally contaminated source of drinking water and after accounting for data on water quality, as required by the SDGs, the estimated population with access to safely managed drinking water is substantially lower<sup>5,6</sup>. But the association between *E. coli* counts in drinking water and diarrheal disease is often weak and variable<sup>7–10</sup>.

Nonetheless, associations between *E. coli* counts and diarrheal disease are often explicitly encoded in risk assessments of distributed drinking water through the use of fecal indicator measurements and waterborne pathogens to indicators ratios to estimate exposure<sup>11–14</sup>. However, the sometimes weak or non-existent correlation between fecal indicator bacteria and waterborne pathogens, including protozoa and viruses, leads to uncertainty in quantifying exposure<sup>12,15,16</sup>. More direct measures of waterborne pathogens in distributed drinking water at counts relevant to infection risks are needed to improve our understanding of the endemic health burden attributable to piped

water supplies. Pairing two recently developed methods in environmental microbiology, dead-end ultrafiltration (DEUF) and droplet digital PCR (ddPCR), holds promise for more sensitive detection and quantification of waterborne pathogens in drinking water at counts informative for risk assessment. Such methods can supplement conventional water safety monitoring methods, such as *E. coli* measurements, and enable application of quantitative microbial risk assessment (QMRA) for distributed drinking water and reveal pathogen-specific data to inform risk management.

Here we report on the application of DEUF and ddPCR to detect and quantify genes associated with waterborne pathogens and fecal indicators in drinking water samples. These molecular measurements are paired with fecal indicator bacteria counts by culture methods and physicochemical measurements to assess the microbial safety of a municipal water supply in Jaipur, India.

Jaipur is the capital of the northern semi-arid state of Rajasthan and receives an average of 600 millimeters of rainfall each year<sup>17</sup>. Until the early 2000s, the residents of Jaipur received their drinking water exclusively from groundwater sources via a network of decentralized municipal tube wells<sup>18,19</sup>. But, over-exploitation of aquifers for irrigation has caused drastic water table decline making sole dependence on groundwater untenable<sup>20,21</sup>. In 2006, Jaipur was allocated surface water from Bisalpur Dam, 120 km southwest of the city, and by 2009 the city was augmenting its groundwater wells with surface water<sup>18</sup>. Before being pumped from the dam to the city, raw surface water undergoes conventional treatment at the Surajpura Water Treatment Plant<sup>18</sup>. The treatment train consists of aeration, pre-chlorination, coagulation and flocculation, rapid sand filtration, and post-chlorination with chlorine gas. Once it arrives in the city, the surface water, average volume of 275 million liters per day (MLD), is distributed to 162 water supply zones via a network of pipelines, elevated storage reservoirs (ESR), and pump stations<sup>18</sup>.

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Groundwater, average volume of 97 MLD, extracted via the previously mentioned network of tube wells, is pumped directly into the distribution network without additional treatment beyond the residual chlorine contact incidental to the distribution network<sup>18</sup>. The distribution network is operated intermittently with each supply zone receiving 1.5–3 h of pressurized water distribution each day<sup>18,22</sup>. In conjunction with a study of geogenic contaminants in groundwater, and with assistance from staff of the Ground Water Department and the Public Health Engineering Department of the Government of Rajasthan, we conducted a cross-sectional sampling of municipal drinking water in Jaipur in May of 2017<sup>23</sup>. The study was implemented to provide an assessment of waterborne pathogen hazards to supplement conventional microbial water safety monitoring.

## RESULTS

### Physicochemical water quality

Of the eight groundwater samples collected from tube wells in which chlorine was measured, all but one was less than the MRL for free chlorine and all but two for total chlorine. Due to possible interference from oxidized iron and manganese, any chlorine value  $<0.1 \text{ mg L}^{-1}$  was denoted as less than the method reporting limit ( $<\text{MRL}$ )<sup>24</sup>. We detected free chlorine in 88% (7 out of 8) of samples from distributed water originating from a surface source and 75% (3 out of 4) of samples from distributed water originating from a mixture of surface and groundwater. We observed less than the India Standard Drinking Water Specification (IS 10500:2012) required  $0.2 \text{ mg L}^{-1}$  of free chlorine in 16.7% of samples collected from the distribution network (excludes groundwater samples from tube wells)<sup>25</sup>. Box and whisker plots summarizing our observations are shown in Supplementary Fig. 1. The pH (range 7.65–9.18) and conductivity (range

$358\text{--}1831 \mu\text{S cm}^{-1}$ ), summarized in Supplementary Fig. 2, reflect distributed water samples originating from two distinct sources – groundwater and surface water, and samples from a mixture of the two.

