Research Note

Optimization and Validation of a Simple Method Using P22::luxAB Bacteriophage for Rapid Detection of Salmonella enterica Serotypes A, B, and D in Poultry Samples

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ABSTRACT

A simple method was developed for the fast and inexpensive detection of Salmonella Typhimurium using a recombinant P22::luxAB phage. All the steps from phage production to detection were considered. A strain of Salmonella Typhimurium harboring the prophage P22::luxAB was grown in batch culture to produce spontaneously the recombinant bacteriophage. Batch production to stationary phase was better for propagation of the phage and led to a total population of 4.3 × 10⁹ (±4.3 × 10⁸) PFU/ml of P22, including only 1.4 × 10⁹ (±1 × 10⁹) PFU/ml harboring the luxAB genes. After preenrichment, a simple four-step bioassay was tested and optimized for several parameters. The detection limit of the luminometer was only 5 × 10² (±1.75 × 10²) CFU Salmonella Typhimurium per ml, but increased to 1.5 × 10³ (±1.17 × 10³) CFU Salmonella Typhimurium per ml when the cells were in a complex matrix. Finally, the bioassay was applied to the detection of Salmonella Typhimurium LT2 in 14 different feed and environmental samples (including duck feed, litters, and feces) spiked either before or after the preenrichment process. It was possible to detect Salmonella Typhimurium LT2 in all samples within 16 h.

Statistics from the Centers for Disease Control and Prevention suggest that, in the United States, food-related infections are responsible for 76 million cases of illness, 325,000 hospitalizations, and 5,000 deaths per year (21). To minimize the impact of foodborne illness, it is essential to develop simple, rapid, inexpensive, and reliable microbiological tests that can be performed in the context of hazard analysis critical control point principles (8).

Current regulations stipulate that no Salmonella should be detectable in 25 g of food. Standard methods require four phases, namely a preenrichment phase, a selective enrichment phase, a reisolation phase, and finally, an identification phase. The maximum total duration for completion of these tests is 66 to 72 h, which is extended to 4 days when the ISO 6579 2002 standard method is used (10).

There has been considerable interest in the use of bacteriophage for the detection of bacterial pathogens (20, 23); however, very few assays based on bacteriophage have been commercialized. One such assay that has yet to fulfill its potential relies on bacteriophages that have been genetically modified to carry reporter genes. In one such application, the specificity of the detection is guaranteed by the use of bioluminescence-inducing bacteriophages (6, 27). For example, the P22::lux phage recognizes the Salmonella lipopolysaccharide antigen through its attachment protein (GP9), which possesses an endorhamnosidase activity (4, 25). The P22 tailspike recognizes the repetitive O antigen in the outer membrane lipopolysaccharide of pathogenic Salmonella species belonging to serogroups A, B, and D1 (3). The recognition is followed by recombinant phage DNA injection and insertion as prophage in Salmonella DNA. Expression of the luxAB genes from Vibrio fischeri or Vibrio harveyi, or the luc genes from fireflies inserted into the genome of the phage leads to the emission of light. In the case of the lux genes, the light is generated after a complex biochemical reaction catalyzed by the enzyme luciferase (22).

Work using bacteriophages was instigated in the 1980s (29). Since then, research has been conducted on Escherichia coli (14), Mycobacterium tuberculosis (12), Salmonella spp. (6, 15, 16, 28), and Listeria monocytogenes (18, 19).

In 1996, Chen and Griffiths (6) developed the P22::luxAB used in this study, but the proposed protocol was not of a commercially viable format, following industrial recommendations. Thus, the present publication describes a
protocol for the development and optimization of a simple and easy-to-use test based on bacteriophage. As a model system, a bioluminescent bacteriophage P22::luxAB for the detection of Salmonella enterica serotypes A, B, and D was used in a disposable format. The assay has been optimized and validated for use with feed and environmental samples commonly tested by the poultry industry using artificially contaminated samples.

