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Phage-based platforms for the clinical detection of human bacterial pathogens

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Bacteriophages (phages) have been utilized for decades as a means for uniquely identifying their target bacteria. Due to their inherent natural specificity, ease of use, and straightforward production, phage possess a number of desirable attributes which makes them particularly suited as bacterial detectors. As a result, extensive research has been conducted into the development of phage, or phage-derived products to expedite the detection of human pathogens. However, very few phage-based diagnostics have transitioned from the research lab into a clinical diagnostic tool. Herein we review the phage-based platforms that are currently used for the detection of *Mycobacterium tuberculosis*, *Yersinia pestis*, *Bacillus anthracis* and *Staphylococcus aureus* in the clinical field. We briefly describe the disease, the current diagnostic options, and the role phage diagnostics play in identifying the cause of infection, and determining antibiotic susceptibility.

Introduction

The ability of bacteriophages (phages) to specifically infect, and lyse its host has been exploited for many decades as a means of uniquely identifying target bacteria. Phage typing is still used to identify and distinguish different strains within a given species when isolated from different origins (disease, food, water, environmental) or geographical locations. In this method, phage dilutions are spotted onto a bacterial lawn; if bacteria are sensitive to the phage, bacterial lysis occurs resulting in an area of clearing. Susceptibility of target bacteria to different phages leads to a characteristic pattern and enables the specific strain to be characterized and epidemiologically identified. Phage typing schemes exist for the majority of clinically relevant pathogens including Brucella,[1] Clostridium,[2] Enterococci,[3] Salmonella,[4,5] Shigella,[6] Listeria,[7] toxigenic *Escherichia coli*,[8] Campylobacter,[9] *Bacillus cereus*,[10] *Vibrio cholerae*,[11] *Mycobacterium tuberculosis*,[12] Proteus,[13] Yersinia[14] and Staphylococci.[13] Phage typing however, requires maintenance of a large number of phage stocks and propagating strains, thus confining its use to select reference laboratories. Therefore, even though a propensity of information on these phage typing schemes exist, they are not used for the identification of bacterial pathogens in a clinical setting. The gold standard for bacterial identification remains traditional culture-based assays but there is a growing need for methods that eliminate the need for primary culture and hence are able to directly detect the pathogen in a variety of clinical matrices.

Phage-based diagnostics (e.g., phage-amplification, reporter phage, phage-labeling, phage capture elements) have the potential to fulfill this gap. Extensive evidence supports this notion since phage diagnostics can rapidly and sensitively detect their specific host in a variety of culture, food, water, clinical and environmental matrices.[15-17] Moreover, in contrast to phage therapy, whereby a myriad of potential inhibiting factors can impede the success of a phage interacting with and ultimately killing its target bacterium, phage-based detection is essentially performed in vitro where environmental conditions can be controlled and manipulated to favor the phage-host interaction. Despite these attributes, there are surprisingly very few phage diagnostic technologies that are used as standard tests in the clinical field.

In this review, we focus on the four bacterial pathogens whereby phage-based diagnostics are currently in use, FDA approved, or a product is available for the detection of clinical isolates; these are *M. tuberculosis*, *Yersinia pestis, Bacillus anthracis,* and *Staphylococcus aureus*. We briefly describe each disease, current diagnosis options, and how phage-based diagnostics are being used and developed to expedite pathogen identification and antimicrobial susceptibility determination. Obstacles and considerations that have impeded the further development of phages into viable diagnostic products are also discussed.

Mycobacterium Tuberculosis

Tuberculosis (TB) is a common and deadly infectious disease caused by the bacillus M. tuberculosis. The World Health Organization (WHO) estimates a global incidence of 128 TB cases per 100,000 people, which is equivalent to 8.8 million new cases per year.[18] The disease is especially prevalent in developing countries, with Asia and Africa accounting for 85% of all cases.[18] Despite many national TB control programs, many developing countries have low case detection rates and once a case is detected, cure may be difficult because of poor case management, high default rates, and insufficient control of drug prescription. These issues are further compounded by the emergence and spread of multidrug resistant tuberculosis (MDR-TB) and extensively drugresistant tuberculosis (XDR-TB).[19] Therefore, rapid, accurate and inexpensive diagnosis of pulmonary TB is essential to ensure

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prompt and appropriate initiation of antibiotic therapy and to prevent further transmission.[20]

