Estimation of concentration ratio of indicator to pathogen-related gene in environmental water based on left-censored data

Tsuyoshi Kato, Ayano Kobayashi, Toshihiro Ito, Takayuki Miura, Satoshi Ishii, Satoshi Okabe and Daisuke Sano

ABSTRACT

A stochastic model for estimating the ratio between a fecal indicator and a pathogen based on left-censored data, which includes a substantially high number of non-detects, was constructed. River water samples were taken for 16 months at six points in a river watershed, and conventional fecal indicators (total coliforms and general *Escherichia coli*), genetic markers (*Bacteroides* spp.), and virulence genes (*eaeA* of enteropathogenic *E. coli* and *ciaB* of *Campylobacter jejuni*) were quantified. The quantification of general *E. coli* failed to predict the presence of the virulence gene from enteropathogenic *E. coli*, different from what happened with genetic markers (Total Bac and Human Bac). A Bayesian model that was adapted to left-censored data with a varying analytical quantification limit was applied to the quantitative data, and the posterior predictive distributions of the concentration ratio were predicted. When the sample size was 144, simulations conducted in this study suggested that 39 detects were enough to accurately estimate the distribution of the concentration ratio, when combined with a dataset with a positive rate higher than 99%. To evaluate the level of accuracy in the estimation, it is desirable to perform a simulation using an artificially generated left-censored dataset that has the identical number of non-detects as the actual data.

Key words | analytical quantification limit, *Bacteroides*, Bayesian estimation, indicator micro-organisms, left-censored data, pathogens

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INTRODUCTION

Fecal indicator micro-organisms, such as coliforms, fecal coliforms, and *Escherichia coli*, are used for determining the sanitary quality of surface, recreational, and shellfish growing waters (Scott *et al.* 2002; Setiyawan *et al.* 2014). The concentration of fecal indicator micro-organisms is often used in quantitative microbial risk assessment for estimating the pathogen concentration based on the indicator/pathogen concentration ratio (Labite *et al.* 2010; Itoh 2013). This indicator/pathogen concentration ratio is acquired based on the quantitative data of a field investigation, in which a pathogen and a fecal indicator are simultaneously quantified from an identical sample (Machdar *et al.* 2013; Silverman *et al.* 2013; Lalancette *et al.* 2014). However, pathogen quantitative data commonly include a substantially high number of non-detects, in which the pathogen concentration falls below the quantification limit (Wu *et al.* 2011; Kato *et al.* 2013). This kind of dataset is called a left-censored dataset, which does not allow us to calculate the indicator/pathogen concentration ratio at each investigation event.

Substitution of the non-detect data with specific values, such as the limit of quantification, the half value of quantification limit, or zero, has been used as a classical approach for dealing with non-detects, but the substitution gives an inaccurate estimation of distribution parameters when the

doi: 10.2166/wh.2015.029

distribution of concentration is predicted (Gilliom & Helsel 1986; Helsel 2006). Alternatively, the Bayesian approach adapted for left-censored data was proposed, and applied to actual left-censored datasets such as pesticide residue concentrations in food (Paulo *et al.* 2005). To study the density of enteric viruses in wastewater, such as those in the genus of *Enterovirus, Heptovirus, Rotavirus*, and *Norovirus* (Bosch *et al.* 2008), we previously applied the Bayesian model proposed by Paulo *et al.* (2005) with a slight modification in which the occurrence of the real zero of virus density is not assumed (Kato *et al.* 2013). In Kato *et al.*'s (2013) model, virus density is assumed to follow a lognormal distribution, which is one of the probabilistic distributions previously modeled for enteric virus density in water (Tanaka *et al.* 1998).

In this study, we employed the extended Kato *et al.* (2013) model to estimate the distribution of the indicator/pathogen concentration ratio, with a further modification of the entry of quantification limit value. The previous model (Kato *et al.* 2013) requires entering an identical quantification limit value within a dataset. However, the quantification limit values change over time due to changes in methods, protocols, and instrument precision even within a single laboratory (Helsel 2006). Those facts motivated us to develop a new Bayesian model to analyze a dataset with a varying quantification limit. Let us refer to the Kato *et al.* (2013) model as the common limit model hereafter. Datasets of concentrations of pathogens and indicator microorganisms were acquired from a watershed, and posterior predictive distributions of these concentrations were estimated with the new Bayesian model for varied quantification limit values. To ensure the accuracy of the prediction, 100 paired datasets were artificially generated, in which the simulated data were assigned to detects and non-detects by setting values of the limit of quantification to obtain the number of detects that was identical to the actual data. Then, the new Bayesian model for varied quantification limit values was applied to the simulated and censored data. The estimated mean and standard deviation were compared with true values by calculating root mean square deviation (RMSD), and the influence of the sample size and positive rate value on the estimation accuracy of the posterior predictive distribution was discussed. Furthermore, a numerical procedure is employed to obtain the distribution of the concentration log-ratio between a fecal indicator and a pathogen by integrating the posterior predictive distribution of the fecal indicator concentration with that of pathogen concentration. The accuracy of the distribution estimation of the fold change was evaluated by Kullback–Leibler (KL) divergence.

