Rapid Identification of *Salmonellae* by Bacteriophage Amplification - Enhanced Immunoassay

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ABSTRACT:

Background:

Bacteria can be detected in clinical specimens with diagnostic bacteriophages that bind to specific receptors on target cells. Upon infection, the input phage directs the production of hundreds to thousands of phage per cell, culminating in a cellular burst event and subsequent release of output phage into the sample. MicroPhage applied this phenomenon to develop a phage amplification assay for the rapid detection of Salmonella, which is suspected to cause more than 2 million foodborne illnesses annually. Current diagnostic methods for this and other bacterial pathogens are slow (often taking 18 hours or more) and do not allow physicians to quickly initiate specific therapies based on etiology.

Methods:

A new phage was isolated from a lawn of Salmonella on an agar plate, and standard plaque assay methods were employed to define its host range and burst characteristics. Phage particles for antibody preparation were filtered through 0.22 um cellulose acetate, PEG precipitated, and purified by size exclusion chromatography. Rabbit polyclonal antibodies were adsorbed against whole heat-killed cells and cell-free extracts of Salmonella to generate necessary specificity in the antibody. A sandwich ELISA was then developed to detect the phage amplification signal.

Results and Conclusion:

Salmonella bacteriophage SS-1 was screened on many strains of *Salmonella* and other bacteria to define its inclusive and exclusive range. The phage is specific for *Salmonella*, exhibiting wide target host range and 2% cross reactivity. Further analysis of screened *Salmonella* bacteriophage demonstrated varying degrees of inclusivity and cross reactivity on a laboratory set of bacteria, up to 96% inclusive and 8% cross reactive.

Burst size is approximately 1000 progeny phage per infected bacterium with a burst time of 90 minutes, which is similar for all studied *Salmonella* bacteriophage.

An anti-SS-1 antibody was used to develop an ELISA to detect the amplified phage. *Salmonella* were detected by measuring the net increase in phage number by ELISA, demonstrating absolute (1 CFU / mL) detection in less than 10 hours. The ELISA will facilitate further assay development planned using a lateral flow detection platform.

INTRODUCTION:

A number of means to detect bacteria in clinical and industrial samples using bacteriophages have been described over the past two decades. The attempt has been to reduce the time to results (TTR) for diagnosis/detection over standard microbiological methods which often take 18 hours or more for many pathogens. This has mostly consisted of utilizing methods which enhance bacterial doubling time, therefore growth time, or increasing the sensitivity of detection platforms with special labels such as fluorescence. To date, the progress has been incremental, saving 3 -10% of overall time with each innovation. Most physicians however are unable to take advantage of most of these incremental advances in diagnostic microbiology due to limitations in office laboratories.

MicroPhage is further developing a platform system, first described at the Colorado School of Mines, to decrease time to results required to assay bacteria from clinical and industrial samples to make results readily available to the physician or the industrial plant operator. Here we review of a number of research projects conducted through late 2004 – early 2005 investigating bacteriophage amplification coupled to an immunoassay detection platform (Enzyme-Linked Immunosorbent Assay, ELISA) for the rapid detection of *Salmonella sp.* from clinical (fecal, blood) and food (chicken wash, chicken meat) samples. Data presented is focused on phage specificity, development of a polyclonal phage antibody, and overall assay performance. Because of increase public awareness of foodborne bacteria and federal regulation for assays to detect *Salmonella sp.* at 1 CFU / 25g sample, it was chosen as a model to demonstrate the MicroPhage platform. Whether the sample is clinical or industrial, the need for rapid detection is the same. Better results faster can enable better clinical decisions.

METHODS:

Bacteria

Bacteria were sourced from the ATCC (Manassas, VA), University of Calgary (*Salmonella*), and the University of Michigan (*E coli*).

Plaque Assay

Bacteria grown to log phase are added to serially diluted phage preparations and plated in soft agar. Plates are examined for plaque formation the following day and phage titer is determined based on plaque formation. Plaque formation is indicative of phage infection.

Phage Purification/ Antibody Development

Phage particles for antibody preparation were filtered through .2 μm cellulose acetate, PEG precipitated, and purified by size exclusion chromatography. Rabbit polyclonal antibodies were adsorbed against whole heat-killed cells and cell-free extracts of *Salmonella* to generate necessary specificity in the antibody.

ELISA Development

Anti-*Salmonella* bacteriophage IgG antibody was bound to a 96-well plate. After washing, a serial dilution of SS-1 *Salmonella* phage was added. Secondary antibody (here the IgG anti phage antibody was again used) is added, followed by incubation and wash. The ELISA was then developed with HRP. Baseline limit of detection of this ELISA was determined to be 8 x 10^6 pfu / mL.

