A Novel Bacteriophage based MRSA/MSSA Acquired Infection Test

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Sponsors:

Dr. Kent J. Voorhees
Professor
Department of Chemistry and Geochemistry
Colorado School of Mines
Golden, CO 80401
303 273 3616

Dr. Drew Smith
Chief Scientific Officer
MicroPhage, Inc.
2400 Trade Center Avenue
Longmont, CO 80503
303 652 5049

Mr. Jack Wheeler
Former Chief Executive Officer
MicroPhage, Inc.
2400 Trade Center Avenue
Longmont, CO 80503

Contact Person:

Dr. Kent J. Voorhees
Department of Chemistry and Geochemistry
Colorado School of Mines
Golden, CO 80401

303 273 3616
kvoorhee@mines.edu

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Recent Milestones:

1. 2007: Colorado School of Mines receives U.S. patent (7,166,425) on phage amplification
2. 2008: MicroPhage receives the ISO 13485 certification as a manufacturer of *in vitro* diagnostics
3. 2009: MicroPhage receives European C mark
4. 2010: MicroPhage begins marketing in Europe
5. 2010: FDA 510(k) approval application filed
6. 2010: Microphage/Colorado School of Mines receives R&D 100 Award
7. 2010: MicroPhage receives IRS/DHS Qualifying Therapeutics Discovery Grant
8. 2011: FDA 510(k) approval for human diagnostic device sale in the United States
9. 2011: Marketing agreement with Cardinal Health, Inc.
10. 2011: First MRSA/MSSA product shipped for U.S. sales

Eligibility: The nominated technology is eligible for both a small business and an academic award.

Focus Areas: The Use of Greener Reaction Conditions and Design of Greener Chemicals.

Development Location: The nominated technology was developed entirely in the United States at the Colorado School of Mines (CSM) in Golden, CO and MicroPhage, Inc. Longmont, CO.

Abstract: The bacteriophage (phage) amplification platform (1) developed at the Colorado School of Mines enables rapid identification of *Staphylococcus aureus* and determination of methicillin resistance (MRSA) and/or methicillin susceptibility (MSSA) without the need for extensive bacterial culturing. As Melvin Weinstein MD, Chief, Division of Infectious Disease, Rutgers University Medical School, stated at an IDSA meeting in Boston concerning an ideal detection method for *S. aureus*, "the identification of the organism is good, but what about susceptibility? As a clinician, that is the key issue" (2). MicroPhage, based on a worldwide license of the CSM phage amplification technology, was founded by Voorhees and Wheeler in 2002 with the goals of providing point-of-care devices which fulfill the needs of the medical profession such as determining *S. aureus* susceptibility and also reduce non-renewable materials in manufacturing and the amount of medical waste generated. Phages are viruses that infect bacteria in a species-specific fashion and then, rapidly multiply. The amplification process can generate as much as a 5-log increase in phage concentration. This allows for reduced incubation times, from one to five hours compared with traditional microbiological culture assays of 24 to 48 hours. The MicroPhage/CSM approach (KeyPath™) is conducted on a milliliter level and has incorporated modern chemical detection approaches. The test resembles a typical immunoassay whereby the sample (blood culture containing a suspected pathogen) is added to two reaction tubes, one containing phage and a nutrient media, and the second, phage, media, and methicillin. The test samples are mixed with the tube contents and incubated, followed by analysis on a dual track lateral flow immunoassay strip. A positive result on the first track indicates the presence of *S. aureus.* A positive result on the second track shows that the *S. aureus* is methicillin resistant. The phage amplification platform is the first and only rapid, direct *in vitro* diagnostic test

approved by the FDA (3) for direct identification of bacteria and determination of antibiotic resistance/susceptibility. The manufacturing and utilization of the KeyPath™ kit addresses principles 1, 3, 6, and 7 of the Twelve Principles of Green Chemistry.

A. Bacteriophage Amplification Process

Bacteriophages (phage) are bacteria-specific viruses. Because of their specificity to only bacteria, they are widely considered safe to humans, animals, and anything that is non-bacterial. Through evolution, phages are capable of efficiently and effectively replicating in the presence of specific live bacteria.

1. The phage infection cycle

The phage infection process (4) can be divided into four steps: adsorption, injection, replication and lysis.

