Isolation of *Campylobacter* sp in surface waters of Taiwan

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**Keywords**
Antibiotics-resistant pathogens; *Campylobacter* sp; Selective enrichment medium

**Background:** *Campylobacter* sp is one of the main causes of human acute enteritis and diarrhea. It is commonly found in bird intestinal canal and pollutes water bodies through bird droppings. Clinically, detection of *Campylobacter* sp is by culture method and most are fecal samples. The objective of this study is to investigate the distribution of *Campylobacter* sp in river water of Taiwan.

**Methods:** The detection of campylobacters in water by the United Kingdom Health Protection Agency was adopted and simplified. Water samples (1 L) were enriched through membrane filters and selective enrichment Bolton broth. The mCCDA medium was used for culturing *Campylobacter* sp. In addition to using the selective media, DNA sequencing for species' identification was also included in this study.

**Results:** *Campylobacter* sp was detected in only 2 of 75 water samples, and 72 suspected strains were isolated from the selective mCCDA medium, in which several pathogenic bacteria included *Escherichia coli* O157.

**Conclusions:** Not only there was pollution of *Campylobacter* sp in water environment but also other antibiotics-resistant pathogenic bacteria, which may cause public health risk.

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**Introduction**

In recent years, *Campylobacter* sp has been proven to be one of the main causes of human acute enteritis and diarrhea.1,2 In Europe and the United States, the impact of *Campylobacter* sp on public health is more serious than that of *Salmonella* sp.3 The infection is not only through food but also through contaminated or unsterilized drinking water.3,4
Campylobacters are slim spirally curved bacilli, with a width of 0.2–0.8 μm and a length of 0.5–5.0 μm. The range of thermophilic growth temperature is 34–44 °C, and it can only grow in microaerobic conditions (O2: 8–9%, CO2: 7–8%). Its infectious dose is 100–500 organisms. It leads to campylobacteriosis. The main symptoms are diarrhea, abdominal pain, vomiting, muscle weakness, and fever, which usually last for 5–8 days. In Taiwan, Campylobacter sp was isolated by culture method from 6,540 clinical samples of diarrhea; the detection frequency was about 2.5%. The above-mentioned cases did not indicate whether they were infected by drinking water or other vectors.

Campylobacter sp is prevalent in the intestinal tract of birds. Many rivers, lakes, and other water bodies in Taiwan are bird habitats; thus, wastes are discharged into the water, leading to an increase of water safety problem. This study adopted and simplified the culture method of Health Protection Agency (HPA) of United Kingdom to detect Campylobacter sp in the water to investigate the distribution of campylobacters in water environment of Taiwan. The water samples were mainly from rivers and water treatment plants.

**Materials and methods**

**Water samples**

Water samples were collected from rivers, including Dahan River, Touchian River, Dajia River, and Gaoping River, which are water sources for drinking water, as well as Han river and Fatze river that flow through Taichung City. Water samples were also collected from source water and sedimentation tank of Fonyuan and Liyutan water treatment plants. In addition, the farmland irrigation water of central Taiwan, Nangan irrigational canal, and Babao irrigation canal were also examined (Table 1). The investigation period was from July 2007 to June 2008, some sampling locations were sampled monthly, and some were sampled seasonally from winter of 2007 to summer of 2008. Han River and Fatze River were randomly sampled twice.

**Detection methods**

The isolation of Campylobacter sp was based on standard detection method of HPA, which was announced in October 24, 2007—Detection of Campylobacter sp in water (Reference no: W8i3). Before that, HPA announced that the detection of Campylobacter sp by membrane filtration (Reference no: W8i2.1) was a method under review. Samples collected before October 2007 (including October) used the method based on W8i2.1 and followed by the method based on W8i3. Both W8i2.1 and W8i3 use the same medium; the main difference is the enrichment conditions. W8i2.1 is enriched at 37 °C and 41.5 °C, respectively, for 22 hours and then is microaerobically cultured in mCCDA medium at 37 °C for 48 hours. W8i3 is enriched at 37 °C for 48 hours and then is microaerobically cultured in mCCDA medium at 41.5 °C for 48 hours. The Bolton broth (BB) enrichment medium contains four antibiotics, that is, cefoperazone, vancomycin, trimethoprim, and cycloheximide, they are mainly to inhibit the growth of gram-negative bacilli other than non-Campylobacter and gram-positive bacilli and the growth of fungi. In mCCDA medium, there are two antibiotics: cefoperazone and amphotericin B, the latter’s role is the same as that of cycloheximide in BB. mCCDA and BB are purchased from Oxoid.

