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A survey on levels and seasonal changes of assimilable organic carbon (AOC) and its precursors in drinking water

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In Japan, customers' concerns about chlorinous odour in drinking water have been increasing. One promising approach for reducing chlorinous odour is the minimization of residual chlorine in water distribution, which requires stricter control of organics to maintain biological stability in water supply systems. In this investigation, the levels and seasonal changes of assimilable organic carbon (AOC) and its precursors in drinking water were surveyed to accumulate information on organics in terms of biological stability. In tap water samples purified through rapid sand filtration processes, the average AOC concentration was 174 μgC/L in winter and 60 μgC/L in summer. This difference seemed to reflect the seasonal changes of AOC in the natural aquatic environment. On the other hand, very little or no AOC could be removed after use of an ozonation–biological activated carbon (BAC) process. Especially in winter, waterworks should pay attention to BAC operating conditions to improve AOC removal. The storage of BAC effluent with residual chlorine at 0.05–0.15 $mgCl_2/L$ increased AOC drastically. This result indicated the possibility that abundant AOC precursors remaining in the finished water could contribute to newly AOC formation during water distribution with minimized residual chlorine. Combined amino acids, which remained at roughly equivalent to AOC in finished water, were identified as major AOC precursors. Prior to minimization of residual chlorine, enhancement of the removal abilities for both AOC and its precursors would be necessary.

Keywords: assimilable organic carbon (AOC); AOC precursors; amino acids; drinking water; advanced water treatment

1. Introduction

Waterworks have been working to improve drinking water quality to reduce health risks caused by man-made pollutants, natural toxins and disinfection by-products. The installation of advanced water treatment processes is one of these efforts and it has been working effectively to supply safe water. However, customers' complaints about drinking water have been increasing even after advanced water treatment processes had been in operation. A questionnaire survey conducted in a distribution area supplied with drinking water treated by advanced treatment processes indicated that more than 40% people who avoided direct drinking of tap water could perceive chlorinous odour [1]. Thus, chlorinous odour has attracted increasing attention as one of important factors to increase customers' satisfaction for drinking water.

Chlorinous odour is produced by the reaction between chlorine and precursors such as nitrogen compounds [2]. Therefore, two types of approach for reducing chlorinous odour may be effective. One is improving water quality by removing its precursors still left even after the application of current advanced processes. Another promising approach is minimization of the chlorine disinfectant itself. However, the latter approach can cause deterioration of microbiological safety of drinking water by allowing bacterial regrowth within the distribution system. In Japan, a residual chlorine level of 0.1 $mgCl_2/L$ should be maintained at each tap, though many waterworks have been trying to keep a residual chlorine level of 0.3–0.4 $mgCl_2/L$ at each tap [3]. This residual chlorine level has worked effectively to inactivate bacteria within distribution systems and to provide a 'biologically stable' state. With lesser disinfection power, bacteria can regrow using a small amount of organics as substrate; therefore, stricter control of organics is needed at the same time with this approach.

The organic fraction, which can be consumed by bacteria, is called biodegradable organic matter (BOM). Assimilable organic carbon (AOC) and biodegradable organic carbon (BDOC) are often used as indicators for

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BOM. Each indicator has advantages and disadvantages. AOC is a very sensitive indicator for bacterial regrowth potential of drinking water, especially distributed drinking water, while BDOC is considered to be linked to chlorine demand or the formation of disinfection by-products [4].

One type of compound of most concern in BOM is amino acids, which contain carbon and nitrogen, and can become good energy sources and nutrients for heterotrophic bacteria. The amino acids have also become a target of researchers' attention because of their high reactivity with chlorine disinfectant [5]. Inorganic or organic chloramines can be formed by the reaction of amino acids with chlorine disinfectant, and it is commonly believed that these products can cause not only a decrease of disinfection efficiency but also an increase of chlorinous odour. Thus, there has been a recent focus on amino acids as key compounds in both chlorinated and chlorine-free water supply systems.

In this investigation, AOC levels in drinking water samples distributed from two different water treatment plants with different treatment processes were determined. AOC removal during advanced water treatment processes was then evaluated. The possibility of AOC increases during water distribution was also examined. Finally, the amino acids as BOM components were determined by analyses of free and combined amino acids, and their contribution to BOM is discussed. This information would be useful in allowing us to improve the microbiological stability of water for water supply systems with minimized residual chlorine.

2. Materials and methods

2.1. Water sampling in distribution systems

Two different distribution areas were chosen.

One is a distribution area (System-A) supplied from a conventional water treatment plant (WTP-A), which includes coagulation–sedimentation, rapid sand filtration (RSF), and chlorination. The raw water of WTP-A is piped from Lake Biwa.

Another distribution area (System-B) is supplied from a water treatment plant (WTP-B), which has advanced processes consisting of coagulation–sedimentation, intermediate ozonation, rapid sand filtration, post-ozonation, biological activated carbon (BAC) and chlorination. The WTP-B purifies surface water taken from the Yodo River, which has a source in Lake Biwa.

Forty water samples and six water samples were taken from each tap in System-A in different seasons, May/June 2007 and January 2008, respectively. Six water samples were also taken from each tap in System-B in January 2008. The water samples after 5 min flashing were collected in carbon-free glass bottles prepared by thermal treatment at 550°C for four hours. The samples for analyses were transported to the laboratory under refrigerated conditions and processed within four hours of sampling.

2.2. Water sampling at a treatment plant

Two samplings were conducted at the above-mentioned advanced water treatment plant (WTP-B) in December 2008/January 2009 (winter season) and June/July 2009 (summer season), respectively. The effluent samples after each treatment process and the water intake were collected and transported to the laboratory under refrigerated conditions. AOC, total organic carbon (TOC) and heterotrophic plate count (HPC) in the samples were analysed within four hours. Those samples such as water intake and BAC effluent that were expected to contain not negligible amounts of microorganism cells were filtrated through an Anodisc 47 membrane filter (GE Healthcare Japan, Tokyo, Japan) to reduce the effect of indigenous bacteria on tested bacterial strains for AOC measurement.

Dissolved amino acids were determined only in samples taken during summer. For determination of dissolved free amino acids (DFAAs), benzalkonium chloride was added immediately to each sample at a final concentration of 0.1% to prevent unintentional biodegradation during transportation [6].

2.3. AOC formation under non-chlorinated and chlorinated conditions

The effluent samples of BAC process at WTP-B were collected in January 2009 as previously described and were stored without any additional treatment at 4°C after being divided into three different carbon-free bottles. The same BAC effluent samples were also stored at 20°C after adding sodium hypochlorite at final concentrations of 0.05 and 0.15 $mgCl_2/L$. After four days storage, the AOC content in each sample was determined after filtration using an Anodisc 47 membrane filter.

2.4. Analytical methods

TOC was analysed using a TOC-5000A or TOC-V_{CSH} analyser (Shimadzu, Kyoto, Japan). HPC bacteria were enumerated using a pour plating procedure with R2A agar (Nippon Pharmaceuticals Co., Ltd, Tokyo, Japan) after seven days incubation at 20°C [7].

AOC was measured according to the standard methods proposed by the Japan Water Works Association [7]. Water samples were pasteurized for 30 minutes at 75°C in a water bath. To make sure that carbon was the only limiting nutrient, nutrients including nitrogen,

phosphorus and other trace elements were supplemented by adding 2 mL mineral solution. Samples were inoculated simultaneously with *Pseudomonas fluorescens* strain P17 (ATCC 49642) and *Aquaspirillum* sp. strain NOX (ATCC 49643) and incubated at 20°C; the growth of each strain was monitored every two or three days during 14–20 days by pour plating on R2A agar at 20°C. AOC was calculated using the average (CV ≤ 20%) or maximum numbers of colonies at steady state and the yield factors determined with sodium acetate as a carbon source. The yield factors in this investigation were determined as 4.53×10^6 and 1.56×10^7 colony-forming units (CFU)/μg of acetate-C for P17 and NOX, respectively.

Free and combined chlorine were analysed using a DPD-ferrous titration method according to standard methods [8].

Amino acids were determined using a liquid chromatography system for amino acids analysis after post-column derivatization with *o*-phtalaldehyde (OPA). This system was equipped with a trapping column for ammonium ion (Shim Pack ISC-30, Shimadzu, Kyoto, Japan) and a cation exchange column (Shim-Pack AMINO Li, Shimadzu, Kyoto, Japan) for separation. The derivatized compounds with OPA were detected using a fluorescence detector (RF-10A, Shimadzu, Kyoto, Japan) at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. For DFAAs analyses, 1/10 volume of dilution buffer (2.5 mol Li/L lithium citrate buffer (pH 2.1)) was added to each sample and filtrated through a 0.22 μm filter (Millex-GP, Nihon Millipore, Tokyo, Japan). For dissolved combined amino acids (DCAAs) analyses, the samples without an antiseptic agent were dried under a stream of N_2 gas and hydrolyzed in 6N hydrochloric acid containing 11.36 mM ascorbic acid at 110°C for 20 hours, according to the method reported by Robertson et al. [9]. Milli-Q water samples treated by thermal hydrolysis in the same way were also analysed as blank samples.

2.5. *Statistical analysis*

Statistical analyses were performed with GraphPad Prism version 4.0 for Macintosh (GraphPad software Inc., San Diego, CA, USA). To compare the differences between two groups of samples, a nonparametric *t*-test was performed. Significant differences were determined with a level of $p<0.01$ in all analyses.

3. Results and discussion

3.1. *AOC levels in tap water samples*

Average AOC levels in two different distribution systems in winter are compared in Figure 1(a). The average AOC in System-B was slightly lower than that in System-A, but the difference was not significant, while the average TOC content in samples taken from System-B (1.3 mg/L) was considerably lower than that in System-A (1.8 mg/L).

