Well water quality in rural Nicaragua using a low-cost bacterial test and microbial source tracking

Patricia Weiss, Tiong Gim Aw, Gerald R. Urquhart, Miguel Ruiz Galeano and Joan B. Rose

ABSTRACT

Water-related diseases, particularly diarrhea, are major contributors to morbidity and mortality in developing countries. Monitoring water quality on a global scale is crucial to making progress in terms of population health. Traditional analytical methods are difficult to use in many regions of the world in low-resource settings that face severe water quality issues due to the inaccessibility of laboratories. This study aimed to evaluate a new low-cost method (the compartment bag test (CBT)) in rural Nicaragua. The CBT was used to quantify the presence of *Escherichia coli* in drinking water wells and aimed to determine the source(s) of any microbial contamination. Results indicate that the CBT is a viable method for use in remote rural regions. The overall quality of well water in Pueblo Nuevo, Nicaragua was deemed unsafe, and results led to the conclusion that animal fecal wastes may be one of the leading causes of well contamination. Elevation and depth of wells were not found to impact overall water quality. However rope-pump wells had a 64.1% reduction in contamination when compared with simple wells.

Key words | developing countries, *E. coli*, low-cost bacterial test, public health, well water quality

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INTRODUCTION

Access to safe drinking water is critical in reducing the incidence of waterborne diseases. In total, 783 million people still lack access to improved drinking water sources. About 2.5 billion people lack access to improved sanitation and some 1.1 billion people practise open defecation (UNICEF & World Health Organization 2012). The majority of these people live in rural areas of developing countries. Even with improved access, which is often wells, water quality testing is not routinely performed. Thus, the water safety of these rural water supplies remains questionable (UNICEF & World Health Organization 2012).

Nicaragua is one of many countries in Central America that face issues of water shortages and water-related diseases. In the late 1990s there was an upsurge of cases of cholera seen in Latin America, impacting Nicaragua the most. Cases increased from 17,760 to 57,106 due to widespread fecal pollution of drinking water sources (WHO 2000). In rural areas in Nicaragua, only 37% of people have improved sanitation, and only 68% of people have access to safe drinking water (Water for People n.d.).

Cases of morbidity and mortality in Pueblo Nuevo, Nicaragua during 2012 were documented, reviewed and compiled by Dr Alejandro Picado, who is the community physician at the free health clinic funded by Nicaragua's Ministry of Health (MINSA) serving the Pueblo Nuevo community and surrounding area. Dr Picado reported 18,061 total cases of illness, of which 5,675 individuals had water-related diarrhea, and 2,850 individuals were infected with parasites (Dr A. Picado, personal communication, 3 March 2013).

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Testing for microbial water quality in low-resource settings such as rural Nicaragua is challenging due to the lack of access to low-cost methods, appropriate materials and laboratories (Stauber *et al.* 2014). Both molecular- and culture-based methods are costly, labor intensive, and must be carried out in a laboratory setting. To overcome this, several low-cost bacterial tests have been developed for water in low-resource settings such as the H2S most probable number (MPN) test for clostridia (McMahan *et al.* 2012) and the compartment bag test (CBT) for *Escherichia coli* (*E. coli*) (Stauber *et al.* 2014). CBT is an MPN chromogenic method for enumerating *E. coli*. This method has been commercialized to provide a low-cost and simple method to detect and quantify *E. coli* in drinking water (Aquagenx n.d.).

One of the limitations of using indicator bacteria is that the sources of microbial contamination in water cannot be determined. Microbial source tracking (MST) is a field that has developed molecular methods to differentiate various sources of fecal contamination (Scott *et al.* 2002). Most of the MST methods utilize genetic markers, which are host-specific, or library independent, and rely mainly on quantitative polymerase chain reaction (qPCR) without cultivation of the microorganisms (Field *et al.* 2003). There are now several molecular markers that allow identification of animal and human fecal sources of contamination in water (Layton *et al.* 2013; Raith *et al.* 2013). However, these MST assays have not been used in rural settings in developing countries.

The objectives of this study were: (1) to determine the efficacy of a new, low-cost bacterial test (CBT) in a low resource setting; (2) to determine the microbial water quality of drinking water wells for residents of the village of Pueblo Nuevo, Nicaragua; and (3) to determine the source of microbial contamination in well water using human- and bovine-specific qPCR assays.