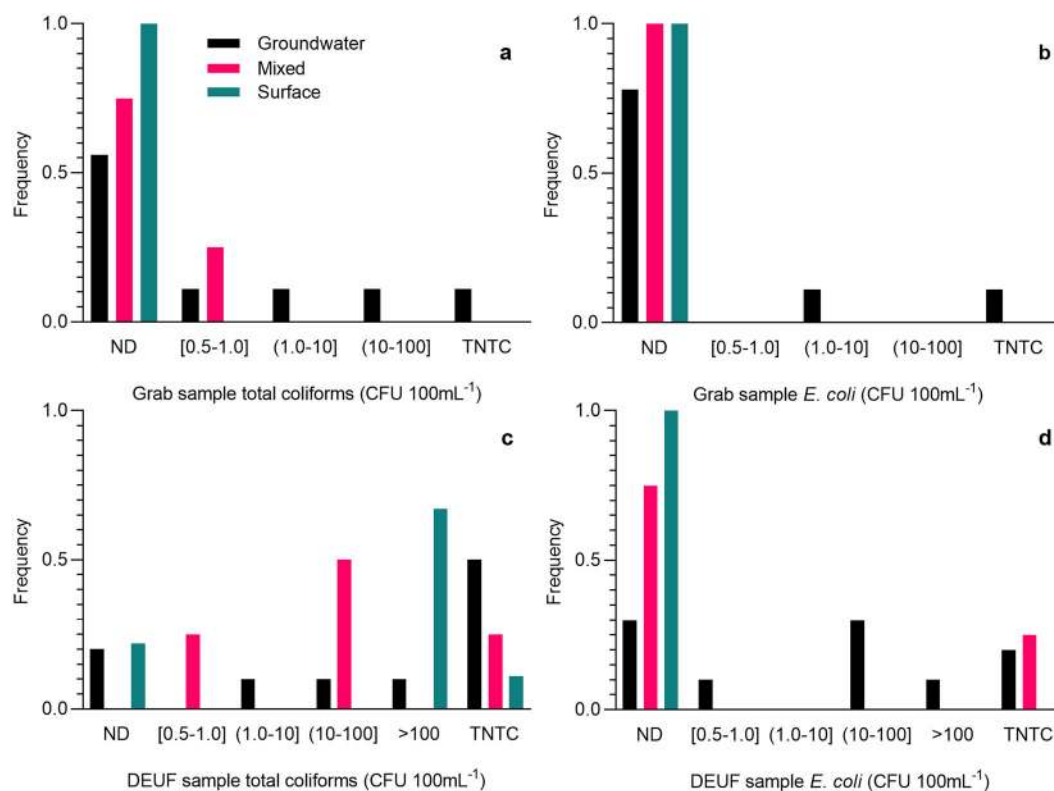
### Fecal indicators by culture

We enumerated total coliforms and *E. coli* in 20 100-mL grab samples (results from three were lost due to incubator power failure) – nine in groundwater from tube wells, seven in distributed water originating from surface water, and four in distributed water originating from a mixture of these two sources. Frequency distributions of the observed counts from grab samples are shown in Fig. 1a, b. We collected 23 DEUF samples (total volume 2407.9 liters) and enumerated total coliforms and *E. coli* from the backflush of 22 (one sample was spilled during analysis). Frequency distributions of total coliform and *E. coli* counts in the drinking water as estimated by DEUF are shown in Fig. 2c, d. During our sampling, 90% of the grab samples were free of *E. coli*.

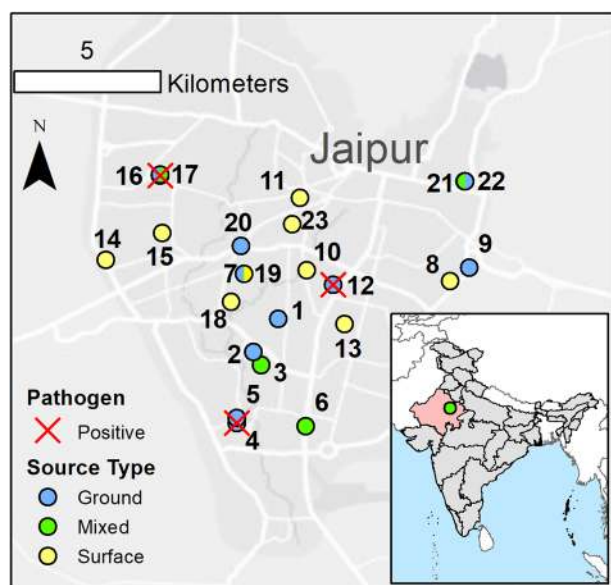
*E. coli* counts were below the detection limit in grab samples and DEUF backflush for all distributed water samples originating from surface water. For mixed-source distributed drinking water, *E. coli* was below the detection limit in all grab samples but only 75% of DEUF backflush samples. In groundwater, on the other hand, *E. coli* was detected in 22% of grab samples and 70% of DEUF backflush samples.

### Jaipur drinking water quality as assessed by ddPCR

Based on our review of the ddPCR data (detailed description in Supplementary Notes), we report our findings concerning the microbial water quality in Jaipur, as observed via molecular evidence, using a strength-of-evidence paradigm as shown in Supplementary Fig. 3. We consider the most credible evidence to



**Fig. 1** Frequency distributions of total coliform and *E. coli* counts. Frequency distributions of total coliform and *E. coli* counts in drinking water from the municipal water supply in Jaipur, India: total coliform (a) and *E. coli* counts (b) as observed in 100mL grab samples from groundwater ( $n = 9$ ), surface source ( $n = 7$ ), and mixed source ( $n = 4$ ) drinking water samples and total coliform (c) and *E. coli* counts (d) as observed in DEUF samples from groundwater ( $n = 10$ ), surface source ( $n = 9$ ), and mixed source ( $n = 4$ ) drinking water samples.



