Potentially pathogenic bacteria isolated from different tropical waters in Sri Lanka
W. M. G. C. K. Mannapperuma, C. L. Abayasekara, G. B. B. Herath and D. R. I. B. Werellagama

ABSTRACT
This study investigated the bacteriological contamination of different water sources in Sri Lanka. Source waters (n = 74) including bottled water, well water and surface water were assessed for enumerating total coliforms and faecal coliforms using the membrane filtration method. The results showed that 18.5 and 14.8% of bottled water samples were contaminated with total coliforms and faecal coliforms, respectively. All the well water and surface water samples exceeded the WHO permitted levels for total coliforms and faecal coliforms. Bacteriological identification using biochemical tests and API 20E identification tests revealed the presence of potentially pathogenic bacteria in all water sources tested. Bottled water mainly contained three Enterobacter species, while well water samples showed the broadest spectrum of bacteria including eleven coliform species belonging to the genera Klebsiella, Escherichia, Enterobacter, Citrobacter, Klyvera, Pantoea, Rautella, and 10 non-coliform species in the genera Pseudomonas, Aeromonas, Salmonella and Acinetobacter. Surface waters contained seven coliform species belonging to the genera Klebsiella, Escherichia, Citrobacter, Rautella and Serratia, and eight non-coliform species in the genera Pseudomonas, Aeromonas and Acinetobacter. Detection of higher bacteriological counts and identification of potentially pathogenic bacteria in different source waters suggest a potential health risk of the water sources used in Sri Lanka.

Key words | Escherichia coli, faecal coliforms, pathogenic bacteria, total coliforms, tropical waters, waterborne diseases

INTRODUCTION
Access to safe drinking water is a vital requirement for all living beings. However, increasing amounts of discharged sewage, progressing urbanization, the chemicalization of agriculture and industry, as well as anthropogenic activities, affect the quality of surface and underground water (Suthar et al. 2009). The final effects of water degradation are the limits as to the use of drinking water reservoirs coupled with microbiological contamination, resulting in the penetration of potentially pathogenic bacteria or microorganisms detrimental to underground waters through the soil (Sjorgen 1995). Furthermore, it has been reported that pathogenic bacteria can survive and also grow in low nutrient aquatic environments such as surface waters or man-made water treatment systems (Camper et al. 1985). According to reports, 2.5 billion people have no access to improved sanitation, and more than 1.5 million children die each year from diarrhoeal diseases (Fenwick 2006). The major pathogenic bacteria responsible for waterborne disease are spread by the faecal–oral route, in which water may play an intermediate role. These enteropathogenic bacteria in water are responsible for a variety of diseases, such as cholera, typhoid, dysentery, bacillary dysentery, etc. in humans (Suthar et al. 2009). Some bacteria, such as Pseudomonas or Aeromonas, may be a threat to human health due
to their ability to multiply in drinking waters (Havelaar et al. 1990). Others, especially those which constitute the natural microflora of human and animal food tracts, can induce acute or chronic gastric diseases (Payment et al. 1991). As a consequence, the mortality caused by water-associated diseases exceeds 5 million people per year (WHO 2004). Of these, more than 50% are microbial intestinal infections, with cholera ahead of other infections (Cabral 2010). Acute microbial diarrhoeal diseases are another major public health problem in developing countries (Seas et al. 2000). Therefore, information related to the presence of pathogens in drinking water is very valuable in the investigation of possible waterborne disease outbreaks (Payment et al. 2005). The WHO has also highlighted that at least 17 different and major genera of bacteria capable of seriously affecting human health can be found even in tap water (WHO 2006).

In Sri Lanka, only 67% of people have access to safe drinking water (Bandara et al. 2008). The rural communities of Sri Lanka obtain their drinking water mainly through well water (66%), springs (7%) and surface water sources, such as rivers and lakes (2%), while only 25% have access to tap water (WHO/UNICEF 2010). Furthermore, most of these water sources are not properly disinfected or treated before consumption. Therefore, the bacteriological quality of water can degenerate and can harbour potentially pathogenic organisms leading to outbreaks of waterborne diseases.

