A Rapid and Simple Determination of Food-Borne Salmonella Strains by Using Multi-Channel Oxygen Electrodes

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Received 8 December 2004/Accepted 5 January 2005

A rapid and simple procedure for the specific detection of Salmonella was developed using a dissolved oxygen measurement device (DOX-96) with anti-Salmonella antibodies. In the DOX-96 system, a gold electrode is located at the bottom of each well, in a 96-hole plate. The gold electrode acts as the working electrode. The anti-Salmonella antibodies are then introduced into the system and immobilized on each well of the plate. Wells contained bound Salmonella Typhimurium cells which were incubated at 37°C, and the oxygen consumption in each well was monitored. It appeared that the oxygen consumption curve was inversely proportional to the growth of S. Typhimurium. In the present method, S. Typhimurium cells with an initial concentration of $2.5 \times 10^2 - 2.5 \times 10^8$ CFU/ml in the sample showed an oxygen consumption curve within 13 h of incubation. Other microorganisms, such as Escherichia coli, Pseudomonas aeruginosa, Corynebacterium aquaticum and Bacillus subtilis did not interfere with the assay system. Thus the present method would be applicable toward a rapid and simple detection of Salmonella in food.

Key words: Multi-channel dissolved oxygen electrode (DOX)/Salmonella typhimurium/Food poisoning/oxygen consumption/immuno-DOX assay.

Food-borne Salmonellosis is still a primary concern due to the frequency at which Salmonella food poisoning cases occur (Simonsen et. al., 1987). Because of these many cases of food poisoning, increasing attention has been focused on the detection of Salmonella. The conventional methods for detection of Salmonella strains in food include pre-enrichment and enrichment incubation. At least 3-4 d are required in these methods to detect Salmonella. Therefore, various other methods have been proposed to shorten the time period for detection of Salmonella by using enzyme immunoassay (Blais et. al., 1998; Cerqueira-Campos et. al., 1986; Prusak-Sochacewski and Luong, 1989), the polymerase chain reaction (PCR) (Chen and Griffiths, 2001; Gooding and Choudary, 1999), quartz crystal microbalance (QCM) (Park et. al., 2000; Wong et. al., 2002), and surface plasmon resonance (SPR) (Oh et. al., 2004).

One method for the detection of Salmonella Typhimurium has been developed by using electrochemical measurement of the cathodic current of oxygen in cyclic voltammetry (Ruan et. al., 2002). The principle of this method is based on the oxygen consumption of growing S. Typhimurium versus time. There are two main advantages that this method has over conventional methods. First, detection sensitivity...
is higher than the PCR and ELISA (enzyme-linked immunosorbent assay) methods. Second, the detection time is within 10 h, making it shorter than in a microbiological method. However, this is not a suitable method for practical use to detect *S. Typhimurium* in food because it cannot measure a number of samples simultaneously.

Another way to detect cell populations, based on the oxygen uptake rate of living cells, is by using a method normally applied for screening anticancer reagents. This method involves multi-channel dissolved oxygen electrodes (DOX-96 system). In the DOX-96 system, the bottom of each well in a 96-hole microplate has three gold electrodes that include a working, counter, and reference electrode. The electrode system is specially designed to measure the dissolved oxygen concentration. This method has allowed the simple detection of the respiratory activity of living cells and simultaneous monitoring of responses of cells against extracellular stimulation (Andreeescu et. al., 2004; Ueda et. al., 1998). The DOX system has not only been applied to evaluate drug susceptibility for screening anticancer reagent (Amano et. al., 1999; Yasuda et. al., 1999), but has also been used for the determination of the cell population of coli-form bacteria and general bacteria in food (Amano et. al., 2001) because of its pre-existing relationship between the respiration of bacteria and the bacterial cell count.

In this study, we report the development of DOX-96 system for a specific, rapid, and simple procedure for the detection of *Salmonella* with anti-*Salmonella* antibodies (immuno-DOX assay). Anti-*Salmonella* antibodies were immobilized on each well of a DOX plate. *Salmonella* cells trapped in the wells were then incubated, and oxygen consumption in the wells was monitored. The optimal conditions for immuno-DOX assay were established and are discussed in detail below.

Anti-*Salmonella* Typhimurium antibodies were obtained from Cosmo Biochemicals Co. (Tokyo, Japan). A multi-channel oxygen electrode system (DOX-96) was obtained from Daikin Environmental Research Ltd. (Tsukuba, Japan). Bovine serum albumin (BSA) was purchased from Sigma Co. (St. Louis, MO). All other reagents and compounds were of analytical grade.

The test bacteria (*Salmonella Typhimurium* NBRC 12529, *Escherichia coli* NBRC 3301, *Bacillus subtilis* NBRC 3009, *Corynebacterium aquaticum* NBRC 12154, and *Pseudomonas aeruginosa* NBRC 3080) were obtained from the Institute for Fermentation, Osaka (IFO, Osaka, Japan).