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**Fig. 2** Sample collection locations from the municipal water supply in Jaipur, India. Water sample collection locations from the cross-sectional sampling of the Jaipur municipal water supply: groundwater source samples are shown in blue, surface source samples in yellow, and mixed source in green. The three groundwater samples positive for a waterborne pathogen associated gene target by ddPCR at the strongest level of evidence are labeled with large red “X” symbols.

be detection by both manual thresholding and model-based thresholding at a density above the 95% limit of detection (LOD) with zero false positive droplets in negative controls. Due to our observation of false positives in negative controls by the model-based thresholding method for the *MS2g1*, *ipaH*, and *hexon* assays, results for these targets are excluded from the strongest evidence category.

At this level of evidence, 19 of 22 DEUF samples across all three source water types are positive for the *E. coli* associated gene *ybbW* with no false positives in no-template controls or sample blanks. This is a much higher proportion of samples positive than observed with culture-based methods where only nine DEUF backflush samples were positive for *E. coli* by culture. This finding could reflect the presence of *E. coli* in a viable but not culturable (VBNC) state, the capture of inactivated but intact *E. coli* cells, or the capture of extracellular DNA during DEUF sampling.

With our interpretation of the strongest evidence, summarized in Table 1, one groundwater sample was positive for *beta giardin* associated with *Giardia duodenalis*, one groundwater sample was positive for the gene encoding human heat-stable toxin (*STh*) associated with enterotoxigenic *E. coli* (ETEC) and one groundwater sample was positive for the *18S rRNA* gene of *Cryptosporidium*. In the sample positive for *beta giardin*, we also observed culturable *E. coli* counts that were too numerous to count in both the 100 mL grab sample and the DEUF backflush. We observed culturable *E. coli* in the DEUF backflush, but not in the 100 mL grab samples, associated with the other two detections by ddPCR. Water quality data stratified by sample combined with ddPCR results at the strongest evidence level are found in Table 2.

At the second strongest evidence level, detection above the 95% LOD by either thresholding method with no false positives in negative controls, three samples were positive for *beta giardin*, six samples were positive for the *18S rRNA* gene of *Cryptosporidium*,

three samples were positive for *STh*, and one sample was positive for the *ipaH* gene associated with *Shigella* as tabulated in Table 1. At this evidence level, the additional number of positive samples stratified by source was four positive groundwater samples, five distributed water samples originating from surface water positive, and two distributed water samples originating from a mixture of ground and surface water positive. Water quality data stratified by sample with ddPCR results at the second strongest evidence level are summarized in Supplementary Tables 1 and 2. We report these detections to provide additional insight into interpreting ddPCR evidence, but for the purposes of the current microbial water quality assessment, we consider only the strongest level of ddPCR evidence.

## DISCUSSION

Implications for drinking water safety in jaipur

Our findings highlight the role of contaminated groundwater as a potential source of waterborne disease in Jaipur. In grab samples and DEUF samples from groundwater, we observed 22% and 70% positive for culturable *E. coli*, respectively. Whereas, for distributed water originating from a surface water source, no grab samples or DEUF samples were positive for culturable *E. coli*. In our interpretation of the ddPCR results at the strongest evidence level, one groundwater was positive for *Giardia duodenalis*, one was positive for *Cryptosporidium* spp., and one was positive for ETEC. Although the groundwater is pumped into the distribution network, where we consistently detected residual chlorine, contact times and concentrations might not be sufficient for inactivation especially for chlorine resistant protozoan pathogens such as *Giardia* and *Cryptosporidium*<sup>26,27</sup>. For each of these samples, the molecular detection was concurrent with the detection of culturable fecal indicator bacteria in the DEUF backflush giving credibility to the potential viability of the pathogens. These detections are consistent with molecular evidence from several other studies in India. In Lucknow, ETEC genes ST1 and LT1 were detected in potable water from the municipal supply, and in Kolkata 9% of *E. coli* isolated from potable water sources were positive for virulence genes including heat-stable toxins associated with ETEC<sup>28,29</sup>. In Chennai, 58% of samples from the piped water supply were reportedly positive for *Cryptosporidium* oocysts by immunofluorescent antibody screening<sup>30</sup>. Our observations are consistent with those of previous studies that have found both urban and rural water supplies in South Asia are frequently contaminated with human enteric pathogens<sup>31</sup>.

Our detection of genes associated with pathogens along with culturable *E. coli* in groundwater in Jaipur draws increasing scrutiny to the frequently made assumption that groundwater is less likely to be contaminated with feces<sup>32</sup>. In urban areas, sewers have been found to be sources of groundwater recharge and to degrade the quality of urban groundwater<sup>33,34</sup>. In Madras, Somasundaram et al.<sup>35</sup> noted microbial contamination in a urban aquifer as indicated by high nitrate levels and the detection of microbes in wells. While a study of groundwater recharge in Hyderabad found that anthropogenic sources of groundwater recharge were ten times greater than natural ones and that leakage from the water distribution system and leakage of sewage from the sewer system comprised a large proportion of the annual recharge volume<sup>36</sup>. In rural Rajasthan, bacterial contamination of groundwater used for drinking has been observed<sup>37</sup>. And a recent study of inorganic contaminants in groundwater in Rajasthan found that elevated levels of dissolved organic carbon and nitrate suggested groundwater contamination from anthropogenic sources<sup>38</sup>. While the previous study and current data cannot implicate human or animal feces as the contamination source, we did not detect human enteric viruses by ddPCR at the strongest evidence level. We did, however, detect genes associated with

**Table 1.** Water Samples Positive for Waterborne Pathogens by ddPCR: number of samples positive and density ranges (gene copies per liter of drinking water) for gene targets in groundwater (G), distributed water originating from surface water (S), and distributed water originating from a mixture of groundwater and surface water (M) samples collected from the Jaipur municipal water supply and assayed by ddPCR.