MATERIALS AND METHODS

Sampling location

Pueblo Nuevo is located on the Atlantic Coast of Nicaragua between the Rio Wawashang and the Kahka Creek Reserve as seen in Figure 1. Water samples were collected during the dry season (3–12 March 2013) from hand dug wells in the village of Pueblo Nuevo as seen on the geographic information system (GIS) map in Figure 1.

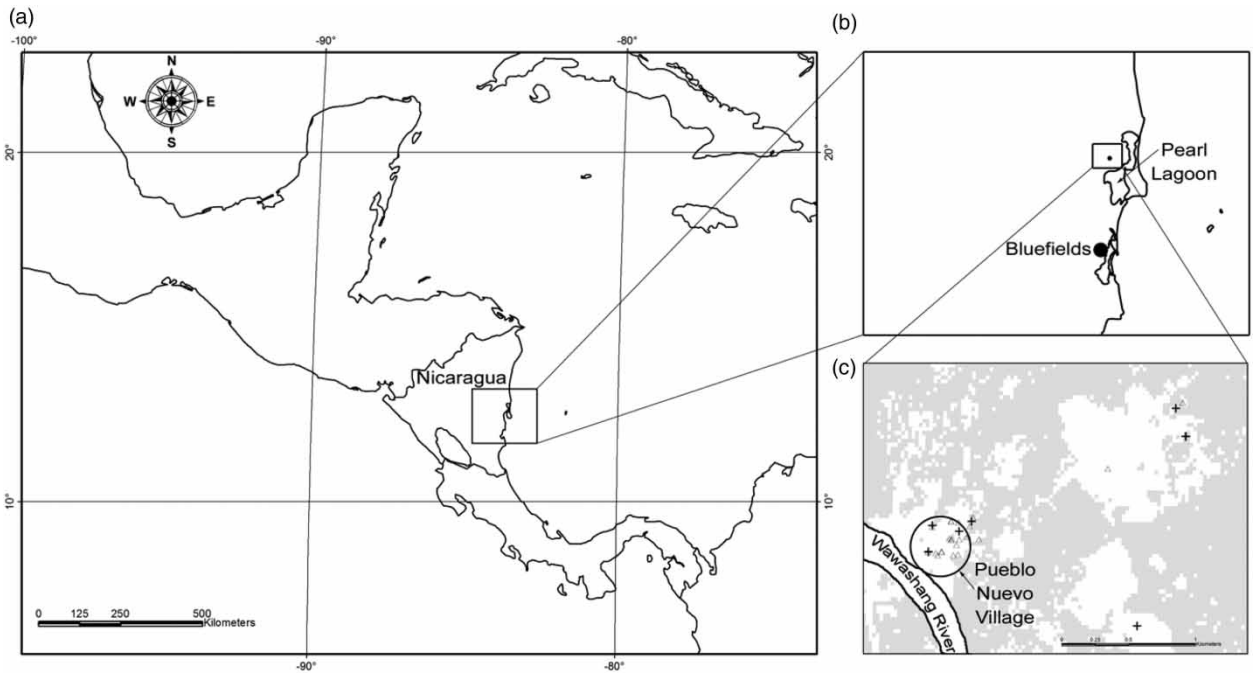

Figure 1 | Location of Pueblo Nuevo, Nicaragua and GIS map showing location of 32 sampled wells. Map (a) shows an entire map of Nicaragua. Map (b) shows Pueblo Nuevo relative to other major areas on the east coast. Map (c) shows well sampling sites within or near Pueblo Nuevo relative to the Rio Wawashang.

Evaluation of different enumeration methods, incubation temperatures, and incubation periods

When incubating samples in a low-resource setting, such as Pueblo Nuevo, Nicaragua, it is unlikely that incubators will be readily available. As such, the ambient temperature and the length of the incubation period must be taken into consideration. Prior to the fieldwork, experiments were conducted in the laboratory to determine the ideal incubation period with average ambient temperature (25 °C) in Nicaragua during the dry season. *E. coli* C-3000 (ATCC 15597) was used with dilutions from 10^{-7} to 10^{-11}. The CBT was used to enumerate *E. coli*. One set of dilutions was incubated at 25 °C and the other set was incubated at 37 °C. No results were seen in either set after 24 hours of incubation. However, after 48 hours of incubation both sets exhibited changes in color. Both sets were found to have an MPN of 8.5×10^{10} per 100 ml after 48 hours of incubation at either incubation temperature. Tryptic soy agar (TSA) plate count and Colilert (IDEXX) assays were also used to compare the CBT to other *E. coli* enumeration methods. Both the CBT MPN and TSA assay (colony forming unit) had a consistent result with an average of 8.5×10^{10} per 100 ml of *E. coli* culture. Raw sewage was then used to compare the CBT to Colilert MPN assay. The Colilert samples had an average MPN of 6.3×10^5 per 100 ml, and the CBT samples had an average MPN $\geq 4.8 \times 10^5$ per 100 ml. As a result, it was determined that the CBT is a viable method that could be used in the field and incubated at ambient temperature.