This study simplified W8i2.1 method by omitting the Preston broth step, enriching the water samples directly in BB at 37 °C and 41.5 °C, respectively, for 22 hours. W8i3 method divides water samples into clean and dirty waters. After filtering the clean water, the membrane is enriched in BB at 37 °C for 44 hours, or 10 mL of clean water sample is directly added into 90 mL of BB, enriched at 41.5 °C for 44 hours. In the case of dirty water, 10 mL of dirty water sample without filtration is added into 90 mL of Preston broth and enriched respectively at 37 °C and 41.5 °C for 22 hours.

In this study, water samples were all filtered, and the membrane was enriched in BB at 37 °C for 44 hours. Not only the physiological and biochemical characteristics of suspected isolates were analyzed but DNA of purified isolates was also identified in this study.

Colonies on mCCDA was isolated based on colony morphology, and physiological and biochemical tests were analyzed. Oxidase, catalase, and aerobic growth were performed.

**Identification of isolate—DNA sequencing**

DNA of purified isolates was extracted with QIAamp DNA Mini kit (Qiagen, Germany), extraction steps are in accordance with the manufacturers’ manual. Extracted DNA was stored at −20 °C until use. DNA was amplified with universal primers of 11f, 5′-GGT TGA TCC TGG CTC AG-3′ and 1492r, 5′-TAC CTT GTT ACG ACT T-3′, using iCycler™ Thermal Cycler (BIO

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**Table 1** Symbols and sampling location for water samples

<table>
<thead>
<tr>
<th>Symbols</th>
<th>Sampling location</th>
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<tbody>
<tr>
<td>Fs</td>
<td>Source water, Fonyuan water treatment plant</td>
</tr>
<tr>
<td>Fd</td>
<td>Sedimentation tank, Fonyuan water treatment plant</td>
</tr>
<tr>
<td>Ls</td>
<td>Source water, Liyutan water treatment plant</td>
</tr>
<tr>
<td>Ld</td>
<td>Sedimentation tank, Liyutan water treatment plant</td>
</tr>
<tr>
<td>C</td>
<td>Dajia River</td>
</tr>
<tr>
<td>N</td>
<td>Nangan irrigation canal</td>
</tr>
<tr>
<td>S</td>
<td>Shigang Dam</td>
</tr>
<tr>
<td>P</td>
<td>Babao irrigation canal</td>
</tr>
<tr>
<td>Fa</td>
<td>Fatze River</td>
</tr>
<tr>
<td>Ha</td>
<td>Han River</td>
</tr>
<tr>
<td>B</td>
<td>Dahan River</td>
</tr>
<tr>
<td>K</td>
<td>Gaoping River</td>
</tr>
<tr>
<td>SL</td>
<td>Touchian River</td>
</tr>
</tbody>
</table>
Detection of campylobacters in water

To improve the Campylobacter sp detection efficiency in water sample, detection method of W813, in which, concentration of water sample with membrane filtration and selective enrichment medium (mCCDA) were used. Figure 1 shows the effect of enrichment on isolation. Under the same dilution rate, the background colonies on medium without enrichment was higher than that with enrichment (Fig. 1A), and the latter had few isolated colonies (Fig. 1B). It indicated that enrichment is more conducive to the detection of campylobacters in water.

Detection of Campylobacter sp in water samples

From July 2007 to June 2008, Campylobacter sp was monitored or detected by culture method. A total of 75 water samples were collected. The characteristics of water samples and number of isolates are shown in Table 2. Water samples collected from the water treatment plants were monitored monthly from October 2007 to June 2008; during the monitoring period, the changes in water temperature were between 16.3–27.1°C; turbidity was 1.05–242 NTU (data not shown). Two suspected strains of Campylobacter were isolated from source water of Fs and Ls water treatment plants. The level of turbidity had no direct relationship with the number of suspected Campylobacter isolates. There were no bacteria cultivated in sedimentation tank of the two plants. Monitoring period included fall, winter, spring, and summer, the average water temperature of each season was 23.5°C, 19.5°C, 19.5°C, and 24.0°C, respectively. The relationship between the number of suspected Campylobacter sp isolates and water temperature was not clear either.

Because the sampling locations of those water samples collected from rivers distributed widely, north to the Dahan River, south to Gaoping, and across the four seasons, the water temperature was between 34.0°C and 16.4°C. Among which, in Fatze River and Han River, spring and summer water temperatures were higher than 24°C, the number of isolates was no more than two in these two seasons. For C, N, S, and P sampling time was mainly in winter and spring, the water temperature was less than 20°C. In addition to the two isolates at C (in December), there was only one isolate. B, SL, and K were monitored respectively in winter, spring, and summer. In winter, K located in southern Taiwan had a water temperature of 21.8°C, which was higher than that of B and SL in northern Taiwan, which were 18.4°C and 19.6°C, respectively, but there was only one suspected strain isolated from each of the three locations. The average water temperature of spring and summer for B and SL were 31.1°C and 29.4°C, respectively, higher than that of the K in the south (25.2°C). There was no suspected strain isolated from B in summer (June), but one to three suspected isolates was isolated from B, SL, and K.