This result suggested that the AOC removal through advanced water treatment processes consisting of ozonation and BAC adsorption achieved only a limited improvement. It is well-known that ozonation increases the AOC fraction by converting high molecular weight organics to low molecular weight and polar compounds, particularly carboxylic acids [6]. Some parts of AOC

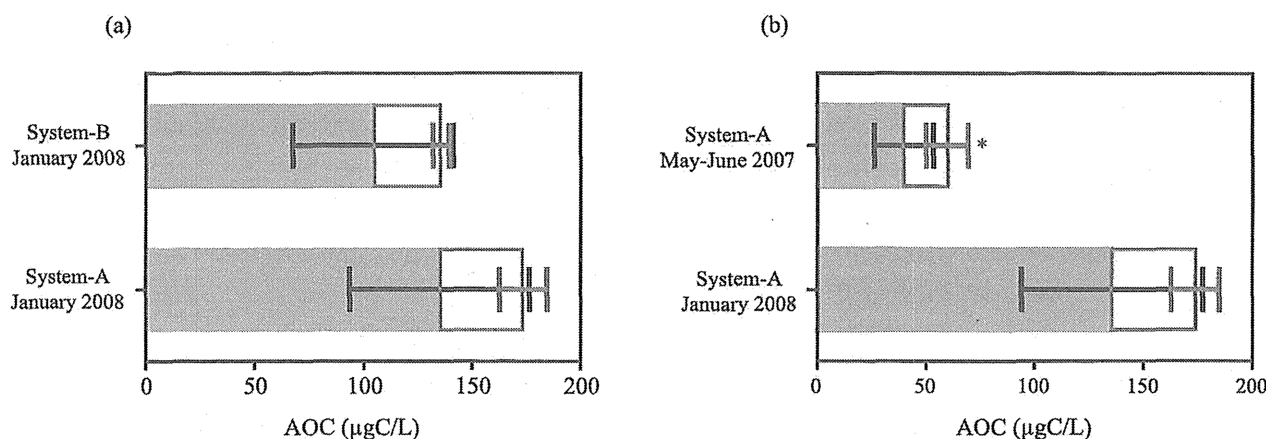

Figure 1. Comparison of AOC levels in tap water samples. Two components of AOC, P17 component (grey solid bar) and NOX component (white bar), were determined by the van der Kooij method [11]. (a) AOC in tap water samples ($n = 6$) purified through different treatment processes, WTP-A (conventional processes) and WTP-B (advanced processes) in winter season. (b) AOC in tap water samples taken from System-A in summer ($n = 40$) and winter ($n = 6$) seasons. The data represented the mean ± the standard deviations. *$p<0.01$.

should be removed during the BAC process by microbial activity on the surface of activated carbon, but in our case the total efficiency of AOC removal was not improved significantly.

On the other hand, as shown in Figure 1(b), the AOC data in the samples taken from the same distribution system (System-A) showed a clear difference between the seasons. The average AOC in summer ($n = 40$) was 59.8 μgC/L, while the average AOC in winter ($n = 6$) was 174 μgC/L. It is widely recognized that the raw water quality of WTP-A has been affected by seasonal changes of water quality in Lake Biwa. Mitamura and Saijo [10] have reported that TOC in the summer season was slightly higher in southern basin of Lake Biwa and that the fluctuation might be caused by phytoplankton blooms. This report suggested that:

- constituents of organics might shift to aquagenic or autochthonous organic matter with higher molecular weight by microbial conversion in the summer;
- the observed seasonal changes in AOC might directly reflect the seasonal changes in quantity and quality of organic fraction in the lake water.

The obtained AOC values were much higher than the AOC level of 10 μgC/L proposed by van der Kooij [11] for biologically stable water in non-chlorinated systems. In the winter season, the AOC levels were higher than 100 μgC/L, which was proposed by LeChevallier *et al.* [12] as a criterion to prevent regrowth of coliforms in chlorinated systems.

The impact of raw water quality and the AOC removal during advanced water processes in WTP-B on AOC levels in finished water are more fully discussed in the next section.

3.2. *AOC removal during advanced water treatment processes*

3.2.1. *Comparison of organic carbon in raw water and finished water*

The averages of AOC concentrations obtained by two samplings in the winter and summer season at WTP-B are shown in Figure 2. The average TOC values and proportion of AOC to TOC are shown in Table 1.

The average AOC levels in raw water were 148 and 32.2 μgC/L in winter and summer, respectively. The AOC level in the winter season was five-times higher than that in summer season, although Huck *et al.* [13] have reported that AOC concentrations in raw water during the winter-fall period were significantly lower than those during summer, and that a similar trend was observed with non-volatile organic compounds.

One of the characteristics of the surveyed WTP-B was that the raw water quality depended on the water qualities of the river water, which has a source in Lake Biwa, and the discharged water from wastewater treatment facilities located upstream of the basin. It is recognized that phytoplankton growth in the summer season can contribute to an increase in TOC in the Yodo River water, because 70% of the water in the river comes from Lake Biwa, where phytoplankton blooms were observed during the season when the water temperature was high. A rough seasonal fluctuation in BOD (higher in winter and lower in summer) was also observed in discharges from wastewater treatment facilities in this basin [14].

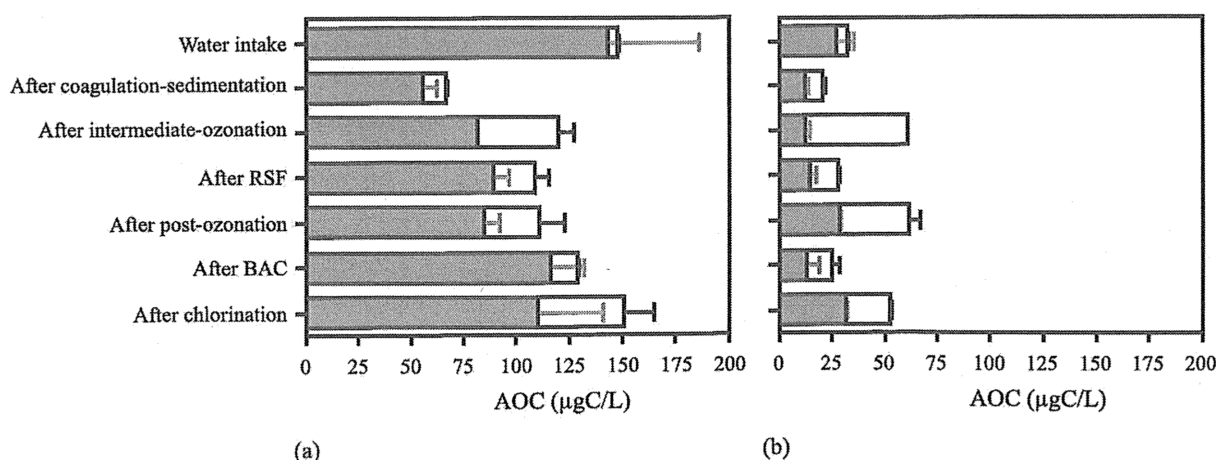

Figure 2. Comparison of AOC changes during advanced water treatment process in (a) winter and (b) summer. Two components of AOC, P17 component (grey solid bar) and NOX component (white bar), were determined. The data represented the mean ± the range of two samplings. BAC = biological activated carbon; RSF = rapid sand filtration.

Table 1. Changes in TOC and AOC/TOC proportions during water treatment processes.

	Winter 2008		Summer 2009	
	TOC (mg/L)	AOC/TOC (%)	TOC (mg/L)	AOC/TOC(%)
Water intake	2.0	6.5	2.0	1.4
After coagulation-sedimentation	1.7	3.5	1.4	1.3
After intermediate-ozonation	1.7	5.8	1.4	4.3
After RSF	1.5	6.4	1.1	2.5
After post-ozonation	1.5	7.6	1.0	6.0
After BAC	1.2	11.9	0.9	3.0
After chlorination	1.1	12.3	0.8	6.7

However, there was no difference in our limited TOC data between the two seasons. This information from the literature supports our assumption (presented in the previous section) that the decrease in AOC in the summer season might reflect the changes in the organic constituents in the raw water. The organic fraction that can be easily consumed by heterotrophic bacteria could, in summer, be converted into biomass or extracellular components with a higher molecular weight such as proteins or polysaccharides, with higher bacterial activity in the natural aquatic environment. The decrease in the proportions of AOC to TOC also indicated that the organic constituents shifted in summer to those considered refractory to biodegradation. In both seasons, the AOC component consumed by the P17 strain preferentially accounted for over 80% of AOC in surface water samples, and the NOX component did not contribute to the seasonal changes in AOC.

After whole treatment processes, the AOC levels in the finished water increased slightly to 151 μgC/L in winter season and 52.7 μgC/L in summer season, respectively. The seasonal difference in AOC levels detected in the finished water was consistent with the results of AOC in tap water samples taken from System-A and AOC in raw water samples as mentioned previously. Therefore, these seasonal changes of organic constituents in raw water could directly affect the AOC levels in finished water.

This result also indicated that the whole treatment processes of WTP-B (including ozonation and BAC adsorption) could not remove AOC at all, whereas more than 40% TOC could be removed constantly in both seasons. The increases in the NOX component were observed during the treatment processes in both seasons. The effect of each treatment process on AOC changes is discussed in the next section.

3.2.2. *Effect of each treatment process on AOC*

Based on Figure 2, the first treatment process, coagulation–sedimentation, was effective for AOC reduction, especially for reduction of the P17 component. The facility uses aluminium sulphate as a coagulant.

There have been conflicting arguments about an efficiency of AOC removal during a coagulation process. Huck *et al.* [13] have pointed out that polyaluminium chloride prolonged the lag phase in the growth of *P. fluorescence* P17 because of its toxicity, but did not impact on maximum colony numbers. However, Lehtola *et al.* [15] have confirmed the inhibitory effect on colony formation of *P. fluorescence* P17 in their surveys at water treatment process plants using aluminium salt. Therefore, there is a possibility of the growth inhibition of *P. fluorescence* P17 being misidentified as AOC removal. On the other hand, Kasahara *et al.* [16] demonstrated that the P17 component of AOC could be reduced by a coagulation process based on their jar test results using polyaluminium chloride as a coagulant. Liang *et al.* [17] also reported that coagulation processes using aluminium sulphate and ferric chloride as coagulants could remove total AOC effectively, especially under the conditions for enhanced coagulation. In general, coagulation is widely recognized as a process suitable for removal of hydrophobic organic fraction with high molecular weight. Those results should be re-evaluated more carefully in terms of an inhibitory effect of alum salt on bacterial strains for AOC determination.

The next step, an intermediate-ozonation, increased a NOX component of AOC significantly. This phenomenon was also observed after the post-ozonation process. It is known that molecular ozone reacts with unsaturated bonds of organic compounds, converting them to lower molecular weight compounds with carbonyl- or carboxyl groups [6]. These newly formed carboxylic acids were measured as NOX components by AOC determination. However, our result indicated that the increased NOX component could be removed quickly during the following rapid sand filtration (RSF) process.

The most noteworthy point in our results was the efficiency of the BAC process for AOC removal.

