Seasonal variation of Legionella in Taiwan's reservoir and its relationships with environmental factors

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Abstract In this study, the presence of Legionella in major water reservoirs of Taiwan was examined with respect to seasonal variation, geographical variation, and water quality parameters using TaqMan real-time qPCR. Water samples were collected quarterly at 19 reservoirs in Taiwan between November 2012 and August 2013. The detection rate for Legionella was 35.5% (277/776), and Legionella was detected in all seasons. The Legionella concentration was relatively high in spring and summer, reaching 3.86×10^3 and 7.35×10^3 cells/L, respectively. By sampling the area, Legionella was detected at a higher proportion in reservoirs in the northern and southern areas, and the difference was consistent in all seasons. Significant association was found between detection of Legionella and various water quality parameters, including conductivity, chlorophyll a, and dissolved oxygen (Mann-Whitney U test, P<0.05). Results of Spearman rank test showed negative correlation for Legionella detection with pH (P=0.030, R=-0.497) and dissolved oxygen (P=0.007, R=-0.596) in full and positive correlation with Carlson’s trophic state index (P=0.049, R=0.457) in spring. The identified species included Legionella pneumophila and Legionella d Joncoursii. The detection of Legionella in reservoirs was indicative of a public health risk and should be further evaluated.

Keywords Legionella • Reservoir • Real-time PCR • Physicochemical characteristics • Species identification • Season

Introduction

The World Health Organization (WHO) estimated that 80% of human illnesses were attributed to waterborne diseases (WHO 1996). Waterborne diseases include a large number of pathogens that may be spread through contaminated water. Legionella is a Gram-negative, aerobic rod-shaped bacterium belonging to the gamma subgroup of Proteobacteria and non-spore-forming genus. Legionella pneumophila and related Legionella species are known to cause Legionnaires’ disease (also known as legionellosis or Legion Fever) and Pontiac fever. Over 90% of Legionnaires’ disease cases are caused by L. pneumophila (Gonzalez-Valero et al. 2009). Legionella spp. can be found in a wide range of aquatic environments, including lakes, groundwater, rivers, hot springs, spa, whirlpool baths, cooling towers, etc., as symbionts of protozoa (WHO 2007; Gonzalez-Valero et al. 2009). In different climatic regions (temperate, tropical, and subtropical), the quality of drinking water from reservoirs may be affected by seasonal changes in environmental conditions
Materials and methods

Sample collection areas and water quality parameters

A total number of 76 samples were collected from 19 reservoirs. The sampling campaign was carried out between November 2012 and August 2013 (four times in a year) at 19 water reservoirs in Taiwan. The sampling locations are presented in Fig. 1. At each reservoir, a 1-L water sample was collected in a sterile polypropylene bottle from within 50 cm from the water surface. The collected samples were transported to the laboratory for analyses within 24 h. At the time of sample collection, several physicochemical water quality parameters were also assessed on site, including pH, water temperature, conductivity, chlorophyll a (Chl-a), and dissolved oxygen (DO) using a portable multi-parameter water quality meter (HI9828, Hanna Instruments Inc., USA). Turbidity was measured using a ratio turbidimeter (Watershed Portable TN160, Eutech Instruments Pte Ltd., Singapore). Several other water quality parameters were also assessed for each sample, including total suspended solids (TSS) by Method 2540 C (APHA 2005), total phosphorus (TP), and NH₄-N using analytical kits (Spectroquant®, 114752 for TP and 106753 for NH₄-N, Merck Millipore, Germany). Further, Carlson's trophic state index (TSI) was evaluated for trophic condition at each reservoir (Carlson 1977). Additionally, a 300-ml water sample was taken in sampling bags (Nasco Whirl-Pak, USA) for microbiological water quality parameters. Total coliforms were measured following the standard method (methods 9222 B) (APHA 2005); heterotrophic bacteria were cultured on M-HPC heterotrophic plate count agar base and measured by spread plate method (Methods 9215C) (APHA 2005).

Sample pretreatment and DNA extraction

Each 1 L sample was concentrated by membrane filtration method with GN-6 Minicel®-mixed cellulose ester membrane disc filters (pore size 0.45 μm, diameter 45 mm, Pall, USA). After filtration, the membranes were scraped, and the collected material was washed with 100 ml eluting fluid consisting of phosphate-buffered saline (PBS; 7.5 mM Na₂HPO₄, 3.3 mM NaH₂PO₄, 0.8 mM NaCl, pH 7.2). The resulting solution was then transferred into two 50-ml centrifuge tubes and centrifuged at 2600×g for 30 min (KUBOTA-Model 2420 Compact Tabletop Centrifuge, Japan). Subsequently, the top 45 ml supernatant fluid was removed, and the remaining 5 ml pellet was resuspended with PBS at 4 °C for DNA extraction.

