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Specific detection of *Salmonella enterica* and *Escherichia coli* strains by using ELISA with bacteriophages as recognition agents

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Abstract

The use of bacteriophages, instead of antibodies, in ELISA-based detection of bacterial strains was tested. This procedure appeared to be efficient, and specific strains of *Salmonella enterica* and *Escherichia coli* could be detected. The sensitivity of the assay was about 10^5 bacterial cells/well (10^6 /ml), which is comparable with or outperforms other ELISA tests detecting intact bacterial cells without an enrichment step. The specificity of the assay depends on the kind of bacteriophage used. We conclude that the use of bacteriophages in detection and identification of bacteria by ELISA-based method can be an alternative to the use of specific antibodies. The advantages of the use of bacteriophages are their environmental abundance (and thus a possibility to isolate various phages with different specificities) and availability of methods for obtaining large amounts of phage lysates, which are simple, rapid, cheap and easy.

Introduction

Since a large number of infectious diseases are caused by pathogenic bacterial strains, it is obvious that such strains should be specifically detected during diagnostic procedures.

Different approaches are used to obtain satisfactory results. The platform used for the detection often depends on the kind of molecular target in the pathogen that has been chosen. When nucleic acids are used, a precise detection of bacteria is possible, but the main disadvantage of this method is requirement for isolation and purification of target molecules prior to their detection. This may be a time consuming step, which makes the whole assay relatively long and expensive [1].

Another approach is to recognize antigens on the surface of detected bacteria by using specific antibodies. This approach does not need any time-consuming initial preparation of the sample, however, antibodies are quite costly and cumbersome in preparation. Their performance, especially affinity and avidity, strongly affects the assay sensitivity. Moreover, an additional drawback is their limited shelf-life, which limits viability of ready-to-use antibody-based sensors [2].

Recently, it was demonstrated that antibodies can be replaced with bacteriophages in assays devoted to detect bacteria [3, 4]. These viruses offer many advantages over standard antibodies in detection procedures, namely, phage proteins engaged in host recognition are extremely stable, phages are easy to isolate and propagate, and they can be stored for relatively long time. These features make bacteriophages a cheap and fully functional tool that can replace primary antibodies in procedures of immunological detection of bacteria [5, 6].

Bacteriophages have already been widely used for identification of bacteria in phage typing tests [7-10]. In these assays, one can employ the relatively narrow host range of some phages, which is limited to one or several strains. When multiple phages with partially overlapping host ranges are used, the ability of the tested bacterial strain to support growth of some of phages from the collection can be considered as a kind of a finger print, allowing for easy and inexpensive strain identification. However, classical phage typing is useful to distinguish bacterial strains, but not to detect them in a clinical or environmental sample.

In this work, we aimed to develop an assay for detection of bacteria using unmodified bacteriophages, which can replace primary antibodies in standard ELISA tests. The main idea of this study was to construct a detection system for quick identification of *Salmonella* and *Escherichia coli* strains with the use of cheap and simple procedures for preparing the reagents and to perform the assay.

Material and methods

Bacteria and bacteriophages

Bacterial strains and bacteriophages used in this work are listed in Table 1.

Antibodies

Anti *E. coli* and *Salmonella* sp. rabbit antibodies were purchased from USBiological (USA). HM serum was obtained from Immunolab (Poland). Anti O1 serum was prepared in the following way: 4 rabbits were immunized using a preparation of O1 phage in PBS (10^{11}

p.f.u./ml). The procedure for immunization was: 0.2 ml injected subcutaneously initially, 0.3 ml injected subcutaneously after one week, and 0.5 ml injected intravenously after another week. At one week after the last immunization, the antibody level was tested and the serum was used for subsequent experiments.