METHODS (Cont'd):

Western Blot

In order to demonstrate the specificity of the polyclonal antibody directed against the SS-1 phage, lysates of bacteria and phage were run on denaturing SDS-PAGE, transferred to PVDF membrane, and stained using the anti-SS-1 IgG. Various bacteria, including the SS-1 host *Salmonella* Typhimurium, were grown to stationary phase. Bacteria were quantified by optical density (A600) using a Spectronic 20D+ (Thermo Electron) with a conversion factor of 1 AU = 1 x 10^8 cfu/mL. Lysates were made directly from cultures using NuPAGE LDS Sample Buffer (Invitrogen) with NuPAGE Sample Reducing Agent (DTT). Phage samples, including purified SS-1, were prepared from phage stocks using NuPAGE LDS Sample Buffer (Invitrogen) with NuPAGE Sample Reducing Agent (DTT).

Matrix Studies

Experiments into bacteriophage amplification in 2 clinical and 2 food matrices were constructed similarly: *Salmonella* Typhimurium (ATCC 14028) were grown in nutrient broth to mid-log phase. Cells were quantified using A_{600} as above. The bacteria were diluted in nutrient broth to produce final spiked concentrations of 1 x 10^6 cfu/mL to 10 cfu/mL in 10-fold series. Phage SS-1 at 1 x 10^5 pfu/mL was used in all reactions. Samples were harvested at each time point by removal of an aliquot from each reaction mixture to a sterile microfuge tube containing 25 μL of chloroform. Chloroform is used to stop phage amplification and has shown previously to have no effect in net amplification. This establishes the endpoint of each reaction. Tubes were vortexed and stored at 4°C until analysis. At time 0, 100 μL of *Salmonella* at 10^1, 10^2, and 10^3 cfu/mL were plated on nutrient agar to enumerate colonies and verify the A_{600} quantification. All samples were measured at the undiluted concentration of cleared supernatant. Visual readout on the ELISA of color change was compared to a standard curve of purified SS-1 phage diluted to cover the range 6 x 10^4 to 1 x 10^9 pfu/mL and the phage equivalent to this curve is reported.

RESULTS:

Primary SS-1 Host Range and Cross Reactivity

We first took a phage isolate from a stock of *Salmonella* Typhimurium and challenged it to a preliminary panel of *Salmonella* and non-*Salmonella* strains to ascertain its infection pattern.

Host range panels were constructed using 2001 US Centers for Disease Control and Prevention (CDC) surveillance data on Salmonella serovars coupled with Salmonella serovars reported in the literature to produce poor or cross-reactive results by other detection methods. Cross reactive strains were chosen based on their likelihood in human fecal and/or food samples. This original phage to which an antibody was developed first demonstrated "good" host range against 10 Salmonella serovars, infecting 7 serovars which included *S.* Enteritidis and *S.* Typhimurium. Conversely, SS-1 showed no infection to the 10 strains it was challenged against. We decided from this and other data that this phage was a good candidate to develop a polyclonal antibody against.

Table 1. *Salmonella* phage SS-1 preliminary host range & exclusivity.

Salmonella serovar	SS-1 phage	Non-*Salmonella* screen	SS-1 phage
S. Typhimurium	1 / 1	*E. aerogenes*	0 / 1
S. Enteritidis	1 / 1	*E. coli*	0 / 1
S. Agona	0 / 1	*S. sonnei*	0 / 1
S. Arizonae	1 / 1	*A. hydrophila*	0 / 1
S. Javiana	1 / 1	*E. hermanii*	0 / 1
S. Mbandanka	1 / 1	*H. alvei*	0 / 1
S. Urbana	0 / 1	*P. vulgaris*	0 / 1
S. Heidelberg	1 / 1	*C. koseri*	0 / 1
S. Anatum	0 / 1	*K. pneumoniae*	0 / 1
S. Newport	1 / 1	*Y. enterocolitica*	0 / 1
Overall coverage	7 / 10	Overall coverage	0 / 10
Percentage	70%	Percentage	0%

Additional *Salmonella* Phage Performance

While developing the SS-1 antibody, we further characterized additionally isolated wild *Salmonella* phage found to contaminate bacterial isolates as well as those found in the food samples. Tables 2 and 3 highlight the host range (HR) and cross reactivity (XR) of a selected number of overall phage tested. Here we were disappointed to see that the preliminary phage, SS-1 did not perform as well as any of these additional phage, demonstrating a low host range of 61% while maintaining high selectivity of only 2%. The 14-1 phage demonstrated the widest host range, infecting 90% of the 41 *Salmonella* strains tested.

Table 2. Six selected secondary *Salmonella* phage host range data.