In addition to the above-mentioned water sources, bottled water is gaining popularity in the country, and is considered as a much safer drinking water source by the majority of people. The microbiological quality of bottled water used in Sri Lanka is monitored according to the guidelines stipulated by the Sri Lanka Standards (SLS 614–1983, SLS 894–2003). SLS permits 1–10 cfu/100 cm<sup>3</sup> of total coliforms in one out of 10 bottles tested. Although there are these standards, bacteriological contamination of bottled water has been reported in several recent publications (Mannapperuma et al. 2011; Chethana & Mannapperuma 2012; Herath et al. 2012). Since bottled water has been implicated as the source of outbreaks of cholera and typhoid fever as well as traveller’s disease in countries such as Portugal and Spain (Battu & Reddy 2009), more concern about the bacteriological quality of bottled water is necessary.

Furthermore, in Canada and other overseas countries, stringent regulations have been implemented in this regard (Battu & Reddy 2009). However, in Sri Lanka, existing regulations are not very strict or specific about the microbial quality of bottled water, since there is not a sufficient amount of research carried out in this area and very little government authority over the matter to ensure that bottled water production is in accordance with the standards (Abayasekara et al. 2007).

Therefore, the aim of this study was to assess the bacteriological contamination of different water sources including bottled water, and to identify the possible pathogenic bacteria present.

**METHODS**

Water samples (n = 74) were collected from different sources (bottled, well and surface water) from five districts, namely Kandy, Nuwaraeliya, Anuradhapura, Kurunegala and Rathnapura in Sri Lanka, which included both rural and urban areas. One-litre bottled water samples (n = 27), shallow (depth around 10 m) well water samples (n = 20) and surface water samples (rivers and streams = 20; lakes = 7) were collected randomly by following the standard procedures (APHA 2000) and analysed (in duplicate) to detect and enumerate indicator bacteria by using the membrane filtration method. Bacteriological analyses were conducted by filtering 100 mL of water samples (undiluted and diluted when necessary) through membrane filters (pore size: 0.45 μm) and placed aseptically on absorbent pads saturated with M-Endo broth (Himedia, India) and M-FC broth (Himedia, India) and incubated at 36 ± 1°C for 24–48 hours and 44–44.5°C for 24–48 hours to detect total coliforms and faecal coliforms respectively. Typical total coliform (pink to dark red colour with a metallic sheen) colonies formed on M-Endo medium and typical faecal coliform (blue colour) colonies formed on M-FC medium were counted separately and converted into cfu/100 mL. Sampling was conducted from July 2008 to June 2009. Bacteriological identification was conducted using biochemical tests as described in Bergey’s Manual of Determinative Bacteriology (Holt et al. 1994), and api 20E identification tests (BioMérieux 2009) for pure isolated...
cultures obtained by subculturing done on tryptic soy agar (TSA) (Oxoid, UK) plates.

Isolation of bacteria

Bacterial isolation was performed by several subcultures on TSA plates. Three basic standard confirmation tests (Gram’s test, oxidase test and catalase test) were conducted to confirm the isolates as coliform bacteria. Stock cultures of all confirmed isolates were prepared by inoculating into brain heart infusion broth (Oxoid, UK) or double strength tryptic soy broth (Oxoid, UK) in Eppendorf tubes and incubated for 24 hours at 37°C. Four replicate stock cultures were prepared from each isolate and stored at −20°C, after overlaying with 40% glycerol.

Identification of bacteria

One or two Eppendorf tubes from each stock culture were thawed, and the tubes were centrifuged for a few seconds to obtain a concentrated cell mass. Subsequently, the TSA plates were streaked with the concentrated cell mass to obtain pure colonies required for identification tests. All plates were incubated at 37°C for 24 hours to obtain pure cultures. The three basic confirmation tests (Gram’s test, oxidase test and catalase test) were performed for further clarification. Subsequently, the other standard biochemical tests used for identification of bacteria were performed as described in Bergey’s Manual of Determinative Bacteriology (Holt et al. 1994). Identification was also performed simultaneously by using commercially available api 20E (BioMérieux 2009) rapid identification strips. Results of both these tests were recorded.

Biochemical tests

The common biochemical tests, generally performed on the isolated pure colonies on TSA plates, for identification of bacteria belonging to the family Enterobacteriaceae were conducted for bacterial identification. Those tests were TSI (triple sugar iron), urease, MR-VP (methyl red-Voges-Proskauer), citrate utilization, hydrogen sulphide production, indole production, ONPG (ortho-nitrophenyl-β-D-galactopyranoside) and the motility tests. However, when non-coliforms were identified (which gave an oxidase positive test), certain additional tests, such as the gelatin liquefaction test, pigment test, growth at different temperatures, etc., were performed. In addition to these tests, an additional Gram staining was performed for certain cultures, which gave anomalous results.