Adsorption- A phage quickly locates specific receptors on the surface of the target bacterial cell and attaches in a two-step process. First, the phage tail fibers create a low-affinity bond to the outer membrane of the bacterium. Second, small tail fibers extend from the baseplate of the phage to form a high-affinity, irreversible bond to the bacterium.
Injection- Once the phage creates an irreversible bond to the bacterium, the phage injects its nucleic acid into the bacterium.
Replication- Following nucleic acid injection, the phage is replicated into hundreds to thousands of progeny phage. The parent phage's nucleic acid inserts itself into the DNA replication process of the bacterial host, halting further bacteria growth and forcing it to produce proteins that will assemble and become phage.
Lysis- Depending on the phage, an additional enzyme is formed following, or in concert with, phage replication. This step produces holing or lysine, which thins the bacterial membrane and allows the progeny phage to break into the open environment.

2. Lateral Flow Immunochromatography

The progeny phage can be rapidly detected on an immunoassay strip, such as lateral flow immunochromatography (LFI) – much like a home pregnancy test. A schematic of a typical lateral flow device is shown in Figure 1. The device is composed of several components starting with a sample pad where approximately 100 microliters of sample are applied. Next, there is a reporter pad which contains analyte-specific antibodies conjugated to colored nanoparticles and different colored nanoparticle conjugated to a control marker molecule such as biotin. There are two capture lines, one, the test line, containing immobilized analyte-specific antibodies and a second, the control line, containing a capture molecule for the marker compound. The immobilized analyte-specific antibodies arrest the analyte-nanoparticle conjugate which if, in high enough concentration, produces a colored line. A colored line on the control line (second collection line) indicates that sufficient time has passed to allow the liquid to migrate down the entire nitrocellulose strip. Finally, an absorbent pad is present to collect liquid as it wicks down the nitrocellulose membrane. The time for the analysis is about 10 minutes.

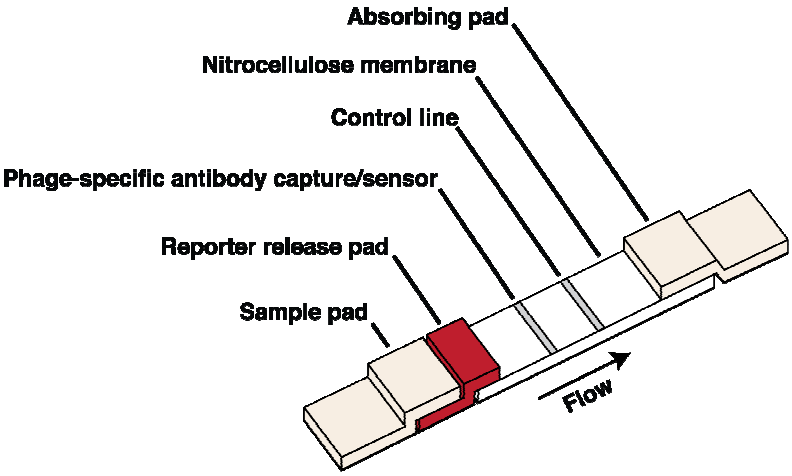

Figure 1. Basic LFI device and application to MRSA/MSSA Analysis.

3. The MRSA/MSSA test

The MicroPhage/CSM phage amplification platform (1) enables rapid identification of *S. aureus* and determination of methicillin resistance (MRSA) or methicillin susceptibility (MSSA). Simple to perform, without the need for expensive equipment or highly technical staff, the test marketed as KeyPath™ makes diagnostics accessible to institutions, large and small.

Antibiotic susceptibility testing is an extension of identification, using a dual strip LFI platform. Susceptibility/resistance testing can be achieved for most antibiotics in a parallel assay to the identification test. Antibiotics act faster in killing susceptible hosts than phage amplification, allowing for a simple means to determine bacterial response to the antibiotic. If the bacteria are determined to be present by having a positive test on the *S. aureus* strip, a positive in the antibiotic strip determines it is a resistant organism, as the bacteria was not inhibited or killed by the present antibiotic. Conversely, if the present bacteria produce no test line from the antibiotic strip, then the user can interpret the organism as susceptible to that antibiotic.

4. KeyPath™ Test Procedure

The phage amplification procedure (Figure 2) involves adding the sample (blood culture with a suspected pathogen) to each of the color coded incubation tubes, set to incubate, followed by running on a detector (color coded dual strip lateral flow immunoassay) to obtain the results. Hands-on time per test run is less than three minutes and performance is comparable to final/definitive identification antibiotic susceptibility test (ID/AST) results.

