Salmonella Detection in Eggs Using Lux+ Bacteriophages

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ABSTRACT

Recombinant bacteriophages specific for Salmonella spp. and containing bacterial luciferase genes were constructed. The phage caused the host cells to luminesce when mixed with Salmonella spp. and the luminescence could be detected using a photon-counting charge-coupled device (CCD) camera, a luminometer, or X-ray film. The initial assay system was capable of detecting Salmonella isolates from group B and group D. Certain isolates from group C could also be detected. With 6 h of preincubation, as few as 10 CFU of Salmonella cells per ml in the original sample could be detected. The minimum time required for the detection of 10^8 CFU/ml was 1 to 3 h with no preincubation, depending on bacteriophage adsorption temperatures. The phage-based assay could be carried out on Petrifilm E. coli Count Plates and the light emission detected within 24 h. The system allowed Salmonella cells to be detected in whole eggs by direct addition of recombinant bacteriophages into the eggs followed by visualization of the luminescent Salmonella cells inside the eggs.

Key words: Salmonella, pathogen detection, phage, bioluminescence, eggs

In order to monitor Salmonella contamination of food, a number of detection procedures have been developed to reduce the time and minimize the number of organisms required to complete the analysis. The techniques used include microbiological, immunological, and molecular procedures.

The conventional procedure for Salmonella detection includes the selection of H2S-positive or lactose-negative colonies from selective media followed by biochemical and serological confirmation. The technique is labor-intensive and time-consuming. It requires multiple subcultures on various selective and indicator media and takes days to complete (10).

The development of immunological methods makes Salmonella detection relatively inexpensive and rapid (6). However, the problem with immunological methods is the lack of specificity for all Salmonella isolates, resulting in the generation of false-positive or false-negative detections (4, 5).

Molecular procedures to detect genetic material instead of gene products have very high specificity. The tests are sensitive and can detect DNA in picogram amounts that equate to 10^2 to 10^3 bacterial cells. However, genetic methods cannot provide all the information that cultural methods provide. Both viable and dead cells are detected. Although the genetic procedure is relatively rapid, an enrichment step is generally required (5, 19).

Recombinant bacteriophages which carry bacterial lux genes can be engineered (18). These genes encode a luciferase enzyme that catalyzes a reaction generating light (9). The recombinant phages lack the metabolic activity required to express the genes and, therefore, remain dark. However, when they are used to infect host cells, following transduction lux genes can be expressed and the host cells become luminescent. A method for the detection of Salmonella spp. based on this phenomenon is sensitive and rapid: as few as 100 Salmonella typhimurium cells per ml could be detected within an hour according to previous studies (12-16). The specificity of the detection is determined by bacteriophage and host specificity and only viable cells are detected.

The present study examines the possibility of applying this detection system directly to foods.

MATERIALS AND METHODS

Bacterial cultures and bacteriophages

Salmonella cultures used in this study were kindly provided by Dr. R. Khakhria, National Laboratory of Enteric Pathogens, Laboratory Centre of Disease Control; Dr. J.-Y. D'Aoust, Health Protection Branch, Health Canada; Dr. C. Poppe, Health of Animals Laboratory, Agriculture & Agrifood Canada; Dr. C. Gyles, Department of Veterinary Microbiology and Immunology, University of Guelph; and Dr. L. Harris, Department of Food Science, University of Guelph.

Bacteriophage P22 was purchased from the American Type Culture Collection (Rockville, MD). Other phages used were induced from Salmonella spp. chromosomes in this laboratory.

Luminescent Escherichia coli cultures were donated by Dr. C. Dion from the University of Laval, Quebec, and Dr. C. Kado from the University of California, Davis.

Bacteriophage induction

Lysogenic bacteriophages located on Salmonella spp. chromosomes were induced using a mitomycin C induction procedure. The
lysenic cultures of *Salmonella* spp. were grown in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI) at 37°C with shaking at 200 rpm. An overnight culture was transferred into BHI broth supplemented with 5 μg of mitomycin C (Sigma Chemical Co., St. Louis, MS) per ml and incubated at 37°C for 4 to 6 h with vigorous shaking at 200 rpm. After the addition of 0.3% chloroform, the bacterial lysates were centrifuged at 4,000 × g and 4°C for 20 min in a Beckman Model J2-MC refrigerated centrifuge (Beckman Instruments (Canada) Inc., Mississauga, ON). The supernatant was removed and stored at 4°C and the bacterial debris was discarded.