Conventional TB diagnostics include clinical assessment, radiology, sputum smear microscopy (Ziehl-Neelsen acid-fast stain) and culture of *M. tuberculosis* on solid or liquid media (such as Löwenstein-Jensen, Kirchner, and various Middlebrook formulations). Although these procedures are irreplaceable they suffer from a number of drawbacks. For example, microscopy of sputum samples for acid-fast bacilli (AFB) shows poor sensitivity (30–50%, $\geq 10^4$ AFB/mL sputum) and presumptive identification by culture on solid media can take up to 8 weeks.[21] Liquid culture analysis with the semi-automated BACTEC 460 instrument and automated systems such as the BACTEC MGIT 960 have reduced turnaround times (10–14 d) but require specialized equipment, costly reagents, and in the case of the BACTEC 460 system necessitate the handling of radioactive material.[21,22] Molecular detection methods such as the FDA-approved Amplified-MTD test (Gen-Probe) and COBAS MTB test (Roche) are rapid and specific; however, nucleic acid amplification techniques can be costly and require technical expertise. Despite these advances alternative or improved diagnostic technologies are desperately needed, especially for low-income areas where resources and funding are limited. Development of such methods is complicated by the slow growth rate (doubling time of 18–24 h) and fastidious growth requirements of *M. tuberculosis*, the presence of non-tuberculosis species in clinical specimens, and the widely divergent levels of *M. tuberculosis* bacilli found in sputum samples from infected patients.

Mycobacteriophage-based detection. Since the first identification of mycobacteriophages over 65 years ago,[23,24] researchers have recognized phage-based assays as a potentially important tool in the identification, diagnosis, and drug susceptibility testing of TB.[25-27] Phage-based assays are particularly attractive since they are rapid, simple, and do not require the use of relatively expensive equipment. There are two main phage-based approaches used to detect *M. tuberculosis*: (1) amplification of phages following infection of TB bacilli and subsequent detection of progeny phages using indicator cells (plaque formation), and (2) detection of light produced by luciferase reporter phages or fluorescent reporter phages following infection of *M. tuberculosis*. Both approaches exploit the specificity of the phage-bacterium interaction and require viable TB bacilli to sustain phage replication or reporter gene expression. Phage-based tests are available as commercial kits (FASTPlaqueTB and PhageTekMB, Biotec Laboratories, UK) and as in-house (laboratory-developed) assays. In house tests use either amplification technology (e.g., phage amplified biologically [PhaB] assay) or reporter phage technology (**Table 1**).

Mycobacteriophage-amplification technology. Phage amplification assays such as FASTPlaqueTB and PhaB utilize the mycobacteriophage D29 to detect the presence of viable *M. tuberculosis* within a clinical specimen.[28-30] D29 is a lytic,

Table 1. Characteristics of phage-based assays

Species	Phage	Detection method	Sample matrix	Time to detection	Sensitivity	Refs.
B. anthracis	γ	Phage amplification (bacterial lysis)	Growth media (pure cultures)	20 h	Concentrated cultures	122
B. anthracis	γ	Phage/DNA amplification (qPCR)	Growth media	5 h	~10^2 CFU/mL	127
B. anthracis	γ	PlyG capture element (dot blot assay)	Growth media	3 h	~10^3	112
B. anthracis	Wβ	Reporter phage (*luxAB*)	Blood	2 h	~10^5 CFU/mL	Schofield et al. unpublished results
Y. pestis	ϕA1122	Phage amplification (bacterial lysis)	Growth media (pure cultures)	20 h	Concentrated cultures	65
Y. pestis	ϕA1122	Phage/DNA amplification (qPCR)	Blood	5 h	~10^6 CFU/mL	89
Y. pestis	ϕA1122	Reporter phage (*luxAB*)	Serum	3 h	~10^3 CFU/mL	Schofield et al. unpublished results
M. tuberculosis complex	D29	FASTPlaqueTB: Phage amplification (bacterial lysis)	Processed sputum	48 h	100 CFU/mL	28
M. tuberculosis	phAETRC201::hsp60-FFlux (TM4ts derivative)	Reporter phage (*FFlux*)	Liquid cultures	3–4 h	8×10^1–1×10^5 CFU/mL	53
M. tuberculosis	phAE87::hsp60-EGFP (TM4 derivative)	Reporter phage (*GFP*)	Liquid cultures	16–18 h	OD_{600} 0.6–1.6, 37–67% positive fluorescence	54
S. aureus	Proprietary phage cocktail	Phage/protein amplification (immunoassay)	Blood	Positive BACTEC™ culture plus 5.5 h	6×10^5 CFU/mL from positive BACTEC™ bottles	145
S. aureus	Proprietary phage cocktail	Phage/protein amplification (immunoassay)	Nasal swab	Positive BACTEC™ culture plus 7 h	Not reported	175