Salmonella serovar / Phage	SS-1	2-1	14-1	3-2	12-1	19-1
S. Typhimurium	24/27	8/10	10/10	9/10	9/10	8/10
S. Enteritidis	4/15	10/14	13/14	4/14	9/14	4/14
S. Agona	0/1	1/1	1/1	1/1	1/1	1/1
S. Arizonae	1/1	1/1	1/1	1/1	1/1	1/1
S. Javiana	3/4	3/4	1/1	3/4	3/4	3/4
S. Mbandanka	1/1	1/1	1/1	1/1	1/1	1/1
S. Urbana	0/1	0/1	1/1	1/1	1/1	1/1
S. Heidelberg	5/5	4/5	5/5	5/5	5/5	5/5
S. Anatum	0/3	2/3	2/3	2/3	2/3	2/3
S. Newport	4/11	2/8	2/4	6/8	6/8	6/7
Overall score	42 / 69	32 / 48	37 / 41	33 / 48	38 / 48	32 / 47
Percentage	61%	67%	90%	69%	79%	68%

Table 3. Six selected secondary *Salmonella* phage exclusivity data.

Challenge Genus / Phage	SS-1	2-1	14-1	3-2	12-1	19-1
Aeromonas	0/1	0/1	1/1	0/1	0/1	1/1
Citrobacter	0/3	0/3	1/3	1/3	1/3	1/3
Enterobacter	0/2	0/2	0/2	1/2	1/2	1 /2
Escherichia	2/90	0/6	0/8	1/6	1/6	1/6
Hafnia	0/1	0/1	0/1	0/1	0/1	0/1
Klebsiella	0/2	0/2	0/2	0/2	0/2	0/2
Listeria	0/1	0/1	0/1	0/1	0/1	0/1
Proteus	0/1	0/1	0/1	0/1	0/1	0/1
Shigella	0/5	0/3	0/3	0/3	0/3	0/3
Yersinia	0/1	0/1	0/1	0/1	0/1	0/1
Overall score	2 / 107	0 / 21	2 / 24	3 / 21	3 / 21	4 / 21
Percentage	2%	0%	8%	14%	14%	19%

Phage Cocktailing

Interested to increase the host rage of assay bacteriophage to more clinically acceptable levels, we explored cocktailing candidate phage. Cocktailing is a practice first described in the phage therapy literature to expand phage coverage for a particular target. We analyzed the host range data by each strain of Salmonella tested and found that while some phage did well with serogroups B and D, they lacked coverage in other serogroups, and that the converse was true. Keeping to a minimal cocktail (pairs only), we revisited the existing data to look at theoretical phage cocktail performance values. Here we took only the union of strains which both phage were individual tested against, and quantitated.

A third generation bacteriophage, F-0 was found to have high infection characteristics for Salmonella serogroups C1 and The results are summarized in Table 4.

Table 4. Three selected *Salmonella* phage cocktail pairs data.

Phage Cocktails	14-1 & SS-1	14-1 & 2-1	14-1 & MF-0
Salmonella infectibility:	93% (61)	94% (61)	96% (47)
Non-*Salmonella* Cross-reactivity:	8% (24)	8% (24)	8% (27)

RESULTS (Cont'd):

Antibody Specificity

Once the anti-SS-1 phage antibody was developed, we went forward to characterize the specificity of the antibody to its target compared with a broad range of bacteria and bacteriophage. Gels were transferred to PVDF membrane and stained with Western Breeze anti-rabbit chromogenic system and polyclonal anti-SS1 IgG (5 ug/mL). Specific staining of immunoreactive SS-1 protein was observed at approximately 43 kDa. It is believed that this represents an SDS-resistant trimerized form of the 13.5 kDa dominant phage protein observed by MALDI-TOF/MS.

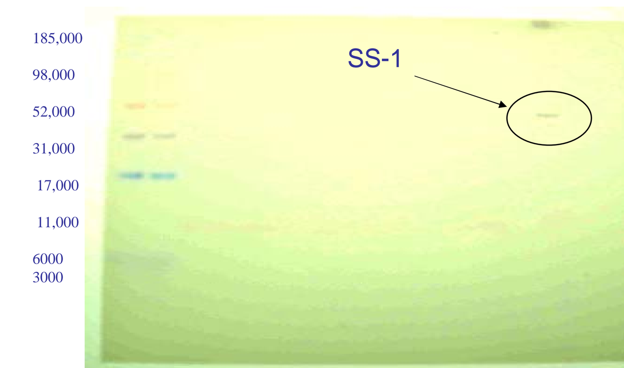


Figure 1. SS-1 Western Blot against bacteria

Ten bacterial strains from the cross-reaction panel were prepared as described and loaded onto the PVDF membrane at a concentration of 7 x 10^5 cells or greater.

Ten additional bacteriophage, comprised of 4 Salmonella phage (MF-0, F-2, P22, ES-18), 3 coliphage (MS2, T4, AR1), Enterobacteriacea phage PRD1, Shigella phage 37, and Staphylococcus aureus phage 47 were prepared and loaded at a concentration of 1 x 10^7 pfu or greater into each lane, as depicted in Figure 2.

