

A Novel Bacteriophage Cocktail with High Specificity to *Escherichia coli*.

BC Smith¹, B Dreiling¹

MicroPhage Incorporated, Longmont, CO 80503 USA

MicroPhage

2400 Trade Centre Ave
Longmont, CO 80503 USA
P: 303-339-1410
F: 303-339-1411
www.microphage.com

ABSTRACT:

Background:

Detection of bacteria has been demonstrated using bacteriophages that bind to target cell receptors. Upon infection, input phage direct production of thousands of progeny per cell, culminating in a release of phage into the sample. Although many *Escherichia coli* bacteriophage have been described with varying degrees of inclusivity to this host, no individual phage or cocktail has been previously described with such high accuracy on such a large collection of bacteria. Here we demonstrate a novel bacteriophage cocktail to *E. coli*, suitable for use in clinical applications.

Materials and Methods:

Hundreds of environmental and clinical samples were collected to source potential bacteriophage candidates, in addition to sourcing from known collections. Bacteriophage were isolated from lawns of host *E. coli* on agar plates. Standard plaque assay methods were employed to define host ranges and burst characteristics on 100 target *E. coli* and 78 non-target Gram Negative clinical isolate strains.

Results:

The four *E. coli* bacteriophage described here individually demonstrate sensitivity and specificity of 79% / 89% (MP-Ec-1), 74% / 92% (MP-Ec-2), 58% / 96% (MP-Ec-3) on more than 175 test strains of bacteria. A novel cocktail of three of these bacteriophage demonstrates a net clinical relevance sensitivity and specificity of 95% and 95% respectively, on 178 clinical isolates.

Conclusions:

Defining host range and specificity is the primary work objective of any bacteriophage-based diagnostic or therapeutic. A bacteriophage cocktail is one means by which target rates of sensitivity and specificity can be achieved. Coupled to an inexpensive detector, such as immunoassays, this cocktail shows promise as a diagnostic tool for detection of *E. coli*. This work supports follow-on clinical research with a prototype detector.

INTRODUCTION:

Here we explored the development of an coliphage-based diagnostic for rapid identification of *E. coli* from clinical samples. Bacteriophage are viruses which infect bacteria. This infection initiates through an interaction of the bacteriophage through the tail fibers and baseplate. Phage nucleic acid is inserted into the bacteria where the host nucleic acid and protein synthesis systems are used to replicate phage components. After assembly, phage protein mediated lysis occurs releasing tens to hundreds of progeny phage. The release of progeny phage can be monitored using a plaques count bioassay or immunologically, via an increase in the response of a test results such as an EIA or Lateral Flow Immunoassay.

This model was used to build understanding of what needs had to be met for the development of any bacteriophage-based diagnostic assay which capitalizes on the inherent specificity and/or replication of the bacteriophage through its lytic life cycle. *E. coli*-specific phage, or coliphage, are well described in the literature, most notably T-4 bacteriophage and the associated T-even and T-odd coliphage. However, we found that the modern literature lacks standardization of infection parameters and well-qualified host range (sensitivity and specificity) descriptions for these phage on broad environmental or clinical strains. Here we describe the development of a three-phage cocktail with high sensitivity and specificity for *E. coli*.

METHODS:

Bacteria

Bacteria were sourced from the ATCC (Manassas, VA), NCTC (Birmingham, UK), and JMI Laboratories (Iowa City, IA).

Bacteriophage

Phage were accumulated from a variety of sources including sewage, fly traps, environmental samples and collections including ATCC and the Universite Laval (Quebec, Canada).

Indicator BioAssay

Bacteria were grown to log phase and mixed with a known concentration of phage. At various time intervals (time = 0-6H) a sample of the suspension was centrifuged to remove any exogenous bacteria and the supernatant is added to phage host strain and plated in soft agar. After overnight incubation plates were examined for phage susceptibility by comparison to input phage concentrations. See Figure 1.

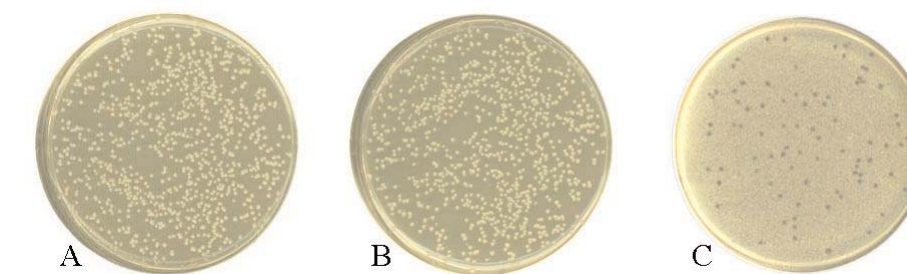


Figure 1. Coliphage indicator BioAssay photos. (A) shows positive growth on a host strain, (B) shows strain susceptibility in a test strain, and (C) shows resistance to amplification on a test strain.