double-stranded DNA phage with a wide host range that is capable of infecting both fast-growing and slow-growing species of mycobacteria (*M. smegmatis*, *M. bovis*, *M. kansasii*, *M. tuberculosis* and *M. leprae*).[31] In this approach a sputum specimen must be processed in order to liquefy the sample and reduce the number of non-target commensal microbes.[32] Sputum samples are typically processed using the *N*-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method;[32] however, a sputum-associated activity that was inhibitory to the phage-based assay can be removed with NaOH alone.[29] After initial processing the sample is washed, concentrated, and the extracted bacilli are cultured in broth for 20 to 24 h prior to inoculation with a mycobacterium-specific phage (Actiphage, D29). Phage-infected cells are then exposed to a chemical virucide that destroys extracellular phage that has not infected a host bacterium. The addition of inactivation compounds such as ferrous ammonium sulfate does not adversely affect the production of progeny phages within the bacilli.[33] Therefore, elimination of exogenous phages means that phage detected in a sample after this treatment results from phage replication. After sequestration of the chemical virucidal agent, progeny phages released by lysis can be detected by infection of a non-pathogenic rapidly growing indicator strain such as *M. smegmatis* (indicator cells). Rapid cycles of phage infection, replication, and cell lysis are seen as zones of clearing (plaques) in the lawn of indicator cells. If a target cell (viable *M. tuberculosis*) was not present in the original sputum sample, phage amplification would not occur, and plaques would not be detected on the indicator test plate (cutoff values: 0 to 19 plaques, negative; $\geq$ 20 plaques, positive). The number of plaques visualized in a given sample is related to the number of viable *M. tuberculosis* in the original sample. Importantly, this test can provide results within 48 h of sample collection. The FASTPlaqueTB test is able to detect 100–300 viable bacilli per mL of sputum,[28,29] which is significantly better than the $\geq 10^4$ bacilli/mL required for the acid-fast smear method, and is equivalent to culture detection methods.[21]

The FASTPlaqueTB and PhaB assays have been evaluated in terms of sensitivity, specificity, and accuracy for the detection of *M. tuberculosis* in clinical specimens.[34-40] In a meta-analysis of 13 published studies,[41] phage amplification assays were found to have high specificity (range 0.83 to 1.00), but modest and highly variable sensitivity estimates (range 0.21 to 0.94). When stratified by smear status, the smear-positive specimens yielded higher estimates of sensitivity compared with the smear-negative specimens (0.29–0.87 and 0.13–0.78, respectively).[41] In head-to-head comparisons, the overall accuracy of the phage-amplification assay was slightly better than sputum microscopy in terms of specificity when compared with the standard culture method (area under the SROC curve, 0.95 and 0.86 for the phage-based assay and sputum microscopy, respectively).[41] A multi-center study found that the overall sensitivity of the PhaB assay was superior to the Löwenstein-Jensen culture and smear microscopy methods for detecting TB in sputum in pulmonary TB patients.[40] Five studies evaluated the performance of the phage-amplification assay using clinical specimens that had been stratified by smear microscopy status.[41] The phage-based assay detected TB with a specificity of 0.60 to 0.88 in smear-positive and 0.89 to 0.99 in smear-negative specimens.[41] Although this data suggests that the specificity of the phage-based assay is higher in smear-negative samples this may simply reflect the sputum collection procedure and study population characteristics. For example, several studies failed to follow the standard two consecutive sputum collection procedure recommended by the WHO and as such may have misclassified smear-positive TB patients as smear-negative. In addition, the sensitivity of smear microscopy is reduced in HIV co-infected patients. The impact of HIV infection on test accuracy could not be determined because none of the studies reported the proportion of HIV infections among the study population.

False-positive test results have been reported for the FASTPlaqueTB and PhaB assays and most likely reflects incomplete neutralization of exogenous phage by the virucidal solution or the presence of other mycobacteria in the respiratory sample.[29] The FASTPlaqueTB assay has been shown to produce positive test results with *M. kansasii*, *M. gastri*, *M. avium*, *M. intracellulare* and *M. fortuitum* isolates.[35,42] False-negative test results may be due to several factors, including delays in specimen transport and processing and carry-over of sputum inhibitory substances. Sputum processing may also damage acid-fast bacilli and reduce phage susceptibility by disrupting phage receptor expression on the cell surface.