Well water sampling and *E. coli* testing using CBT

A total of 32 wells were sampled arbitrarily throughout the village of Pueblo Nuevo. Four disinfected ropes and buckets were used to collect water from the wells as well as measure the depths of the wells. A Magellan SporTrack Color (model 980616-280) was used to determine the elevation of the wells. Sterile buckets and equipment were prepared every evening for the following day of sampling using 5 ml of bleach per bucket (which contained a volume of 13 L). After one hour, the buckets and the equipment were neutralized using 0.05 g sodium thiosulfate per bucket. Tools and equipment, as well as the CBTs, were carefully stored upright, in a redesigned plastic tool kit, and were carried by donkeys to the sampling sites. Once at the sampling site, a water sample (100 ml) was collected using a sterile sampling cup to which a pellet containing chromogenic culture media was added. The water sample was gently swirled for 10 to 15 minutes until all of the media was dissolved, and a white bud was left. After the media had dissipated throughout the water sample, the 100 ml from the sampling cup was emptied into the CBT. The water was then distributed into five compartments, which held volumes of 1, 3, 10, 30 and 56 ml. The bags were sealed using a spring-clip and securely placed into the redesigned carrying case as mentioned above. The bags were left to incubate for 48 hours at ambient temperature (approximately 26 °C). After incubation, bacteria that changed the compartments to a blue color indicated the presence of *E. coli*. Yellow coloration indicated an absence of microbial fecal contamination as seen in Figure 2. A 'Comprehensive Water Quality Rating' chart provided in the CBT kit was then used to assess the quality of the water by determining the MPN per 100 ml. After usage, the bags were decontaminated using chlorine tablets provided in the kits. Half an hour after the tablet was added, the contents of the bags were emptied into a toilet (Aquagenx n.d.).

Membrane filtration for MST

Two liters of water were also collected from each well in sterile plastic bottles that were transported back to the field station. This volume was filtered through a polycarbonate membrane filter (47 mm, 0.45 μm pore size) using a modified plastic garden sprayer that served as a pressure vessel and hand pump connected to a plastic filter holder. A filtration blank control using boiled water was also included for each set of wells. The same hand pump apparatus and filter housing used for the blank control was then used for the sample. Using sterilized forceps, the filter was folded in half with the grid of the filter facing inward, then folded in half again and placed into a zip-lock plastic bag. The filter holders, along with the hand pump, were cleaned and disinfected with bleach and rinsed with boiled water each evening in preparation for the next set of samples. Membrane filters were stored in the Kahka Creek Lodge at 4 °C and then transported

Figure 2 | Examples of positive and negative results that can be seen when running a CBT. Blue indicates the compartment is positive for *E. coli* and yellow indicates it is negative. Photograph (a) shows a test where all compartments except the 1 ml were positive, photograph (b) is a test where only the 30 ml compartment was positive. Please refer to the online version of this paper to see this figure in color.

back to Michigan State University (at ambient temperature) for DNA extraction and qPCR.

DNA extraction and qPCR analysis

For DNA extraction, a membrane filter was unfolded and placed into a 50 ml centrifuge tube containing 50 ml of phosphate buffered water and vortexed for 10 minutes. The tube was then centrifuged for 30 minutes at 4,000 × g. Around 48 ml of the supernatant was discarded, and the remaining sample was mixed by vortexing. The volume was recorded and from this, 200 μL of the pellet was used for DNA extraction with the QIAmp DNA mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