RESULTS:

Screening

More than 100 coliphage sourced from existing collections, environmental samples (sewage and fly traps), and clinical samples. Selecting 14 preliminary *E. coli* strains for wide representation of *E. coli* types (data not shown), phage were first characterized by repeat BioAssay testing as described above. The preliminary sensitivity results of 10 of these coliphage are described to the right in Table 1.

| Screening <i>E. coli</i> / Phage | 8103-S | 11820-F | 498-F | 498-S | 202-S | 202-F | 7867-S | 244-S | 244-F | 3280-S |
|----------------------------------|------------|-------------|-------------|------------|------------|------------|------------|------------|------------|------------|
| <i>Titer</i> | 7.2 x 10e9 | 3.9 x 10e10 | 1.2 x 10e10 | 7.5 x 10e9 | 1.0 x 10e8 | 4.7 x 10e7 | 1.9 x 10e8 | 3.5 x 10e8 | 7.0 x 10e9 | 2.0 x 10e4 |
| <i>Screen Coverage</i> | 2 / 14 | 1 / 14 | 6 / 14 | 7 / 14 | 4 / 14 | 9 / 14 | 5 / 14 | 2 / 14 | 5 / 14 | 2 / 14 |
| <i>Preliminary Sensitivity</i> | 14 % | 7 % | 42 % | 50 % | 28 % | 64 % | 35 % | 14 % | 35 % | 14 % |

Table 1. Screen results of 10 representative coliphage from a number of sources. Preliminary coliphage sensitivity results varied from 7% (host strain only) to more than 70% (not shown).

| Sensitivity Performance | 155 | Fly-002 | MP-498 |
|-------------------------|----------|----------|----------|
| <i>Coverage</i> | 79 / 100 | 74 / 100 | 58 / 100 |
| <i>Sensitivity</i> | 79 % | 74 % | 58 % |

Table 2. Sensitivity of three lead candidate bacteriophage to 100 clinical isolates of *E. coli*. All infections were tested at least twice by the BioAssay method. Positives were measured as amplification of at least 2-logs compared to the input phage.

| Challenge Genus / Phage | 155 | Fly-002 | MP-498 |
|-------------------------------|---------------|---------------|---------------|
| <i>Acinetobacter</i> | 0/3 | 0/3 | 1/3 |
| <i>Aeromonas</i> | 0/3 | 0/3 | 0/3 |
| <i>Bacteroides</i> | 0/1 | 0/1 | 0/1 |
| <i>Citrobacter</i> | 0/4 | 0/4 | 0/4 |
| <i>Enterobacter</i> | 1/14 | 1/14 | 0/13 |
| <i>Escherichia (non-coli)</i> | 1/7 | 0/7 | 0/7 |
| <i>Klebsiella</i> | 2/10 | 1/10 | 0/7 |
| <i>Pasteurella</i> | 1/2 | 1/2 | 1/2 |
| <i>Proteus</i> | 0/9 | 0/9 | 0/1 |
| <i>Pseudomonas</i> | 0/10 | 0/10 | 1/10 |
| <i>Salmonella</i> | 0/3 | 0/3 | 0/3 |
| <i>Serratia</i> | 1/6 | 0/6 | 0/6 |
| <i>Shigella</i> | 3/5 | 3/5 | 0/5 |
| <i>Yersinia</i> | 0/1 | 0/1 | 0/1 |
| Overall score | 8 / 78 | 6 / 78 | 3 / 78 |
| Gross Specificity | 89.7% | 92.3% | 96.1% |

Table 3. Gross specificity of three lead candidate bacteriophage to 78 Gram Negative strains from 14 species.

Sensitivity

Following the gross preliminary screen, coliphage with greater than 50% coverage were brought through a secondary set of BioAssay experiments to determine their sensitivity to 100 additional clinical *E. coli* isolates. Table 2 outlines the sensitivity of three candidate coliphage. Each phage demonstrated a unique host range pattern, infecting different populations of *E. coli*. Table 2 outlines the sensitivity of three candidate bacteriophage following this second effort. As it shows, the actual sensitivity of these phage were generally found to be better when subjected to a large pool rather compared to the screen. As expected, clinically isolated bacteriophage performed more effectively than environmentally- and collection- sourced bacteriophage on the clinical isolates.