MATERIALS AND METHODS

Microbial strains. Bacteriophage P22::luxAB was described by Chen and Griffiths (6). Wild-type bacteriophage P22 (P22WT) was purchased from the American Type Culture Collection (LGC Promochem, France). Salmonella Typhimurium LT2 (CIP 6602) and Salmonella Enteritidis (CIP 81.3) were purchased from the culture collection of the Institut Pasteur (France).

Salmonella Typhimurium LT2::luxAB and Salmonella Enteritidis::luxAB were obtained as follows. Salmonella Typhimurium LT2 or Enteritidis strains were grown overnight at 37°C in Luria-Bertani (LB; Oxoid, Dardilly, France) medium, and then were diluted in fresh LB medium to an optical density at 620 nm (OD620) of 0.6 (about 10⁸ CFU/ml). One hundred microliters was added in a prewarmed 1.5-ml microcentrifuge tube (42°C) to 10 μl of P22::luxAB bacteriophage suspension (10⁵ PFU/ml), and the tubes were incubated at 42°C for 30 min. The mixture was subsequently added to 3 ml of a soft agar LB medium at 45°C containing ampicillin (50 μg/ml final concentration; Sigma-Aldrich, Lyon, France). After 10 min of gentle mixing, the contents were poured on hard agar LB medium plates (plus ampicillin). The plates were incubated for 18 h at 37°C after drying of the surface. Bioluminescent colonies were revealed after addition of 50 μl of pure decanal (Sigma-Aldrich) to the lids of the plates.

Bacteriophage stocks were stored at 4°C in SM buffer (5.8 g of NaCl, 2 g of MgSO4·7H2O [Sigma], 50 ml of Tris-HCl [Sigma] 1 M, pH 7.5), and 5 ml of a 2% (wt/vol) gelatin solution in 1 liter of distilled water, and the pH was adjusted to 7.5. Bacteria were stored at −80°C in 15% (vol/vol) glycerol.

Growth media and solutions. LB medium was prepared according to Atlas (2) and adjusted to pH 7.0. Agar (Biokar Diagnostics, Pantin, France) or agarose (LE, analytical grade, Promega, Charbonnières, France) was added at 7 g/liter and 10 g/liter for soft and hard agar plates, respectively. When it was necessary, ampicillin was added to achieve a final concentration of 50 μg/ml. Buffered peptone water (BPW; MSD, Paris, France) and XLT4 medium (MSD) were prepared according to the manufacturer’s instructions.

The N-decyl aldehyde (decanal) was prepared in distilled water with 2-propanol (Prolabo, VWR International France, Fontenay-sous-Bois, France) at the ratio of 400 μl of 2-propanol to 1 μl of decanal. The final volume was adjusted to 100 ml with distilled water. Stock solutions of decanal were stored at room temperature in amber bottles and renewed each day. The concentration of 2-propanol used for each experiment did not inhibit bacterial growth according to optical density readings (data not shown). All media and solutions were sterilized by autoclaving (100°C, 30 min) except the XLT4 medium, which was filter sterilized. Decanal was not autoclaved and not sterilized.

Pancreatic DNase I (2 mg; Fluka, Sigma-Aldrich) was dissolved in 1 ml of sterile 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM MgCl2. One milliliter of glycerol was added, and the resulting solution was stored at −20°C. Pancreatic RNase (2 mg; Sigma-Aldrich) was dissolved in 2 ml of sterile Tris-EDTA buffer (10 mM Tris–1 mM EDTA, pH 7.6) and then stored at −20°C.

Culture conditions and bacteriophage production. The batch growth of Salmonella strains was performed in a 1-liter flask containing 500 ml of LB medium plus ampicillin, with shaking (Minitron, Infors Sarl, Massy, France) at 250 rpm and at 37°C. Every 20 min, a sample was removed aseptically to follow growth by monitoring OD620. Samples were also removed at exponential (OD620 = 0.9), late-exponential (OD620 = 2), early-stationary (OD620 = 2.6), and late-stationary (OD620 = 3.9) phases of growth for enumeration of bacteria and phage particles.