The presence of human and bovine-specific markers was determined by using qPCR assays as previously described (Shanks *et al.* 2008; Yampara-Iquise *et al.* 2008) with a LightCycler 480 System (Roche Applied Science). The MST assays used in this study have been shown to have an advantage over many other markers in a large fecal source assessment based on specificity, sensitivity, and target abundance (Layton *et al.* 2013; Raith *et al.* 2013). For the human-specific marker, qPCR assay targeting the α-1-6, mannanase gene of *Bacteroides thetaiotaomicron*, used the following sequences: forward primer CATCGTTCGTCAGCAGTAACA; reverse primer CCAAGAAAAAGGGACAGTGG and probe FAM-ACCTGCTG-NFQ. For the bovine-specific marker, the CowM2 qPCR assay used the following sequences: forward primer CGGCCAAATACTCCTGATCGT; reverse primer GCTTGTTGCGTTCCTTGAGATAAT and probe FAM-AGGCACCTATGTCCTTTACCTCATCAACTACAGACA-TAMRA. Amplification reaction mixtures (with a total final volume of 20 μl) contained 5 μl template DNA, 10 μl of 5 × LightCycler TaqMan Master Mix (Roche Applied Science), 200 nM of each primer and 100 nM of probe. The thermal cycling protocols for qPCR were as follows: 15 min at 95 °C for initial denaturation, followed by 45 cycles of three steps consisting of 15 s at 94 °C, 60 s at 60 °C, and 5 s at 72 °C (at a temperature transition rate of 20 °C/s), with a cooling step at 40 °C for 30 s. The detection limit for both human- and bovine-specific qPCR assays was 10 copies per reaction. All samples were each run in triplicate. A negative control (PCR grade water without template) and positive control were included in all PCR runs. For qPCR-negative samples, template DNA was diluted 10-fold to reduce the potential PCR inhibitions, and the qPCR assay was repeated.

Statistical analysis

All statistical analyses were completed using Microsoft Excel 2011 for Macintosh and VassarStats. The significance

level used was $\alpha = 0.05$. The geometric mean was used to determine the mean level of contamination seen in rope-pump wells and simple wells.

Because of the small sample sizes ($n = 23$ for simple wells, $n = 9$ for rope wells), distributions were examined using the Anderson-Darling test for normality and found to be not normally distributed. Therefore, nonparametric statistical tests to compare average MPN levels between simple wells and rope wells utilized the Mann–Whitney U Test using a significance level of $\alpha = 0.05$. Calculations were performed using the VassarStats website (http://vassarstats.net/utest.html). The Spearman correlation test was used to determine the relationship between the elevation of the wells and microbial quality of the water, calculated using Excel 2011 for Macintosh. It was also used to determine the relationship between the depth of the wells and the microbial quality of water as seen in Figures 3 and 4.

RESULTS AND DISCUSSION

Using the CBT in a low resource setting

The CBT made it possible to conduct this study in a more isolated part of the world, where supplies and materials were limited. Incubators were not necessary as the CBT was incubated at ambient temperatures, which, in this case, was between 23 and 28 °C for 48 hours of incubation. As detailed in the methods, CBT samples using *E. coli* cultures were tested and incubated prior to the trip to the laboratory at an ambient temperature of 25 °C. These samples were compared with samples incubated in an incubator at 37 °C using *E. coli*. There was no variation in results between those incubated at ambient temperature and those incubated at 37 °C. When comparing this to TSA and Colilert assays, results were not significantly different. Thus, it was concluded that using the CBT and incubating at

