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Quantification and Distribution of *Salmonella* spp. and *Salmonella typhi* in Wastewater Treatment Plants

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In order to know about distribution of *Salmonella* spp. and proportion of *Salmonella typhi* in raw and treated wastewater, the real-time PCR methods were established and applied to monitoring of influents and effluents from three municipal wastewater treatment plants. *Salmonella typhi* can be well distinguished from non-typhi *Salmonella* by using specific primer set. The detection limit is 4.20 copies for *Salmonella* and 5.28 copies for *Salmonella typhi* per PCR. Statistical analysis showed that the distributions of *Salmonella* spp. and *Salmonella typhi* well followed the log-normal relationship. In influent samples, *Salmonella* spp. ranged 10^3∼10^6 copies/100mL, and *Salmonella typhi* ranged 10^2∼10^4 copies/100mL. Municipal wastewater treatment process can remove around 1 log unit for *Salmonella* and 0.5 log units for *Salmonella typhi* in general. The proportion of *Salmonella typhi* in *Salmonella* spp. was under 5% in most raw wastewater samples. The established methods can be usefully used to quantitative detect *Salmonella* and analyze proportion of *Salmonella typhi* in wastewater. This is useful to further study on *Salmonella* transmission in urban water environment and health risk assessment.

Key words: Distribution; quantification; real-time PCR; *Salmonella* spp.; *Salmonella typhi*; wastewater treatment plant.

Salmonella are a large group gram-negative bacillus composed of more than 2000 different serotypes. Most of these serotypes are believed to be pathogenic to humans and can cause a range of symptoms from mild gastroenteritis to serious illness even death (WHO, 2004). These diseases can be classified into typhoid fever caused by *Salmonella enterica* serovar Typhi (*S. typhi*) and Salmonellosis caused by non-typhi *Salmonella* species. Typhoid fever is fatal disease, which poses high morbidity and mortality in many developing countries and millions of cases are reported in Asia every year (Crump *et al*., 2004). Further, all age groups[1] of humans are susceptible to *Salmonella* infection, Salmonellosis constitutes a major public health and economic burden worldwide (Mansilha *et al*., 2010).

Although infections of *Salmonella* are associated with food products (Mukhopadhyay *et al.,* 2011), water transmission is particularly important to public health and environment (Baudart *et al*., 2000). *Salmonella* can be excreted in feces and may end up in environmental waters through sewage discharge and storm runoff. In general, *Salmonella* species are the most predominant pathogenic bacteria in wastewater (Ho *et al*., 2000). Municipal wastewater treatment system is an important node to investigate

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transmission of *Salmonella* bacteria in urban water environment. With the scarcity of water resource, wastewater has been regarded as an important resource and reused for various usages in the water deficient urban areas. *Salmonella* contamination in water environment may bring health risk to who consume these waters and is a growing concern for public health (Seo *et al*., 2006; Jyoti *et al*., 2010). Microbial safety of treated wastewater is of importance in managing the risk of waterborne disease. Information on the concentration of *Salmonella* in raw and treated wastewater will be useful, not only in understanding their fate but also in assessing the risk of infection through water environment.

Quantitative data is necessary to health risk assessment for water contaminated by *Salmonella* (Ferguson *et al*., 2003). The detection of *Salmonella* has been conducted using culture-based method. It takes four to six days to complete the whole process which includes enrichment, colony isolation, biochemical and serological confirmation, and enumeration (Uyttendaele *et al*., 2003). Obviously, the shortcoming of time-consuming and labor-intensive hampers the application of culture-based method. With the development of molecular technology, real-time PCR has been applied to the detection of pathogen due to its promptness, accuracy and sensitivity. At present, there are a few reports on presence of *Salmonella* in wastewater and effluent (Espigares *et al*., 2006).

In this study, we developed quantitative detection methods for *Salmonella* spp. and *S. typhi* using real-time PCR technology. Through application on influent and secondary effluent of municipal wastewater treatment plant, the suitability and practicability of the methods were verified. It was further investigated the concentrations and distributions of *Salmonella* spp. and *S. typhi* in raw and treated wastewater.