Phage preparation

Phage lysate (2 liters) was centrifuged (3000 g, 20 min) and supernatant was concentrated using 10% polyethylene glycol of molecular weight 8000 Da (PEG 8000) and 2 M NaCl. After overnight incubation, the phage precipitate was centrifuged (8000 g for 20 min), then suspended in 10 ml of TM (10 mM Tris-HCl, pH 7.2, 10 mM MgSO₄) buffer. The remaining PEG 8000 was extracted by addition of 2 ml of chloroform and vortexing. This step was repeated three times. The resulting phage suspension was purified on a cesium chloride gradient by ultracentrifugation (32000 rpm 2h using 50Ti Beckman rotor), and then dialyzed against TM buffer and titrated [13].

Plate coating with phage

Wells of 96-well ELISA plates were coated using 0.1 ml of appropriate phage lysate and incubated overnight at 4°C. The wells were washed three times with PBS buffer (pH 7.4) and blocked for 3 h at 37°C with 5% BSA solution in PBS.

ELISA procedure

Following triple washing of the wells with PBS containing 0.05% Tween 20, bacterial cells in PBS were added. The plates were then incubated for 1 h at room temperature after which the wells were washed three times with PBS containing 0.05% Tween20. HM serum (Immunolab, Poland), suspended in 5% BSA, was added (1:1000 v/v) and incubated for 1 h at room temperature. Following triple washing with the PBS buffer containing 0.05% Tween20, secondary biotinylated antibodies were added (1:2000) and the plates were incubated for another hour at room temperature. Following triple washing with PBS containing 0.05% Tween20, a solution of Extravdine - alkaline phosphatase conjugate (Sigma, USA) (1:500 dilution) was added, and the plates were incubated for 1 h. The wells were washed four times with PBS containing 0.05% Tween20, and 50 μ l of Alkaline Phosphatase Yellow (pNPP) Liquid Substrate (Sigma, USA) were added to each well. After yellow color appeared, the reaction was stopped with 15 μ l of 3 M NaOH, and absorbance was measured at 405 nm wavelength.

Results

We aimed to develop an assay for detection of *Salmonella* and *E. coli* strains by using an ELISA-based method, in which bacteriophages are employed as recognition agents. For

testing the ability of bacteriophages to immobilize bacterial cells specifically on the ELISA plate, we assessed the performance of phages O1, lambda, P1 and T4 to detect cells of *Salmonella enterica ser. Enteritidis* KOS 1663, which were inactivated by formaldehyde treatment, according to Paton et al., [14].

The O1 phage is known to be highly specific to salmonellae [15], while phage lambda is known to infect a relatively narrow range of bacteria including *Escherichia coli* and *Shigella* but not *Salmonella* [16]. The phage T4 host range is restricted mostly to *E. coli* and *Shigella* species [17], and the P1 host range is restricted mostly to Enterobacteriaceae [18]. We expected phage lambda and T4 to be unable to bind *Salmonella* cells used in this experiment, while the other phages should be able to capture and immobilize the bacterial cells in ELISA plate wells, and thus, allow for subsequent detection.

Detection was carried out as follows. Equal amounts of phages (10^8 /well) were adsorbed to polystyrene surface of ELISA plate wells. Next steps involved blocking of remaining binding sites by using skimmed milk and subsequent addition of formaldehyde-fixed *Salmonella enterica ser. Enteritidis* KOS 1663 cells (10^7 /well). Then, primary HM serum was used, and subsequently, biotinylated anti-rabbit secondary antibodies were added. After incubation with Extravidine-Alkaline Phosphatase conjugate, the signal was measured. To assess specificity of the assay, several controls were used, which basically lacked one part of signal-generating complexes: biotinylated secondary antibodies, primary HM serum, phage or bacteria. When any of these factors were lacking, the signal remained weak, in contrast to the wells in which all components of the assay were present (Fig. 1). Contrary to our expectations based on a literature survey [17], we found that T4 phage binds efficiently to *Salmonella enterica ser. Enteritidis* cells (Fig. 1). However, it may indicate, that some phages

can adsorb to bacterial cells without subsequent ability to inject its genetic material or to perform productive infection.