Gene (microbe)	ddPCR evidence level	Ground #positive (n = 9)	Surface #positive (n = 8)	Mixed #positive (n = 4)	Ground (gc per L)	Surface (gc per L)	Mixed (gc per L)
<i>MS2g1</i> (MS2)	Strongest	–	–	–	–	–	–
		–	–	–	–	–	–
	–	1	1	–	29	27	–
<i>ybbW</i> ( <i>E. coli</i> )	Weakest	1	3	–	11	12	–
	Strongest	8	6	3	5600	740	270
		–	–	–	–	–	–
<i>beta giardin</i> ( <i>Giardia</i> )	–	–	–	–	–	–	–
	Weakest	–	1	–	–	7.0	–
	Strongest	1	–	–	36	–	–
1		1	–	85	36	–	
<i>18S rRNA</i> (Crypto.)	–	–	–	–	–	–	–
	Weakest	–	4	–	–	6.9	–
	Strongest	1	–	–	20	–	–
1		3	1	6	6.8	17	
<i>ORF1-2</i> (noro GI)	–	–	–	–	–	–	–
	Weakest	1	1	–	6	66	–
	Strongest	–	–	–	–	–	–
–		–	–	–	–	–	–
<i>ORF1-2</i> (noro GII)	–	–	–	–	–	–	–
	Weakest	2	–	–	8.9	–	–
	Strongest	–	–	–	–	–	–
–		–	–	–	–	–	–
<i>hexon</i> (adeno)	–	–	–	–	–	–	–
	Weakest	4	2	–	13	10	–
	Strongest	–	–	–	–	–	–
–		–	–	–	–	–	–
<i>STh</i> (EPEC)	–	–	–	–	–	–	–
	Weakest	–	–	–	–	–	–
	Strongest	1	–	–	17	–	–
1		1	1	9.7	8.3	26	
<i>ipaH</i> ( <i>Shigella</i> )	–	–	–	–	–	–	–
	Weakest	2	1	–	7.7	5.0	–
	Strongest	–	–	–	–	–	–
1		–	–	320	–	–	
	–	–	–	–	–	–	–
	–	–	–	–	–	–	–
	Weakest	–	2	–	–	6.8	–

Results are stratified by the strength of ddPCR evidence as described in Supplementary Fig. 3.

*Giardia* and *Cryptosporidium* both of which are known to be zoonotic pathogens. Further exploration of the contamination of groundwater in Jaipur via microbial source tracking assays would be of additional benefit for risk management.

The contamination of the drinking water supply in Jaipur, particularly the groundwater itself, is corroborated by events reported in Jaipur. In 2011, an outbreak of waterborne hepatitis E

**Table 2.** Summary of all water quality data stratified by sample with ddPCR results at the strongest evidence level.

Sample	Location <sup>a</sup>	Source type <sup>b</sup>	Free CL (mg/L)	Total CL (mg/L)	pH	Temp (F)	Cond. (us/cm)	TDS (ppm)	GS TC (CFU/100 mL)	GS EC (CFU/100 mL)	DEUF TC (CFU/100 mL)	DEUF EC (CFU/100 mL)	MS2 (MS2gt1)	E. coli (ybbW)	Giardia (beta giardin)	Crypto. (18S rRNA)	Shigella (ipaH)	ETEC (STh)	Noro GI (ORF1-2)	Noro GI (ORF1-2)	Adeno A-F (hexon)	#
1	TW	G			8.15	85.3	1080	540	<0.5	<0.5	<0.51	0.51	# <sup>c</sup>	#	#	#	#	#	#	#	#	#
2	TW	G	<MRL	<MRL	8.06	85.6	874	437	<0.5	<0.5	<0.35	<0.35	+	+	-	-	-	-	-	-	-	-
3	ESR	M	<MRL	0.13	8.80	89.0	722	361	<0.5	<0.5	13	<0.48	-	+	-	-	-	-	-	-	-	-
4	TW	G	0.10	0.10	8.29	85.3	1096	540	<0.5	<0.5	TNTC	154	-	+	-	-	-	-	-	-	-	-
5	TW	G	<MRL	<MRL	7.96	85.0	1380	686	9.5	4.5	536	32	-	+	-	-	-	-	-	-	-	-
6	ESR	M	0.28	0.55	9.11	90.7	938	419	0.5	<0.5	0.91	<0.25	-	-	-	-	-	-	-	-	-	-
7	TW	G	<MRL	<MRL	8.12	84.3	774	387	<0.5	<0.5	43	<0.35	-	+	-	-	-	-	-	-	-	-
8	T	S	0.28	0.70	8.82	86.4	405	202	<0.5	<0.5	TNTC	<0.27	-	+	-	-	-	-	-	-	-	-
9	TW	G	<MRL	<MRL	7.65	90.1	1166	584	0.5	<0.5	TNTC	23	-	+	-	-	+	-	-	-	-	-
10	ESR	S	0.87	1.08	8.86	86.0	394	197	<0.5	<0.5	14	<0.24	-	-	-	-	-	-	-	-	-	-
11	T	S	1.18	1.49	8.96	92.0	361	181	<0.5	<0.5	60	<0.19	-	-	-	-	-	-	-	-	-	-
12	TW	G	<MRL	<MRL	8.18	90.0	962	481	10.5	<0.5	TNTC	0.88	-	-	-	+	-	-	-	-	-	-
13	T	S	0.71	0.92	8.91	91.7	359	180	<0.5	<0.5	<0.31	<0.31	-	+	-	-	-	-	-	-	-	-
14	ESR	S	0.73	1.15	8.90	88.8	360	180	<0.5	<0.5	33	<0.30	-	+	-	-	-	-	-	-	-	-
15	HHS	S	<MRL	0.14	9.11	95.0	388	194	<0.5	<0.5	32	<0.29	+	+	-	-	-	-	-	-	-	-
16	TW	G	<MRL	0.10	8.11	90.4	926	443	TNTC	TNTC	TNTC	TNTC	-	+	+	-	-	-	-	-	-	-
17	T	M	0.47	0.49	8.52	93.4	897	446	<0.5	<0.5	TNTC	TNTC	-	+	-	-	-	-	-	-	-	-
18	ESR	A	0.10	0.23	9.03	88.9	358	179	***	***	0.48	<0.26	-	+	-	-	-	-	-	-	-	-
19	ESR	S	1.51	1.85	9.18	87.1	362	180	***	***	<0.27	<0.27	-	+	-	-	-	-	-	-	-	-
20	TW	G	<MRL	<MRL	7.81	88.7	1509	755	***	***	TNTC	TNTC	-	+	-	-	-	-	-	-	-	-
21	T	M	0.14	0.23	8.23	89.9	1161	580	<0.5	<0.5	34	<0.29	-	+	-	-	-	-	-	-	-	-
22	TW	G	<MRL	<MRL	7.68	90.2	1831	899	<0.5	<0.5	5.3	33	-	+	-	-	-	-	-	-	-	-
23	T	S	0.72	0.95	9.05	94.2	363	181	<0.5	<0.5	16	<0.27	-	+	-	-	-	-	-	-	-	-