Although many studies have demonstrated relatively high efficiency (37–86%) in AOC removal during biological filtration processes [18–21] such as BAC or biofiltration, our results suggested that BAC operated in this facility did not remove AOC at all in the winter season although it could remove 50% of AOC in the summer season.

One reason for the low efficiency of the BAC process in the winter season could be an immature bacterial community on the surface of activated carbon because of lower water temperature. In fact, the heterotrophic plate counts (HPC) in the BAC effluent during the winter season were less than 10 CFU/mL, while the HPC in the summer season averaged 105 CFU/mL. In addition, the bacterial community on the activated carbon might have lower activity to assimilate organic carbon in the winter than that in the summer.

Persson *et al.* [22] reported that the specific respiratory activity of biofilter biomass was dependent on water temperature. Their results suggested that there was a crucial point between 13°C and 8°C for the respiratory activity of bacteria. The average water temperature during the period of our winter survey was reported as 8–12°C; such a low water temperature had a significant impact on bacterial activity in the BAC process. Therefore, some improvement in operation such as the longer contact time for BAC treatment would be needed to improve AOC removal efficiency during the winter season.

The final step, chlorination, also increased AOC slightly in the summer season and drastically in the winter season. This suggested that the finished water in the winter season contained a larger fraction of AOC precursors even after BAC treatment, which could be readily converted into the AOC fraction by chlorination.

3.3. AOC formation under non-chlorinated and chlorinated conditions

AOC levels after storage, with and without residual chlorine, are compared in Figure 3. The BAC effluent samples at WTP-B (AOC 149 μgC/L) taken in winter season were stored at 4°C for four days. AOC levels determined after storage were 269–289 μgC/L and an average increase of 87% was observed. This result proved that AOC precursors, which could easily release AOC into aqueous phase not only through a chemical reaction but also by microbial activity, remained abundant in the winter season even after ozonation–BAC treatment processes.

A small count of bacteria, which was detected in BAC effluent samples and did not increase during storage in every sample, might contribute to the newly formed AOC in these samples despite no intentional inoculation. Although the samples without residual

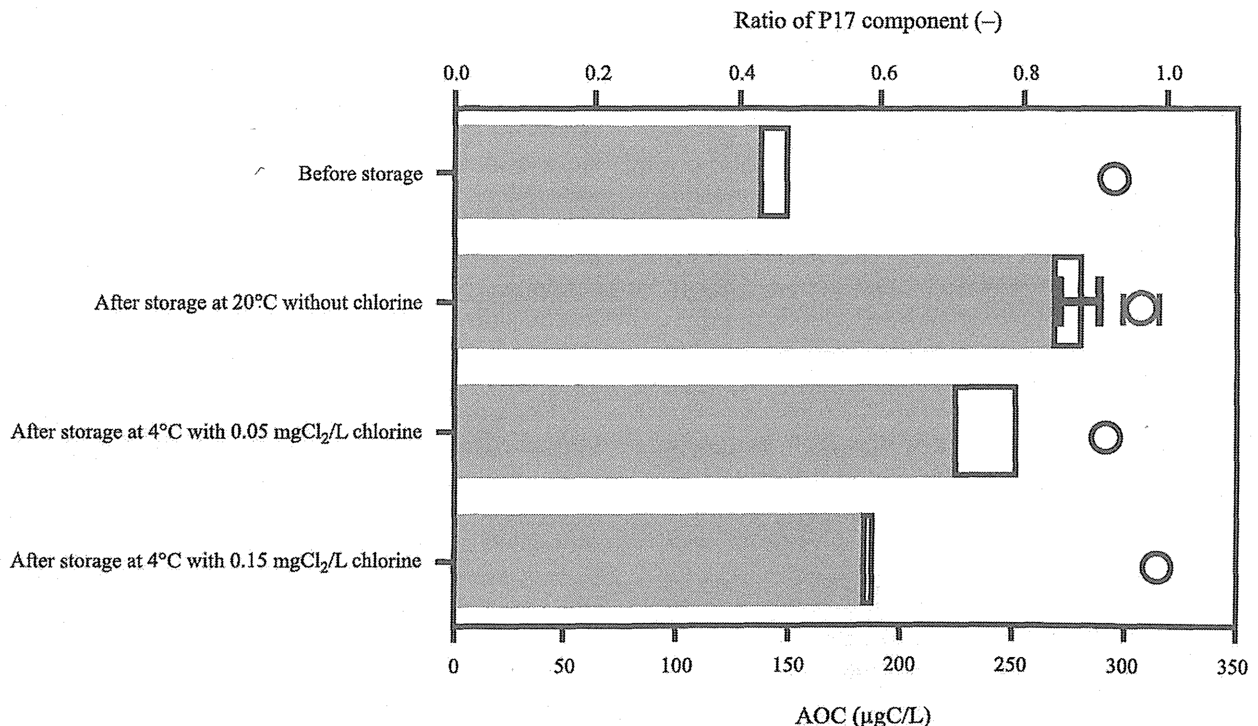

Figure 3. Effect of residual chlorine on AOC changes during sample storage. AOC concentrations after storage (P17 component, grey solid bar; NOX component, white bar) and the ratios of P17 component (open circle) are shown. Only stored samples without residual chlorine were measured in triplicate and represented the mean ± the standard deviation.

chlorine were kept in a refrigerator and no bacterial regrowth was detected under this storage condition, surprisingly, the hydrolysis of organics by microbial activity progressed very quickly. This result strongly suggested that the AOC increase was caused by enzymatic hydrolysis of bacteria remaining in the BAC effluent even at 4°C. Based on the AOC increment during storage, the hydrolysis rate of BOM to produce AOC was estimated to be much greater than the microbial assimilation rate of organic carbon at 4°C.

In contrast, smaller increases in AOC were observed in the samples stored at 20°C for four days with residual chlorine, and the increments were smaller at higher residual chlorine. This result also could be evidence that bacteria in the status of 'resting cells' played an important role in enzymatic hydrolysis of BOM remaining in the BAC effluent to AOC even under unfavourable conditions for bacterial regrowth such as chlorinated conditions or at lower water temperature.

According to the comparison of AOC levels in finished water at WTP-B with those in tap water taken from System-B presented in previous sections, significant AOC increases were not however observed in an actual distribution system (System-B). This discrepancy was attributed to different levels of residual chlorine because the waterworks had been maintaining a minimum residual chlorine concentration of 0.4 mgCl_2/L during water distribution in System-B. But our data shown in Figure 3 suggested the possibility that the AOC increase could be occurring during water distribution at 20°C if lower residual chlorine than the present level applied. As mentioned in the Introduction, minimization of residual chlorine is a promising option for reducing chlorinous odour to an acceptable level for customers and it should be widely applied to water distribution systems in the near future. Under these circumstances, the waterworks should pay much more attention not only to AOC levels in finished water but also to levels of AOC precursors.

The component that contributed to the increase in AOC was identified as the P17 component. In all samples, only P17 components increased dramatically while NOX components did not change significantly. This result was completely different from the specific increase in NOX components (including acetate, formate and oxalate) observed during oxidation processes such as ozonation and chlorination [6,23]. While organics containing unsaturated bonds can become predominant AOC precursors during these oxidation processes, our results suggest that hydrophilic AOC precursors such as poly- or oligosaccharides, peptides or proteins, which can be converted into P17 components by microbiological hydrolysis, could cause a drastic increase in AOC during water distribution with minimized residual chlorine. Considering that Jo [24] reported that hydrophilic fractions accounted for approximately 50% of DOM in the Yodo River water and that their percentage was increased after an ozonation–BAC process, the removal of hydrophilic organics during water treatment could be key to the production of biologically stable water. Thus, organic constituents that could increase AOC during water distribution should be also identified in order to improve biological stability of the finished water.

3.4. Determination of amino acids in processed water samples

Dissolved free amino acids (DFAAs) and dissolved combined amino acids (DCAAs) were analysed separately in order to determine the contribution of amino acids, peptides and proteins to AOC precursors. Their concentrations in processed water samples at each sampling point are shown in Table 2.

DCAAs were detected in both samples during water treatment processes. However, the concentrations of DFAAs were less than detection limits in both samplings. These results are consistent with our assumption that the AOC fraction could be easily converted into organics with a relatively higher molecular weight by microorganisms in this basin.

In our analysis, the samples to which an antiseptic agent had been added immediately after sampling were provided for free amino acids analysis, while the samples without antiseptic agent were provided for combined amino acids analysis. It was expected that DFAAs in the samples without an antiseptic agent would be readily degraded by bacteria during transportation to our laboratory and therefore overlap of DFAAs and DCAAs was considered to be negligible in our analysis.

The interesting finding is that the concentration of DCAAs rose drastically on occasion. We have not yet been able to identify the causes or sources for these spikes in the concentration of amino acids.

DCAAs were removed during water treatment processes efficiently, even though we observed a large difference between the two samplings of raw water. The concentrations of total DCAAs in finished water were quite stable at around 1 μM in both samplings and were equivalent to 24.6–65.0 μgC/L. Their percentages in TOC in finished water were not so large at less than 8%. However, if the concentrations of combined amino acids were compared to AOC levels, their fractions were relatively large (approximately 50–100%).

Needless to say, direct comparison of organic carbon levels derived from amino acids with AOC levels is difficult because the AOC was calculated as the acetate-based amount of organic carbon from biomass. However, these results indicated that the combined amino acids could be one of the major AOC precursors,

Table 2. Changes in concentration of amino acids during water treatment processes.

	June 25, 2009		July 6, 2009	
Sampling points	DFAA (μM) <(μgC/L)>	DCAA (μM) < (μgC/L)>	DFAA (μM) < (μgC/L)>	DCAA (μM) <(μgC/L)>
Water intake	N.D.	87.2 <4332.2>	N.D.	3.60 <165.5>
After coagulation–sedimentation	N.D.	3.01 <137.7>	N.D.	1.44 <54.0>
After intermediate–ozonation	N.D.	5.17 <237.0>	N.D.	1.11 <35.9>
After RSF	N.D.	2.48 <111.4>	N.D.	1.32 <48.2>
After post-ozonation	N.D.	1.34 <50.0>	N.D.	1.79 <69.8>
After BAC	N.D.	1.95 <82.2>	N.D.	3.24 <149.1>
After chlorination	N.D.	0.76 <24.6>	N.D.	1.39 <65.0>

N.D. = not detected.

which could be converted by *P. fluorescens* P17 as observed in Figure 3. Our data was limited in the summer season in that AOC was relatively low. Further data in other seasons should be accumulated because information on the constituents of AOC and its precursors could be crucial in optimizing water treatment processes in terms of biological stability.

