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Detection of Multiple Antibiotic–Resistant *Salmonella enterica* Serovar Typhimurium DT104 by Phage Replication–Competitive Enzyme-Linked Immunosorbent Assay

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ABSTRACT

A phage replication–competitive enzyme-linked immunosorbent assay (PR-cELISA) was developed for the detection of multiple antibiotic–resistant *Salmonella* Typhimurium DT104. In the PR-cELISA procedure, a phage, BP1, was inoculated into a log-phase bacterial culture at a ratio of 1:100. After a 3-h incubation of the mixture, BP1 replication was measured by cELISA based on the competitive binding between BP1 and biotinylated BP1 to *Salmonella* Typhimurium smooth lipopolysaccharide. Among the 84 *Salmonella* strains and 9 non-*Salmonella* strains that were tested by PR-cELISA, BP1 detected 39 of 40 *Salmonella* Typhimurium strains, 2 of 10 *Salmonella* non-Typhimurium somatic group B strains, and 5 of 18 *Salmonella* somatic group D_1 strains. With the addition of chloramphenicol to the culture medium, PR-cELISA detected all 27 multiple antibiotic–resistant *Salmonella* Typhimurium DT104 and none of the other *Salmonella* strains or non-*Salmonella* strains tested. The results demonstrated that PR-cELISA has potential applications for the detection of multiple antibiotic–resistant *Salmonella* Typhimurium DT104.

Multiple antibiotic–resistant *Salmonella enterica* serovar Typhimurium definitive type 104 (MAR DT104) emerged as a world health problem in the mid-1980s, and over the last two decades the incidence of its infection in humans and animals continuously increased in Europe and North America *(1, 6, 15)*. Most of MAR DT104 isolates demonstrated resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) and were widely distributed in humans, most meat-producing animals, exotic birds, and various foods, including meat, dairy products, and salad ingredients *(14)*. Rapid detection methods specific for this pathogen are critical in any strategies to control its spread.

Recently, phages (also called bacteriophages) have been used as biological agents for rapid detection of bacteria due to their specific host range of adsorption and replication. Target bacteria were detected based on the adsorption of fluorescent-labelled phages by microscopy or cytometry *(5, 11)* or based on the phage replication. The replication-based methods involve enumerating phage by plaque assay *(12)* or mass spectrometry *(9)*, measuring enzymatic activities released from bacteria by phage lytic action *(2, 10, 13, 16)*, or monitoring the expression of reporter genes introduced into the target bacteria by phages *(4, 7, 8)*.

The aim of the present study was to develop an assay for the detection of MAR *Salmonella* Typhimurium DT104 using a lytic phage BP1 that was isolated from swine fecal samples. BP1 replication was more specific than its adsorption for detection of *Salmonella* Typhimurium, and a BP1 replication-based competitive enzyme-linked immunosorbent assay (cELISA) was developed. The cELISA provided a high-throughput platform for measuring BP1 replication based on the competition of BP1 and biotinylated BP1 for binding to *Salmonella* Typhimurium smooth lipopolysaccharide (LPS). With the addition of chloramphenicol into the culture medium for BP1 replication, phage replication (PR)–cELISA was found to be specific for the MAR DT104 strains tested.

MATERIALS AND METHODS

Bacterial strains and phage. The bacterial strains used in this study are shown in Table 1 and include strains from the culture collection of the Ottawa Laboratory Fallowfield and the Ottawa Laboratory Carling of the Canadian Food Inspection Agency (Ottawa, Ontario, Canada), and the Health Canada Laboratory (Guelph, Ontario, Canada). Phage BP1 was isolated by Biophage Pharma Inc. (Montreal, Québec, Canada) from a swine farm in the province of Québec. BP1 had a 63-nm head, a tail measuring 111 by 7 nm with terminal spikes, and a double-stranded DNA genome.