Total DNA extraction was done with the concentrated pellet (1 ml) using MagPurix Bacterial DNA Extraction Kit ZPD2006 and automated DNA extraction by MagPurix 12 s Automated Nucleic Acid Purification System (Zinser Life Science Corp., Taiwan) according to manufacturer’s specifications. The resulting solution (with final volume at 100 μl) was analyzed for Legionella-specific genes with PCR and real-time qPCR.

PCR molecular identification of Legionella

For genetic typing of Legionella, 16S rRNA gene was amplified with primers LEG 225 (5'-AAGATTAGCGCTGCACCG AF-3') and LEG 858 (5'-GTCAGCATATTGCGCCTTCGCT-3'). The PCR solution was prepared with 3 μl of the DNA templates and PCR mixture to a total volume of 25 μl. The PCR mixture included 2.5 μl 10× PCR buffer (20 mM MgCl₂), 0.5 μl 40 mM dNTPs, 0.5 μl each of the primer (10 μl), 0.2 μl Taq DNA Polymersase (Vigene, 5 U/μl), and DNase-free deionized water (MIYAMOTO et al. 1997). The PCR products (654 bp) of Legionella were identified by gel electrophoresis on a 2 % agarose gel (Bio Basic Inc., Canada) with 5 μl of the reaction solution. The DNA fragments were
confirmed using ethidium bromide staining and visualized under UV light. PCR products were directly analyzed through DNA sequencing for species identification and for qPCR for quantification (only for positive samples through molecular technique).

Real-time qPCR detection of *Legionella*

Real-time qPCR assay was performed to quantify *Legionella* concentrations with 23S-5S rRNA gene sequence using primers sets LegF (5'-CTAATTGCTGTTGTCTTGAC-3') and LegR (5'-GGCGATGACTACTTCGG-3'). The TaqMan probe used in this study was the Leg Probe (5'-VIC-CGAACTCAGAAGTGAAAC-3') (Heipers et al. 2003). The real-time qPCR assay was performed with ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Singapore) with a total reaction volume of 20 μL. Each reaction mix contained 3.5 μL sterile PCR grade deionized water, 0.5 μL each of the primer, 0.5 μL TaqMan probe, 10 μL Probes mix, and 5 μL template DNA. For each assay, the threshold cycle (Ct) value, defined as the real-time qPCR cycle at which the fluorescence signal exceeded the background threshold, was determined to quantify each DNA product. Negative DNA controls (template DNA replaced by double distilled water) and positive control DNA (*L. pneumophila* ATCC 43661) were included with each qPCR run. A Taqman® Exogenous Internal Positive Control (IPC, Applied Biosystems) was co-amplified in each qPCR reaction with target DNA according to manufacturer’s instruction.

**Copy number standard curve of the 23S-5S rRNA gene in *Legionella***

A standard curve was constructed to determine the *Legionella* 23S-5S rRNA gene copy number with yT&A clone vector kit (Yeastern Biotech Corporation, Taipei, Taiwan). Recombinant plasmid DNA was purified in triplicates using a HiYield™ plasmid mini kit (Real Biotech Corporation, Taiwan). Following purification, the plasmid DNA concentration was determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, USA). The number of construct copies in the plasmid solution was calculated based on plasmid and insert sizes. A plasmid-based standard curve was generated with serial tenfold dilutions of plasmid solutions containing the target 23S-5S rRNA gene sequence. The standard curve, with a concentration of 2.7 × 10⁹ copies/μL for the dilution with the highest copy number, was used for determining the copy number of the 23S-5S rRNA gene in *Legionella*. 
Statistical evaluation

The Mann-Whitney U and Spearman rank tests were used to compare association between physicochemical water quality parameters and Legionella. The statistical software used was STATISTICA® version 6.0 (StatSoft, Inc., USA).

Results and discussion

Detection and quantification of Legionella spp. in reservoirs

A total of 76 water samples were collected from 19 reservoirs, with one sample from each season at each reservoir. As shown in Table 1, Legionella spp. were detected in 27 (35.5%) of the 76 samples. The 27 positive samples were from 15 reservoirs. The global detection rates for Legionella spp. in different aquatic environments ranged between 8.7 and 69.6% (Shen et al. 2005; Hou et al. 2009; Qin et al. 2012; Wang et al. 2012; Garcia et al. 2013). The detection rates showed that Legionella spp. may be detected in all seasons in Taiwan’s reservoirs. The detection rate for Legionella spp. was highest among samples collected in fall. Seasonal rainfall may have some effect on the detection rate for Legionella (Tung et al. 2013). The number of reservoirs with Legionella spp. was higher in fall, but the concentration of Legionella was highest in spring (Table 1) when the seasonal share of rainfall increased with low water level registered in reservoirs during the years 2012 and 2013. Statistics from the past few years showed that cases of legionellosis in Taiwan and USA occurred mostly in summers and falls (Neil and Berkelman 2008; CDC 2011; Taiwan Centers for Disease Control 2014). The elevated legionellosis risk may be a result of seasonally higher Legionella levels.