The P22::luxAB bacteriophages were processed after batch growth of Salmonella Typhimurium LT2::luxAB under the conditions described above. After 14 to 16 h, chloroform was added to the bacterial culture during a 30-min period to achieve a final concentration of 4% (vol/vol). The suspension was then centrifuged at 5,000 × g (10 min, 4°C) and the supernatant was filtered through a 0.22-μm Analypore membrane (Millipore, Goyancourt, France). The filtrate was treated with DNase and RNase solutions (1 μg/ml) at 37°C for 30 min.

Microbial measurements. Counts of Salmonella Typhimurium LT2 or Enteritidis were performed in duplicate after serial dilution in 0.01 M MgSO4 solution. One hundred microliters was spread on LB medium with or without ampicillin. The plates were incubated at 37°C for 24 h.

Counts of Salmonella from the poultry environmental and feed samples were obtained by plating on XLT4 medium. Serial dilutions were made in 0.01 M MgSO4. The plates were incubated at 37°C for 24 h.

The total phage count (PFU total) was measured after serial dilution of the bacteriophage suspension in SM buffer. Ten microliters of the bacteriophage dilutions was added to 100 μl of a sensitive Salmonella Typhimurium LT2 suspension (OD620 = 0.6), and the mixture was transferred after 30 min at 42°C to 3 ml of soft LB agar medium (without ampicillin). After 10 min at 45°C, the contents of the tubes were poured on hard agar LB medium plates (without ampicillin). The plates were incubated at 37°C for 24 h.

The P22::luxAB counts were obtained using the same protocol, except that ampicillin was added to both the soft and hard LB media. The infection of Salmonella Typhimurium LT2 with the P22::luxAB in the medium containing ampicillin led to bioluminescent and ampicillin-resistant colonies which were then counted.

Bioluminescence measurements. Bioluminescence was monitored with a luminometer (Minitron LB 96V, EG&G Berthold, Bad Wildbad, Germany) in white-opaque 96-well microtiter plates (Nunc, Roskilde, Denmark). Each well contained 100 μl of the bacterial sample (with or without a preenrichment). For the bioassay, bioluminescence was measured in a single tube with a Lumat LB 9507 luminometer (EG&G Berthold). The bioluminescence was revealed after addition of decanal (80 μM final concentration). The results were expressed as a light production; that is, the ratio between bioluminescence at each sampling time to the bioluminescence at t = 0.

Bioassay description. Preenrichment of both Salmonella Typhimurium LT2 (positive control) or poultry samples was carried out in BPW medium incubated for 12 to 14 h at 37°C. One hundred microliters of the bacterial suspension was transferred to six plastic tubes of 5 ml, and then 100 μl of P22::luxAB bacteriophage (3.15 × 10⁵ [± 1.15 × 10⁴] PFU/ml) was added (the control Sal-
monella Typhimurium LT2 contained about $10^6$ to $10^7$ CFU/ml, final concentration). After incubation of the tubes for 1 h at 37°C without agitation, 300 μl of a 1.65× BPW medium containing ampicillin was added. Three tubes were incubated at 37°C, without agitation for 2 or 4 h. Bioluminescence was recorded after addition of decanal as described above. The steps of the bioassay are illustrated in Figure 1.