METHODS

Water samples and measurement of water quality parameters

River water samples (10 L of surface water) were collected from Toyohira River (Site 1), Kamogamo River (Site 2), Nopporo River (Site 3), Atsubetsu River (Site 4), and Motsukisamu River (Site 5) in Sapporo City, and Atsubetsu River (Site 6) in Ebetsu City, Hokkaido, Japan (latitude–longitude locations are listed in Supplementary data, Table S1, available in the online version of this paper). River water samples were collected about twice a month from January 2012 to April 2013. The total sample number was 144. No major fecal contamination sources were located near Site 1. On the other hand, effluents from wastewater treatment plants were discharged in proximity to Sites 3 and 5. Wild waterfowl, such as wild ducks, were observed in Sites 2 and 5. Domestic stock farms were located near Sites 4 and 6, so contamination by animal feces is expected in these sites. Total coliforms and general *E. coli* were measured for each water sample according to the standard method using a defined substrate (APHA 2005).

Recovery of bacterial cells from water samples

To monitor the DNA loss during the bacterial cell recovery and DNA extraction processes, *E. coli* MG1655 Δlac::kan was used as the sample process control (SPC) for genetic markers and pathogenic bacteria (Kobayashi *et al.* 2013b). One hundred microliters of *E. coli* MG1655 Δlac::kan were added into 5 L of river water before the recovery of bacterial cells. Bacterial cells in 5 L of river water were collected by pressure filtration with a 0.22 μm-pore-size polyethersulfone membrane filter (Millipore). Bacterial cells on the membrane filter were eluted by soaking in 30 mL of sterile phosphate-buffered saline (PBS) with a gelatin

buffer (NaH_2PO_4: 0.58 g, Na_2HPO_4: 2.5 g, NaCl: 8.5 g, and gelatin: 0.1 g per liter) and vigorously shaken by a vortex mixer (Ishii *et al.* 2014b). Suspended cells in the PBS with a gelatin buffer were collected by centrifugation at 10,000 ×*g* for 15 min at 4 °C, and the pellet was re-suspended in 0.8 mL of distilled MilliQ water.

DNA extraction and quantitative PCR assays

Total DNA was extracted from bacteria cell suspensions (200 μL) obtained from water samples by using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). The concentrations of total and human-specific genetic markers (Total Bac and Human Bac, respectively), and genes of pathogenic bacteria (*ciaB* for *Campylobacter jejuni* and *eaeA* for enteropathogenic *E. coli*) were quantified using quantitative polymerase chain reaction (qPCR) methods previously developed (Okabe *et al.* 2007; Ishii *et al.* 2013). The *E. coli* MG1655 Δlac::kan gene was quantified using a qPCR method, which does not amplify indigenous bacterial genes (Kobayashi *et al.* 2013b). The virulence gene *ciaB* encodes a 73 kDa secreted protein (CiaB), which enhances the internalization of *C. jejuni* into epithelial cells (Konkel *et al.* 1999). The virulence gene *eaeA* encodes a 94–97 kDa outer membrane protein that mediates adherence of enteropathogenic *E. coli* to epithelial cells (Frankel *et al.* 1995). Levels of PCR inhibition were evaluated by the addition of internal amplification control (IAC) to the sample DNA prior to qPCR. We used Chicken-Bac plasmid (Kobayashi *et al.* 2013a) as the IAC in this study. Amplification efficiencies of IAC were calculated as quantitative values of IAC in environmental DNA samples divided by the quantitative value of IAC in a pure plasmid solution. All primers and probes used in this study are shown in Supplementary data, Table S2 (available in the online version of this paper).

The qPCR assays were performed using SYBR Green chemistry to quantify the Total Bac, *E. coli* MG1655 Δlac::kan, and Chicken-Bac (IAC). In SYBR Green assays, each PCR mixture (25 μL) was composed of 1 × SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), 1 × ROX Reference Dye (Takara Bio), 400 nM each of forward and reverse primers, and 2 μL of template DNA. A TaqMan qPCR assay was also performed to quantify Human Bac, in which each PCR mixture (25μl) was composed of 1x Premix Ex Taq (Takara Bio, Otsu, Japan), 200 nM each of forward and reverse primers, 200 nM of fluorogenic probe, and 2.0 μL of template DNA. PCR reactions using SYBR Premix Ex Taq II (Takara Bio) and Premix Ex Taq (Takara Bio) were performed in MicroAmp Optical 96-well reaction plates with Applied Biosystems 7,500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction was carried out by heating at 95 °C for 30 sec, followed by 40 cycles of denaturation at 95 °C for 5 sec, and annealing and extension at 60 °C for 34 sec. Following the amplification step, melting curve analysis was performed for SYBR Green assays to confirm that no unexpected PCR products had been obtained.

The TaqMan qPCR assay was also applied to the quantification of *ciaB* of *C. jejuni* and *eaeA* of enteropathogenic *E. coli*, in which each PCR mixture (20 μL) was composed of 1× FastStartTaqMan Probe Master (Roche, Germany), 900 nM each of forward and reverse primers, 250 nM of fluorogenic probe, and 2.0 μL of template DNA. PCR reactions using the FastStart TaqMan Probe Master (Roche) were performed in MicroAmp Optical 96-well reaction plates with the ABI PRISM 7,000 sequence detection system (Applied Biosystems). The reaction was carried out by heating at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, and annealing at 60 °C for 1 min.