The occurrence of Legionella spp. in reservoirs was further compared by geographic region and sampling season. As shown in Fig. 2, the sampling sites were grouped into northern (seven sites) and central and southern regions (six sites each). Legionella spp. were found at higher proportion in fall and spring (57.1%, 4/7) in northern reservoirs. Legionella was not detected at all among central reservoirs in spring and was more prevalent in winter. The weather in the southern region is relatively warm year-round, and the Legionella detection rate was relatively stable across all seasons. The weather patterns differed by region and season and may likely affect the presence of Legionella in different water reservoirs (Garcia et al. 2013).

The results of TaqMan real-time qPCR analyses for Legionella-positive water samples are also summarized in Table 1. Lower Legionella spp. concentrations were found in fall, which ranged from 160 to 2.6×10³ and averaged 1.2×10³ cells/L. In Taiwan, rainfall mainly occurs in the summer with frequent thunderstorms and occasional typhoons. The seasonal variations in rainfall seemed to be unrelated to Legionella spp. concentrations. Nonetheless, the highly variable numbers found in some reservoirs suggested potential sources of contamination in the water supply systems. Further evaluation on water treatment plants may be needed, especially with respect to potential health threats from water containing L. pneumophila.

Identification of Legionella spp.

The Legionella-positive samples (n=28) detected by PCR were subjected to DNA sequencing for species identification. The DNA sequences of sample strains were compared with Legionella reference strains from the NCBI GenBank to determine the likelihood of specific strains. As shown in Table 1, the dominant Legionella species differed by season and sampling location. In this study, three Legionella species were identified. The most commonly identified species was uncultured Legionella sp. (AB455091, AY924069, AY924070, AY924135, AY924146, FJ480933, HE616182, HQ121251, JN318005, n=20), followed by Legionella sp. (JN983403, n=3), and Legionella duncanii (NR026335, n=1). The results showed that Legionella is widespread in Taiwan’s reservoirs, and uncultured Legionella sp. is most frequent among the studied reservoirs. L. pneumophila is the most pathogenic Legionella species, which attributed to approximately 80-90% of all reported legionellosis cases worldwide (Yanaz et al. 2005; Gomez-Valero et al. 2009). In this study, L. pneumophila was found only in fall, which was in agreement with the fact that cases of legionellosis in Taiwan and USA occurred primarily in fall and summer (Neil and Berkelman 2008; CDC 2011; Taiwan Centers for Disease Control 2014). The results provided some evidence that reservoirs in Taiwan may be a potential source of Legionella contamination in potable water supply systems. The raw water must undergo treatment before use and Legionella is sensitive to disinfection. However, it is possible that the presence of protozoan hosts may protect parasitic Legionella from disinfections (Gomi et al. 2014). In this event, they can get to the water distribution system and present a risk of aerosolized and inhaled. Various studies have also demonstrated the ability of free-living amoebae to protect Legionella from biocides and disinfectants (Barker et al. 1992; Gomi et al. 2014). Therefore, it is necessary to ensure the treatment efficiency of potable water supply plants in the expected requirements when considering Legionella infection risk. In addition, long-term monitoring on the presence of Legionella may be necessary to determine the severity of Legionella infestation in reservoirs in Taiwan.
Table 2: Average and range of water quality parameters at the reservoirs during four seasons