**Sample collection.** Fourteen samples including duck feed ($n = 11$), feces ($n = 1$), and litters ($n = 2$) were collected at two poultry companies in the Vendée and Mayenne departments of France. Samples were collected using aseptic techniques, transported to the laboratory on ice, and then used for the bioassay during the same day. Twenty five grams of the sample was added to a sterile plastic bag containing BPW medium to achieve a weight of 300 g as recommended by the Association Francaise de Normalisation method (NF U 47-100) (1). The bag was closed and incubated for 12 to 14 h at 37°C, without agitation. If samples were spiked with Salmonella Typhimurium LT2 before the preenrichment, then 5 to 10 μl of an overnight culture ($OD_{620} = 2$, corresponding to $5 \times 10^9$ CFU/ml) of the strain in BPW was added in the bag with the sample. When the sample was spiked after the preenrichment, a specific volume of the overnight culture of Salmonella Typhimurium LT2 was added to achieve the desired concentration. The negative controls were unspiked samples (poultry samples plus P22::luxAB). A positive control (Salmonella Typhimurium only) was added to each bioassay, and the analyses were conducted with Salmonella Typhimurium only and P22::luxAB.

**RESULTS AND DISCUSSION**

**Bacteriophage production.** The effect of culture conditions on bacteriophage production was investigated. Salmonella Typhimurium LT2 harboring the prophage P22::luxAB was cultivated over time in batch mode, and samples were withdrawn at different points in the growth cycle. The stationary phase was the most efficient for phage production leading to a population of $4.3 \times 10^9 \pm 4.3 \times 10^9$ PFU/ml of all P22 phage particles (P22_total). Of these, only $1.4 \times 10^6 \pm 10^6$ PFU/ml harbored the luxAB genes (P22lux). More lipopolysaccharide is produced by the Salmonella strain in stationary phase (7, 9), and this may increase the number of adsorption sites for the phages. The total production of the recombinant bacteriophage was nevertheless close to that found for the parent P22 phage. The production of a mixture of P22lux and P22 wild type may be due to the production of inactive phage P22 (11), demonstrating that during induction of the prophage P22, both active phage (entire phage able to propagate) and active heads (containing the DNA but lacking the base plate necessary for propagation) were produced. Alternatively, the mechanism of DNA encapsidation during the P22 life cycle (5, 13, 26) during which a concatemer is cut, allowing the packaging of a DNA copy into the capsid, could be influenced by the 3.5 kb insertion of the luxAB.

**Effect of the prophage P22::luxAB on the growth of Salmonella.** Step 3 of the bioassay (Fig. 1) involves growth of Salmonella after infection with the P22::luxAB bacteriophage to overcome potential inhibition of bacterial growth due to the energy requirement for bioluminescence. Consequently, Salmonella could be out competed by other ampicillin-resistant strains utilizing the carbon source of the peptone water. Growth and bioluminescence of Salmonella Enteritidis were monitored in peptone water before and after infection with the P22::luxAB bacteriophage. Growth was not altered by the prophage integration into the host DNA when Salmonella Enteritidis was grown in peptone water in the presence or absence of ampicillin. The growth rates ($\mu$) were 1.49, 2.23, and 1.69 h$^{-1}$ for the uninfected strain, the infected strain grown in the presence of ampicillin, and the infected strain grown in the absence of ampicillin, respectively. Bioluminescence was also not affected by the presence of ampicillin.

**Detection limits of the current bioassay.** To determine the detection limit of the assay, we considered (i) the lowest number of bioluminescent Salmonella cells that could be de-
ected with our luminometer (DL_L), and (ii) the lowest Salmonella concentration detected after addition of the bacteriophage after preenrichment of the sample (DL_APE).

DL_L was determined both for Salmonella Typhimurium luxAB diluted in MgSO_4 solution and in a complex matrix from a 12-h preenrichment of a fecal sample (Fig. 2). In the MgSO_4 solution, bioluminescence produced by only 5 × 10^2 (±1.75 × 10^2) CFU/ml was detected, but with the fecal sample, the detection limit increased to 1.5 × 10^4 (±1.17 × 10^4) CFU/ml. The latter was considered to be the DL_L for the bioassay.