**Construction of luminescent Salmonella cultures**

Luminescent cultures used in this study were obtained by either electroporation or conjugation. Lysogenic *S. enteritidis* GCDE was obtained by electroporation of wild-type *S. enteritidis* cells with *luxAB* plasmid pHDB30 (3). Other luminescent cultures were constructed by bacterial conjugations. Four pairs of conjugations were performed. The first was between *E. coli* HB101(pUC6263) and *S. typhimurium*. *E. coli* HB101(pUC6263) was nalidixic acid sensitive but carried two antibiotic-resistance markers (for ampicillin and chloramphenicol) on the plasmid and the entire lux operon from *Vibrio fischeri* situated on transposon Tn4431 (tetracycline resistant) was also located on the plasmid (11). At first, nalidixic acid-resistant spontaneous mutants of *S. typhimurium* were selected and used as recipients. The conjugation was performed on 0.45-μm-pore-size nylon membrane (Boehringer Mannheim, Laval, Quebec, Canada) at 30°C for 18 h. Nalidixic acid and tetracycline double-resistant transconjugants that were also luminescent were viewed by using a BIQ Bioview Image Quantiﬁer (Cambridge Imaging, Cambridge, UK) and selected.

Matings were also performed between *E. coli* WA803(pDB30), a luminescent culture containing Tn3 (kanamycin resistant) as well as *luxA* and *luxB* genes from *V. harveyi* (1), and three recipients: *S. infantis*, *S. thompson*, and *S. hadar*. Since *E. coli* WA803 has a streptomycin-resistant residue on its chromosome, the first step of the mating was to select the streptomycin-resistant spontaneous mutants of *S. infantis*, *S. thompson*, and *S. hadar*. Matings were performed under the same conditions described above and streptomycin- and kanamycin-resistant luminescent transconjugants were selected.

**Production of transducing bacteriophages**

Recombinant bacteriophages used in the detection were obtained either by infection of luminescent *Salmonella* strains with wild-type bacteriophages or by bacteriophage induction from luminescent, lysogenic bacterial cells. Recombinant bacteriophages MJB and MJc were produced by bacteriophage induction from luminescent *S. typhimurium* and *S. infantis*, respectively, using the mitomycin C induction procedure. Another *lux*+ recombinant phage, MJD, was obtained by infection of luminescent *S. enteritidis* culture with bacteriophage P22. Following cell lysis and after the addition of 0.3% chloroform, the bacteriophages were collected by centrifugation at 4,000 × g and 4°C for 20 min. The bacteriophage suspensions were then treated with DNase and RNase (each at a final concentration of 200 μg/ml) at 37°C for 1 h. The enzymes were removed by chloroform extraction, allowing the recombinant bacteriophages to be used for the detection of *Salmonella* spp.

**Detection of Salmonella spp. using *lux*+ bacteriophage transfections**

*Salmonella* spp. were grown in BHI broth at 37°C until the absorbance of the culture at 600 nm in a Novaspec II spectrophotometer (Pharmacia, Uppsala, Sweden) reached 0.9 to 1.0. The bacterial cells (100 μl) were mixed with 10 μl of the bacteriophage stock (10⁹ PFU/ml) and incubated at 42°C for 30 min without shaking to allow bacteriophage adsorption. The mixture was inoculated on the surface of a selective medium composed of BHI agar containing kanamycin (100 μg/ml) and tetracycline (50 μg/ml) and the plates were incubated at either 30°C (for transfectants containing lux genes from *V. fischeri*) or 37°C (for transfectants containing lux genes from *V. harveyi*) for 18 h. The transduced cells were luminescent when analyzed with the BIQ Bioview Image Analyzer. All experiments were performed in duplicate.

