**Pseudomonas aeruginosa** in bottled drinking water in Sri Lanka: a potential health hazard
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**ABSTRACT**

*Pseudomonas aeruginosa*, a food- and water-borne opportunistic pathogen, constitutes a health risk mostly to immunocompromised patients, and also affects the taste, odour and turbidity of potable water. In order to detect *P. aeruginosa* in bottled water in Sri Lanka, 36 bottled water brands were collected randomly from retail and supermarkets island wide. *P. aeruginosa* was detected by the membrane filtration technique, using cetrimide agar supplemented with nalidixic acid. The isolates were subjected to confirmatory tests, viz: ISO 16266:2006 methods and growth at 4 and 42°C, and API 20NE followed by DNA sequencing. Presumptive isolates of *P. aeruginosa* were observed on cetrimide agar in 50% of brands. Among these isolates, ISO procedures confirmed 58% as *P. aeruginosa*. Thirty-nine randomly selected isolates were identified as *P. aeruginosa* by the API 20NE, three of which were further confirmed by DNA sequencing. The presence of *P. aeruginosa* in bottled water raises health concerns since *P. aeruginosa* is an indicator of inferior water quality. Therefore, strict regulations and regular monitoring of bottling plants are recommended in order to supply safe and acceptable bottled drinking water to the Sri Lankan market.

**Key words** | bottled water, cetrimide agar, *Pseudomonas aeruginosa*

**INTRODUCTION**

Bacteria belonging to the genus *Pseudomonas* are widespread in the environment and often opportunistic pathogens for many episodes of infections. *Pseudomonas aeruginosa* is associated with food- and water-borne diseases and outbreaks of infection caused by *P. aeruginosa* are widespread (Clesceri *et al.* 1998; Srinivasan *et al.* 2003; Trautmann *et al.* 2005). *P. aeruginosa* infects the pulmonary tract, urinary tract, burns and wounds, and also causes other blood infections in immunocompromised individuals. The organism expresses virulence factors such as, exotoxins, a phagocytosis resistant slime layer, various enzymes and hemolysins that degrade host tissues (Vachee & Leclerc 1995).

This species can even grow in low-nutrient water (Moreira *et al.* 1994) and, therefore, can colonize bottled waters and also survive for long starvation periods (Legnani *et al.* 1999). The growth of *P. aeruginosa* during storage could lead to a risk for consumers, especially the immunologically weak, infants and the elderly (Legnani *et al.* 1999). Although examination of *P. aeruginosa* in drinking water is not recommended as a routine procedure according to WHO guidelines, it can be used as an indicator of the quality of drinking water (Gerba 2000; WHO 2006). According to drinking water criteria of the European Union, *P. aeruginosa* should be absent in 250 mL for bottled water (European Communities 2007). However, in Sri Lanka, although 2 cfu/250 mL of *P. aeruginosa* is permitted for natural mineral water, it is not included in bottled drinking water standards (SLS 894: Part 2: 2003 and SLS 1038: Part 2: 2003). *P. aeruginosa* has been found in some mineral waters in Brazil, Canada, France, Germany, Spain and the United States (Schindler *et al.* 1995).
The objective of the current study was to detect and identify *P. aeruginosa* in bottled drinking water samples sold in Sri Lanka.

**METHODS**

**Sample collection**

Thirty-six brands of bottled water (source: natural springs – 13 brands, wells – 12 brands, unknown source – 11 brands, five bottles of 500 mL per brand), within its shelf life, were randomly selected in the years 2010–2011 from retail and supermarkets covering the entire island of Sri Lanka, and subjected to the determination of *P. aeruginosa*.

**Microbiological analysis**

**Detection of *P. aeruginosa* in bottled drinking water samples**

Membrane filtration technique was used to detect *P. aeruginosa* in water samples. One hundred millilitres of each bottled water sample was passed through sterilized membrane filters (Sartorius, Germany, 0.45 μm) in a membrane filtration apparatus (Pyrex, Germany). Filters were incubated on cetrimide agar plates supplemented with 15 mg/L of nalidixic acid at 37 °C for 44 ± 2 h. Colonies that showed a bluish/greenish pigmentation or fluorescence under UV light (364 nm) were selected as presumptive *P. aeruginosa* isolates. Pure cultures of presumptive colonies made on nutrient agar plates were incubated at 37 °C for 22 ± 2 h (Casanovas-Massana *et al*. 2010). *P. aeruginosa* (ATCC 27853) was used as a reference control in all the assays performed in the study. Three bottles from each brand were analysed taking duplicate samples from each bottle, resulting in six replicates per brand. Stock cultures of all isolates were used for further identification.