TW tube well, ESR elevated storage reservoir, T tap, HHS household storage, G groundwater, M distributed water originating from mixed source, S distributed water originating from surface water, <MRL less than method reporting limit, TNTC too numerous to count.

<sup>a</sup>Location – TW, ESR, T, and HHS.

<sup>b</sup>Source type – G, M, and S.

<sup>c</sup>ddPCR results unavailable due to sample spillage during processing.

<sup>d</sup>\*\*\*culture results unavailable due to incubator power failure.



was blamed on leaks from aging sewer lines<sup>39,40</sup>. In May and June of 2017, the time of our sampling, hospitals in Jaipur reported 7–10% increases in cases of diarrhea and vomiting<sup>41,42</sup>. This seasonal increase in diarrheal disease was also reported in the summer of 2019<sup>43</sup>. Our microbiological findings in Jaipur are consistent with these observations and indicate that the groundwater should not be neglected as a source of waterborne disease<sup>32</sup>. Currently, the municipal government reports that groundwater accounts for 26% of daily water supply. If extraction of groundwater increases during the summer season, it could explain the seasonal increase in diarrheal disease.

To manage the risk of waterborne disease associated with contaminated groundwater, the municipal government could treat the groundwater prior to distribution. Disinfection with chlorine might be considered, but the high concentrations of halides and organic contaminants observed in the groundwater increase the likelihood of forming disinfection byproducts<sup>38</sup>. This risk is verified by a recent study of tap water in Jaipur that detected both regulated and unregulated disinfection byproducts that could pose significant risks to public health<sup>44</sup>. Additionally, disinfection using chlorine would be less effective against protozoan pathogens *Giardia* and *Cryptosporidium*, which we detected via molecular assays<sup>26,27</sup>. Further, the distribution of tube wells throughout the city, owing to historic ad-hoc development and subsequent connection via pipelines, would likely require decentralized treatment. Given these constraints, treatment-based risk remediation for the ground water is likely to be cost-prohibitive.

A compelling alternative to treating the groundwater would be preventing its contamination in the first place. In 2016, local news reported a master plan for implementing improved drainage and sewerage in Jaipur at a cost of 32.6 billion INR that had been under development since 2007<sup>45</sup>. In 2018, replacement of sewer lines in the walled city area was reportedly underway<sup>46</sup>, but as of June 2019 residents of the walled city were still complaining of failing sewers<sup>47</sup>. Our results indicate that contaminated groundwater likely poses a significant risk to public health in Jaipur and that investments in fecal sludge management are warranted<sup>48</sup>. Repairs and improvements to reduce leakage from sewer lines and connect sources that are currently unserved, such as those advocated in citywide inclusive sanitation, could reap the dual benefit of reducing the pathogens and reducing the precursors for disinfection byproducts<sup>49,50</sup>. A quantitative analysis of the trade-offs between disinfection byproducts and waterborne disease would also be useful for decision making in Jaipur and other urban settings.