**Determination of transfection frequency**

*Salmonella* spp. were inoculated in BHI broth and incubated at 37°C to a cell density equivalent to an absorbance at 600 nm of 0.9 to 1.0. The culture was serially diluted from 10⁻¹ to 10⁻⁹ in phosphate-buffered saline (PBS) at pH 7.4. Bacterial dilutions (100 μl) to which had been added bacteriophage suspension (10 μl containing 10⁶ PFU/ml) were incubated at 37°C for 30 min. BHI-kanamycin (100 μg/ml) plates were inoculated with the bacteria/bacteriophage suspension and incubated at 37°C for 16 h. The number of phage-infected *lux*+ *Salmonella* colonies were counted and the transfection frequency was calculated as the ratio of the number of these colonies to the total *Salmonella* population used in the transfection.

**Determination of minimal time required for the detection of *Salmonella***

A subculture of *Salmonella* spp. (absorbance at 600 nm of 0.9 to 1.0; 100 μl) was mixed with recombinant bacteriophage stock (10⁹ PFU/ml; 10 μl) and incubated at 37°C for 30 min. The experiment was repeated but, this time, bacteriophage adsorption was performed at 42°C for 30 min. BHI broth (500 ml) containing 100 μg of kanamycin per ml was added to each tube and shaken at 37°C. Luminescence was measured at hourly intervals using a Biotrace Multilite luminometer (Biotrace, Bridgend, UK). The earliest time when a positive luminescence reading was measured (i.e., 2× background reading) was recorded as the minimum time required for detection of the *Salmonella* spp. All experiments were performed in duplicate.

**Determination of minimum number of *Salmonella* cells detected by the assay**

*S. enteritidis* cells were grown in BHI broth and incubated at 37°C until the absorbance at 600 nm reached about 0.95. The bacterial culture was serially diluted in PBS from 10⁻¹ to 10⁻⁸ and incubated at 37°C for 6 h. Samples (100 μl) from each preincubated bacterial dilution were mixed with recombinant bacteriophage (10⁶ PFU/ml; 10 μl). After incubation at 42°C for 30 min, the mixtures were inoculated on BHI agar (1.5%, w/vol) containing kanamycin (100 μg/ml) by dropping an aliquot (100 μl) onto the surface of the plate. The number of luminescent transfectants was recorded and the minimum number of bacterial cells that could be detected after the preincubation was determined.

**Biochemical and serological characterization of transfectants**

Luminescent strains of *Salmonella* spp. obtained from recombinant bacteriophage transfectants were tested for biochemical properties using the Vitek Jr. system (bioMérieux Vitek Inc., Hazelwood, MS). The cultures were grown on BHI agar plates at 37°C. Colonies were characterized by using a Gram-Negative-Identification card (bioMérieux Vitek Inc.) according to the procedures recommended by the manufacturer.

Dehydrated antisera (Difco) were resuspended into saline according to the manufacturer’s instruction. The serology of the
transfectants was confirmed, using slide agglutination assays, on overnight cultures of Salmonella spp. from BHI plates.

**Genotypic characterization of transfectants using polymerase chain reaction**

The nucleotide sequences of the *lux* gene of both *V. harveyi* (Accession no. X06758) and *V. fischeri* (Accession no. X58791) were retrieved from GenBank. The primers designed for the *lux* gene from *V. harveyi* were 20 bp in length with a GC content of 50% and melting temperatures (Tm) of 65 and 70°C. The up and down primer sequences were located at positions 24 to 43 and 1034 to 1053 and the size of the product was 1030 bp. The primers for the *lux* gene of *V. fischeri* were also 20 bp in length; however, the GC content was 50% and 55% and Tm was 69 and 68 for the up and down primers, respectively. The up primer sequence was located at position 34 to 53 and that of the down primer at 903 to 924. The product was 924 bp in length.

The nucleotide sequences of primer for the polymerase chain reaction were as follows:

\[
V_h \quad \text{up: cct tct cac tta tca gec ac}
\]

\[
V_f \quad \text{down: tgg cat cac atc aga ctg ga}
\]

\[
V_h \quad \text{up: cgt tct cac cac cag gtg aa}
\]

\[
V_f \quad \text{down: act gct cag gag tgc cta ca}
\]

The experimental conditions used in the PCR reaction were 90°C for 3 min for template DNA denaturation, 50°C for 3 min for primer annealing, and 70°C for 3 min for DNA elongation in the first cycle. In the subsequent 29 cycles, 90°C for 30 s, 50°C for 1 min and 70°C for 1 min were used for template denaturation, primer annealing, and DNA elongation, respectively.