**Confirmation of *P. aeruginosa* (ISO 16266:2006 procedure)**

Presumptive isolates were streaked on nutrient agar plates separately and the plates were incubated at 37 °C for 22 ± 2 h. Well isolated colonies were subjected to confirmation initially by the Gram’s test, oxidase test and the catalase test and, subsequently, relevant assays of ISO 16266:2006 were used for identification of bacteria as described below (Casanovas-Massana *et al*. 2010).

**Cetrimide agar**

Presumptive isolates were further subcultured on Cetrimide agar (Oxoid, UK) supplemented with 15 mg/L of nalidixic acid and incubated at 37 °C for 44 ± 2 h under humid conditions to prevent drying. After 24 ± 2 h of incubation, the isolates showing bluish/greenish pigmentation (pyocyanin production) were further observed following 44 ± 2 h of incubation. Isolates showing reddish brown pigmentation after 44 ± 2 h (pyorubin production) and colonies showing fluorescence under 364 nm UV light (pyoverdin production) were considered positive for the assay.

**Acetamide broth**

Presumptive isolates were inoculated into acetamide broth tubes (Hi-media, India) using a sterile inoculating loop, and the tubes were incubated at 37 °C for 22 ± 2 h. Subsequently, 1 or 2 drops of Nessler Reagent (BDH, UK) were added. Tubes showing a yellow to brick red colour, caused by ammonium production, were considered positive for the assay.

**King B agar**

Presumptive isolates were sub-cultured on King B plates (Fluka, Switzerland) at 37 °C for 5 days in humid containers to prevent drying. The plates were examined daily under 364 nm ultraviolet radiation. The presence of fluorescence (fluorescein production) during the 5 days of observation was considered a positive reaction.

**Growth at 4 and 42 °C**

Presumptive isolates were sub-cultured on nutrient agar plates in duplicate sets (Hi-media, India). One set was incubated at 42 °C and the other set at 4 °C for 5 days in humid containers to prevent drying. The growth of each isolate was observed after incubation.
Identification using API 20 NE identification strips

Forty-seven presumptive isolates were subjected to the API 20 NE system (Biomérieux, France) according to the manufacturer’s instructions, and incubated at 37°C under moist conditions for 24 h. The profiles obtained were identified with the APIWEB™ database.

Molecular characterization

Isolation, PCR amplification and DNA sequencing

DNA was extracted from bacterial cultures using the Promega Wizard Genomic DNA purification kit. Concentration and purity of extracted DNA samples were assessed by spectrophotometric analysis at 260 and 280 nm. The purified DNA was subsequently amplified with universal bacterial primers for Gram-negative bacteria (16S rRNA primers) (Case et al. 2007). Polymerase chain reaction (PCR) primers used for identification included the primer pair fD1 (forward: 5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (reverse: 3′-AAGGAGGTGATCCAGC-5′). The reaction was performed in a thermal cycler (Eppendorf AG Mastercycler gradient, Germany). PCR conditions consisted of 30 cycles of 94°C for 1 min, 50°C for 1.5 min and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The PCR product was sequenced by bidirectional direct sequencing at Asiri laboratory, Colombo, using 16S rRNA universal bacterial primers. The sequence was identified using NCBI nucleotide BLAST tool (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

P. aeruginosa, which is a common bacterium in tropical regions (Downes & Ito 2001), has the capacity to adapt to different hosts and environmental conditions. This ability to activate useful phenotypes under environmental stress has allowed the species to persist in adverse conditions, such as nutrient deprivation, oxygen tension and osmolarity. As bottled water has one or more of the above mentioned conditions, there is a possibility that these waters could harbour P. aeruginosa. The results of the current study provide further evidence of this possibility. Among the brands tested, 18 (50%) brands were confirmed to be contaminated with P. aeruginosa on cetrimide agar (Figure 1(a)), and the isolates were fluorescent when examined under 364 nm UV radiation (Figure 1(b)). Some brands had more than 100 cfu of P. aeruginosa in 100 mL of sample (Table 1).

One hundred and eighty-six isolates on cetrimide agar were considered to be presumptive P. aeruginosa colonies. Out of these isolates, 176 were Gram negative, catalase negative and oxidase positive. These 176 isolates were subjected to ISO 16266:2006 test, viz; production of pyocyanin (Figure 2(a)), pyoverdin (Figure 2(b)) or pyorubin (Figure 2(c)) on cetrimide agar, production of ammonium from acetamide (Figure 2(d)) and production of fluorescein on King B agar (Figures 2(e) and 2(f)) and growth on nutrient agar at 42°C and absence of growth on nutrient agar at 4°C. Out of the 176 isolates, 108 isolates were confirmed as P. aeruginosa. The API 20 NE system identified (in the range of 98 to 99.9% identification) 39 out of 47 isolates as P. aeruginosa.

