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Rapid and Simple Colorimetric Detection of *Escherichia coli* O157:H7 in Apple Juice Using a Novel Recombinant Bacteriophage-Based Method

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In this study, a bacteriophage-based method for the colorimetric detection of *E. coli* O157:H7 in apple juice was investigated. Firstly, a gene encoding Cytochrome c Peroxidase (CCP) chromogenic enzyme was inserted into a wild type PP01 phage genome to construct the recombinant PP01ccp phage that was used in the production of the chromogenic enzyme through specific infection into *E. coli* O157:H7. The method was then examined in the colorimetric detection of *E. coli* O157:H7 in broth, and the appearance of *E. coli* O157:H7 in broth was confirmed by the color change after a few minutes of the enzyme assay. Secondly, the method was investigated in the colorimetric detection of *E. coli* O157:H7 in apple juice. A low *E. coli* O157:H7 concentration as 1 CFU mL⁻¹ was detected in 15 h that was in a shorter time than in previous bioluminescence phage-based methods. Moreover, the method is much simpler compared to other previous phage-based methods since it enables detection without the need for expensive apparatus.

Key words : *E. coli* O157:H7 / Bacteriophage / Colorimetric detection / Apple juice.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) can cause severe foodborne diseases due to the two toxins of Shiga toxin 1 and 2 (Stx1 and Stx2) produced by EHEC. Among serotypes of EHEC, *E. coli* O157:H7 is considered as the most important pathogen in relation to public health. It causes severe bloody diarrhea and hemolytic-uremic syndrome (HUS) in people. In the United States, about 73,000 cases of food-borne illness caused by *E. coli* O157:H7 have been reported per year (Mead et al., 1999). Animal feces are considered as the original source of *E. coli* O157:H7, and via many different routes, *E. coli* O157:H7 can infect human beings (Chekabad et al., 2013). Therefore, from the first outbreak of foodborne illness caused by *E. coli* O157:H7 in 1983, it has become important to detect *E. coli* O157:H7 to prevent such outbreaks. By applying

the Sorbitol-MacConkey agar plate method (Fujisawa et al., 2000; Possé et al., 2008), low *E. coli* O157:H7 concentrations can be detected. However, the agar plate method is time consuming since it takes more than a day for the pre-cultivation and the formation of colonies on the agar plate. One of the approaches considered for shortening the detection time for *E. coli* O157:H7 is the use of polymerase chain reaction (PCR) for amplification of the *stx1* and *stx2* genes (Jinneman et al., 2003; Fode-Vaughan et al., 2003). Although the method is rapid, it is inadequate for distinguishing the living cells from dead cells.

Apple juice is one of the most common fruit juices. While apple juice usually refers to the filtered and pasteurized product of apple pressing, unpasteurized apple juice (or apple cider) is still packed and consumed especially in the apple-producing regions in the world because with its pH of less than 4.6 there is considered to be low risk for the transmission of pathogenic bacteria. However, the infection of *E. coli* O157:H7 from consuming the unpasteurized fresh

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apple juice has been reported (Steele et al., 1982; Besser et al., 1993 and Cody et al., 1999).

The application of bacteriophages for detection of specific bacteria is advantageous owing to the high specificity of bacteriophages in host recognition. Until now, fluorescence- or bioluminescence-based detection methods utilizing bacteriophages for detection of *E. coli* O157:H7 have been investigated (Oda et al., 2004; Brigati et al., 2007). In those studies, the resulting fluorescence or bioluminescence could be detected using an epifluorescence microscope or a luminescence counter, respectively. Although both fluorescence- and bioluminescence-based detection methods allow selective detection of *E. coli* O157:H7 in less than one day, special apparatus are required to evaluate the results. Generally, it is easy and convenient to examine results by the colorimetric examination simply because it can be done visually without the use of specific apparatus, and to quantify the color change by using a spectrophotometer that is more commonly used and easily available compared to an epifluorescence microscope or a luminescence counter. Therefore, in the current study, a recombinant phage carrying the *cytochrome c peroxidase* (*ccp*) gene encoding the CCP enzyme was constructed for application in the colorimetric detection of *E. coli* O157:H7 in apple juice.