A DNA template to generate standard curves for the qPCR assays was prepared using recombinant pCR 2.1-TOPO vector plasmids inserted with target sequences, as described previously (Ishii *et al.* 2013; Kobayashi *et al.* 2013a, 2013b). The standard plasmids for the quantification of these targets were prepared using the TA cloning system. For the standard plasmid for the quantification of *E. coli* MG1655 Δlac::kan, the pUC19 vector carrying the PCR amplicon generated from *E. coli* MG1655 Δlac::kan with the primer set Kan-res-F and DS-Kan-R was used. The ligated products were transformed into *E. coli* TOP10 competent cells (Invitrogen). Plasmids were extracted and purified from *E. coli* cells using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The concentrations of plasmid DNA were adjusted from 10^{-1} to 10^{-8} ng per μL and used to generate standard curves. Standard curves were generated by linear regression analysis between threshold cycles (C_T) and the concentration of the plasmid DNA using Applied Biosystems 7,500 Real-Time PCR

System software version 2.0.4. The quantification limit was defined as the lowest concentration of plasmid DNA that was amplified within the linear range of the standard curve.

Statistical methods

The normality of logarithmic concentrations of detected bacteria was determined by a chi-square goodness-of-fit test at a significance level of 0.01. A Pearson product-moment correlation coefficient at a significance level of 0.01 (two-tailed test) was calculated between datasets that were log-normally distributed. The p-values in the chi-square goodness-of-fit test and Pearson product-moment correlation coefficient were calculated by a chi-square distribution and t-distribution, respectively. All statistical analysis was done using the Microsoft Excel program version 2012 (Microsoft Corporation, SSRI, Tokyo).

Estimation of the posterior predictive distribution of indicators and virulence genes and the concentration ratio of an indicator to a virulence gene

In this study, the common limit model (Kato *et al.* 2013) was extended so that different quantification limits for different observed concentrations were expressed mathematically. Suppose we are trying to measure concentrations of a target organism n times, and each concentration $x_i (i = 1, \cdots, n)$ is non-negative and observed only when the concentration exceeds a quantification limit 10^{θ_i}. It is worth noting that, although the common limit model can express only the case of $\theta_1 = \cdots = \theta_n$, the new model allows a different quantification limit for each sample. The dataset is denoted by n tuples $(x_1, y_1),\ldots,(x_n, y_n) \in \boldsymbol{R} \times \{1, 0\}$, where y_i indicates whether the concentration is detected. If $x_i > 10^{\theta_i}$, $y_i = 1$ is given; $y_i = 0$ otherwise. The value of x_i is unknown if $y_i = 0$.

If n concentrations are assumed to follow according to the log-normal distribution with a mean parameter μ and precision parameter β, the probabilistic density of a detected concentration x_i is represented by the truncated log-normal (TLN) distribution $\mathrm{TLN}(x; \mu, \beta^{-1}, \theta) := \frac{1}{Z(\mu, \beta^{-1}, \theta)x} \exp\left(-\frac{\beta}{2}(\mu - \log_{10} x)^2\right)$ where $Z(\mu, \beta^{-1}, \theta) := \sqrt{2\pi} \ln(10) \cdot (1 - \varphi(\sqrt{\beta}(\theta - \mu)))\beta^{-1}$ and φ is the cumulative density function of the standard normal distribution. Therein, the notation $:=$ has been used to denote a definition. Note that every sample is assumed to be drawn from a probabilistic density function with an exactly equal parameter θ in the common limit model (Kato *et al.* 2013), whereas the new model permits different quantification limits for different samples, leading to probabilistic density functions of different shapes.

Derived from the fact that the normal random variable drawn from $N(\mu, \beta^{-1})$ falls short of the quantification limit θ_i with the probability $\sqrt{\beta}(\theta_i - \mu)$, it is possible to express the probability mass function of y_i as $(\varphi(\sqrt{\beta}(\theta - \mu)))^{1-y_i} (1 - \varphi(\sqrt{\beta}(\theta - \mu)))^{y_i}$. Therein, the indicator variable y_i is treated as a Bernoulli variable where, in the Bernoulli trial, one side of the unfair coin, corresponding to $y_i = 0$, appears with probability $(\varphi(\sqrt{\beta}(\theta - \mu)))$ and the other side $y_i = 1$ appears with probability $(1 - \varphi(\sqrt{\beta}(\theta - \mu)))$. Instead of the Bernoulli distribution, the common limit model uses the binomial distribution (e.g., Cohen 1959), although it is impossible to employ the binomial distribution in the setting of the different quantification limits assumed in this work.

To infer the values of model parameters, Bayesian inference is adopted. So far, many works have employed the maximum likelihood estimation. This estimation method is useful if a large sample is available, although if not, the model parameters often over-fit to the sample. To avoid over-fitting, Bayesian inference is opted for in this study. For Bayesian analysis, a definition of the likelihood function and the prior distribution is required.