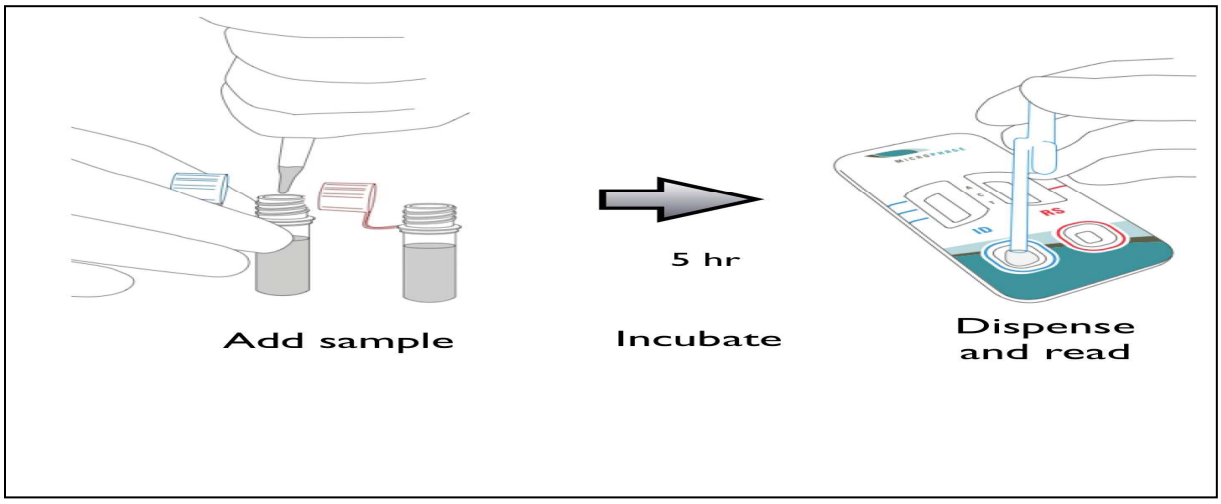

Figure 2. Test Procedure Overview, each tube and the LFI are color coded to prevent mix up.

B. The Health Risk of *S. aureus*

Staphylococcus aureus is the causative agent for most *Staphylococcus* infections. The mutant methicillin resistant form of the bacteria (referred to MRSA) is a major worldwide nosocomial pathogen. The appearance of resistant bacterial form is due to the overuse of antibiotics to which the bacteria has developed a resistance. Over the past 15 years, hospitals have seen a double-digit growth in the number of observed MRSA cases. In 1974, MRSA infections amounted to 2% of the *Staphylococcus* infections. By 2004, the percentage had grown to 63 %. A recent study reported that in 2005 there were nearly 95,000 reported MRSA infections in the U.S. that resulted in 18,650 deaths. In the May 2011 FDA approval press release, the MicroPhage/CSM KeyPath™ test was reported to be greater than 98.9% effective in diagnosing MRSA and 99.4 % accurate for MSSA (3).

C. T**echnology Comparison**

Eighty-five percent of the tests performed for *S. aureus* use traditional microbiology. In this test, suspected samples are plated onto Petri dishes and incubated for 12 to 24 hours. At the end of the incubation, once the plates are read, a significant quantity of biohazard waste is generated (following table) that must be properly treated and incinerated or placed in a landfill. Traditional

microbiology is inexpensive, but in addition to the amount of biohazard waste generated, it also suffers from the time involved to conduct the test. Only two major competitive instrumental based non-FDA approved tests exist and rely on a real-time polymerase chain reaction (RT-PCR) assay. The instrumental approaches amount to about 5% of the total market. All molecular tests require expensive equipment with an expected lifetime of four years. Replacement usually requires disposal of the old instrumentation. RT-PCR produces comparable after use waste to

Product Name	Traditional microbiology	StaphSR Assay	Xpert MRSA/SA BC	MRSA/MSSA Blood Culture Test
Manufacturer	Various	BD Diagnostics	Cepheid, Inc.	MicroPhage, Inc.
Technology	Plate assay	Real-time polymerase chain reaction (RT-PCR)	RT-PCR	Phage amplification-immunoassay
Time to Results	3 days	8-12 Hours	4 Hours	5 Hours
Cost per Test	$24	$35-$45	$40-60	~$55.00
Invalid rates		5-10%	7-12%	0.3%
Estimated waste per Test	~110 g/individual test	unknown	unknown	~60 g/test (see Life cycle Section)

the MicroPhage/CSM phage amplification test, but requires primers to be synthesized which also produce pre-test waste. The primer synthesis is commonly done in a contract laboratory; information on the waste generated is not available. Both the traditional microbiology and the new MRSA/MSSA phage amplification tests require an incubator, but no instrumentation to read the results. Traditional microbiological test clearly generates more after use waste than the MicroPhage/CSM phage amplification test. The cost for the MicroPhage/CSM MRSA/MSSA KeyPath™ test is $55.00 per test.