MATERIALS AND METHODS

Principle of the detection method

The principle of the detection method is schematically shown in Figure 1. In order to detect *E. coli* O157:H7,

FIG. 1. Schematic diagram of principle of the detection method. The recombinant PP01ccp phage is constructed by inserting the *ccp* gene into the genome of PP01 phage. The CCP enzyme will be produced inside the infected *E. coli* O157:H7 cell and is then released after the cell lyses. The existence of *E. coli* O157:H7 in the sample is indicated by the color change caused by oxidation of the substrates through catalysis of the CCP enzyme.

the *ccp* gene was recombined into the genome of PP01 phage to construct a recombinant PP01ccp phage. If the infection of the recombinant phage PP01ccp to *E. coli* O157:H7 occurs, the CCP enzyme will be produced inside the infected *E. coli* O157:H7 cell and is then released together with the newly generated phages after the cell lyses. If the substrate is added to the phage lysate, under catalysis of CCP enzyme, the substrate is oxidized resulting in color change. Therefore, *E. coli* O157:H7 could be detected based on the color change.

Bacterial strain and bacteriophage

E. coli O157:H7 ATCC 43888 that does not produce Stx1 and Stx2 toxins was used as the host for the PP01 phage. The wild type PP01 phage was obtained from Professor Yasunori Tanji (Tokyo Institute of Technology, Japan).

Construction of the recombinant PP01ccp phage

Oligonucleotide primers and probe used in the PCR amplification were designed based on previous study (Oda et al., 2004) where the sequences for the restriction digestion was changed, and wild type PP01 phage (PP01wt) lysate was used as the template. Fragments corresponding to the *ccp* gene and two flanking regions were amplified and inserted into the plasmid vector pCR2.1-TOPO (Invitrogen, CA, USA) to produce the plasmid vector pCRPP01ccp (Figure 2). The *ccp* gene was integrated into the PP01wt genome by homologous recombination. The procedures of homologous recombination and isolation of the recombinant phage PP01ccp were similar to those described in a previous study (Oda et al., 2004).

Evaluation of the activity of CCP produced by the PP01ccp

E. coli O157:H7 was cultivated at 37 °C until an OD₆₀₀ of 0.5 (approximately 1×10^8 CFU mL⁻¹) was attained. Then, the culture was divided into three aliquots, of which two aliquots were mixed with either PP01ccp or PP01wt phage lysate at a multiplicity of infection (M.O.I) of 5.0. One aliquot was left without phage addition. The aliquots were incubated at 37 °C for 1 h and then were passed through a 0.45-μm membrane filter to obtain filtrates. In addition to the filtrates, the LB medium was also used for the assay. The cytochrome c from equine heart (Sigma-Aldrich, Missouri, USA) was used as a substrate for the enzymatic assay, and cytochrome c was reduced prior to the assay in accordance with the protocol described by Spinazzi et al. (2012), with minor modifications. The filtrates or the LB medium was mixed with phosphate buffer (50 mM KH₂PO₄, pH 6.0), cytochrome c, and H₂O₂ to obtain a

ten-fold dilution. The final concentrations of cytochrome c and H_2O_2 were 0.9 μM and 360 μM , respectively. The mixture was incubated at 30 °C and the ABS_{550} of the reaction solution was measured every minute using a spectrophotometer. All the enzyme assays were conducted in triplicate.

