Journal of Infection and Public Health (2016) 9, 362—365

http://www.elsevier.com/locate/jiph

SHORT REPORT

First microbiota assessments of children's paddling pool waters evaluated using 16S rRNA gene-based metagenome analysis

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Received 2 July 2015; received in revised form 29 September 2015; accepted 1 November 2015

KEYWORDS
Children's paddling pool;
Microbiota;
Contamination;
Metagenome;
Small subunit rRNA gene

Summary Insufficient chloric sterilization of children's paddling pool waters increases the risk of diarrheal illness. Therefore, we investigated the microbiota changes after children use pools. First, we applied 16S rRNA gene-based metagenome analysis to understand the dynamics of microbiota in pool water, especially with respect to the bio-contamination by potential pathogens. *Proteobacteria* were major taxa detected in every pool water sample after children spent time in the pool. In more detail, *Gammaproteobacteria* comprised the dominant class, which was followed by *Betaproteobacteria*. Five phyla, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Deinococcus-Thermus* phyla were minor groups. The pool water microbiota are likely to be a consortium of intestinal and skin microbiota from humans. Interestingly, the ratio of *Gammaproteobacteria* and *Betaproteobacteria* differed according to the age of the children who used the pool, which means the pool water was additionally contaminated by soil microbiota as a result of the children's behavior. Furthermore, potential pathogens, such as *Campylobacter* spp.,

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http://dx.doi.org/10.1016/j.jiph.2015.11.008

Comamonas testosteroni and *Burkholderia pseudomallei*, were also found. Considering the standard plate counts, the abundances of these human pathogens are unlikely to be a sufficiently infectious dose. We suggest the importance of sanitary measures in paddling pool waters to reduce bio-contamination from both humans and the environment.

Children's paddling pools serve as a focal point in the transmission of infection and have caused a verotoxin-producing *Escherichia coli* O157 outbreak [1]. The risk of spreading diarrheal illness is increased by the insufficient chloric sterilization of pools [2]. O157 outbreaks in nursery facilities in Japan have been increasing annually; there were seven cases in 2010, four cases in 2011, nine cases in 2012 and 23 cases in 2013 [3,4]. Therefore, the Ministry of Health, Labor and Welfare in Japan published ''Infection measure guideline in child day-care facilities, 2014'', recommending thorough chloric sterilization water quality management for children's pools including a density of free residual chlorine that ranges from 0.4 to 1.0 ppm. On the other hand, chlorine may increase the risk of respiratory organ disease in infants [5]. Additionally, small inflatable and plastic pools for infants are typically filled with tap water that lacks chloric sterilization. In this study, 16S rRNA gene-based metagenome analysis was performed to identify the dynamics of the microbiota in pool water.

Ten water samples (T1 to 7 and F1 to 3) were obtained from children's paddling pools (ca. 270 L) at two nursery facilities (the facility T and F) from July 15 to August 1, 2014. These pools were filled with tap water (chloride density supplied: approximately 1.0 ppm) that had a lower density than 1.0 ppm by volatilization of chlorine. The numbers of children in the pool ranged from four to 16, and the age ranged between zero to six years. In the facility T samples, children from zero to six years old were present in every sample. In the F facility, the pool was used group-by-group; the groups were divided according to the age of the children, e.g., F1, F2 and F3 consisted of one- to two-year-old children three- to five-year-old children and only two-year-old children, respectively.

Indicator bacteria were evaluated in each sample based on standard protocols. *E. coli* counts were estimated using a sheet medium ''Sanita-kun'' (JNC Co., Ltd, Japan). The standard plate counts (SPC), coliforms and *E. coli* were 9.8×10^2 to 4.6×10^3 CFU/mL, $2.0—1.5 \times 10^2$ CFU/mL (except T4) and $0—9.4 \times 10^2$ CFU/mL, respectively. The counts after the children spent time in the pools were beyond the criteria for public health in Japan, including SPC <200 CFU/mL and *E. coli* non-detectable. In previous studies, there was no correlation between the number of bacteria and number of users observed [6,7]. The indicator bacteria were below the undetectable limit in the tap water used in the children's paddling pools.

Five liters of water samples were filtered using a Sterivex filter (Merck Millipore Co.) together with pressure vessels DV-5 (Advantec, Tokyo, Japan) and filter-sterilized nitrogen gas. The microbes that were collected by the filter were treated with lysostaphin (L7386, Sigma—Aldrich) and lysozyme (L7651, Sigma—Aldrich) at 37 °C overnight; then, the bacterial genomic DNA was extracted from the lysate using the Wizard genomic DNA purification system (Promega, Madison, WI). Genomic DNA was used for 16S rRNA gene-based metagenome analysis after measuring the concentration with the Quantus™ and QuantiFluor™ ONE dsDNA System (Promega). Sequencing of the V1-V2 region of the 16S rRNA gene was performed using 454 GS Junior (Roche Applied Science), and 3000 reads per sample were used for the bacterial community comparison with a previously reported method [8]. The sequence data for this study are deposited in DDBJ/GenBank/EMBL under PRJDB4242.

The metagenome analyses revealed three interesting points. First, five phyla (*Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Deinococcus-Thermus*) were detected in the pool water microbiota after the children had been in the pool, accounting for 99.8—100% of the sample (Fig. 1A). *Proteobacteria* was the major phylum in the pool water microbiota, ranging from 76.2 to 100%. In more detail, *Gammaproteobacteria* ranged from 50% to 97%, which was followed by *Betaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria*. The phyla, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*, were minor groups that occurred