When we attempted to attach bacteria directly to the wells in plate (10^7 cells/well), and then added a lysate of bacteriophage O1 (10^8 pfu/well), followed by addition of anti-O1 serum, we were able to detect the bacteria, whereas the signals of the controls were relatively weak (Fig. 2). To assess the specificity of the assay, we tested detection of different strains of *Salmonella* by means of the ELISA test using phages to attach to bacterial cells. The results are summarized in Table 2.

We found that the signals generated during detection of various bacterial strains were different. As expected, among bacteriophages used for testing, phage O1 was the most effective in detection of *Salmonella* strains. However, some *Salmonella enterica* strains e.g. *Salmonella enterica* ser. Tennessee, generated weak signals, indicating that they were not recognized efficiently by this phage. Interestingly, the highest signals were generated by *Salmonella enterica* ser. Anatum (Table 2), which is immune to phage O1 when tested by a plaque test (data not shown). This suggests that this bacteriophage can adsorb efficiently on *Salmonella enterica* ser. Anatum, but is not able to complete its lytic development in this strain. This observation clearly shows that use of phages may create unexpected positive signals, what could be even beneficiary in some cases. Thus, while constructing a detection test, more rigorous experiments, than sole ability of phage to form plaques should be employed, in order to identify properly the specificity of phage binding.

To optimize the method and to determine its sensitivity in respect to the number of phages added to each well, we performed a series of tests with various amounts of phage lysates. Results are summarized in Fig. 3. Generally, the results showed that the highest

signals were obtained at the highest phage densities. Moreover, they showed that it is possible to detect bacterial strains when as few as 10^4 phage particles are deposited in a single well.

To assess the sensitivity of detection of bacteria, we performed the assay using the optimal number of phages, 10^8 pfu/well, and different number of bacterial cell (Fig. 4). In this assay, 10^5 bacterial cells were detected unambiguously when phages T4 or O1 were employed.

We employed the same bacteriophages to detect different strains of *E. coli*. First, we tested efficiency of plating (e.o.p.) of purified phages (Table 3). The differences in e.o.p. values apparently resulted from differences in bacteriophage development in strains used. Phage O1 formed no plaques on *E. coli* strains employed in this assay. This phage is known to form plaques on very few *E. coli* strains and is reported to be specific to the genus *Salmonella* [15]. One strain, *E. coli* O157:H7 86-24, appeared to be insensitive to all four phages. However, in ELISA tests, this strain generated the strongest signals (Fig. 5), which indicated efficient phage adsorption, and thus immobilization on the bacteria in the wells.

Discussion

Results presented in this report suggest that the concept of using bacteriophages instead of primary or secondary antibodies in ELISA for specific detection of bacterial strains may be an effective alternative to classical immuno-detection procedures. The availability of easy methods of phage isolation from natural sources and the relatively low number of

bacteriophages necessary for generation of unambiguous signals indicate that it is reasonable to use phages in this kind of assay. The sensitivity of the assay was also relatively high, nevertheless, we believe that it may be further improved when more advanced platforms are used for signal detection.

On the other hand, a proper selection of bacteriophage strains is not possible without detailed tests of their host range. Our results showed that ability of generating plaques is not always a sufficient feature to choose a proper phage as a recognition agent, and also it is not sufficient to clearly define the range of host strains on which the phage may adsorb.

Recognition of bacterial cells by bacteriophage particles is based on the presence of phage receptors on cell surface. As almost every structure present on the bacterial cell surface can be a potential receptor [19], it is possible to isolate phages varying in the range of bacterial strains which can be recognized. Owing to such a feature of the phage host recognition, it may be possible to construct assays with very generic or very specific recognition patterns, or tests that combine both types of recognition. This might be important if the bacterial phenotype is tightly connected to antigenic specificity, like Shiga toxin production and the O157:H7 serotype of *E. coli* [20]. This approach would be similar to phage typing of bacteria but would differ in that typing by a plaque test would consume much more time than identification by the ELISA test. Moreover, while the plaque test is strongly affected by different ways of phage exclusion, the ELISA assay is affected only by the presence or absence of a phage receptor on the bacterial surface.