#### Fecal indicator bacteria and waterborne pathogen monitoring for water quality assessment

Our work also highlights important considerations for using culture-based fecal indicator measurements and advanced methods such as DEUF and ddPCR to assess microbial water quality. During our study, we interpreted two samples as positive for a waterborne pathogen by ddPCR while the paired 100 mL grab sample was negative for culturable *E. coli*. In these two instances, typical water quality assessment by grab sampling and testing for *E. coli* would indicate the groundwater was safe; whereas, large volume sampling and molecular analysis indicate the groundwater could be a potential source of exposure to waterborne pathogens. In each of these instances, culturable *E. coli* was detected in the larger sample volume afforded by DEUF. These results indicate that increased sample volumes are important for increasing the analytical sensitivity such that microbial risks can be accurately characterized by culture-based or molecular methods. Larger sample volumes have proven effective for detecting etiological agents during outbreaks of waterborne disease<sup>51</sup>, so its importance for accurately

characterizing microbial water quality during routine surveillance follows logically. The requirements of advanced methods such as DEUF and ddPCR, which require expensive consumables such as dialyzers (\$15 US dollars each) and ddPCR supplies (\$3.50 US dollars per reaction) and the technical expertise to conduct and interpret such experiments, suggest that water quality assessments, especially those in low-resource settings, will continue to rely on fecal indicator measurements rather than direct measures of waterborne pathogens. However, the results of our small study in Jaipur highlight that the strategic application of advanced methods can yield meaningful pathogen-specific information for risk management.

Contrary to popular belief, groundwater supplies, especially urban groundwater in the presence of failing or non-existent sewage infrastructure, may present underappreciated risks in municipal water supplies. This is not a new observation as waterborne disease associated with inadequate urban sanitation and groundwater was first noted by Dr. John Snow in his investigation of the Broad Street cholera outbreak in 1854. His conclusion still rings true: “the shallow pump-wells in a town cannot be looked on with too much suspicion whatever their local reputation may be”<sup>52</sup>.

## MATERIALS AND METHODS

### Sample materials

From 18–23 May 2017, we collected 23 paired 500-mL grab samples and DEUF samples (DEUF volume range: 59–161 liters) from throughout the Jaipur municipal water supply as shown in Fig. 2. We selected sampling points in the distribution system such that water originating from different source types including groundwater, surface water, and a mixture of both could be independently characterized. Samples were collected from distribution pipelines between 10:00 a.m. to 3:00 p.m. each day during the water supply period corresponding to that location. Samples collected from ESRs and tube wells were collected between 10:00 a.m. and 3:00 p.m. using the pressure provided by the tube well or ESR without regard for supply timing. At each sampling point, we first collected a 500-mL grab sample and made physicochemical measurements followed by filtration of a large volume DEUF sample. We describe the methodical details of each below. The resulting sample set included 10 samples from groundwater sources (DEUF volume: 59–122.4 liters), 8 distributed water samples originating from a surface water source (DEUF volume: 109.5–161.1 liters), 4 samples from mixed sources (DEUF volume: 63–146.5 liters), and 1 sample from an ambiguous mixture.

### Physicochemical measurements

At the time of sample collection, we measured physicochemical parameters including free chlorine, total chlorine, pH, conductivity, and total dissolved solids (TDS). We measured total and free chlorine using the US EPA DPD Colorimetric Method and a Hach Pocket Colorimeter II spectrophotometer (Hach, Loveland, CO)<sup>24</sup>. We measured pH, conductivity, and TDS by electrode using a Hanna Low Range Combo Tester (Hanna Instruments, Woonsocket, RI)<sup>53</sup>.

### Fecal indicator bacteria by culture

At each sampling location, we collected 500 mL grab samples in Whirl Pak bags pre-dosed with sodium thiosulfate to quench residual chlorine (Nasco, Fort Atkinson, WI). We kept the samples on ice and performed membrane filtration within 8 h of sample collection using a Del Agua Filtration Set (Del Agua, Fyfield, UK). We incubated each replicate along with positive and negative controls at 35 °C for 24 h on Compact Dry-EC plates (Hardy Diagnostics, Santa Maria, CA) and counted total coliforms and *E. coli* on each plate following the manufacturer's instructions for color-based identification and reported the counts in colony forming units (CFU) per 100 mL.

### Dead-end ultrafiltration

DEUF was developed as a simple ultrafiltration configuration for the recovery of microbes from water in field settings<sup>54</sup>. In tests of low- and mid-range turbidity drinking water samples seeded with microbes, DEUF

followed by backflushing led to average recoveries of 93% for *E. faecalis*, 57% for MS2 phage, 94% for *C. perfringens* spores, and 87% for *C. parvum* oocysts<sup>54</sup>.

At each sampling location in Jaipur, we concentrated microbes from large volumes of drinking water (59 liters to 161 liters) by DEUF using a Rexeed 25S dialyzer consisting of a polysulfone membrane with a molecular weight cut off of 30,000 Daltons (Asahi Kasei Kuraray Medical Company, Tokyo, Japan)<sup>54</sup>. Where possible, we connected to water sampling points via sterile Masterflex L/S 36 Platinum-Cured Silicone tubing (Cole Parmer, Vernon Hills, IL) and utilized the pressure in the piped network to force the drinking water through the dialyzer. When pressure was inadequate, we collected drinking water in sterilized plastic buckets and filtered from the buckets using a Geotech Geopump (Geotech, Denver, CO). We recorded the volume of water we filtered using a totalizing flow meter (Clark Solutions, Hudson, MA). Immediately following filtration of each drinking water sample, we flushed the dialyzer with a 500-mL 1% sodium thiosulfate solution to quench any residual chlorine. In the laboratory, we backflushed each dialyzer using a 500-mL 0.5% Tween 80, 0.01% NaPP, and, 0.001% Antifoam Y-30 emulsion solution and collected the resulting backflush in a sterile vessel<sup>54</sup>. From this backflush solution we enumerated total coliforms and *E. coli* using membrane filtration and culture as previously described using serial dilutions to achieve countable plates. Following culture-based enumeration in the backflush, we calculated the number to bacteria in the original drinking water matrix ( $C_{DW}$ ) in colony-forming units per liter using the count observed in the backflush ( $C_{BF}$ ), total volume of backflush ( $V_{BF}$ ), and the total volume of the DEUF sample ( $V_{DEUF}$ ) as shown in Eq. (1) assuming a recovery efficiency ( $\eta$ ) of 100% to estimate a lower bound count.