BP1 adsorption. To demonstrate the binding of BP1 to its hosts, a fluorescent phage adsorption assay was adapted from the procedure of Goodridge et al. *(5)*. Fluorescent BP1 (F-BP1) was prepared as described by Goodridge et al. *(5)*. Bacterial cells in 10 μl of stationary-phase culture of various *Salmonella* strains (approximately 10^9 CFU/ml) were captured with anti-*Salmonella* immunomagnetic beads (Dynal, Lake Success, N.Y.) according to the manufacturer's instructions. Captured bacteria (approximately 10^7 CFU) were incubated with 200 μl of 1.5×10^{10} PFU/ml F-BP1 to allow adsorption of F-BP1 to the bacteria. To remove the unbound F-BP1, the bacteria were washed and filtered through a

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TABLE 1. *Screening of bacterial strains by PR-cELISA and FPAA*

Taxon	No. of strains	Source[a]	No. of positive strains		
			FPAA[b]	PR-cELISA[c] (−) Chl	PR-cELISA[c] (+) Chl
Salmonella					
Somatic group					
B					
MAR DT104 (ACSSuT)	27	A, E	27	27	27
Non-MAR DT104	10	A, E	10	9	0
Non-DT104 Typhimurium	3	A, F	3	3	0
Non-Typhimurium	10	E, F	7	2	0
D_1	18	A, F	18	5	0
A	1	F	1	0	0
C_1	3	F	0	0	0
C_2–C_3	3	F	0	0	0
D_2	2	F	0	0	0
E_1	5	F	0	0	0
E_4	2	F	0	0	0
Alcaligenes faecalis subsp. *faecalis*	1	ATCC 8750	0	0	0
Citrobacter freundii	1	ATCC 8090	0	0	0
Escherichia coli	1	ATCC 25922	0	0	0
Enterobacter cloacae	1	ATCC 13047	0	0	0
Klebsiella pneumoniae	1	ATCC 27736	0	0	0
Proteus mirabilis	1	ATCC 25933	0	0	0
Pseudomonas aeruginosa	1	ATCC 27853	0	0	0
Serratia marcescens	1	ATCC 13477	0	0	0
Stenotrophomonas maltophilia	1	ATCC 13636	0	0	0

[a] A, animals (mainly chicken); E, environment; F, foods; ATCC, American Type Culture Collection.

[b] FPAA, fluorescent phage adsorption assay. Samples with rod-shaped fluorescent halos were considered positive; otherwise, they were considered negative.

[c] PR-cELISA, phage replication–competitive ELISA. Culture of the tested strains was incubated with (+chl) or without (−chl) the addition of 32 μg ml^{-1} chloramphenicol. Positive strains produced inhibition ranging from 43.7 to 75.1%, and negative strains produced inhibition ranging from −2.5 to 12.1%.

0.2-μm black membrane filter (cat. no. GTBP02500, Millipore, Nepean, Ontario, Canada) which was overlaid on a 0.4-μm membrane filter (cat. no. HTTP02500, Millipore, Nepean). The membrane filters were then examined at a magnification of ×1,250 using an Olympus BX 51 epifluorescence microscope equipped with a green fluorescent protein filter set (Olympus, Melville, N.Y.). More than 10 fields in each filter were examined. Each bacterial strain was tested twice, and controls containing only the immunomagnetic beads and no bacteria were included.

Preparation of biotinylated BP1 for PR-cELISA. *Salmonella* Typhimurium DT104 strain SA 97-0963 was used as the bacterial host to replicate BP1. Luria-Bertani (LB) broth (250 ml; Fisher Scientific, Ottawa, Ontario, Canada) containing SA 97-0963 at 5×10^7 CFU/ml and BP1 at 5×10^5 PFU/ml was incubated overnight at 37°C with 250 rpm shaking. Extraction, concentration, and purification of BP1 were performed as described by Goodridge et al. *(5).* The purified BP1 was suspended in phosphate-buffered saline (PBS; 0.32 g/liter NaH_2PO_4, 1.1 g/liter Na_2HPO_4, 8.5 g/liter NaCl, pH 7.2) and was biotinylated with a Biotin Labeling Kit (Roche Diagnostics Canada, Laval, Québec, Canada) according to the manufacturer's instructions. In brief, 1 ml of BP1 suspension (5.0×10^{11} PFU/ml) was mixed with 10 μl of the biotin stock (20 μg/μl). The mixture was stirred gently at room temperature for 2 h and then passed through a Sephadex G-25 column. After the column was washed with 1.5 ml PBS, biotinylated BP1 (B-BP1) was eluted with 3.5 ml PBS. The fractions with optical density at 280 nm (OD_{280}) values higher than 0.4 were collected, pooled, and stored at 4°C. The concentration of B-BP1 was determined with the traditional double-layer plaque-forming assay.