The sensitivity of the test described in this report was estimated to be 10^6 cells/ml. It is worth mentioning that sensitivity of other ELISA tests detecting intact cells show similar or lower sensitivities [21]. As the use of bacteriophages instead of antibodies did not decrease the sensitivity of the assay, and the selection and production of bacteriophages is relatively

easy, cheap and does not involve any work done on live animals, we consider it to be an attractive alternative to traditionally used antibodies. One issue, which should be considered in laboratories preparing the assays, is that bacteriophages may be produced and disseminated accidentally and cause false test results owing to phage contamination [22]. However, it is easy to inactivate bacteriophages using UV light prior to coating of ELISA plates, or after coating. When proper coating conditions will be used, phage re-activation due to multiple infection can be easily avoided. This assumption is strongly supported by the fact, that relatively low number of phage particles deposited in single well was enough to provide good detection sensitivity. Such condition, due to relatively low density of phages on the surface, minimize possibility of high multiplicity of infection during bacterial capture. Thus, the use of phages as a recognition agent in ELISA test may be a safe, sensitive and easy alternative to standard antibody-based tests.

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Figure legends

Fig. 1. Use of phages O1, λ , T4 and P1 as capture agents for *S. enteritica ser. Enteritidis*. In left panel, white bars represent signals from wells, where complete assay was performed, pale gray bars represent signals from wells where biotinylated anti-rabbit antibodies were omitted, gray bars represent signals from wells where HM serum was omitted, dark gray bars represent signals from wells where no bacteria were added, very dark gray bars represent signals from wells where no phage was added. Presented values are average results from 4 experiments with error bars representing standard deviation. Background value from the control employing no phage in detection was subtracted. In right panel, a cartoon showing the assay construction is presented.

Fig. 2. Detection of *Salmonella enteritica ser. Enteritidis* directly adsorbed to ELISA plate wells using phage O1 and anti-phage serum. In left panel, average signal generated in complete assay and in various controls is shown. Presented values are average results from 4 experiments with error bars representing standard deviation. Background value from the control employing no phage in detection was subtracted. In right panel, a cartoon showing the assay construction is presented.

Fig. 3. Detection of *Salmonella enteritica ser. Enteritidis* (10^7 /well) using different number of phage particles per well as a capture agent. In left panel, signal strength in relation to phage number used is presented for phages O1 (circles), T4 (diamonds), P1 (triangles) and λ (squares). Presented values are mean results of 4 experiments with error bars representing standard deviation. Background value from the control employing no phage in detection was subtracted. In right panel, a cartoon showing the assay construction is presented.

Fig. 4. Detection of different number of *Salmonella enteritica ser. Enteritidis* cells using phage particles as a capture agent. In left panel, signal strength in relation to phage number used is presented for phages O1 (circles), T4 (diamonds), P1 (triangles) and λ (squares). Presented values are mean results of 4 experiments with error bars representing standard deviation. Background value from the control employing no phage in detection was subtracted. In right panel, a cartoon showing the assay construction is presented.

Fig. 5. Detection of different *E. coli* strains (B – Black bars, C1a – white bars, 86-24 – pale gray bars, MG1655 – dark gray bars) using different phage strains. Presented values are mean results of 4 experiments with error bars representing standard deviation. Background value from the control employing no phage in detection was subtracted. In right panel, a cartoon showing the assay construction is presented.