Based on the modeling described above, the following likelihood function of two model parameters, μ and β, is employed: $p(X|\mu, \beta) = \prod_{i=1}^{n} (\varphi(\sqrt{\beta}(\theta_i - \mu)))^{1-y_i} ((1 - \varphi(\sqrt{\beta}(\theta_i - \mu)))TLN(x_i; \mu, \beta^{-1}, \theta_i))^{y_i}$. The prior distributions, $\mu \sim N(0, 100)$ and $\beta \sim \mathrm{Gam}(0.01, 0.01)$, are employed, following the original common limit model (Kato *et al.* 2013), where $N(m, v)$ and $\mathrm{Gam}(a, b)$, respectively, denote the normal distribution with mean m and variance v and the Gamma distribution with shape parameter a and rate parameter b.

Applying the Bayesian inference technique to the probabilistic model described above for the concentration datasets of indicators and pathogens, the predictive posterior distributions $p_{pred}(x|X_{ind})$ and $p_{pred}(x|X_{path})$, where X_{ind} and X_{path} are the datasets of indicators and pathogens,

respectively, are estimated. Then, the concentration log-ratio can be obtained as the probabilistic distribution of the difference of the two random variables. The details are referred to in Kato *et al.* (2013).

The Bayesian algorithm is summarized as follows:

1. The posterior parameter distribution $p(\mu, \beta|X_{ind})$ of an indicator's dataset, which is proportional to the product of the likelihood function $p(X_{ind}|\mu, \beta)$ and the prior parameter distribution $p(\mu, \beta)$, is computed.
2. The predictive posterior distribution $p_{pred}(x|X_{ind})$ of an unseen log-transformed concentration x is computed by integrating out the model parameters from the product of the posterior parameter distribution and the model distribution.
3. Similarly, the posterior parameter distribution $p(\mu, \beta|X_{path})$ is estimated from a pathogen's dataset, and its predictive posterior distribution $p_{pred}(x|X_{path})$.
4. The probabilistic distribution of the concentration log-ratio is obtained from two distributions $p_{pred}(x|X_{ind})$ and $p_{pred}(x|X_{path})$.

Accuracy evaluation of the extended Bayesian estimation

To test the accuracy of Bayesian estimation, 100 left-censored datasets were generated for each indicator or pathogen-related gene, and each generated dataset was applied to the extended Bayesian model to estimate distributional parameters. The generation process of a left-censored dataset with 144 total samples including n_v detects is composed of two steps. In the first step, a dataset with 144 total samples, which is equal to that of the actual dataset (Supplementary data, Table S3, available in the online version of this paper), is generated. The model parameters $(\hat{\mu}, \hat{\beta})$ estimated by the maximum a posteriori (MAP) from the posterior predictive distribution (Supplementary data, Table S3,) were regarded as true values, and used to generate 144 data points $x_1, \cdots, x_n$ from the log-normal $TLN(x; \hat{\mu}, \hat{\beta}^{-1}, -\infty)$. In the second step, a detection limit value was chosen randomly from the observed detection limit values in the corresponding actual dataset, and data points below the assigned detection limit value were erased to make the dataset left-censored. These two steps were repeated until the dataset included the target number of detects, n_v. This process was repeated 100 times, which gave 100 left-censored datasets, in which all datasets included the same number of detects, n_v. The generation of 100 left-censored datasets was conducted for each number of detects in the actual datasets (Supplementary data, Table S3). Finally, the dataset was applied to the extended Bayesian model to estimate posterior distributions of distributional parameters, which were used to obtain the posterior predictive distributions of log-concentration of microbes, and log-concentration ratio between an indicator and a pathogen-related gene. The estimated distributions of log-concentration ratio based on the generated left-censored datasets were compared with true distributions by calculating KL divergence (Kato *et al.* 2013).

RESULTS

Characterization of quantitative data

The log-normality of quantitative data was first tested because we assumed a log-normal distribution of the concentration of microbes in the Bayesian estimation. All 144 samples were positive for total coliforms and Total Bac (Supplementary data, Table S3). Normal probability plots of these microbes looked straight (Figure S1(a) and S1(c), available in the online version of this paper) but a chi-square test at the significance level of 1% showed that the logarithmic quantitative data of total coliforms were not normally distributed (p-value was 3.16×10^{-3}; Table S3). The normality of logarithmic concentration values of Total Bac was not rejected by the chi-square test at a significance level of 0.01, with a p-value of 0.03 (Table S3). Only one out of 144 samples was negative in the quantitative data of general *E. coli* and Human Bac (Table S3). The normal probability plot of the 143 logarithmic concentration values of general *E. coli* and Human Bac also looked straight (Figure S1(b) and S1(d), available online), and the normality was not rejected by the chi-square test (p-values were 0.22 and 0.01 for general *E. coli* and Human Bac, respectively; Table S3). Compared to the high positive rate of these indicator micro-organisms and genetic markers, virulence genes were detected at a lower frequency, with 39 and 28 positive samples for *eaeA* of enteropathogenic *E. coli* and *ciaB* of *C. jejuni*, respectively. The linearity of the normal probability plot larger than the

quantification limit values is the necessary condition for the log-normality of the whole dataset of these virulence gene concentrations. The normal probability plot of the logarithmic concentration values of *eaeA* looked straight, but that of *ciaB* did not (Figure S1(e) and S1(f), available online). The chi-square test did not reject the log-normality of the observed values of *eaeA* with a *p*-value of 0.33, but rejected *ciaB* with a *p*-value of 1.16×10^{-4} (Table S3). This does not assure the log-normality of the whole dataset of *eaeA* concentration, but the further analyses were performed under the assumption that the quantified values of *eaeA* concentration are log-normally distributed.