MATERIALS AND METHODS

Water Sample Collection and Concentration

Water samples were collected from influent and secondary effluent in three wastewater treatment plants (WWTPs) of Xi'an city, China. For each sample, one liter of sample was collected in plastic bottle and transported to the laboratory. All samples were stored at 4° before analysis process.

500 mL of water sample was passed through the cellulose mixed-ester microporous filter of 0.22 μm pore size. If the water samples contained a large amount of suspended solid, pre-filtration with the glass filter should be carried out. After filtration, Milli-Q water was used to wash the filter. The eluate was centrifuged at 7000 r/min for 5 min and then the supernatant was removed. Bacterial DNA was extracted by Bacterial Genomic DNA Extraction kit (Geneary Biotech Co., China). The 30 μL of DNA was gained and stored at -20! until real-time PCR amplification.

Real-time PCR for *Salmonella* spp. and *Salmonella typhi*

For *Salmonella* spp., the forward primer 139 5'- GTGAAATTATCGCCACGTTCGGGCAA-3' and reverse primer 141 5'-TCATCGCACCGTCAAAGGAACC-3', were used to amplify *invA* gene which is widely present in *Salmonella* serotype including all subspecies (Rahn *et al*., 1992; Malorny *et al*., 2003). For *S. typhi*, the forward primer ST3 5'-AGATGGTA CTGGCGTTGCTC-3', and reverse primer ST4 5'-TGGAGACTTCGGTCGCGTAG-3' was used to amplify flagellin gene (*H1-d*) of *S. typhi* (Song *et al*., 1993; Kumar *et al*., 2002).

The amplification system was carried out in a total volume of 25 μL. All reaction mixtures contained 2 μL DNA and 12.5 μL 2×SYBR Premix Dimer Eraser (Takara Biotechnology, China), 0.3μmol/L forward primer and 0.3μmol/L reverse primer. For *Salmonella* spp., real-time PCR was carried out in iQ™5 thermal cycler(Bio-rad, USA) with temperature profiles of 95! for 1 min and followed by 40 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 30s, and fluorescent signal acquirement at 82°C. For *S. typhi*, real-time PCR consisted of an initial denaturation at 95°C for 30s followed by of 40 cycles of denaturation at 95°C for 10s, annealing at 59°C for 30s, extension at 72°C for 30s and fluorescent signal acquirement at 81°C. Melting curves were generated following amplification by gradual increase from 65°Cto 95°C with continuous fluorescence measurement.

Each sample was performed in duplicate within the same run. Because of *invA* gene and flagellin gene are single-copy in *Salmonella* genome, the concentration of organism in sample

was calculated according to corresponding target gene copies and concentration process. Genomic DNA of *Salmonella typhimurium* (clinical isolation strain) and *Salmonella typhi* (CMCC 50071) was extracted and amplified by normal PCR, respectively. The length of specific amplified product was 284 bp for *Salmonella* spp. and 343 bp for *S. typhi*, which was verified in a 1.5% (wt/vol) agarose gel electrophoresis. PCR product was purified and cloned into pMD19-T vector (Takara Biotechnology, China). After transformation into DH5á competent cell, the positive colony was selected and incubated in Luria-Bertani (LB) broth at 37!, 200 r/min for 12h. The recombinant plasmid DNA was extracted by Plasmid DNA Extraction kit, and quantified by ND-1000 spectrophotometer (Nanodrop, Thermo Finnigan, USA). Serial ten-fold dilutions of the recombinant plasmid DNA were prepared in nuclease free water and used to generate the standard curve.

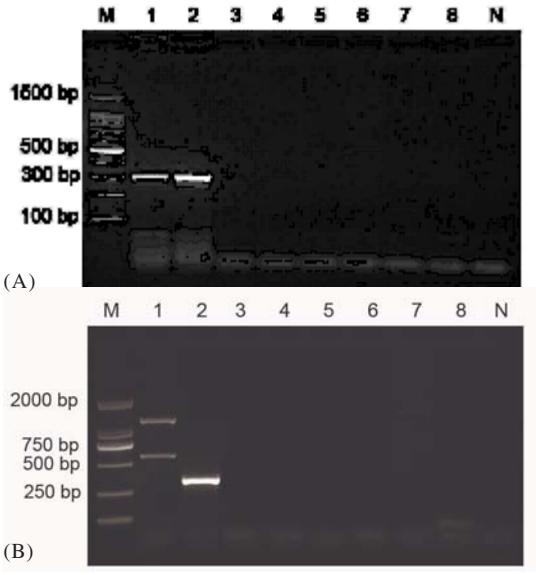
Statistical Analysis

The statistical treatment of the data was done using the SPSS for Windows software package. One-sample of Kolmogorov-Smirnov (K-S) Test was used to test whether the data follow presumptive distribution or not.

