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The application of bioluminescence assay with culturing for evaluating quantitative disinfection performance

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ABSTRACT

Various methods, including bioluminescence assay, were investigated regarding their suitability for quantitatively evaluating the disinfection performance. Although bioluminescence assay itself has been widely reported as a rapid, easy and suitable method for analyzing live microorganisms, the limited sensitivity of its measurement (approximately 10^3–10^4 cells/assay vial), which is insufficient for disinfection study, requires further study.

Among three methods (amplifying by enzymatic method, membrane filtration, and amplification by culturing) examined for increasing the detection sensitivity, amplification by culturing showed the best performance as *Escherichia coli* was employed as an indicating microorganism. Even with a short culturing time of 4 h, the detection limit of *E. coli* measurement was successfully improved 200-fold, and the analytical results were not dependent upon the state of *E. coli* growth (stationary state with *E. coli* stock suspension vs. growth state with *E. coli*). In addition, the analytical integrity of bioluminescence assay with culturing was further demonstrated in comparison with spread plate method as free chlorine and UV irradiation were employed in the disinfection study.

1. Introduction

Disinfection is an important process in water treatment systems (EPA Guidance Manual, 1999, 2003; EPA Toolbox Guidance Manual, 2003; Cho et al., 2003a, b) for providing safe water. A wealth of research has been accumulated regarding the disinfection ability of specific disinfecting agents, such as free chlorine, ozone, and UV, etc., and the effect of water parameters (pH, temperature and dissolved organic compounds (DOC), etc.; Montgomery, 1985; Joret et al., 1997; EPA Guidance Manual, 1999, 2003; Craik et al., 2001; Khadre and Yousef, 2001; Lewin et al., 2001; Oguma et al., 2001; Shin et al., 2001; Huffman et al., 2002; Kim et al., 2002; Linden et al., 2002; Cho et al., 2003a, b) on disinfection performance. Free chlorine plays a central role not only in inactivating bacteria and viruses in the disinfection process of water treatment plants but also in providing a residual disinfectant during water distribution (Montgomery, 1985; Sobsey et al., 1988), whereas, ozone and UV are recommended as a viable option to control free chlorine resistant pathogenic microorganisms, such as oocysts of protozoan parasites (e.g., *Cryptosporidium parvum*, etc.) and spores of vegetative bacteria (e.g., *Bacillus subtilis* and *Bacillus anthracis*), in many water conditions (EPA Guidance Manual, 1999, 2003; EPA Toolbox Guidance Manual, 2003).

However, effective disinfection study has been greatly hampered by the limitation of conventional microbial analysis which requires generally long analytical time, large cost, etc., and sometimes does not include measuring non culutrable but possibly injured microorganism (Andrew and

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Roberts, 1993; Sobsey et al., 1988; LaRossa, 1998; Cowan and Falkinham III, 2001; Kong et al., 2002). For example, the analysis of *Cryptosporidium parvum*, one of the most important pathogens in water treatment plant, with animal infectivity assay, cell culture, or in vitro *excystation*, etc., requires long analytical time and high cost and frequently shows a large experimental deviation depending upon the level of analytical skill (Oguma et al., 2001; Shin et al., 2001; EPA Toolbox Guidance Manual, 2003). Even spread plate method (colony on agar method), which is easy and one of the most popular assays widely used for analyzing agar culturable microorganisms, such as *Escherichia coli* and *Pseudomonas aeruginosa*, etc., in disinfection study, also requires at least a further 24 h until counting (Cho et al., 2004).

Recently, as a suitable alternative analysis to overcome these limitations, bioluminescence assay was suggested by Squirrell et al. (2002) as an effective method for rapidly and easily detecting the viable bacteria. Furthermore, in disinfection study, Cowan and Falkinham III employed this method for measuring the inactivated *Mycobacterium avium* on a chlorine disinfection system (Williams et al., 1999; Cowan and Falkinham III, 2001).