Pearson's product-moment correlation coefficients between the virulence gene *eaeA* and three indicators were calculated (Supplementary data, Table S4, available in the online version of this paper). The coefficient value between general *E. coli* and *eaeA* was 0.24 with a *p*-value of 0.17. This result means that the quantification of general *E. coli* fails to predict the presence of the virulence gene from enteropathogenic *E. coli* in the study area and period. Therefore, in the subsequent analyses, the quantitative data of general *E. coli* were not used. On the other hand, genetic markers (Total Bac and Human Bac) gave higher correlation coefficient values, which were 0.72 and 0.62 with *p*-values of 2.31×10^{-7} and 2.74×10^{-5}, respectively.

Predictive distribution of the concentration of genetic markers and *eaeA*

Since significant correlations were detected between genetic markers (Total Bac and Human Bac) and the virulence gene *eaeA*, the posterior predictive distributions of the concentration of two genetic markers and *eaeA* were estimated individually. The posterior mean values of μ and log (σ), where $\sigma = \beta^{-1/2}$, of Total Bac were 3.45 and 0.03, respectively (Table 1). These values were identical to those calculated from raw data and estimated from the normality probability plot (Table S3). Since the genetic marker of Total Bac was detected in 100% (144/144) of the samples, the posterior mean and SD (Figure 1(a)) and the predictive distribution of Total Bac concentration (Figure 1(b)) were accurately estimated. To clarify the extent of accuracy of predictive distribution with 100% positive samples (144/144), 100 datasets were simulated using the posterior mean values of μ and log (σ). Quantile values and KL divergence are shown in Table 1. When there are 100 datasets of 144 quantified values, 100-times estimation of the posterior predictive distribution gave a KL divergence of 0.04 or less.

The genetic marker of Human Bac was detected 143 times out of 144 total samples. The posterior mean values of μ and log (σ) of Human Bac were 3.04 and 0.03, respectively (Table 2). These values were similar to those estimated from the normality probability plot (Table S3), which means that one non-detect out of 144 does not affect the accuracy of the estimation. The estimation of the posterior predictive distribution of Human Bac concentration (Figures 1(c) and 1(d)) was also regarded as accurate, because the KL divergence of 100-times simulated datasets using the posterior mean values of $\mu = 3.04$ and log (σ) = 0.03 was 0.04 or less (Table 2), which was the same accuracy level as 100% positive datasets (Table 1).

Table 1 | Estimation accuracy of μ and log (σ), and the predictive distribution of Total Bac

	μ			log (σ)			
	Posterior mean	RMSD	Posterior SD	Posterior mean	RMSD	Posterior SD	Kullback–Leibler divergence
μ and log (σ) estimated by the Bayesian approach	3.45	–	–	0.03	–	–	–
Minimum	3.17	0.08	0.08	−0.02	0.03	0.03	0.00
25%tile	3.40	0.09	0.09	0.02	0.03	0.03	0.00
Median	3.45	0.11	0.09	0.03	0.03	0.03	0.00
75%tile	3.51	0.13	0.09	0.04	0.04	0.03	0.01
Maximum	3.74	0.30	0.10	0.08	0.06	0.03	0.04