RESULTS

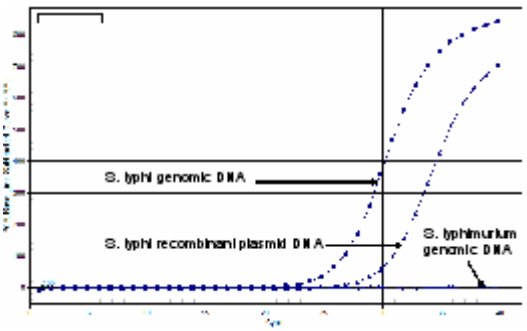
PCR primer specificity

The specificity of primer was tested in normal PCR conditions by using target and non-target DNAs. The electrophoresis results were shown in Fig 1. Using primer set 139-141, *S. typhimurium* and *S. typhi* produced obvious fragment expected site, while other organisms were all negative. Using specific primer set ST3-ST4, *S. typhi* produced light specific fragment, while *S. typhimurium* produced non-specific fragments. In real-time PCR detection and melting curve analysis, it was easy to find difference between *S. typhi* and non-typhi *Salmonella* species. It was found that *S. typhimurium* genomic DNA can not contribute fluorescent signal in real-time PCR detection above-mentioned (Fig 2 A). Melting temperature of *S. typhi* genomic DNA and *S. typhi* recombinant plasmid DNA were 84!, which was different with that of *S. typhimurium* genomic DNA (Fig 2 B). It indicated that the real-time PCR detection of *S. typhi* can not be affected by other organisms

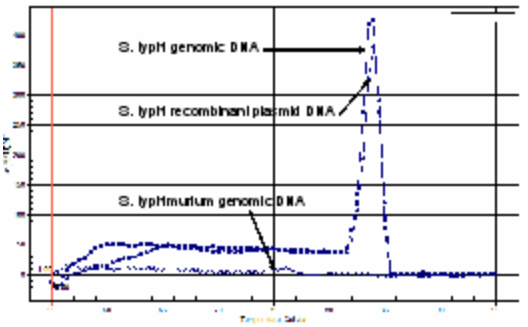


A: using primer set 139-141; B: using primer set ST3-ST4 M: DNA marker; 1: *S. typhimurium* (clinical isolation strain); 2: *S. typhi* (CMCC 50071); 3: *Escherichia coli* (ATCC 25922); 4: *Staphylococcus aureus* (ATCC 25923); 5: *Staphylococcus aureus* (ATCC 29213); 6: *Pseudomonas aeruginosa* (ATCC 27853); 7: *Shigella* sp. (clinical isolation strain) ; 8: *Bacillus subtilis*(clinical isolation strain) ; N: negative control

Fig.1. Specificity of primer sets test results



a) real-time PCR amplification profile



b) melting curve of PCR product

Fig. 2. Real-time PCR amplification and melting curve using primer set ST3-ST4

including *S. typhimurium*. Therefore, the real-time PCR methods established in this study could well differentiate *Salmonella* spp. and *S. typhi* from other microorganisms, respectively.

Sensitivity and precision of the real-time PCR

The recombinant plasmid DNA of *S. typhimurium* was prepared in a series of 10-fold dilutions of 4.20 copies to 4.20×10^6 copies. They were amplified to generate standard curve and evaluated the sensitivity of real-time PCR. When the cycle thresholds (C_T) were plotted as a function of the recombinant plasmid DNA copy, there was a good linear relationship between the C_T values and the logarithm of the input copy numbers over a range encompassing 7 orders of magnitude (r^2 = 0.993). The detection limit of the real-time PCR for *S.* spp. was determined to be 4.2 copies, which is approximate as low as single copy. Amplification efficiency (E) was estimated by the slope of the standard curve and calculated as E= ($10^{"1/slope}$) "1= 103%. When the same standard curve test was applied to *S. typhi* recombinant plasmid DNA, an identical standard quantification curve was produced. The detection limit was determined as 5.28 copies. E was calculated as 93%.