Nutrient broth (Difco, Detroit, MI, USA) was used to grow bacterial cultures. It was also used as a pre-enrichment medium for the incubation of food samples. Nutrient agar (Difco, Detroit, MI, USA) was used to maintain the bacterial stock on slopes at 4 °C. It served as a plating medium for viable bacterial counts. L-B broth (Difco, Detroit, MI, USA) was used as the basal media for bacteria growth, and all bacterial strains were grown overnight at 37°C. The M broth and Rappaport-Vassiliadis R10 broth (Difco. Detroit, MI, USA) were used as selective broth for *Salmonella*. Saline was used for preparing bacterial dilutions. All media were autoclaved at 121°C for 15 min.

The 96-holes of the microplate for the DOX system with three electrodes (counter, reference, and working electrodes) installed in the bottom of each well (Daikin Environmental Research Ltd.) were coated with 100 μl of 100 μg/ml anti-*Salmonella* antibodies in 0.1 M phosphate buffer (pH 7.4) containing 137 mM NaCl and 2.7 mM KCl (PBS, phosphate-buffered saline) for 1 h at 25°C. Antibodies were immobilized on the microplate which was made of polyethylene terephthalate by adsorption. The wells were then washed three times with PBS containing 0.01% Tween 20 (PBST) and blocked by incubating 200 μl/well of 10% polyethyleneimine in PBST for 1 h at 25°C. After blocking, wells were washed three times with PBST and used for assays. The decimal dilution of sample (100 μl) was added to the wells and incubated for 1 h at 25°C. The wells were washed with PBST three times, and then LB broth was added to the wells. The Dox-plate was incubated at 37°C for 18 h without shaking.

The electrode DOX system is a multi-channel system that enables the measurement of cell respiratory activity via the consumption of dissolved oxygen (Katayama, 2000). The applied potential was set at −0.6 V vs. gold electrode and the cathodic current was monitored and logged on a personal computer. Detection time (DT) was defined as the time it took for the cathodic current to reach a steady state and was calculated from the inflection point in the current shift curve.

Ground beef and whole eggs were purchased locally. Samples artificially contaminated with appropriate amounts of *S. Typhimurium* were also used for the assay. Food samples (each 10 g) were processed with a stomacher (SKM-713, As One Co., Tokyo, Japan) for 1 min in 100 ml of saline and decimal diluted for the assay. The numbers of cells in food samples were also confirmed by determination of colony forming units as described as follows. Samples (0.5 ml) were inoculated with 10 ml of the RV agar medium and incubated for 1 to 2 d at 37°C (Vassiliadis, 1983).
Bacteria were grown in nutrient broth at 37°C for 24 h. Decimal dilutions of bacteria were prepared in PBS and counts of colonies were done using pour plates of nutrient agar incubated at 37°C for 1 d. Each developed colony was assumed to have grown from one viable unit.

When a sample solution containing *S. Typhimurium* was applied to a well in which anti-*Salmonella* antibodies were immobilized and incubated in LB broth, the cathodic currents based on the dissolved oxygen in each well decreased in approximately 6 to 11 h and then reached a steady state (Fig. 1A). The order of the cathodic current increase depended on the initial concentration of *S. Typhimurium*. However, *S. Typhimurium* grew not only in antibody-immobilized wells but also grew in the wells without antibodies (data not shown). The non-specific adsorptions of *S. Typhimurium* to the antibodies were observed under the standard conditions. To avoid the non-specific adsorption for *S. Typhimurium* to the antibodies, the immuno-DOX assay was examined with the selective medium of *Salmonella* sp.

Fig. 1B and C show the cathodic current changes from different initial concentrations of *S. Typhimurium* on the Rappaport-Vassiliadis (RV) broth and M broth. Both media did not show non-specific growth of *S. Typhimurium* (data not shown). Selective media might improve the specificity of immuno-DOX assay, because the experiments without antibodies also showed a weak response of *S. Typhimurium* and other microorganisms (data not shown). However, the multiplication of *S. Typhimurium* was slow with RV broth. It took more than 13 h to detect *S. Typhimurium*. Based on these results, M broth was chosen as the optimum medium for detection of *S. Typhimurium*.

To examine the influence of cross-activities of other microorganisms against the immuno-DOX assay, *E. coli* and *B. subtilis* were used. As shown in Fig. 2A, the cathodic current based on the dissolved oxygen in the well decreased for the growth of *E. coli* at an initial concentration of 62000 CFU (colony forming unit) /ml. For the concentration of 6200 CFU/ml of *E. coli* and 50000 CFU/ml of *B. subtilis*, oxygen consumption curves were not observed (Fig. 2A and B, respectively). *E. coli* and *B. subtilis* did not interfere with the assay system when their concentrations were below the order of 10⁴ CFU/ml. Neither *C. aquaticum* nor *P. aeruginosa* showed any current response due to oxygen consumption (data not shown).