DL_APE was calculated following serial dilution of Salmonella Typhimurium LT2 wild type with or without a 12-h preenrichment of a fecal sample. Each dilution was then infected with 3.15 × 10^5 (±1.15 × 10^5) PFU/ml P22::luxAB, and bioluminescence was recorded. For Salmonella Typhimurium LT2 only, DL_APE was 6.5 × 10^3 (±1.5 × 10^3) CFU/ml, but when Salmonella Typhimurium LT2 cells were in a complex matrix (fecal sample), DL_APE was increased 100-fold (DL_APE = 1.65 × 10^5 [±0.15 × 10^5] CFU/ml); an extended incubation time did not improve the detection limit (Fig. 3).

The detection limit after the preenrichment (DL_APE) is sufficient for commercial application since the Salmonella concentration after a preenrichment in peptone water ranges from 10^6 to 10^8 cells per ml. This DL could be decreased by using a higher P22::luxAB phage concentration to compensate for possible nonspecific adsorption of the phages.

Application of the bioassay to the detection of Salmonella in poultry environmental and feed samples. Fourteen different feed and environmental samples (duck

FIGURE 2. Detection limit of the luminometer (DL_L) for the detection of Salmonella with the bioassay. Salmonella Typhimurium LT2::luxAB was either diluted in 0.01 M MgSO_4 or in a complex matrix coming from a 12-h preenrichment culture in peptone water of a fecal sample. Bioluminescence was monitored after addition of decanal (80 μM final concentration). Each experiment was repeated four times. The average P22::luxAB concentration was 3.15 × 10^5 (±1.15 × 10^5) PFU/ml.

FIGURE 3. Detection limit for the detection of Salmonella after preenrichment (DL_APE). Salmonella Typhimurium LT2 wild type was grown in LB medium, diluted in PBS buffer, or added to a complex matrix (12-h preenrichment culture in peptone water of a fecal sample). Each dilution received 3.15 × 10^5 (±1.15 × 10^5) PFU/ml P22::luxAB suspension, and the bioassay was performed as described in “Materials and Methods.” The experiments were performed in duplicate at 37°C.
feed, litters, feces) were inoculated with *Salmonella Typhimurium* LT2 either before or after the preenrichment process, and the bioassay was carried out. For samples spiked after the preenrichment, *Salmonella Typhimurium* LT2 wild type was introduced at a concentration above the spiked after the preenrichment, *Salmonella Typhimurium* LT2 (ST) in spiked industrial poultry samples. Duck feed (n = 11), feces (n = 1), litters (n = 2). The negative controls were unspiked samples (poultry samples plus P22::luxAB). A positive control (ST only, n = 21) was added to each bioassay and was conducted with *Salmonella Typhimurium* only and P22::luxAB. The average P22::luxAB concentration was 3.15 ± 10^5 (±1.15 ± 10^5) PFU/ml.

Figure 4 shows the results obtained with the bioassay for the negative control and all the industrial samples after 2 and 4 h of incubation. In all cases, the bioassay did not lead to false-positive results and matched the results obtained with the standard method. When *Salmonella Typhimurium* LT2 was added to the samples, neither the indigenous microflora nor the sample matrix prevented detection by P22::luxAB, and all the tests were positive after an incubation time of 4 h.

Even though these results are promising, our current bioassay suffers from two main drawbacks related to the use of P22, (i) it is unable to detect all *Salmonella* (only serotypes A, B, and D1), and (ii) it is a temperate phage. In 1995, Schicklmaier and Schmieger (24) demonstrated 95% of the natural strains of *S. enterica* Typhimurium harbored prophages in their chromosome. *Salmonella* is considered immune to infection after exposure to phage and, thus, P22::luxAB would not be able to reinfect a formerly infected *S. enterica* Typhimurium, reducing the spectrum of application of the bioassay and leading to false negative results. Nevertheless, this phenomenon was not observed in our study, and the proposed protocol could be easily applied to other phages, or phage cocktails could be employed to increase the host range.

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