Specificity

In addition to performing sensitivity testing, specificity testing was performed to define the exclusivity of the bacteriophage to bacteria other than *E. coli*. Here we found the literature lacking in data that demonstrated exclusivity to a wide variety of similar hosts, assuming a natural state of high specificity.

Again, the BioAssay was performed in addition to binding experiments (data not shown) to determine the specificity of candidate bacteriophage. Again, a preliminary panel of 10 Gram Negative bacteria were used to first screen phage, and those with greater than 85% specificity (15% cross-reactivity) were selected for further study on 78 Gram Negative clinical isolates (data not shown). Gross specificity of these three candidates are shown to the right in Table 3.

Specificity Weighting

Since the specificity panel was constructed without regard to clinical significance, a weighting scheme was developed to assess the true specificity of the bacteriophage in clinical settings. Working from Karlowski *et al.* who defined the prevalence of organisms from more than 82,000 hospital blood cultures at 286 hospitals, MicroPhage weighted the specificity data of its findings for lead candidate bacteriophage to the twenty five bacteria defined in this study. The top 10 organisms are outlined in Table 4 to the right.

Weighting for specificity produced results as expected in the data, raising results to greater than 95% for each of the three bacteriophage described in Table 5.

Cocktailing

Although not studied, weighting sensitivity data to be more reflective of the *E. coli* strains found in blood cultures is one approach to predict individual phage sensitivity. However, it was determined that the literature lacked sufficient description of these strains consistently nation-wide, so a cocktailing approach was employed instead.

Previously described, cocktailing bacteriophage allows for enhanced sensitivity of a phage-based approach to exist, without concern for competing effects. A cocktail takes advantage of host range specificity of complementary phage to produce a greater result. Table 5 shows the performance of a novel 3-phage cocktail is described beside its constituent members.

CONCLUSIONS:

In first developing the *E. coli* assay several lessons were learned that can be applied to other bacterial targets. First, lytic bacteriophage are a tool that can be used to identify bacteria on a species level. Second, bacteriophage cocktailing is one means to effectively increase the sensitivity of an assay as use of a single phage for detection is unlikely to cover all strains necessary for a clinical product.

Host range and cross reactivity of bacteriophage vary a good deal, however it has been demonstrated that developing a cocktail with highly specific bacteriophage sets is achievable. Further work applying this technology to clinical samples is needed to better understand truer rates of target and non-target infection.

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

OPPORTUNITIES:

By incorporating phage amplification into immunoassays, it appears that an expanded menu can be accomplished for bacteria that have otherwise eluded immunoassays, such as *E. coli*. The method holds promise for additional bacterial targets, such as *S. aureus*, *S. pneumoniae*, *Enterococcus sp.*, and *Pseudomonas sp.*, all with lytic phage candidates available for development.

ACKNOWLEDGEMENT:

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REFERENCE:

Karlowski, et al. Prevalence and antimicrobial susceptibilities of bacteria isolated from blood cultures of hospitalized patients in the United States in 2002. *Annals of Clin. Microbiol and Antimicrobial*. 2004, 3:7.

| Rank | Bacteria | Isolates | Percentage (of total) |
|------|-------------------------------------|---------------|-----------------------|
| 1 | Coagulase-Negative <i>Staph. sp</i> | 34,640 | 42.0 |
| 2 | <i>Staphylococcus aureus</i> * | 13,618 | 16.5 |
| 3 | <i>Enterococcus sp.</i> | 9,919 | 12.0 |
| 4 | <i>Escherichia coli</i> | 5,942 | 7.2 |
| 5 | <i>Streptococcus sp.</i> | 5,299 | 6.4 |
| 6 | <i>Klebsiella sp.</i> | 3,412 | 4.1 |
| 7 | <i>Pseudomonas aeruginosa</i> | 2,030 | 2.5 |
| 8 | <i>Enterobacter sp.</i> | 1,962 | 2.4 |
| 9 | <i>Serratia marcescens</i> | 814 | 1 |
| 10 | <i>Acinetobacter baumannii</i> | 733 | .9 |
| | TOTAL | 78,369 | 95% |

Table 4. The top ten bacteria found in hospital blood cultures. This and additional data was used to construct a weighting scheme to predict performance.

| Sensitivity Performance | 155 | Fly-002 | MP-498 | 3-Phage Cocktail |
|-----------------------------|------|---------|--------|------------------|
| <i>Sensitivity</i> | 79 % | 74 % | 58 % | 95 % |
| <i>Gross Specificity</i> | 90 % | 92 % | 96 % | 90 % |
| <i>Weighted Specificity</i> | 96 % | 98 % | 99 % | 95 % |

Table 5. Sensitivity and Specificity of the Novel 3-Phage cocktail compared to its constituent coliphage members.