<table>
<thead>
<tr>
<th>Water quality parameters</th>
<th>Fall</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC (CFU/mL)</td>
<td>4450 (20-27,500)</td>
<td>4516.7 (5-14,150)</td>
<td>9315.4 (650-60,500)</td>
<td>429,2 (5-11,955)</td>
</tr>
<tr>
<td>Total coliforms (CFU/100 mL)</td>
<td>1992.3 (0-15,500)</td>
<td>18.2 (0-206)</td>
<td>392 (0-3100)</td>
<td>256.3 (0-2120)</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>5.5 (1-25)</td>
<td>7.1 (4-9.45)</td>
<td>5 (2-11)</td>
<td>9.2 (2-40)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>25.6 (22.6-28.1)</td>
<td>20.1 (17-23.7)</td>
<td>25.3 (21.4-29.5)</td>
<td>29.2 (24-37.4)</td>
</tr>
<tr>
<td>pH</td>
<td>8.2 (7.6-8.4)</td>
<td>8.3 (7.8-8.5)</td>
<td>8.4 (7.7-8.1)</td>
<td>8.5 (7.9-8.4)</td>
</tr>
<tr>
<td>CTSI</td>
<td>443 (23.2-80.8)</td>
<td>46 (34-78.6)</td>
<td>49.7 (41.6-75.9)</td>
<td>50.2 (42.7-74.1)</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>345.7 (136-590)</td>
<td>380 (171-651)</td>
<td>375.3 (172.5-595)</td>
<td>327.5 (136-461)</td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>7.5 (4.2-8.7)</td>
<td>8.7 (2.5-12.2)</td>
<td>8.5 (3.8-16.6)</td>
<td>8.6 (7.2-11.9)</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>6.3 (1.3-28.5)</td>
<td>7.4 (1-21)</td>
<td>5.8 (2.3-14.8)</td>
<td>10.6 (3.4-49.2)</td>
</tr>
<tr>
<td>Chl-a (μg/L)</td>
<td>10 (3-36.9)</td>
<td>3.2 (1.6-11.6)</td>
<td>5.5 (3-29.2)</td>
<td>10.3 (1.8-59.5)</td>
</tr>
<tr>
<td>TP (μg/L)</td>
<td>0.038 (0.002-0.372)</td>
<td>0.094 (0.006-0.994)</td>
<td>0.015 (0.002-0.027)</td>
<td>0.033 (0.008-0.429)</td>
</tr>
<tr>
<td>Nlt-N (mg/L)</td>
<td>0.18 (0-1.025)</td>
<td>0.47 (0.01-5.41)</td>
<td>0.1 (0.01-5.5)</td>
<td>0.11 (0.01-5.2)</td>
</tr>
</tbody>
</table>

Note: HPC = heterotrophic plate count, CTSI = Carbon's trophic state index, DO = dissolved oxygen, TSS = total suspended solids, Chl-a = concentration of chlorophyll a, TP = total phosphorus

Table 3: Nonparametric test results for differences and correlations for Legionella spp. in terms of water quality parameters

<table>
<thead>
<tr>
<th>Water quality parameters</th>
<th>Fall</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC (CFU/mL)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>Total coliforms (CFU/100 mL)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>pH</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>CTSI</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>Chl-a (μg/L)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
<tr>
<td>TP (μg/L)</td>
<td>M-W U test</td>
<td>Spearman test</td>
<td>M-W U test</td>
<td>Spearman test</td>
</tr>
</tbody>
</table>

Note: M-W U test = Mann-Whitney U test, Spearman test = Spearman rank test, HPC = heterotrophic plate count, CTSI = Carbon's trophic state index, DO = dissolved oxygen, TSS = total suspended solids, Chl-a = concentration of chlorophyll a, TP = total phosphorus
pH value ($R^2=0.497$) and DO ($R^2=0.296$) in fall and with CTSI ($R^2=0.463$) in spring. The results also supported previous reports of correlation between DO, pH value, and Legionella spp. in the aquatic environment (Ortiz-Roque and Hazen 1987; Wang et al. 2012).

As shown in Table 3, it was evident that DO was an influential environmental factor for the detection of Legionella spp. in fall. The significant correlation between CTSI and detection of Legionella spp. in the aquatic environment has not been mentioned previously. Issues in reservoir water quality usually involve the occurrence and severity of eutrophication. In Taiwan, reservoir water quality may be affected by seasonal rainfall and typhoons. The correlation between CTSI and Legionella spp. in reservoirs in spring may be due to seasonal and nutrient factors. The results also suggested that seasonal characteristics may have more effects on the presence and concentration of Legionella spp. than physical, chemical, and microbiological water quality parameters in reservoirs.

Conclusions
In this study, seasonal variations in the presence, species, and concentration of Legionella spp. in 19 reservoirs were examined. The overall detection rate for Legionella spp. was 35.5%. The detection rate was higher in fall, but concentrations were higher in spring. By sampling location, Legionella spp. was detected at higher rates in the northern and southern reservoirs in all seasons. *L. pneumophila* was found in all, and the finding was consistent with seasonal variations in occurrence of legionellosis in Taiwan. Finally, several water quality factors (conductivity, DO, pH, Chl-a, and CTSI) were correlated with concentration of Legionella spp. Further studies are needed also to identify the presence of Legionella in water treatment plants and in the systems of water distribution and artificial systems and compare them with reservoirs to see its relationship.

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