After the optimization of operating conditions, serially diluted *Salmonella* broth (from 2.5 x 10⁰ to 2.5 x 10⁸ CFU/ml) was tested in this system. A linear relationship between the DT and the logarithmic value of the initial concentration of *Salmonella* cells was found for cell concentrations ranging from 1x10⁰ to 1x10⁸ CFU/ml with a correlation coefficient of -0.996 (Fig. 3). The detection limit reached 1x10¹ cells/ml *Salmonella* for pure culture and was from 1 to 3 orders of magnitude lower than the ELISA (enzyme-linked immunosorbent assay) method [1x10³ CFU/ml, (Prusak-Sochacewski and Luong, 1989)], Enzyme-linked immunomagnetic electrochemical detection (8x10⁰ CFU/ml, (Abdel-Hamid et. al., 1999)), and also below that obtained with SPR method with

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**FIG. 1.** Effects of selective medium on the dissolved oxygen consumption profile based on the respiration of *S. Typhimurium* growth. Electrochemical measurement was carried out under the standard conditions with various media: (A) LB broth, (B) RV broth, and (C) M broth. Initial *S. Typhimurium* cell concentrations were as follows.

- , 62000CFU/ml;
- , 6200CFU/ml;
- , 620CFU/ml;
- , 6.2CFU/ml;
- , Control.
The cross-activities on the immuno-DOX assay. 

**FIG. 2.** The cross-activities on the immuno-DOX assay. M broth was used for cultivation. Other conditions were the same as the standard conditions.

(A) *E. coli*, initial cell concentrations:
- 62000 CFU/ml; 6200 CFU/ml;
- 62 CFU/ml; 6.2 CFU/ml;
- Control.

(B) *B. subtilis*, initial cell concentrations:
- 500000 CFU/ml; 50000 CFU/ml;
- 500 CFU/ml; 50 CFU/ml;
- 5 CFU/ml; Control.

**TABLE 1.** Detection of *S. Typhimurium* in food samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Present method</th>
<th>Brilliant Green Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sliced beef</td>
<td>N.D.*</td>
<td>N.D.</td>
</tr>
<tr>
<td>Ground beef</td>
<td>3.00 × 10⁴</td>
<td>1.11 × 10⁴</td>
</tr>
<tr>
<td>Whole egg</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Whole egg*</td>
<td>1.91 × 10⁴</td>
<td>12.8 × 10⁴</td>
</tr>
<tr>
<td>Soil</td>
<td>3.99 × 10⁴</td>
<td>N.D.</td>
</tr>
<tr>
<td>Soil*</td>
<td>2.05 × 10⁴</td>
<td>4.00 × 10⁴</td>
</tr>
</tbody>
</table>

Numbers indicate *S. Typhimurium* CFU/g-sample.

*Samples were artificially contaminated by *S. Typhimurium.*

not detected.

Samples of sliced meat, ground beef meat, and egg were obtained from local market in Fukui city. Soil and whole eggs inoculated with *Salmonella* cells were also used for the assay. The results were compared to those obtained by the selective medium (Brilliant Green Agar, BGA) assay in Table 1. In most of the cases, *Salmonella* was not detectable in the BGA method. The immuno-DOX assay showed high sensitivity and advantage compared with the BGA method, because the immuno-DOX assay took less than 13 h for detection of *Salmonella*. This method would be useful for the simple and the rapid detection of *Salmonella* in meat and eggs.

In conclusion, an antibody-immobilized 96-hole microplate in which gold electrodes were installed could be used for the simple and rapid quantification of *Salmonella* in some kinds of food. Under optimized conditions, *Salmonella* cells at an initial concentration of 2.5 × 10⁴-2.5 × 10⁵ CFU/ml in the sample showed oxygen consumption curves within a 13 h incubation. The other microorganisms (below 10⁴ CFU/ml), such as *E. coli*, *B. subtilis*, *P. aeruginosa* and *C. aquaticum* did not interfere with the assay system. We demonstrated the usefulness of the immuno-DOX system developed for a *Salmonella* assay in some kinds of food.

**ACKNOWLEDGMENTS**

S. Suye gratefully acknowledges support for this research by a Grant-in-Aid for the prevention of food quality accidents from the Ministry of Agriculture, Forestry and Fisheries of Japan. The authors also wish to thank Dr. Junichiro Arai and Mr. Hideo Katayama, Daikin Environmental Laboratory Ltd. for their helpful discussions. Thanks are also due to Daikin Environmental Laboratory Ltd. for their generous gift of a DOX-96 system.

**REFERENCES**


The application of the present method for the determination of *S. Typhimurium* in food was evaluated.