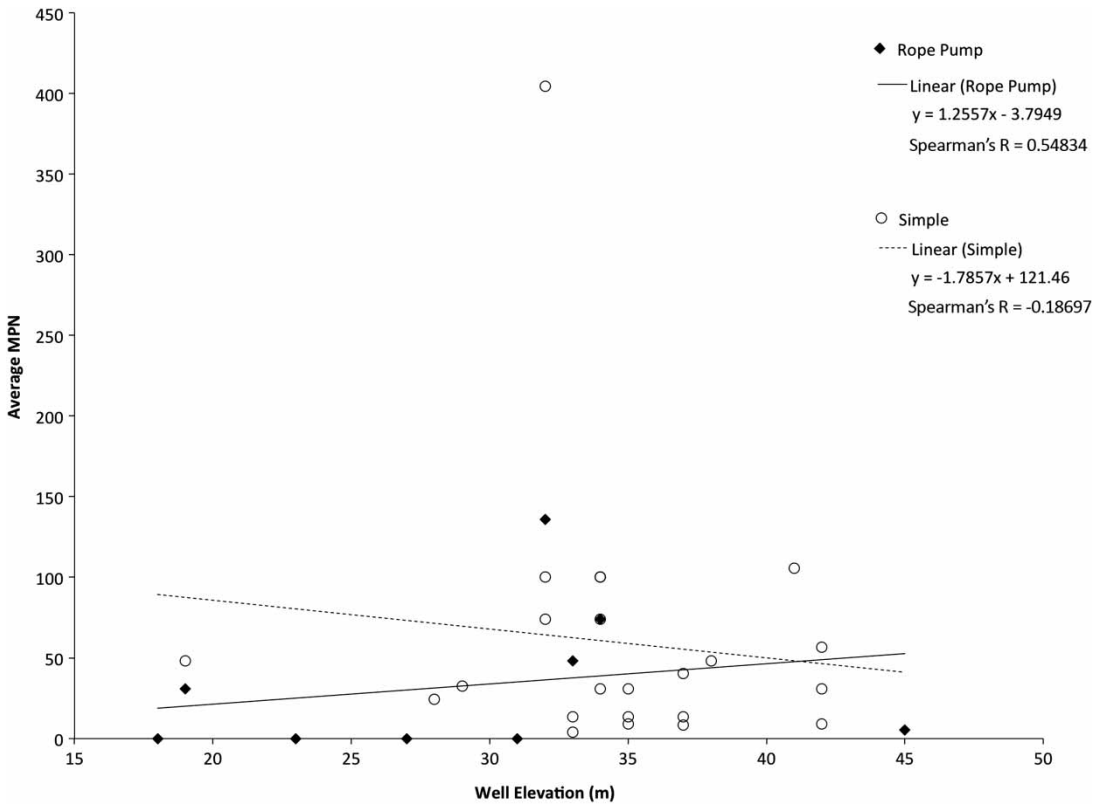

Figure 3 | MPN of tested wells in relation to elevation of wells. Solid line indicates regression for rope-pump wells (diamonds), dashed line is a regression for simple wells (open circles).

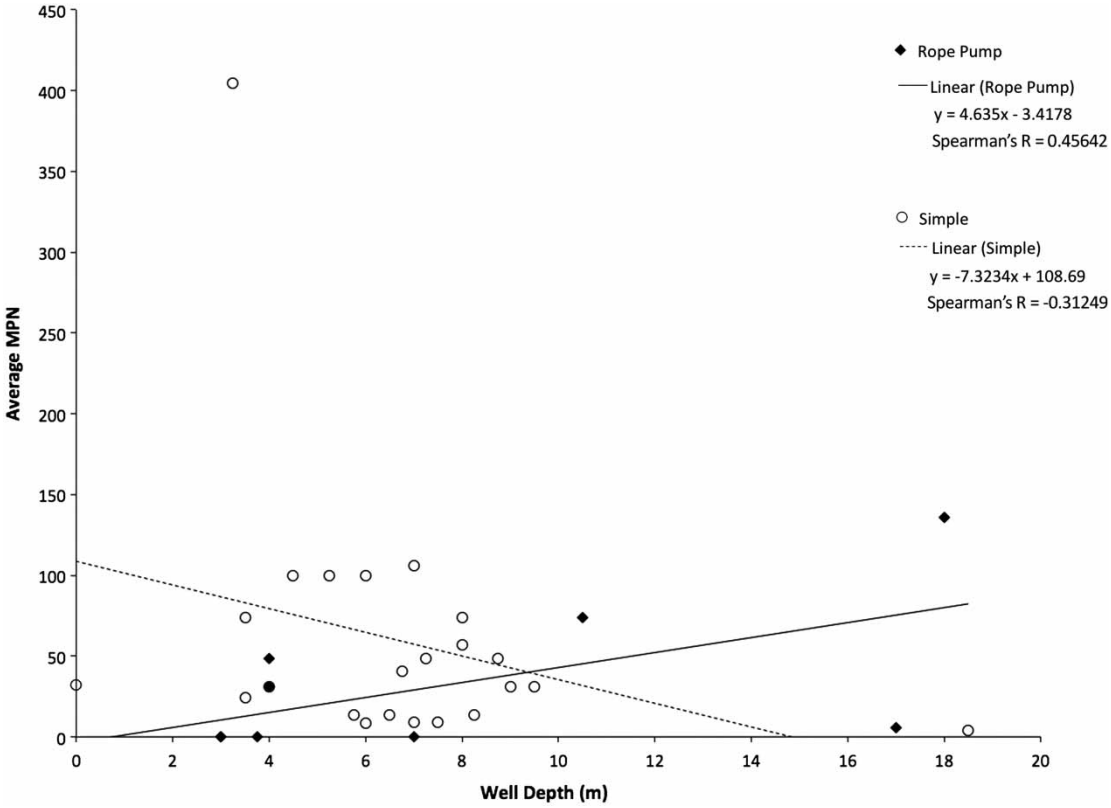

Figure 4 | MPN of tested wells in relation to depth of wells. Solid line indicates regression for rope-pump wells (diamonds), dashed line is a regression for simple wells (open circles).