Identification using api 20E identification strips

Pure colonies (well separated) on TSA plates were selected and emulsified in 5 mL sterilized distilled water, in a sterilized tube, and mixed well using a vortex machine (Fisher FB 65000, UK) to obtain a homogenous suspension. Using a micropipette, this bacterial suspension was inoculated into 20 mini test tubes of the api 20E strip, following the manufacturer’s instructions; both the tube and the cupule were filled for the tests marked as CIT, VP and GEL. Only the tube was filled in the remaining tubes; anaerobiosis was created for the tests ADH, LDC, ODC, H2S and URE by overlaying with sterilized mineral oil. To create a humid atmosphere, 5 mL of sterilized distilled water was distributed in honey-combed wells on the tray. The incubation box was covered with the lid and incubated at 37°C for 18–24 hours.

After incubation the strip was read using the ‘reading table’ provided by BioMérieux, Inc., USA. Spontaneous reactions were recorded, and the TDA, VP and IND tests were performed by addition of TDA, VP 1 + VP 2 and IND reagents, respectively, and the results were recorded. On the results sheet, the tests were separated into groups of three sets and values summarized as instructed. By adding together the values corresponding to positive reactions within each group, a seven-digit profile number was obtained for the 20 tests of the api 20E strip.

After this, identification was performed using the database (V 4.0). The numerical profile was looked up in the Analytical Profile Index. The seven-digit numerical profile was entered manually via the keyboard into the identification software and submitted.

RESULTS AND DISCUSSION

The different water sources and their water usage percentage of some districts in Sri Lanka are depicted in Table 1. Mean bacteriological counts obtained for all water types
exceeded the WHO permissible levels for both total coliforms and faecal coliforms (Figure 1). It was shown that 18.5 and 14.8% of bottled water samples were contaminated with total coliform bacteria (counts ranging from $10^2$ to $10^3$ cfu/100 mL) and faecal coliforms (counts 0 to $10^2$ cfu/100 mL), respectively. This finding also agrees with previous studies by Abayasekara et al. (2010), Mannapperuma et al. (2014) and Chethana & Mannapperuma (2015) on bottled water quality in Sri Lanka. This study identified potentially pathogenic coliform bacteria Enterobacter sakazaki, Enterobacter cloacae and Enterobacter spp. (Table 2), while Herath et al. (2012) have reported identification of Klebsiella pneumoniae ssp. pneumoniae and Enterobacter cloacae. In addition, they also identified non-coliform species Pseudomonas aeruginosa and Pasteurella haemolytica in bottled waters used in Sri Lanka. Khaniki et al. (2010) have also suggested the possibility of contamination of bottled waters by pathogenic microorganisms when they are positive for coliform bacteria. Therefore, an improved surveillance system is required for the bottled water industry in Sri Lanka.

One hundred per cent of well water samples analysed in peri-urban and rural areas of the country exceeded the WHO permissible levels for total coliforms and faecal coliforms, compared to 89.5% in Kathmandu, Nepal (Prasai et al. 2010), indicating the frequent faecal contamination in the peri-urban and rural areas in Sri Lanka. Total coliform counts and faecal coliforms counts ranged from $10^2$ to $10^4$ cfu/100 mL in well water samples, producing the broadest spectrum of bacteria among all water sources including five faecal coliform species, three total coliform species and eight non-coliform species (Table 2). Identification of potentially pathogenic Enterobacteriaceae species, namely Klebsiella pneumoniae, Klebsiella pneumoniae ssp. pneumoniae, Klebsiella oxicota, Enterobacter sakazaki, Citrobacter braakii and Citrobacter freundii and the non-coliform spp. such as Pseudomonas (three spp.), Aeromonas (three spp.), Salmonella (three spp.) and Actinobacter sp. in drinking well waters (Table 2), suggested a considerable health risk for people using well water without any treatment. However, these findings could not be compared due to the lack of information on bacteriological identification in well waters in Sri Lanka. Some work done in Finland has identified Erwinia spp., Hafnia alvei, Rahnella aquatilis, Serratia fonticola, Serratia liquifaciens, Serratia enterolitica, Yersinia enterolitica and Yersinia spp. (Niemi et al. 2001) and Yersinia enterolitica, Escherichia coli and Clostridium

