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Microbial respiration-based detection of enrofloxacin in milk using capillary-tube indicators

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ABSTRACT

A simple method was developed for detecting enrofloxacin in milk using microbial respiration and capillary-tube indicators. A glass vial containing *E. coli* ATCC 11303 in LB was spiked with enrofloxacin and sealed with a screw cap supporting a silicon septum. A capillary tube with a drop of ink was inserted through the septum, and the ink-level change was measured. The growth of *E. coli* produced CO_2 gas, which increased the pressure inside the vial and raised the ink level in the capillary tube; in this manner, small changes in the gas volume were translated into large changes in the ink level. The increase in the ink level was inversely proportional to the concentration of enrofloxacin, which suppressed the growth of *E. coli*. The detection limit of enrofloxacin was found to be 10 ng/mL using the naked eye after the microbial culture had been permitted to grow over 2 h.

1. Introduction

Antibiotics are chemical compounds used for the treatment and prevention of bacterial infections in humans and livestock. The discovery of antibiotics was an epoch-marking event in the battle to protect against bacterial infection; however, the overuse of antibiotics has prompted bacteria to develop resistance mechanisms [1,2]. As bacteria evolve resistance to antibiotics faster than humans are able to develop novel antibiotics capable of killing these strains, the United States and the European Union have elected to pass regulations limiting the maximum residual level of antibiotics permitted in food samples in an effort to prevent the emergence of antibiotic-resistant bacteria [3]. These regulations can only be made effective by developing a simple method for monitoring residual antibiotics in food samples on-site.

The standard techniques used to detect residual antibiotics include high-performance liquid chromatography (HPLC) [4–10] and mass spectrometry (MS) [11–14] owing to their high sensitivity and accuracy. HPLC and MS, however, are not practical for on-site-detection applications because they require expensive instruments and experienced staff members. Microbial-inhibition tests [15–19] provide good alternatives to instrumental analysis methods. These tests offer easy and cost-effective screening of antibiotic residues in food samples [20–23]. However, microbial-inhibition tests rely on the growth of bacterial population and suffer from the drawback of a long assay time, requiring several days for a microbial culture.

The problems associated with other current techniques are circumvented here by developing a novel microbial-respiration method that does not rely on bacterial population growth but rather upon bacterial respiration. Most bacteria produce CO_2 gas during respiration [24,25]. As antibiotics suppress the growth of bacteria, the presence of residual antibiotics may be easily determined by measuring the amount of CO_2 gas produced over time. The production of CO_2 due to bacterial respiration may be measured using conventional Durham tubes, which comprise a small test tube inserted upside down within a large test tube [26,27]. The small tube is initially filled with a solution containing bacteria, and a CO_2 bubble becomes trapped inside the small tube during incubation. Although the generation of CO_2 gas can be identified with the naked eye, the assay time required for use of a Durham tube is usually > 24 h because the size of the small glass tube is large compared with the amount of CO_2 produced during fermentation.

We addressed this problem by adopting a glass vial with a capillary tube containing a drop of ink [28]. A vial containing *E. coli* ATCC 11303 in LB was spiked with various concentrations of enrofloxacin and sealed using a screw cap through which a capillary tube containing a drop of ink had been passed. Enrofloxacin, a second-generation fluoroquinolon antibiotic, was selected for this study owing to its broad-spectrum antimicrobial activity. The production of CO_2 gas during the growth of *E. coli* increased the pressure inside the glass vial and raised the ink level in the capillary tube. This increase in the ink level was found to be inversely propor-

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Table 1
Comparison of various methods for the detection of residual enrofloxacin.

Methods	LOD (ng/mL)	Time	Cost	References
HPLC-DAD	0.5–10	~ 1 h	High	[26]
Luminescence	0.06	~ 2 h	High	[27]
Microbiological assay	109	5.5 h	Low	[28]
Bacterial respiration	10	2.5 h	Low	This paper

tional to the enrofloxacin concentration. The detection limit of the assay using the naked eye was found to be 10 ng/mL enrofloxacin after microbial culturing over 2 h. Table 1 compares the sensitivities and assay times of various methods in the detection of residual enrofloxacin [29–31].

2. Materials and methods

2.1. Materials

E. coli ATCC 11303 was obtained from the Korea Culture Center of Microorganisms (KCCM). Lysogeny broth (LB), enrofloxacin, and safranin O were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (18.3 MΩ cm) was obtained using a reverse-osmosis water system (Human Science, Korea). 2-mL gas-tight glass vials and screw caps with silicon septa were purchased from Agilent Technologies (Santa Clara, CA, USA). Capillary tubes with an inner diameter of 1.02 mm were obtained from Hirschmann Laborgerate (Eberstadt, Germany).