A total of 72 suspected Campylobacter sp colonies were isolated from the 75 water samples. They were identified according to the tests of catalase, oxidase, and aerobic growth (Table 3). Campylobacter sp. was detected in source water (Fs) and river waters (C) but not in other locations. All of Campylobacter had positive reaction in oxidase test except C gracilis, and generally all Campylobacter could not be cultured aerobically.7,16 The biochemical characteristics of isolates from Fs and C were consistent with Campylobacter sp.; they were also identified as C jejuni through DNA sequencing. Many non-Campylobacter strains were also isolated from mCCDA, such as E coli, Proteus mirabilis, Aeromonas hydrophila, E coli O157, Ochrobactrum sp, Pseudomonas putida, Acinetobacter sp, Shigella sonnei,
The campylobacters isolated is mainly determined by colony morphology, which is changeable on mCCDA, including flat white glossy colony, transparent droplets of fluid, low and convex with a dull surface colony, or spreading film. The colony morphology may be similar to other antibiotics-resistant isolates, so it is prone to errors in the identification and results in false negative results.

### Isolation frequency of Campylobacter sp and other antibiotics-resistant bacteria

A total of 72 suspected Campylobacter colonies were isolated from mCCDA medium of which six were without further identification, but they were non-Campylobacter sp through physiological and biochemical tests. After sequencing for the remaining 66, there was one C jejuni, one C jejuni subsp jejuni, which showed that the isolation frequency of Campylobacter sp. by culture method was only 2.8%. Eleven species of antibiotics-resistant bacteria were also isolated (Table 4), including: E coli (30.6%), E coli O157 (13.8%), Arcobacter sp (8.3%), A hydrophila (1.4%), Acinetobacter sp (4.2%), K pneumoniae subsp pneumoniae (5.5%), Ochrobacterum sp (2.7%), P mirabilis (1.4%), S sonnei (4.2%), S boydii (9.7%), S maltophilia (1.4%), and Enterobacter sp (6.9%). Among them, except E coli, Ochrobacterum sp, P mirabilis, and Enterobacter sp belong to nonpathogenic bacteria, others were all human pathogens. Dolejska et al.\(^\text{24}\) in 2007 isolated 257 E coli from cloacal smears; after the detection of antibiotics resistance, there were 30% of the E coli samples resisting one or more of the antibiotics, including ampicillin (12%), cefalothin (4.3%), tetracycline (12%), sulphonamides (7.8%), and chloramphenicol (1.9%). In water samples of this study, those antibiotic resistant strains isolated were resistant to four kinds of antibiotics; among them, E coli O157 was worth noting. E coli O157 can cause enterohemorrhagic E coli, which is the second legal infectious disease announced by the Center for Disease Control (CDC) in Taiwan.\(^\text{23}\) According to CDC data, in 2006, there were 109 cases
resulting from eating fresh spinach contaminated by \textit{E coli} O157 in the United States and even causing death.\textsuperscript{22} Based on this survey, \textit{E coli} O157 also presents in the surface waters of Taiwan, its impact on public health should be concerned.

After enrichment, the culture method is more conducive to the detection of \textit{Campylobacter} sp in water. A total of 72 suspected \textit{Campylobacter} strains were isolated according to colony morphology.

After DNA extraction and sequencing, only two were \textit{Campylobacter} sp, which were respectively from the Dajia River and source water from Fonyuan water treatment plant, indicating that these two water bodies might be contaminated by the campylobacters. Other isolates belonged to 11 species, all of which could resist to cefoperazone, vancomycin, trimethoprim, and cycloheximide. They were pathogens, such as \textit{E coli} O157, \textit{Arcobacter} sp, \textit{A hydrophila}, \textit{Acinetobacter} sp, \textit{K pneumoniae} subsp \textit{pneumoniae}, \textit{S sonnei}, \textit{S boydii}, and \textit{S maltophilia}, respectively; it suggested that the antibiotics-resistant bacteria were widespread in surface water of Taiwan.

Because most of \textit{Campylobacter} sp in environmental water bodies were dead or in a viable but nonculturable state, it resulted in a low isolation frequency (2.8%). \textit{Campylobacter} were detected in Dajia River and source water from Fonyuan water treatment plant, indicating that there were live campylobacters. Although its detection was through the enrichment process, the water quality of water treatment plant was still affected.

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**References**