Tab. 1 Phages and bacterial strains

Strain	Source/reference
Phage T4 CGSC#12143	<i>E. coli</i> Genetic Stock Center, Yale, USA
Phage P1vir	Collection of Dept. Molecular Biology UG ^a
Phage λ	[11]
Phage O1	Collection of Salmonella Microorganisms ^b
<i>Escherichia coli</i> O157:H7 86-24 Δ stx2	Gail Christie, Department of Microbiology & Immunology, Virginia Commonwealth University
<i>Escherichia coli</i> MG1655 CGSC#6300	<i>E. coli</i> Genetic Stock Center, Yale, USA
<i>Escherichia coli</i> B KOS 1466	Collection of Salmonella Microorganisms ^b
<i>Escherichia coli</i> C1a	[12]
<i>Salmonella enterica</i> ser. London KOS 76	Collection of Salmonella Microorganisms ^b
<i>Salmonella enterica</i> ser. Panama KOS 73	Collection of Salmonella Microorganisms ^b
<i>Salmonella enterica</i> ser. Heidelberg KOS 16	Collection of Salmonella Microorganisms ^b
<i>Salmonella enterica</i> ser. Anatum KOS 78	Collection of Salmonella Microorganisms ^b
<i>Salmonella enterica</i> ser. Tennessee KOS 142	Collection of Salmonella Microorganisms ^b
<i>Salmonella enterica</i> ser. Reading KOS 19	Collection of Salmonella Microorganisms ^b
<i>Salmonella enterica</i> ser. Enteritidis KOS 1663	Collection of Salmonella Microorganisms ^b

^a UG, University of Gdańsk, Poland

^b Collection of National Salmonella Centre at Medical University of Gdańsk, Poland

Tab. 2. Detection of different *Salmonella enterica* strains by using indicated phages as capture agents in ELISA

Phage	Signal ^a									
	<i>S.</i> London	<i>S.</i> Panama	<i>S.</i> Heidelberg	<i>S.</i> Anatum	<i>S.</i> Tennessee	<i>S.</i> Reading	No secondary antibodies	No primary antibodies	No bacteria	No phage
O1	0.362 ± 0.068	0.104 ± 0.071	0.258 ± 0.202	0.533 ± 0.164	0.042 ± 0.020	0.083 ± 0.070	0.013 ± 0.004	0.011 ± 0.010	0.011 ± 0.006	0.052 ± 0.074
T4D	0.154 ± 0.057	0.036 ± 0.018	0.037 ± 0.031	0.112 ± 0.102	0.050 ± 0.035	0.038 ± 0.018	0.008 ± 0.014	0.014 ± 0.004	0.013 ± 0.014	0.006 ± 0.012
P1vir	0.054 ± 0.040	0.023 ± 0.017	0.034 ± 0.020	0.090 ± 0.063	0.049 ± 0.014	0.041 ± 0.022	0.003 ± 0.009	0.004 ± 0.009	0.016 ± 0.014	0.036 ± 0.067
λclb2	0.053 ± 0.028	0.026 ± 0.011	0.028 ± 0.012	0.068 ± 0.048	0.065 ± 0.061	0.034 ± 0.012	0.005 ± 0.017	0.004 ± 0.015	0.022 ± 0.031	0.004 ± 0.020

^a The presented values are mean results from 4 experiments ± standard deviation.

Tab. 3. Results of titration of phage lysates on bacterial strains used in this study.

Phage	p.f.u./ml determined on <i>Escherichia coli</i> strains ^a			
	<i>E. coli</i> B	<i>E. coli</i> C1a	<i>E. coli</i> 8624	<i>E. coli</i> MG1655
O1	< 10 ¹	< 10 ¹	< 10 ¹	< 10 ¹
λcIb2	4.0 × 10 ⁵	1.2 × 10 ¹⁰	< 10 ¹	8.0 × 10 ¹⁰
T4D	1.2 × 10 ¹¹	2.4 × 10 ⁹	< 10 ¹	2.0 × 10 ¹¹
P1vir	4.0 × 10 ⁶	8.0 × 10 ⁹	< 10 ¹	1.2 × 10 ⁸

^a p.f.u., plaque forming units