PR-cELISA. Single colonies from cultures of various bacterial strains were inoculated in LB broth and grown to log phase in 2 h. The cultures were standardzed to an OD_{600} of 0.5 by dilution with LB broth as necessary. Bacteria (5×10^6 CFU) and BP1 (5×10^4 PFU) were inoculated into 1 ml of fresh LB broth with or without supplement of 32 μg/ml chloramphenicol. Controls containing only bacteria and no BP1 were included. The mixture was incubated at 37°C for 3 h with 200 rpm shaking. The cultures were centrifuged at 10,000 × *g* for 5 min to remove the bacterial debris, and the supernatant was subjected to plaque-forming assay and cELISA as described below.

Salmonella Typhimurium smooth LPS (1.0 μg/ml; cat. no. L6511, Sigma, St. Louis, Mo.) in 100 μl 1× coating solution (cat. no. 50-84-01, Kirkegaard & Perry Laboratories [KPL], Gaithersburg, Md.) was used to coat microtitration plates (Maxisorp, cat. no. 446612, Nunc, Roskilde, Denmark) overnight at 4°C. The plates were then blocked with 100 μl 1× blocking solution (cat. no. 50-61-01, KPL) for 1 h at room temperature, and then washed three times using 1× wash solution (cat. no. 50-63-01, KPL). Culture supernatant (100 μl) of each bacterial strain with or with-

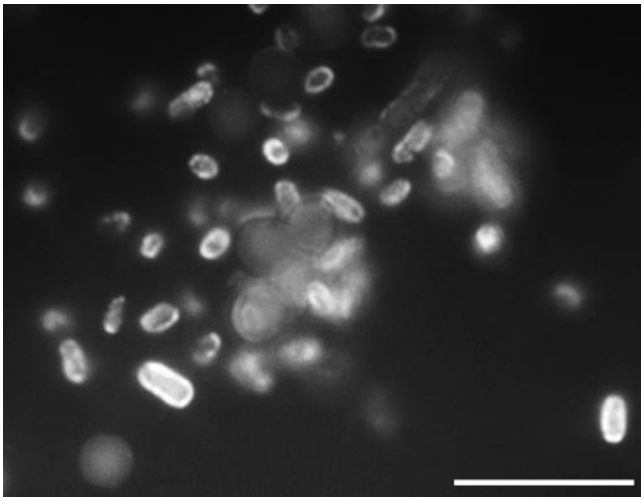

FIGURE 1. *Fluorescent phage BP1 adsorption to* Salmonella *Typhimurium DT104 cells (rod-shaped halos) captured by immunomagnetic beads (discs). Bar = 10 μm. Magnification = ×1,250.*

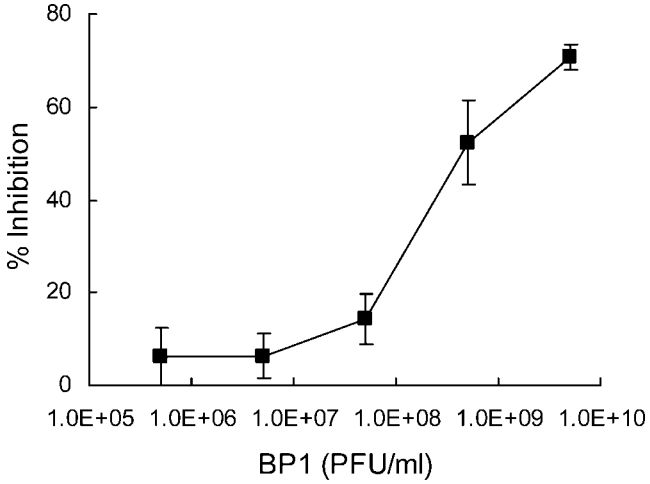

FIGURE 2. *Average percent inhibition with standard deviation in cELISA using various concentrations of phage BP1 and 2.5 × 10^9 PFU/ml of biotinylated phage BP1.*