4. Conclusions

A survey of AOC levels in drinking water samples was conducted in two different distribution areas. In tap water samples purified through rapid sand filtration processes, the average AOC was 174 μgC/L in the winter season and around 60 μgC/L in the summer season. The limited effect of advanced water treatment processes on AOC removal was also noted. Removal of AOC and combined amino acids during advanced water treatment processes was also evaluated. We concluded that very little or no AOC was removed throughout the whole advanced water treatment processes and that AOC levels in the finished water reflected the seasonal fluctuation of AOC in the natural aquatic environment. Especially in winter, waterworks should pay attention to BAC operation to improve AOC removal.

The AOC increase during storage with minimized residual chlorine implied that AOC precursors remained abundant in the finished water and that they could contribute to a drastic AOC increase during water distribution. Analysis of amino acids identified combined amino acids, which remained at roughly equivalent to the AOC level in finished water, as major AOC precursors. These results indicated that the removal abilities of water treatment processes for hydrophilic organics, not only AOC but also its precursors, should be largely enhanced prior to minimization of residual chlorine.

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〈原著論文—Original Paper〉

疫学調査に基づいたCampylobacter jejuni感染における感染−発症割合の推定

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Estimation of Illness-to-Infection Rate of *Campylobacter jejuni* Based on Epidemiological Survey

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Abstract

Disability Adjusted Life Years (DALYs) are good estimates of the effects caused by waterborne pathogens in drinking water. The illness-to-infection rate of *Campylobacter jejuni* is one of the most important factors for estimating DALYs. In this study, the illness-to-infection rate of *Campylobacter jejuni* was estimated on the basis of seroepidemiological survey. From the data of serum antibody levels against *C. jejuni*, the cutoff levels for asymptomatic infection were set on the basis of medical data from previous studies and using a statistical procedure. From these cutoff levels, the percentage of people with asymptomatic infection was estimated to be 7.9 - 35.0% and 12.1 - 13.6%, respectively. By using the numbers of people with symptomatic infection estimated using the number of laboratory-confirmed cases, the illness-to-infection rate of *C. jejuni* was estimated to be 12.1 - 53.8% and 31.3 - 35.1%, respectively.

Keywords: Disability Adjusted Life Years; Waterborne disease; *Campylobacter jejuni*; Illness-to-infection rate; Seroepidemiological survey

1. はじめに

水道水の塩素消毒は，副生成物による健康リスク問題を生じさせるほか，カルキ臭を生成することから，現在の日本では水道水離れが問題視されている[1]。将来は塩素注入量を低減しつつ，より満足度の高い水道システムを構築する必要がある。しかし，残留塩素濃度の低減は病原微生物による微生物リスク増大に直結しうることから，同時に微生物リスク管理手法の高度化を進めることが重要となる。

微生物リスク管理の高度化は，許容リスクレベルを設定した上で，各病原体による感染確率と引き起こされる健康影響の大きさを指標として定量化したリスク値を踏まえて，そのマネジメントを行う。しかし，一般に病原微生物が引き起こす水系感染症は，症状の種類，重篤度，持続期間が多様であるため，感染症による健康影響の大きさ全体を評価する指標が必要となる。ここで，疾病要因による多種多様な健康影響の大きさを表す指標として障害調整生存年数（Disability Adjusted Life Years: DALYs）がある。飲料水の安全評価分野では，WHO（世界保健機関）が数種類の微生物と化学物質を取り上げてDALYsの定量を実施している[2]。

水道水を介した水系感染症の主要原因菌のひとつに *Campylobacter jejuni* がある。日本では塩素消毒の不備により，*C. jeuni* による感染症が発生したケースが確認されており[3]，残留塩素を低減した場合，*C. jejuni* による微生物リスクが増加すると考えられる。さらに *C. jejuni* 感染症は，下痢症だけではなくギラン・バレー症候群や反応性関節炎のような重篤な健康影響を引き起こす可能性があるため，DALYsを用いた評価は有益である。

病原体曝露後の感染確率に基づいてDALYsを評価する場合，感染者から発症者の発生する割合（感染−発症割合）を設定する必要がある。しかし，*C. jejuni* 感染による感染−発症割合に関する有用な情報は乏しいのが現状である。Tompkins ら（1999）[4] や De Wit ら（2001）[5] による腸管感染症の発生率から推定した不顕性感染者（感染者）数に対する顕性感染者（発症者）数の割合がそれぞれ1.2%，0.8%であるという報告[6]がある一方で，多くの用量反応モデルで発症の確率が最大100%であることから感染−発症割合を100%と仮定している[6]ケースもある。そのため，感染−発症割合は0.8〜100%の広範囲で設定されているのが現状であり，これらの感染−

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発症割合の情報に基づいて感染確率からDALYsを推定する場合，感染－発症割合の設定によりDALYs推定値の差が最大120倍程度生じることになる。

オランダのある浄水処理施設を対象として，定量的微生物リスク評価（Quantitative Microbial Risk Assessment; QMRA）によって*C. jejuni*曝露による感染確率を求め，それに基づいてDALYsを試算した試み[7]がある。その試みでは，取水する原水の種類の影響，浄水処理における除去能に対する水温の影響，病原微生物数と指標微生物の比の影響，用量反応モデルの影響，処理前後の微生物濃度データのペアリング方法の影響，感染－発症割合の影響に関して，DALYs推定に対する不確実性分析を行った結果，感染－発症割合がDALYs推定において最も大きな不確実性を持つ因子であると判定している[7]。精度の高いDALYs推定には信頼できる感染－発症割合に関する情報が不可欠といえる。

一般的に病原体を曝露し感染が成立した後は，発症が認められた状態（顕性感染），あるいは発症に至らないままの状態（不顕性感染）となる。ここで顕性感染者は発症者であり，感染者は感染が成立した者，すなわち顕性感染者と不顕性感染者を含んでいる集団である。そして感染－発症割合を推定していく上では一般集団における感染者数と発症者数の把握が必要となる。また発展途上国では先進国に比べ，*C. jejuni*の曝露頻度が多く免疫レベルが高いため，無症状あるいは穏和な影響を示すことが多い[2]。つまり*C. jejuni*による感染および発症には，*C. jejuni*の曝露量や曝露頻度，そして感染時に獲得した免疫による感染防御効果が大きく関わっていると考えられ，これらを踏まえた推定方法が望まれる。

感染者数については，食品安全評価委員会により*C. jejuni*の主な感染経路である鶏肉の曝露量・曝露頻度に基づいて年間感染確率・感染者数を推定した試みが報告されている[8]。しかしこの推定方法は，感染時に獲得した免疫による感染防御効果について一切考慮していない。一般的に病原体の感染を受けた人が再び病原体に曝露された場合，初回感染時に産生された抗体が感染した病原体に速やかに結合し，病原体を排除し破壊するといった免疫獲得による感染防御効果を得ることとなる。特に高頻度で病原体に曝露された場合に，免疫獲得による感染防御効果が高いと予想され，対象病原体に対する感染自体も生じない可能性が高い。そのため，*C. jejuni*の主要な感染源である鶏肉の喫食頻度，特に生食の喫食頻度が高い[8]ことを考慮すると，*C. jejuni*感染に対する免疫獲得による感染防御効果が高いと予想されるため，免疫獲得による感染防御効果を考慮していない推定方法では感染者数を過大評価している危険性がある。

そこで本研究では*C. jejuni*感染による感染－発症割合の把握を目的とし，*C. jejuni*感染成立後に症状の有無に関わらず体内で起こる免疫応答に着目して，血清疫学調査により得られた*C. jejuni*の血清抗体価データに基づいて感染者数の推定を試みた。一方，病原体検出情報に記載された*C. jejuni*分離報告数に基づいて一般集団中の発症者数を算出し，*C. jejuni*感染による感染－発症割合の推定を行った。

2. 方法

2.1 血清疫学調査の対象

国立感染症研究所が管理・運営する国内血清銀行に保管されている血清の中で，京都府で一般集団を対象として2006年～2008年に採取された血清140サンプルを対象とした。なお，年齢区分ごと（5～9歳，10～19歳，20～29歳，30～39歳，40～49歳，50～59歳，60歳以上）に20検体，男女比が1：1になるように設定した。感染性腸炎研究会の調査で，1996年から2000年までの5年間における*C. jejuni*による感染性腸炎入院患者数が0～4歳，5～9歳と同程度であることが報告されている[9]。そのため，血清が得られなかった年齢区分0～4歳における*C. jejuni*感染の感染・発症レベルは年齢区分5～9歳と同程度であるとみなした。なお，本調査の実施に先立ち，京都大学大学院工学研究科研究倫理委員会の審査・承認を受けた。

2.2 *Campylobacter jejuni*に対する血清中の抗体価の測定

対象とする抗体クラスは，immunoglobulin A (IgA)，IgG，IgMとした。なお抗体価測定には，SERION ELISA classic *Campylobacter jejuni* IgG/IgA/IgMキット（Virion/Serion）を使用した。

2.3 *Campylobacter jejuni*による感染－発症割合の推定

2.3.1 一般集団中の*Campylobacter jejuni*感染者割合の推定

感染者数については，2.2により得られた抗体価測定結果に基づいて推定した。まず得られた抗体価分布に対して感染者と健常者を判別する陽性カットオフ値を設定し，抗体価陽性と判断された集団を感染者の集団と定義した。そして，140サンプル中の感染者サンプル割合を一般集団における感染者割合とみなした。

医学的には感染者集団と健常者集団をそれぞれ設定し，各集団の測定した血清抗体価分布データに基づいて感染者と健常者を判別する陽性カットオフ値を設定している。しかし食中毒のケースを活用する場合，不顕性感染者に関しては症状がないことから，多くの病原微生物に対する血清抗体価測定において，感染者集団と健常者集団をそれぞれ設定し抗体価を測定することは困難である。そこで感染者と健常者を判別する陽性カットオフ値の設定方法として，客観的根拠として既往研究の抗体価データを用いて推定する手法について検討を行った。また客観的根拠が得られなかった場合も想定して，統計学的観点からカットオフ値を推定していく手法についても検討し，両推定方法を用いて感染者割合の推定を行った。

(1) 既往研究の医学データに基づいた陽性カットオフ値の設定

抗体価測定試薬に記載されている発症者判定に用いるカットオフ値は，およそ400人の血清を分析した結果から得られた値であり，急性胃腸炎の糞便検査を同時に行うことで，発症者を示すカットオフ値として感度，特異性ともに妥当であることが示されている[10]。そこで抗体価測定試薬に記載されているカットオフ値を，発症者判定カットオフ値として採用した。