**Combination of lux* phage assay with Petrifilm for convenient detection of Salmonella spp.**

Different methods for detecting light emission were investigated. Conditions for the adsorption of recombinant bacteriophage to the bacterial cells was as described above. The procedure was then modified so that 1 ml of PBS-kanamycin (100 μg/ml) solution was added to the bacteria-recombinant bacteriophage suspension and inoculated on Petrifilm *E. coli* Count Plates (3M Health Care, St. Paul, MN). Following overnight incubation of the Petrifilm at 37°C, luminescent *S. enteritidis* cells could be visualized with the Biq Bioview Image Quantifier. Alternatively, X-ray (Kodak Diagnostic Film X-Omat™ AR) autoradiography of the Petrifilm containing transduced *Salmonella* colonies could be performed using a 5-min exposure time at room temperature. The X-ray film was developed using developer and fixer supplied by Eastman Kodak (Rochester, NY).

Liquid selective media were also used. BHI-kanamycin broth (500 μl) was added to the bacteria and recombinant bacteriophage mixture and incubated at 37°C with shaking. The luminescence produced by phage-infected lux* S. enteritidis* cells was measured with a Biotrace Multilite luminometer.

**Detection of Salmonella enteritidis in eggs**

Wild-type *S. enteritidis* cells were grown in BHI broth and incubated at 37°C with shaking until the absorbance at 600 nm reached about 0.95. The bacterial culture was then serially diluted and 100 μl of the 10^-5, 10^-6, and 10^-7 dilutions were used to inoculate the white of whole eggs by injection through the shell with a sterile syringe needle; the hole was sealed with sterile tape. The exact number of *S. enteritidis* cells in each of the dilutions was determined by plate count. The infected eggs were incubated at 37°C for 24 h. Recombinant bacteriophage was then added into the egg through the inoculation hole. Bacteriophage adsorption was allowed at 42°C for 30 min. After the addition of the selective agent (50 μl of 100 μg/ml kanamycin solution), the eggs were incubated at 37°C. Images of the luminescent eggs after overnight (about 16 h) and 24 h of incubation were obtained using a Mitsubishi Color Video Processor Model CP110U (Mitsubishi Electronics America, Inc., Cypress, CA).

**RESULTS AND DISCUSSION**

**Recombinant bacteriophages**

Three recombinant bacteriophage were constructed in this study: bacteriophage P22 containing *lux* and *luxB* genes (MJd); an uncharacterized bacteriophage containing the entire *lux* operon of *V. fischeri* induced from lysogenic *S. typhimurium* chromosome (MJb); and an uncharacterized bacteriophage carrying *lux* and *luxB* genes of *V. harveyi* induced from lysogenic *S. infantis* chromosome (MJc). The two latter bacteriophages were obtained using the mitomycin C induction procedure.

**Detection of Salmonella strains**

Seventy-nine bacterial cultures were tested (57 *Salmonella* spp. and 22 nonsalmonella cultures) (Table 1). All 57 *Salmonella* isolates (100%) were successfully detected by the recombinant phage assay and none of the nonsalmonella (i.e., 0% false positives) were detectable. The *Salmonella*