out BP1 was added to duplicate plate wells. Positive (BP1 at 2.5 × 10^9 PFU/ml in LB broth) and negative (uninoculated LB broth) controls were added to quadruplicate plate wells. The plates were incubated for 1 h at room temperature and washed three times. B-BP1 suspension (100 μl, 2.5 × 10^9 PFU/ml) in 1× diluent solution (cat. no. 50-61-01, KPL) was added to the plate wells. The plates were incubated for 1 h at room temperature, and then washed three times. To monitor the competitive binding between BP1 and B-BP1 to *Salmonella* Typhimurium smooth LPS, 100 μl of streptavidin-peroxidase conjugate (25 mU/ml, Roche Diagnostics Canada) in 1× diluent solution was added to the wells. Following a 1-h incubation at room temperature, the plates were washed three times, and 100 μl 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (cat. no. 52-00-00, KPL) was added to the wells. The plates were then incubated 15 min in the dark at room temperature, and the reaction was stopped by adding 100 μl of TMB stop solution (cat. no. 50-85-05, KPL). The OD_{450} was read by ELISA plate reader (Multiskan, ThermoLabSystems, Helsinki, Finland). Plates were considered valid if the negative control wells had an average OD_{450} of >0.700, and the positive control wells had an average OD_{450} of 0.300 to 0.500. A percentage of inhibition value was calculated using the following equation for each sample:

$$\% \text{ Inhibition} = 1 - \text{OD of the sample/OD of the negative control}$$

where OD of the sample is the mean value of two wells, and OD of the negative control is the mean value of four wells.

RESULTS

The fluorescent phage adsorption assay illustrated the binding of BP1 to *Salmonella* Typhimurium DT104 captured by immunomagnetic beads (Fig. 1). The bacterial cells appeared as green fluorescent rod-shaped halos around the red-orange beads under the epifluorescent microscope equipped with the green fluorescent protein filter set. Uniform fluorescent halos with an intact rod shape indicated that BP1 adsorbed to somatic receptors that are evenly distributed on the surface of *Salmonella* Typhimurium DT104. Among the 84 *Salmonella* strains tested by using the adsorption assay, 47 of 50 somatic group B strains, 18 of 18 group D_1 strains, and the single group A strain were positive. All nine non-*Salmonella* strains and all 15 *Salmonella* strains from somatic groups E_4, E_1, C_1, C_2–C_3, and D_2 were negative (Table 1).

Based on BP1 adsorption to somatic receptors of *Salmonella* Typhimurium, both smooth and rough *Salmonella* Typhimurium LPS were evaluated as a BP1 receptor by ELISA. As BP1 adsorbed to the smooth LPS but not to the rough LPS (data not presented), smooth *Salmonella* Typhimurium LPS was used as a BP1 capture reagent to coat the microtitration plates for developing a cELISA to measure BP1 replication level. Various concentrations of the smooth LPS and B-BP1 were tested for production of an OD greater than 1.2 with minimum B-BP1, and 1.0 μg/ml of the smooth LPS and 2.5 × 10^9 PFU/ml of the B-BP1 were chosen for the cELISA. When this cELISA condition was used, BP1 at a concentration of 5 × 10^8 PFU/ml or higher competed strongly with B-BP1 and produced an average inhibition greater than 50%. When the BP1 concentration was reduced to 5.0 × 10^7 PFU/ml or lower, the inhibition decreased to 14.2% or lower (Fig. 2). BP1 showed excellent replication capabilities at 37°C as well as at room temperature (data not presented). A strong inhibition in the c-ELISA was observed after a 3-h or longer incubation of 5 × 10^4 PFU/ml BP1 in a log-phase *Salmonella* Typhimurium culture at 5 × 10^6 CFU/ml (Fig. 3), and the increase of inhibition in the cELISA corresponded to BP1 replication (data not presented).