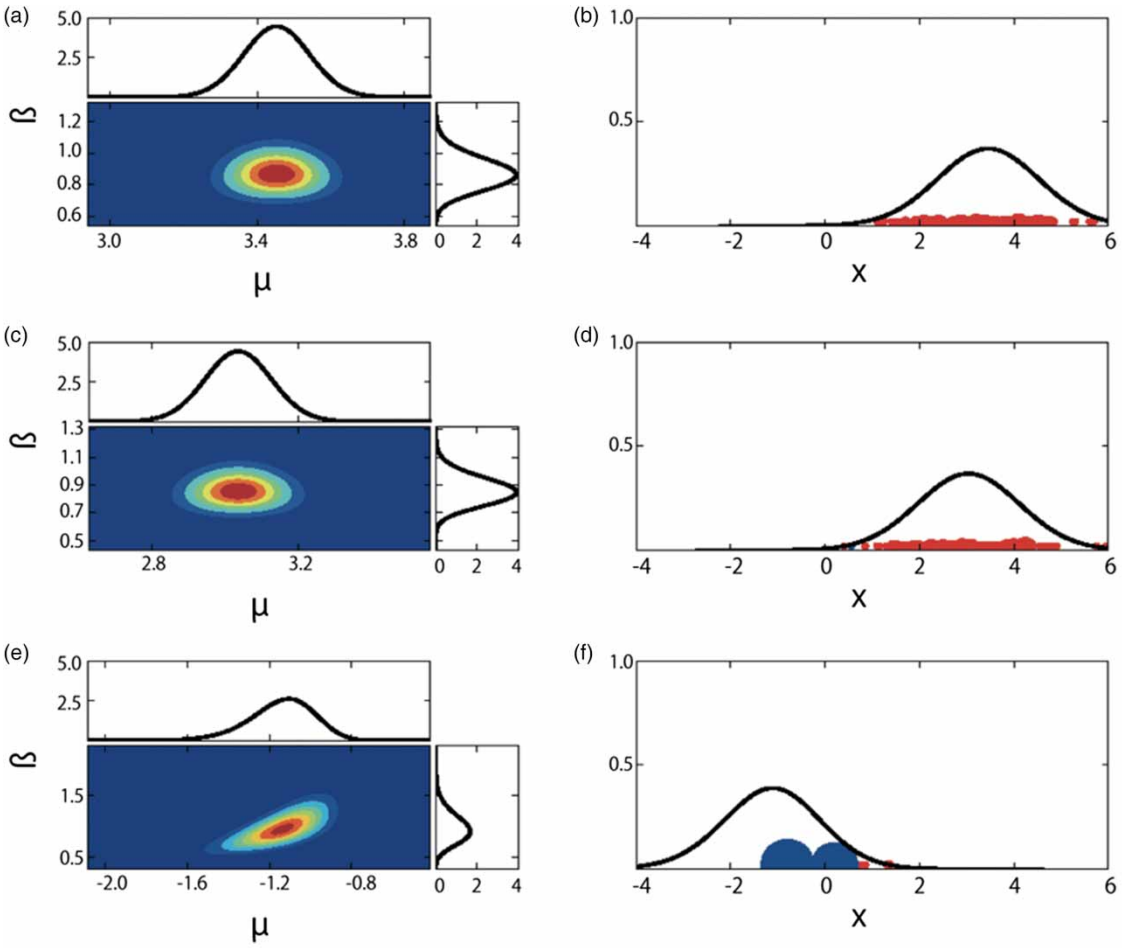

Figure 1 | Posterior distribution of μ and log (σ) and posterior predictive distribution of concentration in environmental water. (a) Posterior distribution of μ and log (σ) of Total Bac. (b) Posterior predictive distribution of Total Bac concentration. (c) Posterior distribution of μ and log (σ) of Human Bac. (d) Posterior predictive distribution of Human Bac concentration. (e) Posterior distribution of μ and log (σ) of *eaeA*, a virulence gene of enteropathogenic *Escherichia coli*. (f) Posterior predictive distribution of *eaeA* concentration. Red circles are the observed concentrations, and blue circles are the detection limits. The area of each red circle is proportional to the number of observations at the value. The area of each blue circle is proportional to the number of undetected concentrations with the detection limit at the position. Since there were two values of analytical quantification limit for *eaeA* (data not shown), there are two blue circles in panel (f).

Table 2 | Estimation accuracy of μ and log (σ), and the predictive distribution of Human Bac

	μ			log (σ)			
	Posterior mean	**RMSD**	**Posterior SD**	**Posterior mean**	**RMSD**	**Posterior SD**	**Kullback–Leibler divergence**
μ and log (σ) estimated by the Bayesian approach	3.04	–	–	0.03	–	–	–
Minimum	2.85	0.08	0.08	−0.04	0.03	0.03	0.00
25%tile	2.98	0.10	0.09	0.03	0.03	0.03	0.00
Median	3.07	0.12	0.09	0.04	0.03	0.03	0.00
75%tile	3.11	0.13	0.09	0.05	0.04	0.03	0.01
Maximum	3.29	0.27	0.11	0.11	0.08	0.03	0.04

The proportion of positives for *eaeA* (27.1%) was significantly lower than those of Total Bac (100%) and Human Bac (99.3%). The virulence gene was detected 39 times out of 144 total samples (Table S3). The small number of positives resulted in a posterior distribution with low entropy and relatively large RMSDs, as shown in Figure 1(e) and Table 3. The posterior mean values of μ and log (σ) of *eaeA* were −1.14 and 0.01, respectively (Table 3), while those estimated from the normality probability plot were −1.73 and 0.07, respectively (Table S3). To clarify the estimation accuracy, 100 simulated datasets with 27.1% positives were created using the posterior mean values of $\mu = -1.14$ and log $(\sigma) = 0.01$. The maximum KL divergence was 0.12, while 75% of estimation gave KL divergence values less than 0.02 (Table 3). It is impossible to define how accurate is accurate enough in the estimation. However, the maximum KL divergence of 0.12 is lower than that obtained when 100% positives were obtained in the total sample number of 12 (Kato *et al.* 2013), which means that the posterior predictive distribution was estimated with relatively high accuracy by using 39 detects out of 144 total samples (Figure 1(e) and 1(f)).

Distributions of the concentration ratio of genetic markers to *eaeA*

The distributions of the concentration ratio between genetic markers and *eaeA* are depicted in Figure 2. Since the posterior mean of μ for Total Bac (3.45, Table 1) was larger than that for Human Bac (3.04, Table 2), the distribution of Total Bac/*eaeA* (Figure 2(a)) shifts to the right compared to that of Human Bac/*eaeA* (Figure 2(b)). These distributions were regarded to be very accurate, because the maximum values of KL divergence were 0.02 when 100 simulated datasets of genetic markers and *eaeA* were used to estimate the distributions of the concentration ratio (Table 4). This level of KL divergence is comparable with that obtained for a pair of 100% positives with the total sample number of 48 (Kato *et al.* 2013). These results indicated that the combination of datasets with the positive rates of 27.1% (39/144) and 99.3 (143/144) gave high estimation accuracy of the distribution of the concentration ratio.