**TABLE 1. Organisms used to test the specificity of the assay method**

<table>
<thead>
<tr>
<th><em>Salmonella</em> sp.</th>
<th>Non-<em>Salmonella</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. agona</em> (2)</td>
<td><em>Bacillus cereus</em> (3)</td>
</tr>
<tr>
<td><em>S. berta</em> (2)</td>
<td><em>Lactobacillus brevis</em></td>
</tr>
<tr>
<td><em>S. braenderup</em> (1)</td>
<td><em>Lactococcus lactis</em></td>
</tr>
<tr>
<td><em>S. brandenburg</em> (2)</td>
<td><em>Leuconostoc lactis</em></td>
</tr>
<tr>
<td><em>S. enteritidis</em> (22)</td>
<td><em>Listeria monocytogenes</em> (3)</td>
</tr>
<tr>
<td><em>S. gallinarum</em> (1)</td>
<td><em>Staphylococcus aureus</em> (3)</td>
</tr>
<tr>
<td><em>S. hadar</em> (1)</td>
<td><em>Enterobacter aerogenes</em></td>
</tr>
<tr>
<td><em>S. heidelberg</em> (2)</td>
<td><em>Escherichia coli K-12</em></td>
</tr>
<tr>
<td><em>S. indiana</em> (1)</td>
<td><em>Escherichia coli O157:H7</em> (2)</td>
</tr>
<tr>
<td><em>S. infantis</em> (3)</td>
<td><em>Klebsiella pneumoniae</em></td>
</tr>
<tr>
<td><em>S. panama</em> (1)</td>
<td><em>Proteus vulgaris</em></td>
</tr>
<tr>
<td><em>S. reading</em> (3)</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td><em>S. saintpaul</em> (2)</td>
<td><em>Serratia marcescens</em></td>
</tr>
<tr>
<td><em>S. schwarzengrund</em> (1)</td>
<td><em>Shigella dysenteriae</em></td>
</tr>
<tr>
<td><em>S. sendai</em> (1)</td>
<td><em>Yersinia enterocolitica</em></td>
</tr>
<tr>
<td><em>S. thompson</em> (1)</td>
<td></td>
</tr>
<tr>
<td><em>S. typhi</em> (1)</td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em> (10)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses refer to number of strains tested.
spp. tested included S. typhimurium, S. hadar, S. enteritidis, S. heidelberg, and S. thompson, which made up to 63.2% of the Salmonella cultures isolated between 1983 and 1993 according to the national survey conducted by Laboratory Centre of Disease Control (Dr R. Khakhria, personal communication). S. enteritidis strains detected by the recombinant bacteriophage included isolates from human, bovine, porcine, avian, food, and unknown origin. The common phage types of S. enteritidis in North America, phage types 8, 8a, 13, and 13a, were successfully detected by the system. An untypable phage type of S. enteritidis was also detected.

Transfection efficiency and minimum time required for detection of salmonellae

Cells of Salmonella spp. were mixed with transducing bacteriophage and adsorption was allowed. Luminescent transduced colonies were grown on selective media overnight at 37°C. The number of transfectants were counted and the transfection efficiency was calculated as $2.2 \times 10^{-4}$ to $3.0 \times 10^{-4}$ under these experimental conditions.

Different isolates did not have equal transfection efficiencies; therefore, the times required for the detection of different Salmonella isolates were not exactly the same. When the bacteriophage adsorption was performed at 37°C for 30 min, the minimum time required for the detection of approximately $10^8$ CFU/ml of Salmonella sp. was 3 h (Fig. 1). However, if the adsorption was allowed at 42°C for the same period of time, the detection could be accomplished within 1 h without preincubation (Fig. 1). Experiments showed that the adsorption performed at 42°C for 30 min increased the transfection efficiency by 17% and 72% when compared with that obtained at 37°C and 30°C for the same period of time, respectively (Fig. 2).

Sensitivity of the detection method

Salmonella cultures were diluted to obtain suspensions containing about 1,000, 100, 10 and 1 CFU/ml. Preincubation at 37°C for 6 h increased the sensitivity of the detection system and a positive response was obtained at the bacterial dilutions containing 1,000, 100 and 10 CFU/ml. Thus, adoption of the preincubation procedure allowed reliable detection by the recombinant phage of as low as 10 CFU/ml in the original sample (Fig. 3). Kodicara et al. (8) used a group of phage with a broad specificity for Enterobacteriaceae which they engineered to carry the lux gene. They analyzed 141 swab samples from a meat-processing plant and showed that the detection limit for the assay was $10^2$ CFU/g or cm$^2$. A similar increase in sensitivity of the assay to that observed in the present study was brought about by the inclusion of a 4-h enrichment step at 37°C before infection by the phage. This allowed detection of as few as 10 enteric bacteria per cm$^2$ in the original sample.