Detection of *E. coli* O157:H7 in apple juice

Apple juice was purchased from a local supermarket and kept at 4 °C. When *E. coli* O157:H7 culture reached OD_{600} of 0.5, cell pellets were obtained by centrifugation at 4,600 $\times g$, 7 minutes. The pellets were suspended by adding an equal volume of apple juice. A ten-fold dilution series was performed in apple juice down to approximately 1 CFU mL^{-1} . Then, pre-warmed LB medium was added into the mixture following a ratio of apple juice to medium as showed in a previous study (Brigati et al., 2007). Each mixture was cultivated at 40 °C, 200 rpm for a certain time and used in the phage assay with PP01ccp infection or without phage addition as shown above.

RESULTS

Construction of PP01ccp

The *ccp* fragment and the two flanking regions were inserted into the vector pCR2.1-TOPO to produce the vector pCRPP01ccp (Figure 2). Then, PP01ccp was produced by the homologous recombination between the vector pCRPP01ccp with the genome of PP01wt. The positive plaques were picked and suspended in the SM buffer, and PP01ccp was isolated from the suspension by repeated plaque hybridization. Integration of the *ccp* gene into the genome of the PP01 phage was confirmed by sequencing the *ccp* gene and the adjacent two regions in the PP01ccp

FIG. 2. Schematic diagram of homologous recombination between the constructed plasmid vector and PP01wt genome. Double crossover events occur in the g56 and socmrh2 regions of the plasmid vector and PP01wt genome, resulting in the fusion of the *ccp* gene into the PP01wt genome to produce the recombinant phage PP01ccp

genome (data not shown). It indicated the success of the construction of the recombinant PP01ccp phage. Next, the activity of CCP enzyme produced from PP01ccp genome was examined by the detection of *E. coli* O157:H7 in broth.

Activity of CCP expressed from PP01ccp genome

In the enzyme assay using the lysates obtained by the PP01ccp and PP01wt infections of *E. coli* O157:H7, the change in the color of the reaction solution could be visually perceived (Figure 3). The color change of the assay using either the lysate obtained by the PP01wt infection of *E. coli* O157:H7 or the filtrate of the *E. coli* O157:H7 culture without phage addition was almost identical to that obtained using LB medium without any bacterial inoculation (data not shown). It was confirmed that the presence of *E. coli* O157:H7 or the lysis of *E. coli* O157:H7 by the infection of PP01wt did not affect the oxidation of the substrate. In other words, the CCP expressed from the PP01ccp genome contributed substantially to the oxidation of cytochrome c. Therefore, detection of *E. coli* O157:H7 in broth could be conducted by using the PP01ccp phage.

Detection of *E. coli* O157:H7 in apple juice

The detection efficiency was examined with apple juice containing *E. coli* O157:H7 with a concentration range from 10^8 to 1 CFU mL^{-1} . After pre-cultivation if needed, PP01ccp was added or not added to the apple juice sample to carry out the phage assay. At the

FIG. 3. Visualization of the detection based on the enzyme assay of the lysates obtained by the PP01ccp and PP01wt infections of *E. coli* O157:H7 against cytochrome c/ H_2O_2 after 3 min. The oxidation of cytochrome c under catalysis of the CCP enzyme resulted in the color change from the original red color to the orange-yellow color in the PP01ccp tube. The original red color showed almost no change in the PP01wt tube.

FIG. 4. Response time profile of the detection of *E. coli* O157:H7 in apple juice. Detection time involved the time in pre-cultivation and the phage assay. Error bars indicating 95% confidence intervals for the averaged values ($n = 3$) are not graphically detectable at some points as the intervals were too narrow.

concentration of 10^8 CFU mL⁻¹, the phage assay could detect *E. coli* O157:H7 without the pre-cultivation step. At lower concentrations, the pre-cultivation step was needed. Detection time for the whole process (involving pre-cultivation and phage assay) is described in Figure 4. Time required to detect the highest and lowest concentrations of *E. coli* O157:H7 was about 1 h and 15 h, respectively.