A 3-h incubation of 5.0 × 10^6 CFU/ml of each test bacterium and 5.0 × 10^4 PFU/ml of BP1 were used in PR-cELISA for screening of 93 bacterial strains, and the screening results were verified by the plaque-forming assay. BP1 replicated in 39 of 40 Typhimurium strains, 2 of 10 non-Typhimurium somatic group B strains, and 5 of 18 group D_1 strains tested by PR-cELISA (Table 1). None of the other *Salmonella* strains or non-*Salmonella* strains tested were positive. The positive strains produced an average inhibition of 68.5% (±6.6%), and the average inhibition of

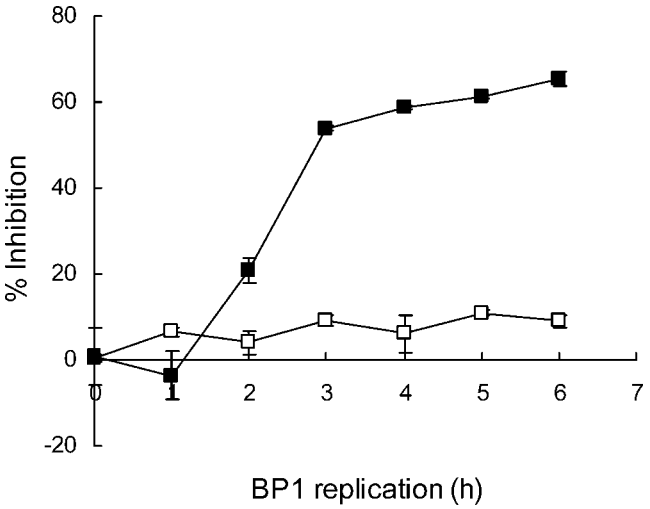

FIGURE 3. *Average percent inhibition with standard deviation in cELISA using 2.5 × 10⁹ PFU/ml of biotinylated phage BP1 and culture supernatants that were collected at intervals during the incubation of* Salmonella *Typhimurium DT104 cultures with (■) or without (□) phage BP1 inoculation.*

the negative strains was 4.5% (±3.9%) in RP-cELISA. To improve the specificity of the assay for detection of MAR DT104, chloramphenicol was added to the culture to inhibit the growth of nonresistant strains, and only the MAR DT104 strains with the R-type ACSSuT pattern were positive using PR-cELISA (Table 1).

DISCUSSION

The use of *Salmonella* Typhimurium smooth LPS as a BP1 capture reagent in the cELISA resulted in little interference of the test culture with the assay and allowed direct testing of culture supernatant without further treatment. Based on the competitive binding between BP1 and B-BP1 to the smooth LPS, the cELISA provides a high-throughput platform for measuring BP1 replication level. Because the concentration of BP1 used as the inoculum was well below the cELISA detection level, the percent inhibition results could clearly distinguish positive and negative strains by measuring the BP1 replication after a 3-h incubation of the test culture. BP1 recognition of *Salmonella* Typhimurium LPS O antigen was demonstrated by both the fluorescent phage adsorption assay and the PR-cELISA. The PR-cELISA detected 39 of 40 Typhimurium strains and 7 of 44 non-Typhimurium *Salmonella* strains. In comparison, the phage adsorption assay detected 40 of 40 Typhimurium strains and 26 of 44 non-Typhimurium *Salmonella* strains. PR-cELISA provided more specific detection of Typhimurium strains than the adsorption assay, as more sophisticated interactions with the host were required for phage replication than for phage adsorption *(3).* Addition of chloramphenicol to the culture medium allowed PR-cELISA to identify chloramphenicol-resistant *Salmonella* Typhimurium strains. However, most of such strains isolated in the last two decades were indeed MAR DT104 strains with the R-type ACSSuT pattern *(6).* Thus, in combination with selective bacterial enrichment, the PR-cELISA would have potential diagnostic applications for the detection of multidrug-resistant *Salmonella* Typhimurium DT104 in food and environmental samples.

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