$$C_{DW} = \frac{C_{BF} * V_{BF}}{\eta * V_{DEUF} * 10 \frac{100 \text{ mL volumes}}{\text{liter}}} \quad (1)$$

### PEG precipitation and ultracentrifugation

After performing membrane filtration for culture-based enumeration on a subsample of the backflush solution, we further concentrated 300 mL of the remaining backflush using polyethylene glycol (PEG) precipitation and ultracentrifugation<sup>55,56</sup>. We added PEG 8000, sodium chloride, and bovine serum albumin in succession with gentle hand shaking until the reagents were dissolved to achieve a 12% PEG 8000, 0.9 M sodium chloride, and 1% bovine serum albumin solution and incubated the samples overnight at 4 °C. Following incubation, we centrifuged 300 mL of the PEG-precipitated solution in six 50 mL centrifuge tubes at 10,000 times gravity at 4 °C for 30 minutes. We then poured off the supernatant, resuspended each individual pellet using a PBS-Tween80 solution, and combined the resuspended pellets from each tube with a resulting volume of resuspended concentrate from 3 to 4 mL. We stored the DEUF concentrate at -20 °C until we pre-treated using UNEX lysis buffer and bead beating as described in the next section. Following pre-treatment, we transported the samples at room temperature for ~36 h to the laboratory in Atlanta, GA where we froze them at -80 °C until extraction and further molecular analysis.

### Nucleic acid extraction

We performed nucleic acid extraction using a universal extraction buffer, UNEX (Microbiologics, St. Cloud, MN), as developed and implemented on DEUF concentrates by the CDC<sup>55,57,58</sup>. For pre-treatment, we added 500  $\mu$ L of DEUF concentrate, 500  $\mu$ L of UNEX buffer, and 5  $\mu$ L of Inforce 3 Bovine Vaccine (Zoetis, Parsippany, NJ), our process control containing bovine respiratory syncytial virus (BRSV), to an SK-38 bead tube (Bertin Corp, Rockville, MD) and then bead beat the mixture for 2 min. After transport and prior to proceeding with extraction, we vortexed and then centrifuged the bead beating tubes and completed the extraction following the protocol for nucleic acid extraction from parasites in water samples per the manufacturer's instructions. We stored the purified nucleic acid from each

**Table 3.** Primer and probe sequence information for qPCR assays as adapted to ddPCR for interrogation of DEUF concentrate from drinking water samples collected in Jaipur, India.

Microbe	Gene	Gen bank accession	Sequence	Sequence position	Ref	
Adenovirus A-F	<i>hexon</i>	AC_000008	Forward	GGACGCCTCGGAGTACCTGAG	18,895–18,915	80
			Reverse	ACNGTGGGGTTTCTGAACCTTGTT	18,990–18,968	
			Probe	CTGGTGCAGATTCGCCCGTGCCA	18,923–18,944	
Norovirus GI	<i>ORF1-ORF2 junction</i>	MG049693.1	Forward	GCCATGTTCCGNTGGATG	5266–5283	81
			Reverse	TCCTTAGACGCCATCATCAT	5361–5342	
			Probe	TGTGGACAGGAGATCGCAATCTC	5303–5325	
Norovirus GII	<i>ORF1-ORF2 junction</i>	AF145896.1	Forward	CARGARBCNATGTTYAGRTGGATGAG	5003–5028	82
			Reverse	TCGACGCCATCTTCATTACA	5100–5080	
			Probe	TGGGAGGGCGATCGCAATCT	5048–5067	
<i>Shigella/EIEC</i>	<i>ipaH</i>	M76445.1	Forward	ACCATGCTCGCAGAGAAACT	1345–1364	83
			Reverse	TACGCTTCAGTACAGCATGC	1525–1506	
			Probe	TGGCGTTCGGGAGTGACAGC	1401–1421	
ST-EPEC	<i>STh</i>	M29255.1	Forward	TCCTGAAAGCATGAATAGTAGCAATTAC	171–198	84
			Reverse	TTAATAGCACCCGGTACAAGCA	243–222	
			Probe	ACAACACAATTCACAGCA <sup>a</sup>	199–216	
<i>Cryptosporidium</i> spp.	<i>18S rRNA</i>	AF093491.1	Forward	GGTGTATTATTAGATAAAGAACCA	197–223	85,86
			Reverse	AGGCCAATACCTACCGTCT	322–303	
			Probe	TGACATATCATTCAAGTTTCTGAC <sup>a</sup>	268–291	
<i>Giardia duodenalis</i>	<i>beta-giardin</i>	AY072727	Forward	GGCCCTCAAGAGCCTGAAC	402–420	87
			Reverse	GGGCGATCGTCTCCTTCTC	544–526	
			Probe	CTCGAGACAGGCATC <sup>a</sup>	424–438	
MS2	<i>MS2g1</i>	NC_001417	Forward	TGGCACTACCCTCTCCGTATTACCG	160–185	74
			Reverse	GTACGGGCGACCCACGATGAC	258–237	
			Probe	CACATCGATAGATCAAGGTGCTACAAGC	201–229	
<i>E. coli</i>	<i>ybbW</i>	NC_000913.3	Forward	TGATTGGCAAATCTGGCCG	538,033–538,052	70
			Reverse	GAAATCGCCAAATCGCCAT	538,224–538,243	
BRSV (extraction/RT control)	<i>beta actin</i>	AF092942.1	Forward	GCAATGCTGCAGGACTAGGTATAAT	2120–2144	88
			Reverse	CACTGTAATTGATGACCCCATCT	2243–2219	
			Probe	ACCAAGACTTGTATGATGCTGCCAAAGCA	2171–2199	

<sup>a</sup>Minor groove binder.

sample and the associated negative and positive controls at  $-80^{\circ}\text{C}$  until reverse transcription and ddPCR.