ambient temperature in Nicaragua would be feasible. According to a study by Murcott *et al.* (2015), in which different microbial water quality tests were used and compared in the field, the CBT was found to have the best overall general statistical values. The CBT was used in Ghana and compared with Petrifilm, Easygel Card, and Lab-made H2S. The CBT not only did well in terms of overall general statistical values but also did reasonably well when detecting both true positive and true negative results. The sensitivity and specificity were found to be 92% and 73%, respectively. Due to this, the CBT was recommended when conducting *E. coli* enumeration in the field. These results were in agreement with those reported by Stauber *et al.* (2014).

Higher variability might be expected in the field, where ambient temperatures fluctuate over the diurnal cycle. In this study, duplicates were created for each well to ensure accuracy. Of the 32 wells sampled where duplicates were done ($n = 32$), 75% yielded the same result. The other 25% resulted in inconclusive results or an MPN exceeding the limit. In these cases, the wells were sampled again. If the MPN exceeded the limit, a 10-fold dilution was used. Also, the CBT may be limited to a warmer climate if an incubator is not available, as a certain temperature range is necessary for the appropriate growth of *E. coli*.

The CBT bag is fragile, and leakage may occur if not properly sealed. Special preparation to avoid such an incident was necessary. To overcome this, our team created a plastic box fitted to the CBT to store and transport the bags in a safe manner in order to reduce leakage, cross-contamination and exposure to sunlight when collecting and transporting large amounts of samples over great distances and varying terrain throughout the day. The CBT has a low learning curve that involves a simple set of steps and only a short introductory training is needed. Thus, this method can be used by individuals with minimal training and in a variety of settings, particularly in developing countries and natural disaster areas.

A major limitation of the CBT used in this study was the upper detection limit of this method of 100 per 100 ml. However, this was overcome by having additional sampling

days in order to collect more samples from wells that showed the MPN exceeded 100 per 100 ml. Samples were diluted to determine the MPN of these wells. Since samples were taken during the dry season, the MPN was generally below the upper detection limit of the CBT. However, more preparation and additional sampling may be needed during the rainy season.

Microbial quality of well water in rural Pueblo Nuevo, Nicaragua

A total of 32 wells were sampled to assess the microbiological quality of water using the CBT method. Of these 32 wells, nine were categorized as rope-pump wells and 23 were categorized as simple wells. The wells served a range of four to 20 people and one to four families. Overall 87.5% of the wells were contaminated with *E. coli*. The concentrations of *E. coli* in contaminated wells ranged from 5.5 to 404.5 MPN per 100 ml, with an overall geometric mean of 25.8 MPN per 100 ml. The water could be deemed safe, likely safe, possibly safe, possibly unsafe, likely unsafe, and unsafe according to the Comprehensive Water Quality Rating Manual (CBM) which was provided in each kit. These ratings were assigned based on MPN per 100 ml ranges. An MPN of 0 was deemed safe. A range of 1–3 was determined to be likely safe, a range of 3.1–9.6 was determined to be possibly safe, a range of 13.7–17.1 was deemed possibly unsafe, a range of 32.6–48.3 was deemed likely unsafe, and anything over 100 was unsafe. Of all the samples, 12.5% were found to be safe, 0% likely safe, 15.6% possibly safe, 12.5% possibly unsafe, 28.13% likely unsafe, and 31.25% were found to be unsafe. However, these ratings were confusing when describing the results to the local outpost clinic nurse. While the 'likely safe' and 'likely unsafe' categories were clear, it is uncertain what to do with the information in the 'possibly safe' or 'possibly unsafe' categories. Thus, it is recommended that this description is not used and that the information is conveyed as meeting WHO guidelines or country standards or not meeting them.