次に既往研究の*C. jejuni*抗体価に関する医学データの

発症者集団の吸光度平均値と感染者集団の吸光度平均値の中間値が設定した発症者判定カットオフ吸光度値に該当すると仮定した。一方，同様に感染者集団の吸光度平均値と健常者集団の吸光度平均値の中間値が，感染者判定カットオフ吸光度値に該当すると仮定した。そして，感染者カットオフ吸光度値に対する発症者カットオフ吸光度値の上昇率を計算し，その比率を用いて抗体価測定試薬に記載された発症者判定カットオフ吸光度値から感染者判定カットオフ吸光度値を算出した。最後に吸光度と抗体価の関係式を用いて陽性カットオフ値を抗体価として表示した。

(2) 統計学的手法に基づいた陽性カットオフ値の設定

統計学的手法を用いてある疾患のカットオフ値を設定する場合，カットオフ値により生じる偽陽性，偽陰性の2種類の過誤による平均損失を最小にする値を対象疾患陽性判断のカットオフ値として設定している[11]。しかし，血清抗体価試験で得られた分布は，健常者分布と感染者分布が複合的に存在する状態であるため，得られた分布を２つのクラスに領域分割する必要がある。

まず得られる１つの分布を複合分布モデルと見なして最尤推定の観点から２つのクラスに領域分割する手法である最大尤度しきい値選定法があり，その方法には２つの推定方法が挙げられている[12]。１つの方法は，各クラスの分布が平均値は異なるが同じ分散を持つ正規分布であるという仮定のもとで，条件付き分布の尤度を最大とするしきい値を求める方法（推定方法1）である。もう１つの方法は，各クラスの分布が異なる平均値と分散を持つ正規分布であるという仮定のもとで，同時分布の尤度を最大とするしきい値を求める方法（推定方法2）である。各推定方法には最大対数尤度関数が設定されている[12]。そして最大対数尤度が最大となるしきい値を，血清疫学調査により得られた抗体価分布を低抗体価分布設定用データ群と高抗体価分布設定用データ群の２つに分割するしきい値と設定した。

抗体価分布をしきい値で２分化した際，各集団のデータ群に偽陽性，あるいは偽陰性のサンプルデータが含まれている可能性がある。そこで低抗体価データ群と高抗体価データ群に対して当てはめた分布と，偽陽性，偽陰性の２種類の過誤による損失の関係式[11]から陽性カットオフ値の推定を行った。カットオフ値の推定式は以下の通りである。

$$Y(a)=\frac{Y\{d_1\int_a^{\infty}f(x)dx+d_2\int_{-\infty}^{a}g(x)dx-2N\int_a^{\infty}f(x)dx\bullet\int_{-\infty}^{a}g(x)dx\}}{1-\int_a^{\infty}f(x)dx-\int_{-\infty}^{a}g(x)dx}\quad\cdots(1)$$

f（x），g（x）は，それぞれ低抗体価データ群と高抗体価データ群に対しての確率密度関数である。また d_1，d_2 は，カットオフ値 a の場合に健常者および感染者と判定されたデータ数，Y は過誤による平均損失，N は全サンプル数とした。最後に推定方法 1，推定方法 2 により設定した分布のパラメータと式(1)を用いて陽性カットオフ値を推定した。

なお統計学的手法に基づいた推定方法に関しては，仮説検定を行う際に PASW Statistics 17（IBM）を用いて，両側有意確率が 0.05 以下の場合に仮説が棄却されると判断した。

2.3.2 *Campylobacter jejuni* 感染症発症者数および感染－発症割合の推定

対象地域の発症者数を地方衛生研究所等のホームページに記載されている病原体検出情報から推定した。病原体検出情報では，指定された定点医療機関を受診し採取された患者検体を調査した結果をまとめている。そこで，病原体検出情報に記載された *C. jejuni* 分離報告数を *C. jejuni* 感染により消化器疾患を発症した患者数と設定した。

より正確な発症者数を推定するためには，病原体分離報告数が正確である必要がある。ヒアリング調査により，定点医療機関から地方衛生研究所への *C. jejuni* 菌体分離数の報告率は 100％であることを確認できた神戸市を感染－発症割合推定の対象地域として選定した。また，*C. jejuni* 感染は，主要な汚染源の一つである養鶏場などの畜産業が盛んな地域で多いと予想されるが，血清採取地域である京都府と神戸市の産業形態の違いは小さいとみなし，神戸市の定点医療機関，神戸市環境保健研究所，神戸市中央市民病院から報告されている *C. jejuni* 分離報告数[13]を実患者数推定に用いた。

しかし，実際には *C. jejuni* 感染により消化器疾患を発症したとしても症状が軽いため医療機関を受診しないといったように，発症していながら分離報告数に含まれないケースが多く存在すると考えられる。そのため，分離報告数の集計データのみでは実際の発症実態を把握できない。そこで，宮城県で実施された急性下痢症疾患の実被害者数推定研究の考え方[14]に従い，対象地域の *C. jejuni* 感染症実患者数を推定した。具体的には，検査機関が対象地域の受診者人口をカバーしているかを示す指標である人口カバー率を推定し，神戸市の医療機関全体の *C. jejuni* 検出数を算出した。次に医療機関における医師の糞便検査率に基づいて，*C. jejuni* 感染症発症者の医療機関受診者数を推定した。最後に患者の医療機関受診率に基づいて，*C. jejuni* 感染症実患者数を推定した。なお，保菌者が便検査で陽性となる割合については 100％とみなした。

まず外来患者延数を用いて，対象地域である神戸市の全医療機関と定点医療機関の外来患者延数から人口カバー率を求めた。糞便検査率と医療機関受診率については，宮城県で実施された急性下痢症疾患の実被害者数推定研究のデータ[14]に基づいて設定した。そして *C. jejuni* 分離報告数を基にして，人口カバー率，糞便検査率，医療機関受診率の要因を加味することで得られた *C. jejuni* 感染症実患者数を，発症者数と設定した。血清抗体価分布から推定した一般集団中の感染者割合を神戸市に適用し，神戸市における３年間の *C. jejuni* 感染者数を算出し，神戸市における３年間の *C. jejuni* 感染症実患者数から，感染－発症割合を推定した。

3. 結果および考察

3.1 一般集団中の *Campylobacter jejuni* 感染者割合の推定

3.1.1 各抗体クラスにおける血清抗体価分布

140 サンプルについて，各抗体クラスの血清抗体価を測定した結果を **Fig. 1** に示す。血清抗体価は，IgA 抗体で 0.03 ～ 20.6 $U \cdot mL^{-1}$，IgG 抗体で 0.6 ～ 31.5 $U \cdot mL^{-1}$，

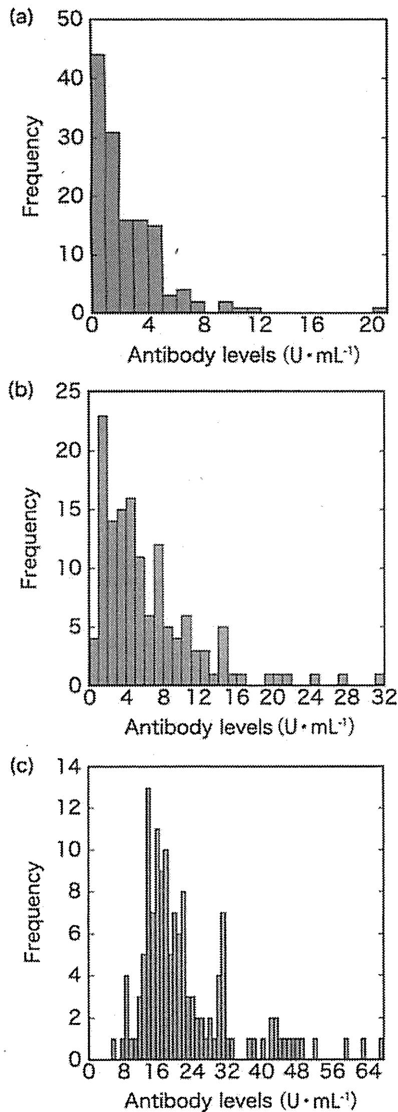

Fig. 1 The distributions of the antibody levels. ((a) IgA (b) IgG (c) IgM)

IgM 抗体で 5.6 ～ 66.4 U・mL^{-1} となり，健常者集団においても全ての抗体クラスにおいて抗体価が幅広く分布することがわかる。抗体価測定試薬には，発症者を判定するカットオフ値（IgA 抗体：25 U・mL^{-1}，IgG 抗体：30 U・mL^{-1}，IgM 抗体：60 U・mL^{-1}）が設定されているが，今回の測定サンプル中にも IgG 抗体，IgM 抗体についてはこのカットオフ値を上回るサンプルが存在した。Blaser ら（1984）や Black ら（1988）は，ボランティア試験などにより顕性感染者集団，不顕性感染者集団，健常者集団の順に抗体価レベルが低下する傾向にあると報告している[15,16]。140 サンプルの中に不顕性感染者あるいは発症者に由来するサンプルが存在する可能性が高い。そこで全血清サンプル中の感染者サンプル割合を推定するために，血清抗体価の測定データを用いて感染者を判定する陽性カットオフ値の設定を試みた。

3. 1. 2　既往研究の医学データに基づいた陽性カットオフ値の設定

Blaser ら（1984）と Black ら（1988）は，発症者，感染者，健常者集団の血清抗体価をそれぞれ ELISA 法により測定し，その吸光度分布を表すとともに各集団間における吸光度の差を確認した[15,16]。そこで，Blaser ら（1984）と Black ら（1988）の *C. jejuni* 抗体価に関する医学データ[15,16]を本研究に用いる感染者判定陽性カットオフ値設定用の医学データと設定し，2. 3. 1 (1)の方法に従い，感染者判定陽性カットオフ値の設定を行った。

その結果，Blaser ら（1984）のデータ[15]を用いた場合，吸光度上昇率は IgA 抗体で 2.6 倍，IgG 抗体で 1.8 倍，IgM 抗体で 2.6 倍となり，感染者に対する陽性カットオフ値は，IgA 抗体で 8.2 U・mL^{-1}，IgG 抗体では 13.2 U・mL^{-1}，IgM 抗体では 21.9 U・mL^{-1} と計算された。一方 Black ら（1988）のデータ[16]を用いた場合，吸光度上昇率は IgA 抗体で 2.4 倍，IgG 抗体で 1.7 倍，IgM 抗体で 1.3 倍となり，感染者に対する陽性カットオフ値は，IgA 抗体で 9.0 U・mL^{-1}，IgG 抗体では 14.9 U・mL^{-1}，IgM 抗体では 47.3 U・mL^{-1} と計算された。