### Reverse transcription

Prior to ddPCR assay for RNA targets (*MS2g1*, *Noro GI ORF1-2*, *Noro GI ORF1-2, beta actin*), we performed reverse transcription of RNA to cDNA using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ThermoFisher Scientific, Waltham, MA) per the manufacturer's instructions.

### ddPCR

Another promising development is the use of ddPCR for the absolute quantification of nucleic acid templates<sup>59,60</sup>. Droplet digital PCR has been used to quantify *Salmonella* in river sediments, Shiga-toxin-producing *E. coli* (STEC) in bovine feces, foodborne pathogens in soft cheeses, and fecal indicators in environmental water<sup>61–63</sup>. The method shows great promise for the detection of waterborne pathogens in drinking water due to its reported sensitivity and resilience to inhibitory substances<sup>64–66</sup>.

Based on their associations with diarrheal disease in South Asian settings during the Global Enteric Multicenter Study (GEMS) and the Malnutrition and Enteric Disease Study (MAL-ED), we selected *Shigella*/enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* with heat-stable toxin (ST-EPEC) as bacterial pathogens of interest, *Cryptosporidium* spp. and *Giardia* spp. as protozoan targets, and norovirus GI, norovirus GII, and adenovirus A-F as viral targets<sup>67,68</sup>. We did not select rotavirus as a viral target due to the rollout of rotavirus vaccination programs in India which could confound our detection of wildtype rotavirus in drinking water via ddPCR<sup>69</sup>. We adopted previously published qPCR assays, as summarized in Table 3, and adapted them to ddPCR as described in the Supplementary Notes.

In addition to waterborne pathogens, we also performed molecular analysis for genes associated with microbial indicators *E. coli* and male-specific coliphage (MS2). For *E. coli*, we selected a qPCR assay targeting the *ybbW* gene, a putative allantoin transport protein, for adaptation to ddPCR because the assay has demonstrated 100% sensitivity and specificity for the bacteria as compared to 16S and 23S assays which demonstrate lower specificities for *E. coli*<sup>70–73</sup>. For the detection and quantification of MS2, we selected an assay targeting the *MS2g1* gene, which encodes a maturation protein, as the ddPCR assay based on published use of MS2 as an internal control in reverse transcriptase polymerase chain reaction (RT-PCR) assays for viral targets<sup>67,74</sup>. All assays and relevant information including GenBank Accession numbers, sequences, and alignment positions are summarized in Table 3 for pathogens and the BRSV control assay.

We performed all ddPCR workflow following the manufacturer's recommended protocol using a QX200 Droplet Generator, PX1 PCR Plate Sealer, C1000 Touch Thermal Cycler, and QX200 Droplet Reader (Bio-Rad, Hercules, CA)<sup>75</sup>. Reaction conditions are detailed in the Supplementary Notes. We estimated the 95% limit of detection (LOD) for each ddPCR assay using positive control materials prepared in a serial dilution series and probit analysis<sup>76</sup>.

### Interpreting ddPCR results

In ddPCR, the density of a gene target in the reaction mix is estimated by means of a most probable number according to a Poisson distribution as detailed in the Supplementary Notes. Droplets are classified as either positive or negative for the gene target based on their measured fluorescence amplitude following thermal cycling and a fluorescence threshold<sup>77</sup>. However, user-based thresholding creates opportunity for bias in the interpretation of ddPCR results<sup>78</sup>. We estimated the density of target genes in our ddPCR assays using both a manual user-determined threshold in QuantaSoft (V1.7.4; Bio-Rad, Hercules, CA) and a model-based classification called Umbrella implemented in RStudio (Version 1.1.456; RStudio Team, 2015)<sup>79</sup>. The Umbrella package reports an estimate of the density based on the regression estimated proportion of droplets negative ("robust estimate") and an estimate based on the number of droplets with a <5% probability of being negative, which we report as the Umbrella threshold<sup>79</sup>.

### DATA AVAILABILITY

The datasets generating during and/or analyzed during the current study are available in the OSF.IO repository, <https://osf.io/4mwzh/>, <https://doi.org/10.17605/OSF.IO/4MWZH>.

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### AUTHOR CONTRIBUTIONS

A.B. and J.B. designed the study and methods. A.B. and R.C. completed the field sampling. A.B. and S.L. completed the laboratory analyses. A.B., J.B., S.L., M.B., P.L., and H.M.M. completed the data analysis. A.B. composed the manuscript drafts and J.B., S.L., M.B., P.L., and H.M.M. provided comments and revisions to the drafts.

### COMPETING INTERESTS

The authors declare no competing interests.

### ADDITIONAL INFORMATION

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