DNA samples extracted from three isolated bacterial cultures confirmed as P. aeruginosa were amplified with 16S rRNA universal bacterial primers to identify...
*P. aeruginosa.* *Pseudomonas* spp. were detected by a 1,500 bp fragment (Figure 3). According to bidirectional direct sequence data, the DNA sequence was confirmed as *P. aeruginosa* using NCBI nucleotide BLAST tool [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/).

Obiri-Danso et al. (2003), Venieri et al. (2006) and Da Silva et al. (2008) reported contamination of bottled water with *P. aeruginosa* in different countries. Manaia et al. (1990) reported that 83% of carbonated bottled water samples tested were contaminated by *Pseudomonas* spp. in Portugal, while Momtaz et al. (2011) reported the detection of *P. aeruginosa* in 1.97% of 152 bottled mineral water samples investigated from Isfahan province, Iran.

The presence of *P. aeruginosa* in bottled water could be due to contamination during the bottling process or because the source water was initially polluted by organic material (Rosenberg 2005; Bartram et al. 2004). Moreover, it has been documented that if these microorganisms are not adequately removed during processing and bottling, bacterial multiplication may occur 1–3 weeks after bottling, and the bacterial count can reach $10^3$–$10^4$ bacteria mL$^{-1}$ at 37°C.

Tamagnini & Gonzalez (1997) reported that *P. aeruginosa* was isolated from commercial products bottled in returnable plastic containers due to the contamination from the
containers and the subsequent multiplication utilizing trace nutrients. Further, *P. aeruginosa* showed a high doubling time (26 h) due to competition from the accompanying microflora. In the absence of competing flora *P. aeruginosa* reached a higher density than the maximum reached by aerobic flora, with a doubling time of only 3.6 h. It is also reported by Tamagnini & Gonzalez (1997) that after 30 d storage, this micro-organism was predominant. The storage temperature of bottled water has also been demonstrated to affect the rate of multiplication and survival of microorganisms (Bischofberger et al. 1990).

*P. aeruginosa* is used as an indicator organism in water contamination (Gerba 2000) and it has been suggested as a surrogate indicator for the presence of other opportunistic pathogens (Geldreich 1992). In most countries, *P. aeruginosa* is also used as a parameter of bottled drinking water standards. According to the European Union bottled drinking water standards, *P. aeruginosa* should be absent in 250 mL for bottled water (European Communities 2007), while the standards in the United Kingdom (Barrell et al. 2000) and Canada (Health Canada 2001) and in the East African Standards (EAS 2009) indicate that *P. aeruginosa* should be absent in 100 mL of bottled water. In Greece *P. aeruginosa* is included as one of the unacceptable microbiological criteria in bottled water and is used as a process management indicator during production (Papapetropoulou 1998). However, in the Sri Lankan Standards, although *P. aeruginosa* is a criterion for natural mineral water standards, it is not included for bottled drinking water.

In a previous study done by the same authors on the quality of bottled water in Sri Lanka (Herath et al. 2012), it was observed that many brands tested exceeded the levels permitted by the Sri Lanka Standards Institution for total coliforms (<10 cfu per 100 mL) and fecal coliforms (FC) (0 cfu per 100 mL). In addition, in the current study, the API 20 NE system identified *Pseudomonas fluorescens*, *Aeromonas sobria*, *Aeromonas hydrophila* and *Burkholderia cepacia*. Armas & Sutherland (1999) and Venieri et al. (2006) reported the presence of *P. fluorescens*, *Aeromonas sp.*, *Pasteurella sp.*, *Citrobacter sp.*, *Flavobacterium sp.*, *Providencia sp.* and *Enterococcus sp.* in bottled water.

The high numbers of total and FC and other non-coliforms including *P. aeruginosa* detected in bottled water in Sri Lanka may be due to improper UV treatment, faulty UV systems, clogging of filters and cross-contamination or the use of unsuitable source water for bottling. In the Sri Lankan bottled water industry, it is recommended that UV radiation and filtration be employed during the bottling process. However, it was not possible to determine the efficiency of UV treatment, as bottle water manufacturers are very reluctant to grant permission for outsiders to visit their processing plants.

**CONCLUSIONS**

According to the results of the current study, *P. aeruginosa* was observed in 50% of the bottled water brands investigated, leading to health concerns, since *P. aeruginosa* is an indicator of inferior water quality. Use of suitable source water and regular monitoring of bottling plants are recommended in order to provide safe bottled drinking water to the Sri Lankan market.

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