本研究では，Blaser ら (1984)[15] および Black ら (1988)[16] の報告に示された吸光度上昇率をともに使用したため，陽性カットオフ値は一意的に定まらなかった。そこで，今回推定された 2 つの陽性カットオフ値を，医学データに基づいて推定した陽性カットオフ値として採用した。

3. 1. 3　統計学的手法に基づいた陽性カットオフ値の設定

(1)　最大尤度しきい値選定法による血清抗体価分布の領域分割

最大尤度しきい値選定法の推定方法 1 でしきい値を算出した結果，IgA 抗体で 3.9～4.1 U・mL^{-1}，IgG 抗体で 10.6～10.8 U・mL^{-1}，IgM 抗体で 27.6～27.9 U・mL^{-1} となった。

一方，推定方法 2 を用いてしきい値を算出した結果，IgA 抗体で 6.6～7.0 U・mL^{-1}，IgG 抗体で 11.5～12.3 U・mL^{-1}，IgM 抗体で 32.1～36.1 U・mL^{-1} となった。なお，ある抗体価の範囲でデータが存在しなかった場合，各推定方法の判定に用いる最大対数尤度がしきい値が変動したとしても変動しないケースがあるため，2 つの推定方法ともにしきい値が一点では決定できず範囲が生じた。

最大尤度しきい値選定法では各分布設定用データ群が正規分布に従うと仮定している。そこで 2 種類の推定方法から得られたしきい値により 2 分化したデータ集団に対して，コルモゴロスースミノフ検定を行った。推定された正規分布のパラメータと有意確率を **Table 1** にまとめる。IgA 抗体の高抗体価分布設定用データ群（推定方法 1）と IgA 抗体の低抗体価分布設定用データ群（推定方法 2）に関しては，「正規分布に従う」という仮説が棄却された。そのため，今回測定した IgA 抗体データ群には，この手法は不適切であると判定された。その理由として IgA 抗体の高抗体価データ数が極端に少ないことが影響していると考えられ，IgA 抗体に関してはデータ数を増やすことで今回提案した手法でしきい値が推定可能となると思われる。

以上を踏まえて陽性カットオフ値推定には，IgM 抗体，IgG 抗体に対して正規分布に従うと仮定し，これらの設定分布に基づいて検討を行っていくこととした。また IgA 抗体に対しても，不確実性要素として取り上げ，**Table 1** に示した正規分布を設定し，陽性カットオフ値の推定を行っていくこととした。

Table 1 The parameters of normal distribution.

	Estimation method 1		Estimation method 2	
Antibody class	IgA		IgA	
Data group	Low antibody levels	High antibody levels	Low antibody levels	High antibody levels
Data	107	33	131	9
Parameters of normal distribution				
Mean values ($U \cdot mL^{-1}$)	1.6	6.4	2.2	10.4
Standard deviation	1.06	3.32	1.62	4.24
Significance probability	0.563	0.041	0.025	0.418
Antibody class	IgG		IgG	
Data group	Low antibody levels	High antibody levels	Low antibody levels	High antibody levels
Data	116	24	120	20
Parameters of normal distribution				
Mean values ($U \cdot mL^{-1}$)	4.4	17.0	4.7	18.1
Standard deviation	2.65	6.21	2.87	6.16
Significance probability	0.248	0.123	0.139	0.147
Antibody class	IgM		IgM	
Data group	Low antibody levels	High antibody levels	Low antibody levels	High antibody levels
Data	107	33	123	17
Parameters of normal distribution				
Mean values ($U \cdot mL^{-1}$)	16.8	38.7	18.5	46.9
Standard deviation	4.34	10.6	6.03	8.59
Significance probability	0.967	0.080	0.251	0.530

(2) ２種類の過誤による平均損失に基づいた陽性カットオフ値の推定

式(1)と各抗体価データ群に対する設定分布を用いて，陽性カットオフ値を算出した。その結果，推定方法１を用いた場合，本手法により推定された陽性カットオフ値は，IgA抗体で5.1 $U \cdot mL^{-1}$，IgG抗体で11.2 $U \cdot mL^{-1}$，IgM抗体で30.8 $U \cdot mL^{-1}$と計算された。一方，推定方法２を用いた場合，IgA抗体で10.3 $U \cdot mL^{-1}$，IgG抗体で13.0 $U \cdot mL^{-1}$，IgM抗体で34.0 $U \cdot mL^{-1}$と計算された。本研究では，推定方法１と推定方法２で異なる前提条件を用いて推定を行っており，陽性カットオフ値を１点に決定することはできなかった。そこで，今回推定された２つの陽性カットオフ値を，統計学的手法に基づいて推定した陽性カットオフ値として採用した。

3.2 感染者判定の抗体クラスの選定および血清抗体価分布に基づいた一般集団中の感染者割合の推定

感染者を判定する上でもう一つの重要な要素が，感染者判定に用いる抗体クラスの選定である。抗体クラスは，クラスごとに異なる *C. jejuni* 感染に対する抗体価上昇パターンを示す。IgA抗体，IgM抗体は感染後短期間で抗体価が上昇し，２ヶ月程度で健常者と同レベルまで低下する傾向を示す[17]。一方で，IgG抗体は抗体価の持続期間が長く，１年以上も高い抗体価を持続するケースもある[17]。Angら（2007）は，IgG抗体は若年層で上昇を示さないケースがあり，IgA抗体とIgM抗体の方が発症者の指標として適切であると報告している[18]。そこで，得られた各抗体クラスの血清抗体価分布から，本研究の感染－発症割合推定方法に適切な抗体クラスを選定する必要がある。

まず抗体価測定結果に対して，各抗体クラスの年齢群ごとの抗体価分布を調べた。その分布をFig. 2に示す。若年者ではIgM抗体が高い抗体価を示しており，一方でIgA，IgG抗体は30－39歳でピークを示し，その後徐々に低下する傾向となった。そのため若年者の判定にはIgM抗体が有効であり，年配者の判定にはIgA抗体あるいはIgG抗体が有効と判断した。

IgA抗体とIgG抗体に関して抗体価の上昇，持続パターンが大きく異なることから，感染者判定の際に片方のみあるいは両方の抗体クラスを用いるかを選定する必要がある。そこで，同一検体におけるIgA抗体価とIgG抗体価の関係を調べた。その結果，IgA抗体とIgG抗体は年齢による分布が同傾向であるにも関わらず，IgA抗体，IgG抗体のみが高い抗体価を示すケースが存在した。

IgA抗体のみ抗体価が高いケースは，*C. jejuni* 曝露後抗体価が上昇するまでの期間がIgA抗体の方がIgG抗体と比較して短いため，直近の感染履歴があることを表すと考えられる。IgG抗体に関しては，Stridら（2001）が *C. jejuni* 感染症患者の抗体価変動を２年間追跡した結果，２年間が経過しても高い抗体価を示すケースがあることが示されている[17]。しかし，それ以降の抗体価変動は明らかでないため，感染あるいは発症により高いIgG

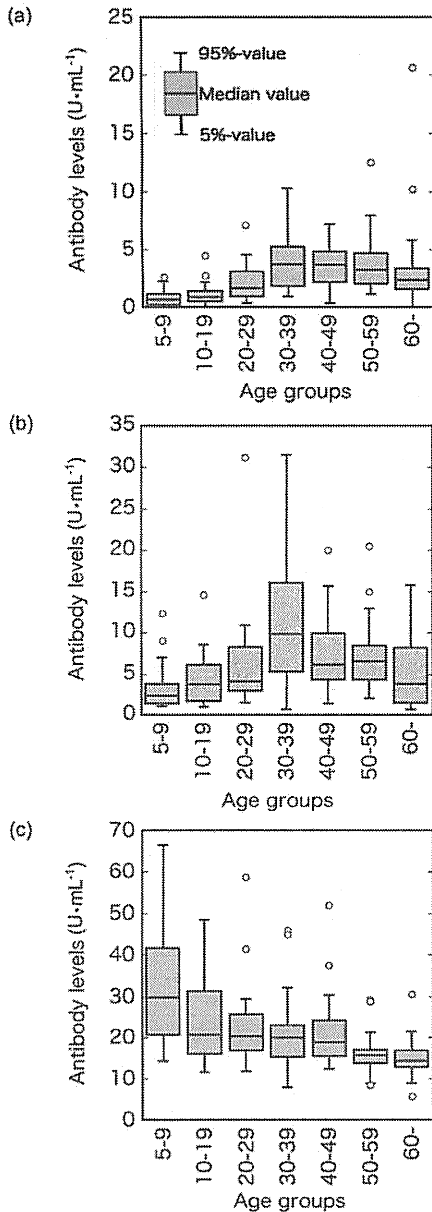

Fig. 2 The distributions of antibody levels in age groups. ((a) IgA (b) IgG (c) IgM)

抗体価を保有した場合に，健常者レベルまでに抗体価が減少する期間が予測できない状況にある。つまり，IgG抗体のみが高い抗体価を持つ人の感染時期を特定することができないため，調査期間より以前に感染した人を調査期間内に感染したものと判断してしまう可能性がある。

一方でIgA抗体あるいはIgM抗体を判定に用いる場合でも，3年間の調査期間を考慮した場合に，調査期間に感染したにも関わらずIgA抗体およびIgM抗体の抗体価が健常者レベルまで低下している人を見逃してしまう可能性は否めない。しかしIgG抗体とは異なり，IgA抗体，IgM抗体は抗体価が変動する期間が感染後2ヶ月程度である[17]ため，IgA抗体あるいはIgM抗体が陽性であれば本研究で設定した調査期間に感染したと見なすことができると考えられる。本研究ではこれらの特徴を重視し，IgA抗体およびIgM抗体を直近の感染履歴判定に用いることとした。そして，IgA抗体，IgM抗体が3.1で設定した感染者に対する陽性カットオフ値以上の抗体価を示したサンプルを，採血年に感染した経歴を持つサンプル（感染者サンプル）と判定した。