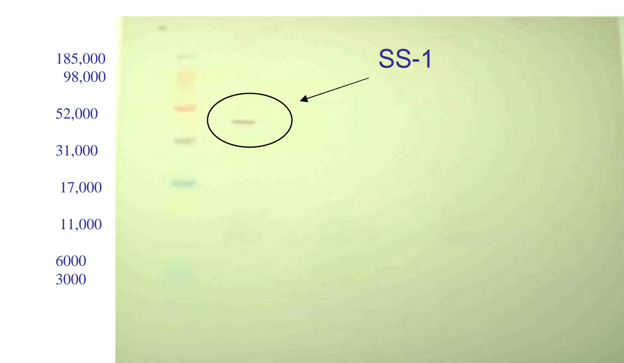


Figure 2. SS-1 Western Blot against phage

Sensitivity / Time to Results

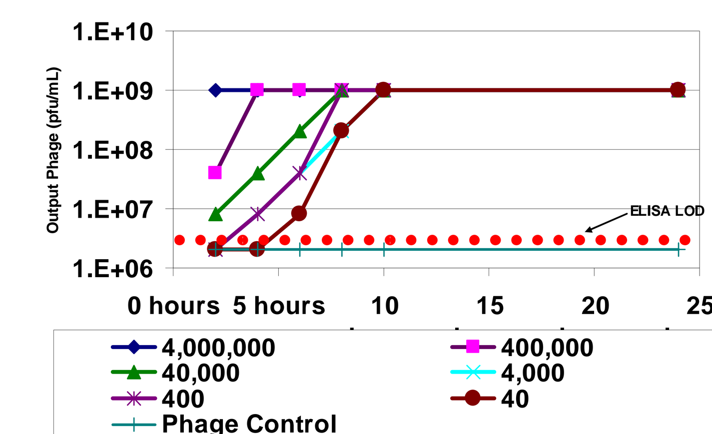
Following development of the SS-1 ELISA, we revisited phage amplification experiments in clinical and food matrices to determine overall performance of the assay. Presented here as industrial samples (chicken matrix), the phage-enhanced ELISA was able to detect low concentrations of spiked Salmonella in 6 hours. Similar results have been demonstrated in human fecal and bovine blood experiments using the same protocol.

Phage amplification of various concentrations of input *Salmonella* over time, measured by SS-1 ELISA is illustrated in Table 5 and Figure 3. All *Salmonella* concentrations were detectable by 6 hours of amplification. Detection of 40 cfu/mL *Salmonella* was achieved after 6 hours of amplification in chicken matrix. While contaminating non-Salmonella bacteria from the matrix were at a low level (10^3 cfu/mL), this was 100-fold higher than the lowest amount of spiked *Salmonella*. By the 6 hour point, the matrix only control was visibly cloudy due to growth of the endogenous bacteria. These bacteria did not interfere with *Salmonella* detection by phage amplification.

Table 5. *Salmonella* phage SS-1 ELISA Data

Spiked *Salmonella* Concentration (cfu/mL)	Output Phage Concentration (pfu/mL Equivalent) ELISA LOD = 8 x 10^6 pfu/mL			
	2 hours	4 hours	6 hours	8 hours
4 x 10^6	1 x 10^9	1 x 10^9	1 x 10^9	1 x 10^9
4 x 10^5	4 x 10^7	1 x 10^9	1 x 10^9	1 x 10^9
4 x 10^4	8 x 10^6	4 x 10^7	2 x 10^8	1 x 10^9
4 x 10^3	-	8 x 10^6	4 x 10^7	1 x 10^9
4 x 10^2	-	8 x 10^6	4 x 10^7	1 x 10^9
4 x 10^1	-	-	8 x 10^6	2 x 10^8
Salmonella only Control (4 x 10^6)	-	-	-	-
Phage Only Control (1x10^5)	-	-	-	-

Figure 3. *Salmonella* phage SS-1 ELISA Data



CONCLUSIONS:

As previously demonstrated in environmental samples, the phage amplification method is shown here to be effective in additional samples and with an additional bacteria / host pair. Given the relatively high limit of detection of this experimental ELISA (8x10^6), we believe that overall assay time can further be reduced with a more sensitive detector, as are most of today's automated immunoassay systems and lateral flow immunochromatographic (LFI) detectors.

Host range and cross reactivity of bacteriophage vary a good deal, however it has been demonstrated that developing an assay with highly specific bacteriophage is achievable. Further work applying this technology to clinical and industrial samples is needed to better understand rates of target and non-target infection. We believe that these posted rates can still be considered artificial.

This work supports further development by MicroPhage to develop an assay using a more sensitive detector (LFI) and supports viability to expand the technology's menu to other clinically relevant bacteria.