All viable microorganisms have a fixed specific quantity of adenosine triphosphate (ATP) and the luminosity of produced light by chemical reactions (Eqs. (1) and (2)) is proportional to that of ATP in the microorganisms (LaRossa, 1998).

$$\mathrm{ATP} + \mathrm{Luciferin} \xrightarrow{\text{Firefly Luciferase, } Mg^{2+}} \mathrm{Adenyl} - \mathrm{Luciferin} + \mathrm{PP_i}, \quad (1)$$

$$\mathrm{Adenyl} - \mathrm{Luciferin} + \mathrm{PP_i} \rightarrow \mathrm{Oxyluciferin} + \mathrm{AMP} + \mathrm{CO_2} + \mathrm{Light}. \quad (2)$$

This luciferase-based method for the detection of live microorganisms via quantitative ATP assay is called bioluminescence assay. Since bioluminescence assay is known to have the detection limitation of 10^{-15} mol of ATP, and one typical bacterium contains only approximately 10^{-18} mol of ATP (Andrew and Roberts, 1993; LaRossa, 1998; Squirrell et al., 2002), a concentration larger than 10^3–10^4 cells/assay vial can be quantitatively measured. Considering the level of disinfection of 2–4 log (99–99.99%) inactivation, frequently employed in disinfection study (EPA Guidance Manual, 1999, 2003; EPA Toolbox Guidance Manual, 2003), this sensitivity becomes the greatest barrier to its application in water disinfection study.

Therefore, it is no wonder that previous studies which applied this assay to disinfection field showed limited success because of the narrow window of microbial inactivation up to only 0.6 log (Cowan and Falkinham III, 2001). This limitation has prompted study into the development of other methods, including bioluminescence assay, in terms of their analytical sensitivity.

Three methods for increasing the detection sensitivity are examined in this study: (i) amplification by enzymatic method (adenylate kinase bioluminescence assay, Squirrell et al., 2002), (ii) concentration of cells by membrane filtration, and (iii) amplification of cell concentration by culturing. In addition, the analytical integrity of the bioluminescence assay suggested in this study is further demonstrated in comparison with the spread plate method as free chlorine and UV irradiation were applied in the disinfection study.

2. Materials and methods

2.1. Preparation and analysis of *E. coli*

E. coli (ATCC strain 8739), used as the test microorganism, was prepared by inoculating in Tryptic Soy Broth and then incubating in a shaking incubator (SI-600R, Jeio Tech Co., Korea) at 37 °C for 18 h as described previously (Cho et al., 2004). After incubation, the grown *E. coli* was centrifuged at 3000*g* for 10 min in a 50 ml conical tube and washed twice with phosphate buffered saline (PBS, 150 mM sodium phosphate+150 mM sodium chloride, pH 7.2, P4244, Sigma Co., USA). The spread plate method (colony on agar method) with nutrient agar (BD-0003-17, Difco Co., USA), most frequently used in disinfection study, was used to analyze the stock *E. coli* concentration and compare it with the modified methods including bioluminescence assay. As previously described, sampled *E. coli* was diluted 1/1–1/10000 and three replicate 0.1 ml samples of these diluted samples were spread on nutrient agar (Cho et al., 2004). After incubation in an incubator (IB-25G, Jeio Tech Co., Korea) at 37 °C for 24 h, the colonies, matched as viable *E. coli*, were counted. This method showed good reproducibility within 10% standard deviation.

2.2. Bioluminescence assay

In bioluminescence assay for measuring the quantity of ATP (LaRossa, 1998; Squirrell et al., 2002; Delahaye et al., 2003; Yang and Irudayaraj, 2003), 0.1 ml of prepared *E. coli* was transferred to a 1.6 ml tube containing 0.1 ml detergent lysing agent. The contents were vortexed for 10 min and then 0.1 ml samples were transferred to 96-well luminometer cuvettes containing 1/10 ATP assay mix (FL-AAM, Sigma Co., USA), diluted with dilution buffer (FL-AAB, Sigma Co., USA).

Control experiments to assay the standard ATP concentration were conducted with ATP stock solution (FL-AAS, Sigma Co., USA). The emitted lights were detected in the 96-well plates using luminometer (Lumino Ascent, Thermo Electron Co., USA). Light was integrated for 1 s, following a 1 s delay, with the reading presented as relative light unit (RLU).

2.3. Three methods for increasing the detection sensitivity

Out of the three methods for increasing the detection sensitivity, firstly, adenylate kinase (AK) bioluminescence assay, which is an enzymatic amplification step, was conducted using the procedure described by Squirrell et al. (2002). In this assay, 0.1 ml of diluted *E. coli* was added in the 1.6 ml tube containing 0.1 ml of magnesium acetate (15 mM), to which was mixed 0.1 ml of detergent lysing agent with 0.3 mM purified ADP. After 5 min of reaction time, the quantity of ATP was determined by the luminometer. In control experiments,