この判定基準を用いて一般集団における感染者割合を推定した結果，医学データを用いた推定方法では，7.9〜35.0%と推定された。統計学的手法に基づいた推定方法では，IgA抗体を使用することができないため，ここではIgM抗体のみで感染者の判定を行った。その結果，一般集団における感染者割合は12.1〜13.6%と推定された。参考としてIgA抗体を含んだ場合の感染者割合を推定すると14.3〜23.6%となり，IgA抗体による判定の有無で感染者割合が変動することが確認された。これは，IgM抗体が若年者の判定に，IgA抗体が年配者の判定に有効であるという特性が影響していると考えられる。IgM抗体のみでは年配者の抗体価上昇があまり見られず，年配者の直近の感染履歴判定が難しいため，IgM抗体のみで感染者を判定する場合に年配者に対して誤陰性を示してしまう可能性がある。そのため、年配者の直近の感染履歴判定としてIgA抗体が取り上げられており，より正確に感染者割合を推定するためにはIgA抗体による感染者判定も必要であるといえる。

3.3 病原体分離報告数に基づいた *Campylobacter jejuni* 発症者数の推定および感染−発症割合の推定

神戸市における *C. jejuni* 感染症実患者数を推定するために，まず人口カバー率，糞便検査率，医療機関受診率について推定を行った。アンケート調査により13の対象医療機関の外来患者延数を調査した結果，定点医療機関における平成20年度の外来患者延数が2,566,012人であった。そして病院報告・医療施設調査で報告されている精神病院を除いた神戸市全体の医療機関の外来患者延数[19]が8,086,015人であり，人口カバー率は31.7%となった。

次に糞便検査率と医療機関受診率の設定を行った。宮城県で行われた下痢症患者の実被害者数把握研究では，全国の電話調査の結果と宮城県の電話調査の結果が，ほぼ同程度であることを報告している[14]。また，人口の年齢別分布が宮城県と神戸市ではほとんど変化がなかったため，人口分布補正の影響は無視できると考えられる。そこで，宮城県での電話調査により推定された糞便検査率10.9%[14]と医療機関受診率32.0%[14]を，本研究での *C. jejuni* 感染症実患者数推定に用いることとした。

実患者数推定のために2006年から2008年の3年間の分離報告数を調査した結果，2006年から2008年の3年間の分離報告数は2,159件となった。なお，神戸市では対象医療機関からの報告率は100%である。この報告数と設定した人口カバー率，糞便検査率，医療機関受診率を用いて実患者数を推定したところ，3年間の実患者数は195,262人となり，年間10万人あたり4,252人が *C. jejuni* 感染症を発症したと推定された。

C. jejuni 感染症実患者数において日本国内では大きな差はないと予想されるものの，実患者数推定に必要な人口カバー率，糞便検査率，医療機関受診率，そしては定点医療機関から地方衛生研究所への *C. jejuni* 菌体分離数の報告率といった要因の設定方法を変更した場合，実患者数の推定値は大きく変動する可能性がある。そのた

Table 2 The parameters for estimating the illness-to-infection rate of *C. jejuni*.

	Estimation method based on medical data	Estimation method based on statistical method
The rate of asymptomatic infections (%)	7.9~35.0	12.1~13.6
The number of asymptomatic infections (person)	362,759~1,607,161	555,619~624,496
Correction factor		
Population coverage rate (%)	31.7	31.7
Consultation rate (%)	32.0	32.0
Stool sampling rate (%)	10.9	10.9
Laboratory-confirmed cases	2,159	2,159
The number of symptomatic infections (person)	195,262	195,262
The illness-to-infection rate of *C. jejuni* (%)	12.1~53.8	31.3~35.1

め，各要因の設定方法に関して様々な地域の情報を用いて検討していく必要がある。しかし，定点医療機関から地方衛生研究所への *C. jejuni* 菌体分離数の報告率の設定は，地方衛生研究所，あるいは定点医療機関へのヒアリング調査が必要となる。報告率は病原体検出情報の *C. jejuni* 分離報告数の正確性に関与しているため，実患者数推定に大きな影響を及ぼすと考えられる。そのため，神戸市以外の地域で *C. jejuni* 感染症実患者数を推定する場合は，人口カバー率，糞便検査率，医療機関受診率といった要因を患者数のデータなどで設定する一方で，*C. jejuni* 菌体分離数の報告率に関してもヒアリング調査を行うことで情報を集めることが重要である。そして対象地域においてそれらの情報が得られた場合に，本研究で用いた推定方法によって *C. jejuni* 感染症実患者数を推定し，感染－発症割合を算出することが可能であると考えられる。

最後に，感染－発症割合推定に必要なパラメータと推定結果を Table 2 に示す。その結果，感染－発症割合は，既往研究での医学データを用いた推定方法で 12.1～53.8%，統計学的手法に基づいた推定方法で，IgM 抗体のみを判定基準にした場合に 31.3～35.1%と推定された。一方 IgA 抗体を判定基準に組み込んだ場合は，感染－発症割合が 18.0～29.7%と推定された。

IgM 抗体は若年者の判定に有効である一方で年配者に対しては誤陰性を示す可能性がある。年配者の判定に IgA 抗体を用いていることから，全年齢群における感染－発症割合を推定するためには IgA 抗体の判定が必要である。そのため IgA 抗体の抗体価に関するデータ数をより多く確保することで，統計学的手法に基づいた推定方法で IgA 抗体による判定が可能か否かを判定する必要があるといえる。

また Black ら（1988）が行ったボランティア試験結果では感染－発症割合は 0～60.0%の範囲に存在しており[16]，2つの推定方法で得られた推定値も共にこの範囲内にあった。本手法を用いて感染－発症割合を推定することで，現状の感染－発症割合の設定値である 0.8～100%と比較してその範囲を大幅に小さくでき，結果として DALYs 推定の不確実性を小さくすることが可能である。また他の病原微生物による感染－発症割合を推定する際に，血清抗体価測定情報に対する対象者の病原微生物感染状況といった付随する情報が限られる場合において，本研究で確立した推定方法により感染－発症割合を推定することが可能であるといえる。そして本研究で確立した推定方法により感染－発症割合を推定する際には，医学データの有無，十分な血清抗体価のデータ数の確保，*C. jejuni* 分離報告数の正確性などの諸条件を考慮した上で，感染－発症割合推定に必要なデータの取得および推定方法を選択していく必要がある。

4. まとめ

水道水の微生物リスク評価指標として DALYs を取り上げ，*C. jejuni* を対象として，その計算過程において不確実性が高い因子である感染－発症割合を血清疫学調査を用いて推定した。血清疫学調査により，一般集団における感染者割合は医学データを用いた推定方法では 7.9～35.0%，統計学的手法に基づいた推定方法では，12.1～13.6%と推定された。一方，神戸市の *C. jejuni* 分離報告数を基に推定した発症者数から *C. jejuni* 感染による感染－発症割合を求めた結果，医学データに基づいた推定方法では 12.1～53.8%，統計学的手法に基づいた推定方法では 31.3～35.1%と評価され，感染－発症割合の範囲を大幅に小さくすることができた。そのため血清疫学調査による感染－発症割合の推定により，DALYs 推定の精度向上が可能になる。

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[論 文 要 旨]

水道水を介した水系感染症による健康影響を表す指標として, 障害調整生存年数 (Disability Adjusted Life Years: DALYs) に着目し, *Campylobacter jejuni* を対象として, その評価値に最も影響を与える因子である感染－発症割合を, 血清疫学調査に基づいて推定した。まず血清疫学調査で得られた血清抗体価データに対して, 既往研究の医学データ, 統計学的手法を用いて感染者を判定する陽性カットオフ値を設定し, 一般集団における感染者割合を求めた。その結果, 7.9～35.0% (医学データ), 12.1～13.6% (統計学的手法) と推定された。最終的に *C. jejuni* 分離報告数に基づいて推定した発症者数から *C. jejuni* 感染による感染－発症割合を求めた結果, 12.1～53.8% (医学データ), 31.3～35.1% (統計学的手法) と評価された。

キーワード：障害調整生存年数；水系感染症；カンピロバクター・ジェジュニ；感染－発症割合；血清疫学調査

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Comparison of inflammatory responses in human cells caused by lipopolysaccharides from *Escherichia coli* and from indigenous bacteria in aquatic environment

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The endotoxic activities of lipopolysaccharides (LPSs) in water samples are usually determined using a *Limulus* amoebocyte lysate (LAL) assay, but it is known that the determined activities do not always represent their inflammatory potency in humans. In this investigation, the inflammatory responses in three different human cells stimulated with *Escherichia coli* LPS, keratinocyte, $CD14^+$ monocyte, and THP-1, were compared using cytokine secretion as biomarkers to develop novel *in vitro* assay systems for detecting changes in inflammatory potencies of endotoxins in aquatic environment. Only THP-1 with 6-h stimulation showed dose-dependent responses in the range of normal endotoxin levels in aquatic environment. Then, the inflammatory potency of environmental LPS, which was purified from river water, was tested using THP-1. The levels and patterns of cytokine secretion after the environmental LPS stimulation were completely different from *E. coli* LPS. Interleukin 8 (IL-8) secretions after the environmental LPS stimulation were approximately 10-fold higher than those after *E. coli* LPS stimulation. The environmental LPS also induced much higher levels of TNF-α secretions in THP-1. These results suggest that a diversity of LPS structures in aquatic environment could contribute to stronger and different inflammatory responses. This investigation indicated that the proposed THP-1 assay system could be useful for detecting the changes in inflammatory potencies caused by aquatic bacteria.

Keywords: Lipopolysaccharide (LPS), endotoxin, human keratinocyte, human blood cells, cytokine secretion, aquatic environment.

Introduction

It is widely known that lipopolysaccharides (LPSs), which are outer membranes of gram-negative bacterial or cyanobacterial cells, can cause strong innate immune reactions in humans via Toll-like receptor 4 (TLR-4) on cell surfaces. Their biological activities are also called endotoxin. In aquatic environments or manmade water systems, there are various situations in which endotoxins increase: cyanobacterial blooms in water resources[1], bacterial regrowth in piped water systems[2], water reuse[3] and so on.

An increase of endotoxins caused by cyanobacterial blooms requires enhancement of physicochemical removal process, such as coagulation-sedimentation or media filtration. The endotoxins in piped water can be increased with detachment of accumulated biofilm inside pipe systems. The bacterial communities inside pipes varying from systems to systems have not yet been elucidated, but Norton and LeChevallier[4] reported that gram-negative bacteria were dominant in biofilm.