DISCUSSION

E. coli O157:H7 causes approximately 73,000 cases of illness in USA annually with the main epidemiological symptoms of severe diarrhea and HUS. The largest *E. coli* O157:H7 outbreak was reported in January, 1993 with more than 700 who became ill people and 4 children who died (Rangel et al., 2002). *E. coli* O157:H7 outbreaks have been also reported in other developed countries (Isaacson et al., 1993; Chapman et al., 1989; Armstrong et al., 1996). In Vietnam, information of *E. coli* O157:H7 contamination in environmental and food samples is still very limited. However, it is expected that contamination of *E. coli* O157:H7 in those samples in Vietnam with poor hygiene conditions is more prevalent than in other developed countries. Therefore, the development of simple and cheap methods used to detect *E. coli* O157:H7 would play an important role in preventing serious diseases caused by *E. coli* O157:H7.

The detection method developed in this study can be considered as the first successfully performed phage-based colorimetric detection of *E. coli* O157:H7. The

enzyme assay was conducted in few minutes against cytochrome c/H₂O₂ and the color change could be easily recognized by the naked eye without the need for any apparatuses. In addition, the color change could be quantified using a spectrophotometer that is relatively commonly used and more easily available compared to an epifluorescence microscope or a luminescence counter that are required in the previously developed phage-based methods. The convenience is a strong point compared to the other phage-based detection methods.

Animal feces are considered as the original source of *E. coli* O157:H7. The feces are normally treated by composting to produce compost that is used on farms as a fertilizer to cultivate plants. During the composting process, *E. coli* O157:H7 in the feces is eliminated by high temperatures generated inside the composting zone. The composting process is usually performed by farmers especially in developing countries and may not result in mature compost that completely eliminates *E. coli* O157:H7 from the original feces. At apple farms, during harvesting season, *E. coli* O157:H7 from the land can easily be transmitted to apples. Unpasteurized fresh apple juice is still consumed especially in the apple-producing regions in the world because with its pH of less than 4.6 it is considered to be of low risk for the transmission of pathogenic bacteria. However, it was demonstrated that *E. coli* O157:H7 can survive at a pH as low as 2.0 (Miller & Kaspar, 1994; Conner & Kotrola, 1995). In addition, the infection of *E. coli* O157:H7 via consuming the unpasteurized fresh apple juice has been reported (Steele et al., 1982; Besser et al., 1993; Cody et al., 1999).

In the current study, apple juice was artificially contaminated by *E. coli* O157:H7 and addition of pre-warmed LB medium in the suspension step would favor the growth of *E. coli* O157:H7. The ratio of the volume of the pre-warmed LB medium to that of the contaminated apple juice followed the ratio employed in a previous study (Brigati et al., 2007). The method in the current study enabled the detection of concentrations of *E. coli* O157:H7 as low as 1 CFU mL⁻¹ in apple juice, while the phage-based bioluminescent method (Brigati et al., 2007) could not detect the *E. coli* O157:H7 at less than 10^4 CFU mL⁻¹ due to the interference of the apple juice to the system. To overcome the interference of the apple juice in the detection of such low concentrations of *E. coli* O157:H7, Ripp et al. (2008) centrifuged the apple juice to discard the supernatant to obtain and to concentrate the cell pellet that was then suspended in LB medium prior to the pre-cultivation. In this way, such a low concentration of *E. coli* O157:H7 as 1 CFU mL⁻¹ could be detected after 22 h.

Compared to previous phage-based bioluminescent methods, the method in the current study is more advantageous in detection of *E. coli* O157:H7 in apple juice. Firstly, the method could detect *E. coli* O157:H7 even at 1 CFU mL⁻¹ without a centrifugation step. Secondly, the method just takes about 15 h to detect the *E. coli* O157:H7 at a concentration as low as 1 CFU mL⁻¹ while the phage-based bioluminescent method needs about 22 h as mentioned above. Therefore, the method was demonstrated to be faster and simpler than previous phage-based bioluminescent methods in the detection of *E. coli* O157:H7 in apple juice. In future studies, the method will be examined in the detection of *E. coli* O157:H7 in other food samples such as milk, vegetables, meats, etc.

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