The Inter-assay coefficient of variation (CV) obtained by reproducibly detecting the same sample for five times was used to evaluate the precision of real-time PCR. In order to compare detection results on different times, C_T values were obtained in the universal standard of threshold line 20 relative fluorescence units. At low (4.20×10 copies), middle(4.20×10^3 copies) and high (4.20×10^5 copies) amounts of template, inter-assay CVs were all below 5%, which showed the real-time PCR methods for *Salmonella* spp. and *S. typhi* were very high precision.

Concentrations of *Salmonella* spp. and *S. typhi*

The real-time PCR methods were applied to 21 sample sets of influent and secondary effluent collected from three WWTPs. Geometric means of concentrations of *Salmonella* spp. and *S. typhi* in influent of WWTP-A, WWTP-B and WWTP-C were 2.31×10^4 copies/100mL and 6.59×10^2 copies/100mL, 1.47×10^5 copies/100mL and 9.69×10^2 copies/100mL, and 5.31×10^4 copies/100mL and 8.57×10^2 copies/100mL respectively. Compared to the others, influent of WWTP-B contained more *Salmonella* spp. and *S. typhi*, which could be caused by receiving landfill leachate in influent. Table 1 showed the concentration ranges of *Salmonella* spp. and *Salmonella typhi* in all influent and secondary effluent samples.

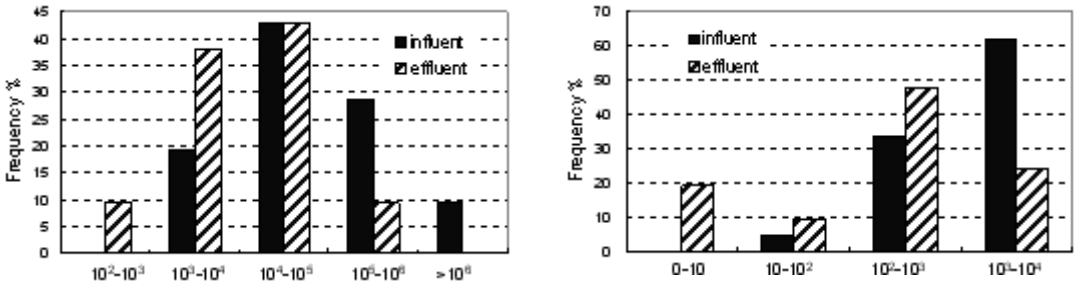
Table 1. Concentration ranges of *Salmonella* spp. and *Salmonella typhi* (copies/100mL)

Sample	Organism	Max	Min	Geometric mean
Influent	*Salmonella*	1.59×10^6	1.96×10^3	5.65×10^4
	S. typhi	5.27×10^3	6.95×10	8.17×10^2
Effluent	*Salmonella*	3.74×10^5	1.39×10^2	9.06×10^3
	S. typhi	3.14×10^3	0	3.86×10^2

Although *Salmonella* spp. and *S. typhi* in municipal wastewater are partially removed after wastewater treatment, the removal efficiency is not always stable as shown in Fig 3. Similarly as other parameters, it could be affected by influent quality and operation condition. High concentration of *Salmonella* in secondary effluent would commonly occur if influent contains a large amount of *Salmonella* bacteria. In most cases, the decreases in *Salmonella* spp. and *S. typhi* between influent and secondary effluent were approximate 1 log unit and 0.5 log units respectively.

Distributions of *Salmonella* and *S. typhi* in wastewater

The occurrence frequencies of different concentrations in influent and effluent were analyzed (Fig 3). For *Salmonella* spp., around 43% (9/21) influent samples were with the concentration ranging from 10^4 to 10^5 copies/100mL, and 29% (6/21) influent samples with 10^5~10^6 copies/100mL, while about 81% (17/21) secondary effluent samples with 10^3~10^5 copies/100mL. For *S. typhi*, around 62% (13/21) influent samples were with the concentration ranging 10^3~10^4 copies/100mL, and



A) *Salmonella* spp B) *S. typhi*

Fig. 3. Frequency of concentration in influent and effluent of WWTPs

48% (10/21) effluent samples with 10^2~10^3 copies/100mL.