Table 1 | Different water sources and their use in different districts (Demographic and Health Survey 2007/2008)

<table>
<thead>
<tr>
<th>Province</th>
<th>District</th>
<th>Total population (million)</th>
<th>Tap water</th>
<th>Well water</th>
<th>Surface water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>Kandy</td>
<td>1.2</td>
<td>43</td>
<td>26.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Central</td>
<td>Nuwaraeliya</td>
<td>0.7</td>
<td>10</td>
<td>9.4</td>
<td>27.6</td>
</tr>
<tr>
<td>Sabaragamuwa</td>
<td>Rathnepura</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>North Central</td>
<td>Anuradhapura</td>
<td>0.9</td>
<td>20.1</td>
<td>62.5</td>
<td>10</td>
</tr>
<tr>
<td>North Western</td>
<td>Kurunegala</td>
<td>1.5</td>
<td>2.7</td>
<td>83.3</td>
<td>8.3</td>
</tr>
</tbody>
</table>

aData not available.
C. perfringens (Pitkänen et al. 2011) in Finnish well water samples. Another study conducted in Poland has identified coliform species *Citrobacter freundii*, *Erwinia herbicola*, *Escherichia coli*, *Proteus vulgaris* and *Serratia arcescens* and non-coliform *Pseudomonas aeruginosa* and *Aeromonas hydrophila* as contaminants in well water (Golas et al. 2002). Contamination found in the current study might be due to faecal contamination through contaminated subterranean water flow (due to toilet pits built relatively close to wells), heavy rainfall and the sloping terrain patterns (which can produce surface faecal contamination) and the higher abstraction rates. Although well waters are disinfected in most developed countries by chlorination (Niemi et al. 2004), it is not generally practised in developing countries including Sri Lanka. Therefore, at least boiling water before consumption would be the safest way of using well water for drinking purposes in Sri Lanka.

All surface water samples assessed in this study were heavily contaminated by total coliforms ranging from $10^2$ to $10^5$ cfu/100 mL and faecal coliforms ranging from 10 to $10^5$ cfu/100 mL, respectively (Figure 1). They contained five potentially pathogenic Enterobacteriaceae spp. in three genera, *Klebsiella*, *Escherichia* and *Citrobacter* and non-coliform spp., such as *Pseudomonas* (three spp.), *Aeromonas* (two spp.), *Acinetobacter* (two spp.) and *Salmonella choleraesuis* spp. *arizonae* (Table 2). Eleven isolates of the genus *Salmonella* have been identified in high quality surface waters in Colorado (Fair & Morrison 1967). Since 1.8% of surface water is consumed by the rural population in Sri Lanka (WHO/UNICEF 2010), the potential for waterborne disease outbreaks are higher.

### CONCLUSIONS

Most of the water sources analysed in the current study were contaminated with potentially pathogenic bacteria of faecal origin. Isolation of potentially pathogenic *Enterobacter* spp. in bottled waters is a serious issue, requiring an improved surveillance system for the bottled water industry in Sri Lanka. One hundred per cent of the well water was faecally contaminated, indicating the potential health risk of consuming well water without any treatment. Therefore, at least boiling water before consumption is recommended. Furthermore, adoption of proper disinfection programmes is urgent in preventing diseases which could result from pathogenic bacteria, especially in immunocompromised people. Faecal contamination of all the studied surface water bodies indicates contamination of water
intakes used for public water supply. Therefore, higher treatment efficiency should be maintained to meet the water quality standards but this requires costly treatment operations. On the other hand, use of contaminated surface waters without treatment can cause waterborne disease outbreaks. Since disposing of sewage pollutes both surface and ground water bodies, awareness and proper sewage management programmes should be introduced in the country. Furthermore, proper management and maintenance of both surface water bodies and water-sheds through government-mediated community participation could be recommended. Finally, regular monitoring and assessment of the bacteriological quality of all source water types and studies on the surveillance of pathogenic bacteria in both raw water and treated water are highly recommended.

REFERENCES