All waste for any of the tests for *S. aureus* must be disposed as biohazard waste.

D. Life Cycle Analysis

The following Table provides information on waste generated from antibody production, LFI production, and the actual MicroPhage/CSM KeyPath™ test. The kit weighs about 50g, all of which eventually end up as waste.

Production step	Source of Starting materials	Amount of waste	Type of waste
Phage for antibody production	Renewable sources	10 L of media*	Spent microbiological media
PEG 8000 for phage purification	Petroleum	500g per mouse	polymer
CsCl for ultra centrifuge purification	Mineral	25g per mouse	Inorganic salt
Antibody production at contract laboratory	Biological synthesis	<20g per mouse	Biological and organic non-RCRA
LFI Kit	Petroleum	11g/device	Polystyrene plastic for cassette
	Wood	<1g per device	nitrocellulose
	Petroleum	6.5g per test	Polyethylene for 2 vials and 2 medicine droppers
	Wood	<0 The cost for the MicroPhage/CSM MRSA/MSSA KeyPath™ test is $55.00 per test. .5g per device	Cellulosic pads
Media for phage amplification	Renewable sources	4.2g per device	Microbiological media

* 200,000 LFI strips can be made from each mouse injected. The 10L waste figure represents production of phage to inject 5 mice.

Petroleum is the source for the polystyrene, polyethylene, and the polyethylene oxide polymers used in manufacturing components for the kit. The production quantity of polystyrene need for Petri dishes and other components for traditional microbiology testing requires 85g of polystyrene compared to 11g for the new MRSA/MSSA test. The market for all *S. aureus* tests is approximately 3.2 million per year in the U.S. Considering a conservative number of one million LFI tests performed, the polystyrene savings for component production based on traditional microbiology using 93.5 tons versus 12.1 tons for the MRSA/MSSA test is 81.4 tons. It is estimated that each pound of molded polystyrene requires 11.9 kWh/lb. Both the quantity of polystyrene and the energy needed for molding are significantly reduced in manufacturing of the MRSA/MSSA test.

The remainder of the components for the LFI device is produced from renewable materials. For example, the cellulosic pads and nitrocellulose membranes are prepared from wood. Energy and waste figures for these two materials were not available from the suppliers.

The chemical components used in antibody production are not regulated and are considered as aqueous or organic non-RCRA wastes. Tissue culture materials, cultures, and mice are handled as biological waste.

The information generated by the KeyPath™ test allows the physician to rapidly differentiate MRSA from MSSA to better direct antibiotic therapy. In the case of MSSA, the physician is allowed to "de-escalate" from the more toxic, broad-spectrum antibiotic such as Vancomycin to a more effective and safer beta lactam antibiotic like Nafcillin. Since MSSA represents more than 40% of most *Staphylococcus* infections, rapid identification of the organism and assessment of antibiotic resistance results in shorter hospital stays and significantly lower mortality rates. Improper treatment also creates environmental and indirect synthetic waste associated with the manufacturing of the incorrectly prescribed antibiotic. Any addition of antibiotics into the environment also creates the possibility of developing new antibiotic resistant bacteria. Pharmaceuticals in the environment have become an important issue (5), but the general population is not sensitive to the amount of waste (E-factor of 100 to 150) that is produced to prepare an antibiotic.

E. Conclusion

The KeyPath™ diagnostic product provides information on both the identification of the *Staphylococcus aureus* bacterial as well as its susceptibility to specific antibiotics. This test is the only MRAS/MSSA test that has received FDA approval (3). Four principles of the Twelve Principles of Green Chemistry, 1: Prevention, 3: Less Hazardous Chemical Synthesis, 6: Design for Energy efficiency, and 7: Use of Renewable Feedstocks, are addressed in the manufacturing and utilization of the KeyPath™ diagnostic product .

F. References

1. A.J. Madonna, S. Van Cuyk and K.J. Voorhees (2003) Rapid Commun. in Mass Spectrom., 17, 257.
2. M. Weinstein (2011) Infectious Disease Society of America Annual Meeting, Boston, Massachusetts
3. U. S. Food and Drug Administration, Press Release, May 6, 2011.
4. C.K. Mathews (1971), "Bacteriophage Biochemistry," Van Nostrand Reinhold Co., New York.
5. K. Kummer (2003) J. Antimicrobial Chemotherapy, 52, 5.