According to previous report (Tanaka *et al*., 1998), log-normal distribution was considered as the presumptive distribution. One-sample of K-S test of the logarithms of data excluding the value of measurement as zero were to be conducted. The distributions of *Salmonella* spp. and *S. typhi* well followed the log-normal relationship in influent and secondary effluent of WWTPs (p>0.05).

Proportion of *Salmonella typhi*

Because of health concern, the amount of *S. typhi* in wastewater was always easy to draw more attention. By analysis of detection results, in 38% (8/21) influent samples and 76% (16/21) effluent samples, the concentration of *S. typhi* was under 10^3 copies/100mL. Compared with *Salmonella* spp., the concentration of *S. typhi* was relative stable. High concentration of *Salmonella* spp. did not mean high concentration of *S. typhi*. (Fig 4). In most influent samples, the proportion was lower than 5%. The results indicated that non-typhi *Salmonella* species was commonly prevalence in municipal wastewater.

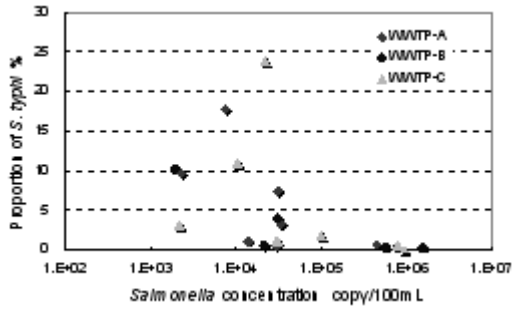


Fig.4. Proportion of *S. typhi* in influent of WWTPs

DISCUSSION

Compared with culture-based methods, real-time PCR method is more accurate, sensitive and rapid. Because of providing quantitative information of microorganism, it can play an important role in quantitative microbial risk assessment (QMRA).

In this study, we eliminated adverse effect by optimization including mixture adjustment and signal acquirement in high temperature. The established real-time PCR methods using SYBR Green could work extremely well. Molecular Beacon and TaqMan probe-based real-time PCR methods were generally believed highly sensitive and specific. These assay, targeting *invA* gene, the detection limits are about 1~100 copies or the equivalent CFU of *Salmonella* per PCR (Novinscak *et al*., 2007; Hadjinicolaou *et al*., 2009; Jyoti *et al*., 2010). Established real-time PCR using SYBR Green could detect 4.20 copies for *Salmonella* and 5.28 copies for *S. typhi*, which are better than some probe-based real-time PCR methods. Besides, SYBR Green-based real-time PCR has advantages of inexpensive and easy to use, which are important to practical application.

There is less research on *Salmonella* in water environment, especially their concentration and distribution in municipal wastewater and effluent. In the United States, *Salmonella* numbers in wastewater were estimated in the range from 10^2 to 10^4 organisms/100 mL, but much higher concentrations have been reported in developing countries (Bitton *et al*., 2005). Our study provides quantitative information of *Salmonella* spp. and *S. typhi* in municipal wastewater and secondary effluent, which is useful to know the contamination

level of *Salmonella* in developing countries. Monitoring results show that common wastewater treatment process can not completely eliminate *Salmonella*. The effluents containing *Salmonella* are finally received by urban surface water. It indicates that human population could be at risk of infection caused by *Salmonella* due to contact with water or inhalation aerosol. Besides, the results of study are useful to further study on *Salmonella* transmission in urban water environment and health risk assessment.

CONCLUSIONS

The real-time PCR methods for detection of *Salmonella* spp. and *S. typhi* were established and proved suitable to wastewater sample by a series of tests and practical monitoring. It is commonly believed that concentration of *Salmonella* spp. was 10^3~10^6 copies/100mL, and *S. typhi* ranging 10^2~10^4 copies/100mL in influent sample. After wastewater treatment, the concentration of *Salmonella* spp. and *S. typhi* decreased around 1 log unit and 0.5 log units respectively. The concentrations of *Salmonella* spp. and *S. typhi* followed log-normal distribution in wastewater. The proportion of *S. typhi* is commonly low in raw wastewater.

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