Two types of wells were found in Pueblo Nuevo and tested: simple wells and rope-pump wells. Simple wells were found to have a geometric mean of *E. coli* of 34.4 MPN per 100 ml, and it was determined that rope-pump wells had a geometric mean of 12.3 MPN per 100 ml. A 64.1% reduction of *E. coli* was seen between simple wells and rope-pump wells. The results of the Mann–Whitney *U* Test comparing MPN levels for simple wells ($n = 23$) with rope wells ($n = 9$) showed a significantly lower MPN for rope wells ($U = 63.5$, $P = 0.0485$). This is consistent with results found by Gorter *et al.* (1995), which indicated that there was a 62% reduction in terms of the geometric mean of fecal coliform contamination as a result of the installation of a rope-pump well. However, according to a study conducted by Bennett *et al.* (2010) in Cambodia, no significant difference in water quality was found between open wells (simple wells) and rope-pump wells, suggesting alternative household treatments to the water rather than relying on well type to improve water quality (Bennett *et al.* 2010). This discrepancy could be due to the fact that rope-pump systems are often installed upon existing open wells in Cambodia according to the study, whereas this is not the case in Pueblo Nuevo, Nicaragua.

The Spearman R and resulting probability values when looking at the relationship between elevation of wells and MPN was found to be $R = 0.09429$, $P = 0.6077$ (all wells), $R = -0.18697$, $P = 0.393$ (simple wells), and $R = 0.54834$, $P = 0.125$ (rope wells) (Figure 3), demonstrating a lack of a relationship between the elevation of the wells and contamination levels. When looking at the relationship between depth of wells and MPN, the R and P values were found to be $R = 0.0093$, $P = 0.960$ (all wells), $R = -0.31249$, $P = 0.147$ (simple wells), and $R = 0.45642$, $P = 0.0217$ (rope wells) (Figure 4), which similarly shows a lack of relationship between depth and MPN. However, after studying Figure 4, it can be seen that two out of the three deepest wells had extremely low levels of contamination. This contamination suggests a relationship may be present, but the sample size was not large enough due to a lack of wells above a depth of 16 m. A conclusion cannot be drawn from this data, and further research must be conducted concerning the depth of wells and contamination levels.

Determining the source of contamination

In this study, a low qPCR-positive rate was observed for human- and bovine-specific markers in well water samples. The human-specific *Bacteroides* marker was not detected

in any well water samples. Bovine-specific CowM2 marker was detected in four out of 31 (12.9%) well water samples, with an average concentration of 4.3×10^2 gene copies per 100 ml. This suggested potential bovine fecal pollution in these well water samples.

The low qPCR detection rate of these molecular markers, particularly the absence of the human-specific marker in this study, could be due to the small well water sample volume collected (2 liters). This is consistent with another groundwater study, which suggested the inability to extract sufficient good quality DNA from a small volume of sample (Zhang *et al.* 2014). In addition, these markers may not persist for a long time in the tropical environment, and the detection level may have decreased during transport of the filters from Nicaragua to the laboratory in the United States at ambient temperature. A recent study showed that filtering water prior to transportation of samples to the laboratory on a membrane filter and storing at low temperature (i.e. 4 °C) could increase the persistence of bacterial DNA markers measured by qPCR (Brooks *et al.* 2015). Temperature has been suggested as the main factor affecting *Bacteroides* persistence in freshwater environments (Ballesté *et al.* 2010). However, further study is needed to determine the occurrence and persistence of human- and bovine-specific markers in the tropical well water with larger sample volumes.

CONCLUSION AND RECOMMENDATIONS

- The CBT made it possible to test for *E. coli* contamination and obtain viable results in the field in a rural location where supplies and laboratory equipment were limited. However, limitations were found such as a low upper detection limit and the potential of the CBT bag to break or leak during transportation, which were overcome with additional preparation. Also, the water quality rating system was found to be confusing.
- Overall the well water was deemed unsafe in this small rural area in Nicaragua. Rope-pump wells contained significantly less *E. coli* contamination than simple wells. As such, more rope-pump wells should be built and used if possible, to reduce the pollution potential. Elevation or depth of wells was not found to be associated with the quality of the drinking water source.
- The presence of the M2 bovine marker in the well samples indicated fecal contamination originating from livestock. Educating inhabitants about fecal-runoff and illness associated with this may help reduce the incidence of morbidity and mortality. Physical barriers may be necessary to keep livestock a safe distance from drinking water sources and protect the wells from surface runoff.
- The human marker *B. thetaiotaomicron* was not detected using qPCR and 2 liter sample volumes. Larger sample volumes in the future may be warranted.

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