DISCUSSION

The present study attempted to establish a computational procedure for inferring the concentration ratio between a fecal indicator and a pathogen based on left-censored datasets. Field investigation in a river watershed was conducted for 16 months, and quantitative datasets of conventional fecal indicators, genetic markers, and virulence genes of pathogenic bacteria were obtained. A Bayesian model that was adapted to a left-censored dataset with varying analytical quantification limit values was applied to the quantitative dataset. The posterior predictive distributions of the concentration ratio were predicted, and we found that 39 detects out of the 144 total sample number was enough to accurately estimate the distribution of the concentration ratio, when combined with a dataset with a positive rate higher than 99%.

Table 3 | Estimation accuracy of μ and log (σ), and the predictive distribution of *eaeA*

	Mean			log (σ)			
	Posterior mean	RMSD	Posterior SD	Posterior mean	RMSD	Posterior SD	Kullback–Leibler divergence
μ and log (σ) estimated by the Bayesian approach	−1.14	–	–	0.01	–	–	–
Minimum	−1.43	0.14	0.12	−0.10	0.05	0.05	0.00
25%tile	−1.22	0.16	0.15	−0.02	0.06	0.05	0.00
Median	−1.17	0.17	0.16	0.02	0.07	0.05	0.01
75%tile	−1.12	0.20	0.18	0.05	0.08	0.06	0.02
Maximum	−1.01	0.37	0.23	0.16	0.16	0.06	0.12

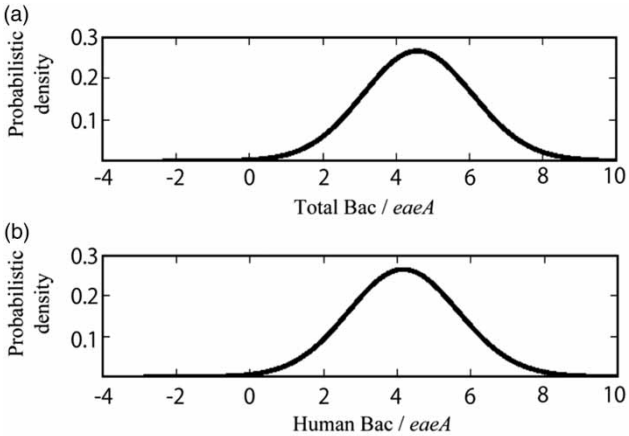

Figure 2 | Distribution of the concentration ratio between a generic marker (Total Bac or Human Bac) and *eaeA*, a virulence gene of enteropathogenic *Escherichia coli*. (a) Total Bac vs. *eaeA*. (b) Human Bac vs. *eaeA*.

Table 4 | Kullback–Leibler divergence of the distribution of the concentration ratio between *eaeA* and genetic markers

	Total Bac vs. *eaeA*	**Human Bac vs. *eaeA***
Minimum	0.00	0.00
25%tile	0.00	0.00
Median	0.00	0.00
75%tile	0.01	0.01
Maximum	0.02	0.02

The most simple and convenient approach to investigate the ratio value must be the data accumulation of the fecal indicator/pathogen concentration ratio. However, this fecal indicator/pathogen concentration ratio is usually difficult to obtain, because the concentration of a pathogen is generally low, and the significant fraction is composed of non-detects (Kato *et al.* 2013), which makes it impossible to calculate the ratio value. The approach proposed in this study overcomes the difficulty in acquiring the concentration ratio between fecal indicators and pathogens in environmental water when left-censored datasets were obtained. The application of truncated probability distribution allows us to estimate the posterior predictive distribution of microbe concentrations based on left-censored data, which can be used for inferring the distribution of the concentration ratio (Paulo *et al.* 2005).

Our analysis has three stages: the first stage checks the log-normality, the second stage confirms the correlation between parameters, and the third stage performs Bayesian analysis; although, one may think that a single framework containing all these steps is more elegant. However, when developing an algorithm for some analysis, in general a trade-off between elegance and reliability is usually faced. In this study, reliability was regarded as more important than simplicity. Unfortunately, few methods that check the normality only in a Bayesian framework are established, well-verified, and widely accepted. Therefore, rather than putting all the analysis steps into a single Bayesian framework, the chi-square goodness-of-fit test and the Spearman's correlation test, which are well-established and widely accepted, are applied for checking the log-normality and the correlation between parameters before Bayesian analysis.

We employed a chi-square test at the significant level of 0.01 for checking the log-normality of quantitative datasets. However, when the dataset is left-censored, it is impossible to test the log-normality of a whole dataset, because only a part of a dataset (values of detected data) can be used in the test. In other words, the acceptance of the null hypothesis in the chi-square test does not ensure that the log-normality assumption is really applicable. The normality check, as the first stage of the proposed approach, is just to exclude datasets that cannot be used in the framework. Under this setting, the significance level is subject to change, and in addition to it other tests such as the Shapiro–Wilk test can be employed for the normality check. Investigators should scrutinize the nature of quantified data carefully in terms of the applicability of the assumption, and can select an appropriate test at an appropriate value of the significance level. In the chi-square test, we found that the null hypothesis was rejected for total coliforms and a virulence gene of *C. jejuni* (*ciaB*) (Table S3). We therefore excluded the total coliforms and *ciaB* in subsequent analyses. Although the chi-square test did not reject the log-normality of Total Bac and Human Bac, *p*-values for these genetic markers are 0.03 and 0.01, respectively (Table S3), which means that the log-normality of datasets of these genetic markers is rejected at a significance level of 0.05 in the chi-square test. It is very apparent that one of the limitations of the proposed approach is the assumption of log-normality of datasets. Since the occurrence of micro-organisms in water is really episodic, the assumption of stationarity in parameters of concentration distribution (e.g., mean and