Reclamation of wastewater in particular is increasingly gaining attention as a solution to reduce imbalances in the quantity and quality of water resources in urban areas. However, treated wastewater usually contains a higher level of endotoxins than virgin water, not only because the sewage itself contains a high level of bacterial substances but because endotoxins are released from activated sludge[5] during the decay process in sewage treatment. Thus, as a result of concern about increasing endotoxin levels in aquatic environments, knowledge of the endotoxin levels and their changes has been accumulating to enhance endotoxin monitoring.

To determine endotoxin levels in water samples, *Limulus* amoebocyte lysate (LAL) assay is widely used. This highly sensitive assay is based on the coagulating reaction of LAL with endotoxin, and can detect trace amounts of endotoxin. In this method, relative endotoxin levels in samples to *Escherichia coli* (*E. coli*) LPS reference standard are determined. However, there has been an on-going argument

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that endotoxic activity determined by LAL assay represents the potency to induce immune responses in humans.[6–7] As the relationships between the endotoxic activity and LPS structures were determined for some enteric/non-enteric bacteria, it became known that the differences in the chemical structures of LPS could also contribute to the potency to induce immune responses in humans.[8]

However, there is still little available information on endotoxin toxicity caused by indigenous bacteria in aquatic environments. Pool et al.[9] and Wichmann et al.[10] attempted to evaluate the inflammatory potency of endotoxins in river water using human whole blood cells or peripheral blood mononuclear cells (PBMCs) from healthy donors. These methods were originally developed as alternative methods to animal testing to detect pyrogenic substances including endotoxins, and were recognized as very sensitive screening tools for pharmaceuticals contaminated with trace levels of pyrogenic substances.[11–12]

Unfortunately, these methods may not always work well in the quantitative detection of endotoxins in aquatic environments because the endotoxin levels are usually much higher than the contaminated pharmaceuticals. There is also a lot of individual variation in the reactivity of blood cells collected from different donors. These disadvantages made it more difficult to compare the obtained data by different assay systems, and therefore novel assay systems for pharmaceuticals using monocytoid cell lines were proposed to mitigate the variation. [13–14] Thus, an appropriate *in vitro* assay system, which would enable us to detect endotoxin contamination and to assess the toxicity in water samples taken from the environment or water systems, is needed.

In this investigation, three different cell lines, normal human epidermal keratinocyte (NHEK) and two types of blood cells were compared regarding their responsiveness to endotoxin stimulation. Keratinocytes make up approximately 95% of the cells in the human epidermis, and it is well known that keratinocytes express cell surface TLR-4 and play a role in cutaneous inflammation and immune responses by dermal contact of bacterial agents[15] as well as acting as a barrier for environmental stimuli.

Based on the comparison results, the most appropriate cell line was chosen to detect changes in toxicity caused by endotoxins in the aquatic environment. Then, LPSs sample derived from indigenous bacteria on river water was purified. The cells were then exposed to the purified environmental LPS, and the cytokine secretions were determined to estimate the relative toxicity of endotoxins derived from bacteria in aquatic environments to that derived from *E. coli*.

Materials and methods

Water sampling

A 21.8 L river water sample was taken in the downstream area of the Yodo River in Osaka (N34.72.48.47, E135.51. 30.37) in December 2010. The water container was soaked with Pyroclean (Alerchek, Inc., Portland, ME, USA) for more than 1 hour to degrade endotoxin contamination, and then rinsed thoroughly with endotoxin-free Milli-Q water. The endotoxin-free Milli-Q water was produced with Milli-Q Academic equipped with a BioPak cartridge as a final filter. The water sample was transported to our laboratory under refrigeration at 4 °C. The culturable and total bacterial cell counts were determined by pour plating using R2A agar plates and fluorescence microscopic enumeration after 4'6-diamidino-2-phenylindole (DAPI) staining, respectively.

Endotoxin extraction

First, the river water sample was filtrated through a glass fiber filter (GA-100, Advantec Toyo Kaisha, Ltd., Tokyo, Japan) and then enriched using an ultrafiltration device with membranes of MWCO 10,000 (Q0100, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). The devices and membranes were washed with 5 N or 1 N NaOH by soaking for more than 3 h to degrade organic contaminants, and then rinsed thoroughly with endotoxin-free Milli-Q water.

After the enriched sample was lyophilized, LPS was extracted according to the method reported by Bernardová et al.[16] A brief description of the procedure follows. An equal volume of hot 90% phenol-water mixture was added to the suspension of lyophilized cells (20.3 mg) in endotoxin-free water, and then stirred at 65–70 °C for 20 min. This extraction procedure was repeated twice.

After centrifugation, the supernatant aqueous phase was collected and dialyzed using Spectra/Por 7 (1000 Da; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against endotoxin-free Milli-Q water for 3–4 days. The dialyzed sample was lyophilized again, and dissolved in 0.1 M Tris-HCl buffer containing 25 μg/mL RNase A (from Bovine Pancreas, Wako Pure Chemicals, Osaka, Japan). After incubation for 16 h at 37 °C to degrade RNA, an equal volume of 90% phenol-water solution was added again, and stirred vigorously. The aqueous phase containing LPS was separated by centrifugation at 9600 $\times g$ and dialyzed sufficiently against endotoxin-free water using Amicon Ultra-15 (MWCO 3 kDa, Millipore Japan, Tokyo). Finally, the dialyzed sample was lyophilized and stored at −80 °C until further assay.

Cell culture

Normal human epidermal keratinocytes (NHEK (B); adult breast skin) were purchased from Kurabo Industries Ltd. (Osaka, Japan), and cultured in serum-free keratinocyte growth medium, EpiLife (Life Technologies Japan Ltd., Tokyo, Japan), supplemented with purified bovine serum albumin, purified bovine transferrin, hydrocortisone, recombinant human insulin-like growth factor type-1, prostaglandin E2, and recombinant human epidermal growth factor. The assays were performed using four passages NHEK.

Cryopreserved $CD14^+$ monocyte was purchased from TAKARA BIO Inc. (Otsu, Shiga, Japan) and cultured in LGM-3 medium (Lonza, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The assays were performed using monocytes after 3–7 days incubation. THP-1 (JCRB0112.1) purchased from the Japanese Collection of Research Bioresources Cell Bank was cultured in RPMI1640 medium with 5% heat-inactivated FBS. The passages of the THP-1 cells were unknown. All culture flasks were incubated at 37 °C in humidified 5% CO_2.

Endotoxin stimulation

Aliquots of 100 μL NHEK suspension at 1.0×10^5 cells/mL were seeded in flat-bottomed 96-well culture plates (Greiner Bio-One Co. Ltd., Tokyo, Japan) and incubated at 37 °C in humidified 5% CO_2 for 24 h prior to endotoxin stimulation. The non-adherent cells, $CD14^+$ monocyte and THP-1 were suspended into each medium at 1.0×10^6 cells/mL. Aliquots of 100 μL cell suspensions were seeded in the same way, and then preincubated for 3 h. LPSs purified from *E. coli* O55:B5 (Sigma Aldrich Japan) or the river water samples were dissolved in endotoxin-free Milli-Q water and filtrated through with a sterilized 0.2 μm syringe filter (DISMIC-25CS, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). After preincubation, the culture medium in each well was removed and 100 μL fresh medium was added. The NHEK was stimulated for 48 h by adding aliquots of 10 μL LPS suspension into each well. The blood cells ($CD14^+$ monocytes and THP-1) were stimulated 6 and 24 h in the same manner.

Determination of cytokine levels and cell counts

After stimulation with LPS, the culture supernatants were removed and stored at −80 °C until cytokines determination. An aliquot of 100 μL fresh medium was added to each well again, and the cells were incubated for 30 min. Viable cell counts were then determined using Cell Counting Kit-8 (Dojin Laboratories, Tokyo, Japan). The cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-8, were measured by indirect sandwich ELISA assays (Diaclone, Gen-Probe Inc., San Diego, CA, USA) following the manufacturer's instructions.

Endotoxin determination

The endotoxins in river water samples were fractionated by centrifugation at 14,000 rpm for 10 min at 4 °C, and the supernatant fractions were used for free endotoxin determination. The endotoxins in the water samples were determined by a kinetic-chromogenic LAL assay using Pyrochrome (Seikagaku Biobusiness Corporation, Tokyo, Japan). The unintentional reaction with β-glucan remaining in the samples was blocked using a Glucashield buffer. LPS purified from *E. coli* strain O113:H10 (Seikagaku Biobusiness Corporation, Tokyo, Japan) was used for calibration, and the results were represented in endotoxin units (EU). Each sample was diluted with endotoxin-free Milli-Q water. Pipette chips and microplates guaranteed to be endotoxin-free were used for assay.

RT-PCR assay for mRNA expression levels of TLR-2

This assay was performed to check an involvement of contaminated substances from gram-positive bacteria in the purified LPS from the river water sample. Toll-like receptor 2 (TLR-2) can recognize the cell components of gram-positive bacteria such like peptidoglycan or lipoteichoic acid, and the expression level changes by stimulation with those compounds. THP-1 was stimulated with LPSs purified from *E. coli* O55:B5 and the river water for 2 h at 0, 500, 1000 EU/mL, and then total RNA was extracted from the cells using RNeasy Plus Micro Kit (Qiagen K.K., Tokyo, Japan). Reverse transcription of the RNA samples to cDNA was performed with PrimeScript II 1st strand cDNA synthesis Kit (TAKARA BIO Inc., Otsu, Shiga, Japan). PCR using primer pairs for TLR-2 was basically performed according to the method reported by Yang et al.[17] using TAKARA PCR Thermal Cycler Dice. Amplified products were visualized on 1.5% agarose gels stained with ethidium bromide under UV light.

Contaminant assay by blocking endotoxin activity

The contribution of contaminated substances in the purified LPS from the river water sample to the changes in cytokines secretion of THP-1 was also evaluated by adding Polymixin B sulfate (Sigma Aldrich Japan) as an inhibitor of endotoxin activity. The LPS samples purified from *E. coli* O55:B5 and the river water (5000 EU/mL) were pretreated with 50 μg/mL Polymixin B for 1 hour at room temperature. Then, THP-1 was stimulated with the pretreated LPSs at 500 EU/mL and the cytokine secretions were determined in the same way as above-mentioned.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Prism Inc., San Diego, CA, USA). Data were compared by *t*-test, and the significant differences were determined with a level of $p < 0.05$ in all analyses.

Results and discussion

Cytokine secretions in three different cell lines stimulated with endotoxin

NHEK. The cell viability of NHEK after stimulation with purified *E. coli* LPS is shown in Figure 1 (a). The final endotoxin concentrations in the culture medium ranged from 0 to 75000 EU/mL. A cytotoxic effect caused by