2.2. Preparation of the E. coli and sample solutions

E. coli ATCC 11303 has been previously used for detecting enrofloxacin (quinolone group) in microbial-inhibition tests [32–34]. After growing a pure culture of *E. coli* ATCC 11303 in 25 mg/mL LB at 37 °C overnight, the culture was centrifuged at 4,000 rpm for 5 min, and the precipitate was dispersed in DI water. As the amount of gas production is affected by the viability of *E. coli*, it is important to use freshly prepared *E. coli* for each experiment. A serial dilution in DI water was prepared to obtain 10^7–10^{10} CFU/mL *E. coli* concentrations, which were determined by measuring light absorption at 600 nm. 100 μL of the *E. coli* solution were added to a 2-mL gas-tight glass vial and mixed with 180 μL of 0.25 g/mL LB and 20 μL of 0.145 g/mL glucose. A volume of the enrofloxacin solution in D.I. water or milk was then added to the vial to make the total volume of the solution 1.82 mL. The residual space inside the vial (0.18 mL) was filled with air to separate the capillary tube from the solution. Once a capillary tube containing a drop of red ink (safranin O in an aqueous solution) had been inserted through the center of the septum and the vial had been sealed tightly with the cap, the glass vial was incubated at 37 °C with gentle shaking. Rapid thermal equilibrium was reached using a homemade aluminum heating block inside the incubator. The ink level was measured using a digital camera every 30 min after allowing 15 min to reach the initial thermal equilibrium.

2.3. Detection of enrofloxacin using microbial respiration and capillary tubes

Fig. 1 shows a schematic of the experimental procedure used to detect enrofloxacin using a capillary tube containing a drop of red ink. The growth of *E. coli* produced CO_2 gas, which increased the pressure inside the glass vial and raised the ink level in the capillary tube (left vial). Spiking the sample solution with enrofloxacin suppressed the growth of *E. coli* and limited the production of CO_2 gas; the increase in the ink level was inversely proportional to the enrofloxacin concentration. Small changes in the CO_2 volume within the glass vial were thus translated into large changes in the ink level within the capillary tube, enabling the detection of small amounts of residual antibiotics. The sensitivity increased as the diameter of the capillary tube decreased or as the amount of sample solution inside the glass vial increased.

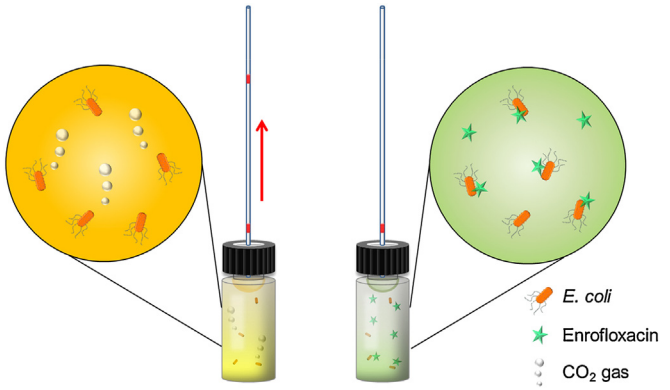

Fig. 1. Schematic of the enrofloxacin detection method using a glass vial and a capillary tube containing an ink indicator. CO_2 was produced during *E. coli* respiration in the absence of enrofloxacin (left) whereas no CO_2 was produced in the presence of enrofloxacin (right).

2.4. Gas chromatography (GC) analysis

Glass vials containing 10^7 CFU/mL *E. coli* in LB solutions were spiked with 1 μg/mL enrofloxacin and incubated for 2 h. The glass vials were sealed with screw caps containing capillary tubes and incubated at 37 °C with gentle shaking. The composition of the gas produced during microbial respiration was analyzed using a YL 6100 gas chromatography apparatus (GC, YL Instrument Co., Korea). After injecting the gas products into the GC, the GC oven was heated from 50 °C to 200 °C at a rate of 20 °C/min, and the solution was maintained at 200 °C for 5 min. Ultrapure helium was used as a carrier gas, and the flow rate was set to 30 mL/min.

3. Results and discussion

3.1. Microbial respiration in the absence of enrofloxacin

E. coli degraded nutrients and produced CO_2 gas during microbial respiration [24,25]. The production of CO_2 gas increased the pressure in the glass vial and raised the ink level in the capillary tube. Fig. 2a shows optical images of the ink level obtained from a glass vial containing 10^7 CFU/mL *E. coli*. The spacing between two adjacent lines was 5 mm. The production of CO_2 gas during the growth of *E. coli* increased the pressure inside the glass vial and raised the ink level in the capillary tube. The ink level increased with incubation time and could be easily measured using the naked eye.

Fig. 2b shows the change in the ink level during the growth of various concentrations of *E. coli* without enrofloxacin. The error bars represent the standard deviation of the measurements. Measurements could be taken after 15 min in thermal equilibrium; this period was required because the placement of a sample solution prepared at room temperature in the incubator increased the ink level owing to temperature increase. The level of ink was measured every 30 min. No noticeable changes were observed in the pure LB or in the *E. coli* solutions with concentrations lower than 10^6 CFU/mL, indicating that no CO_2 was generated from the LB and that the amounts of CO_2 produced from the solutions with low concentrations of *E. coli* over 2.5 h were too small to be detected. Changes in the ink level were observed in the *E. coli* solutions having concen-