A similar assay using lux$^+$ P22 phage has been described for the detection of S. typhimurium LT2 (16). These authors were able to detect as few as $10^2$ S. typhimurium cells in mixed culture. This technique was used to determine the most probable number of S. typhimurium in environmental samples including water, soil, and sewage sludge (17). There was an excellent correlation between MPN obtained by the luminescence method and plate count for all samples, and the lux$^+$ phage method, which could be performed in 24 h, gave no false-positive or false-negative results.

Visualization of transfectants

Attempts were made to simplify the detection procedure. To eliminate the need for media preparation, the experimental procedure was modified and Petrifilm E. coli Count Plates were used to replace agar plates. Results showed that Petrifilm was a convenient alternative to the normal selective media (Fig. 4). To use the system in food-processing plants, a portable luminometer and liquid selective media could be applied to measure the luminescence reading (Fig. 2). For the laboratory which cannot
FIGURE 3. Minimum number of wild-type Salmonella cells required for detection. A Salmonella sp. culture was diluted from $10^{-1}$ to $10^{-8}$ and the diluted bacterial suspensions were preincubated at 37°C for 6 h. Preincubated cultures (100 µl) from each dilution were mixed with recombinant bacteriophage (10 µl; $10^6$ PFU/ml) and incubated at 42°C for 30 min. Luminescent Salmonella cells were identified using the Biq Bioview Image Quantifier and images produced with (A) 1,000 CFU/ml; (B) 100 CFU/ml; (C) 10 CFU/ml; and (D) 1 CFU/ml are shown.

afford instrumentation for light detection, X-ray autoradiography of Petrifilm containing luminescent colonies is the ideal solution (Fig. 5).

**Direct detection of Salmonella in shell eggs**

Twelve eggs were divided into three groups and each group of 4 eggs was infected by a suspension of *S. enteritidis* to give a final concentration of approximately $4 \times 10^3$ CFU per egg, $3.3 \times 10^2$ CFU per egg and $6.3 \times 10^1$ CFU per egg, respectively. The concentration of bacteriophage added to the egg was 15-fold higher than that used in plate assays to increase the chance of contact with and adsorption to Salmonella cells. After overnight incubation (about 16 h), only the eggs infected at a dose rate above $10^3$ CFU per egg became luminescent (Fig. 6). However, when the incubation period was extended to 24 h, eggs infected at all test concentrations emitted light.

The assay offers the ability, not only to detect *Salmonella* contamination in whole eggs, but also to find its location. In the case of the egg pictured in Figure 6, growth of *S. enteritidis* is located in the yolk, and this is in agreement with previous research findings (7) and with results obtained using a luminescent construct of *S. enteritidis* to monitor growth in whole shell eggs (2).

The technique offers a potentially cheap and simple

FIGURE 4. Selection of luminescent Salmonella cells on Petrifilm. Bacteriophage adsorption was performed as described in the legend to Fig. 3: 1 ml of PBS containing a selective agent was added to the bacteria-bacteriophage mixture and this was used to inoculate Petrifilm E. coli Count Plates. The luminescent colonies were identified by monitoring light emission after overnight incubation at 37°C using a Biq Bioview Image Quantifier.

FIGURE 5. Identification of recombinant luminescent Salmonella colonies by X-ray autoradiography. The Petrifilm E. coli Count Plate containing luminescent Salmonella cells was exposed to X-ray film for 5 minutes. Colonies could be observed after developing and fixing.

FIGURE 6. Detection of Salmonella sp. in artificially inoculated eggs. Two-day-old eggs were inoculated with 10, 100, and 1,000 CFU of Salmonella sp. and preincubated at 37°C for 24 h. The recombinant bacteriophage was added to the eggs and adsorption to the bacterial cells was allowed at 37°C for 30 min. The selective agent was then added and luminescence emitted by the recombinant Salmonella cells inside the eggs were observed through the egg shells using a Biq Bioview Image Quantifier.
method of detecting Salmonella spp. in whole eggs. It would substantially cut down on the labor costs involved in conventional cultural methods and is capable of being automated.

Confirmation of transfectants by using a biochemical system and by PCR

Transfectants selected from recombinant bacteriophage infections were biochemically tested using the Vitek Jr.
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