SD) is sometimes inappropriate (Haas & Heller 1988). It is worth preparing other Bayesian estimation algorithms using a different probability distribution, such as gamma distribution and Weibull distribution (Englehardt & Li 2011). The comparison of estimation results between the log-normality-assumed model and other distributions-assumed models may give us insights about the nature of quantified data obtained in field investigations, which should be included in further study.

The Bayesian estimation algorithm used in this study is available for a pair of parameters, even if there is no correlation between them, which means that it is possible to obtain the distribution of the concentration ratio between a pathogen and an unrelated water quality parameter. Needless to say, however, it is meaningless to investigate the concentration ratio between a pathogen and a water quality parameter devoid of the correlation with pathogen occurrence. Thus, the correlation analysis is essential as a component of the process for determining the concentration ratio between an indicator and a pathogen. However, the correlation between an indicator and a pathogen is extremely case-specific, as discussed for a long time (Wu *et al.* 2011). We detected the significant correlations between genetic markers (Total Bac and Human Bac) and *eaeA* (Table S4), which does not mean that these significant correlations can be observed in other watersheds. That is why this study is a case study presenting the estimation process of the distribution of the concentration ratio between an indicator and a pathogen. Although the result of the correlation analysis is devoid of generality, the proposed process must be available in other settings, when the log-normality of datasets is not rejected and a significant correlation is detected between pathogen and indicator concentrations. If a dataset to be analyzed does not follow the log-normal distribution, another Bayesian estimation algorithm using an appropriate probability distribution has to be prepared and applied, as already discussed above.

Accuracy is always the most important issue in the estimation of the distribution of the concentration ratio between a fecal indicator and a pathogen (Kato *et al.* 2013). Since fecal indicators are detected more easily than pathogens because of the relatively high concentration, the accuracy is usually dependent on the number of detects in the quantitative dataset of pathogens. In this study, 39 detects out of 144 total samples was enough to accurately estimate the distribution of the concentration ratio when combined with datasets with a high proportion of positives (100% for Total Bac and 99.3% for Human Bac). In Bayesian analysis, the tolerable accuracy level depends on situations of the scenarios of applications. It may be thus desirable to perform a simulation using artificially generated left-censored data that have the identical number of non-detects with the actual data, for confirming how large KL divergence is obtained.

For accuracy evaluation of our Bayesian algorithm, we generated 100 datasets artificially. Here, a justification of this procedure is described by answering two questions: why 100 datasets are needed and why artificial data are used. If only a single dataset was generated, the estimation result might be much better accidentally or might be much worse. To perform an accurate assessment, repeat experiments are necessary. Furthermore, to use quartile values for assessment, the number of repeat experiments is chosen as 100 in this study. The reason why artificial data are used is that there is no way to know the true distribution of any real-world data. By using artificial data, the assessment can be done by comparison of the estimated distribution to the true distribution that has generated the artificial data.

In the present case study, only two virulence genes from pathogenic bacteria (*eaeA* of enteropathogenic *E. coli* and *ciaB* of *C. jejuni*) were investigated. However, a variety of virulence genes from multiple pathogens are present in water environments (Ishii *et al.* 2014b), which requires us to identify the most important target for analyzing the quantitative relationship with indicators. It may also be necessary to employ appropriate virus markers (Kitajima *et al.* 2011; Fumian *et al.* 2013; Love *et al.* 2014), if the pathogens of concern are viruses. Chemical markers are also available for indicating fecal contaminations (Black *et al.* 2007; Kuroda *et al.* 2012). Pathogens occur in water episodically, and those posing infectious risks may vary from place to place and from season to season in terms of species and strains, which means that the quantification of multiple pathogens and indicators (Wong *et al.* 2013; Ishii *et al.* 2014a) has to be conducted at each study area. Accumulation of multiple quantitative data at each location is a basis for better understanding the quantitative relationship between indicators and pathogens.

CONCLUSIONS

A Bayesian model for estimating the fecal indicator/pathogen concentration ratio was constructed, in which a left-censored dataset with varying analytical quantification limit values was available. When the sample size was 144, numerical simulations concluded that 39 detects was enough to accurately estimate the distribution of the concentration ratio when combined with datasets with a positive rate higher than 99%. To evaluate the level of accuracy in the estimation, it is desirable to perform a simulation using artificially generated left-censored data that have the identical number of non-detects as actual data.

ACKNOWLEDGEMENTS

We thank Ms Rie Nomachi and Reiko Hirano for their technical help. This work was supported by the Japan Society for the Promotion of Science Through Grant-in-Aid for Scientific Research (B) (26303011).

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First received 8 January 2015; accepted in revised form 9 June 2015. Available online 7 July 2015