Reporter mycobacteriophage technology. Reporter phage technology uses the ability of recombinant phages to specifically infect a target cell and upon replication, produce a detectable signal that can function as an indicator of cell viability. Reporter mycobacteriophages have been derived from phages TM4, D29, L5 and Che12.[43-46] A variety of reporter genes have been successfully incorporated into non-essential regions of the parent mycobacteriophage genomes. These include genes encoding firefly luciferase (FFLux), green fluorescent protein (GFP), and yellow fluorescent protein (ZsYellow). Upon infection with the luciferase reporter phage (LRP), metabolically active cells generate the luciferase enzyme, yielding detectable photon production or "light" emission in the presence of cellular ATP and the exogenous substrate luciferin. Light output can be quantified using a luminometer, or with less sensitivity but lower cost, a custom-made box which accommodates a photographic film cassette (Bronx Box).[47,48]

The first generation luciferase reporter phage (phAE40) was developed from the lytic phage TM4 and utilized the *FFLux* reporter gene driven by the strong mycobacterial promoter, BCG hsp60.[43] Phage TM4 has a broad-host range and infects both fast-growing and slow-growing mycobacterial species including *M. tuberculosis*. Therefore, phAE40 was capable of detecting mycobacteria of clinical importance but light output did not always indicate the presence of *M. tuberculosis*.[43,49] For example, light production was observed following infection of all *M. tuberculosis* complex strains (*M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. bovis* BCG), *M. marinum*, *M. chelonae*, *M. aurum*, *M. smegmatis*, *M. phlei*, *M. terrae*, *M. xenopi* and *M. intracellulare*.[49] In order to differentiate between the presence of tubercle bacilli and other mycobacterial species, a modified assay was developed which took advantage of

the compound *p*-nitro-α-acetylamino-β-hydroxypropiophenone (NAP), which has selective inhibitory activity against members of the *M. tuberculosis* complex.[49] Treatment with NAP for 24 h at 5–10 μg/mL was sufficient to rapidly differentiate *M. tuberculosis* complex and non-tuberculosis mycobacterium from BACTEC culture bottles.[49]

New improved TM4 LRPs were subsequently developed which produced sustained, high levels of light production and exhibited increased sensitivity of detection compared with the first generation phage phAE40.[50,51] One such phage (phAE142), which carried the *FFLux* gene driven by the P_{Left} promoter from phage L5, was able to detect mycobacteria directly in subcultured AFB smear-positive sputum samples.[50] The LRP phAE142 exhibited a lower rate of culture detection for primary mycobacterial isolates compared with the MGIT 960 system and Löwenstein-Jensen media (76.1%, 97.2% and 90.1%, respectively); however, the time to detection (TTD) was equivalent to the MGIT 960 system (median 7 to 7.5 d) and faster than the Löwenstein-Jensen method which had a median TTD of 14 d.[52] Using the LRP-NAP test, 94% (47/50) of isolates were correctly identified as tuberculosis complex.[52] Although the performance of the assay was improved by the addition of NAP, it failed to identify isolates beyond *M. tuberculosis* complex and non-tuberculosis mycobacterium.

Additional luciferase reporter phages have been constructed using mycobacteriophages L5,[46] D29[45] and Che12.[53] The luciferase reporter phage phGS18, which was developed from the temperate phage L5, forms a lysogen after infection of *M. smegmatis*, resulting in accumulation of the luciferase protein, sustained increases in light output, and an improved limit of detection;[46] however, the L5 phage does not infect the *M. tuberculosis* complex and therefore LRP phGS18 has limited clinical utility. Reporter phages have been constructed from phage D29, which like phage TM4 is lytic, and can infect *M. tuberculosis* isolates.[45] The D29-derived LRP, phBD8, produced light faster than TM4-derived LRP (phAE40), but light output from phAE40-infected cells quickly surpassed that of phBD8.[45] Improved signal output was observed for phBD8 following superinfection of a L5 lysogen of *M. smegmatis*, suggesting that suppression of the D29 lytic cycle may improve the sensitivity of this reporter phage.[45] It has been suggested that the poor sensitivity of LRP assays is due to the lytic nature of the phages, which lyse the bacteria leading to rapid loss of cellular ATP.[45,51,53] Consequently, LRP reporter phages derived from the temperate phage Che12 and a TM4 temperature-sensitive (TM4ts) mutant, which behaves as a temperate phage at its restrictive temperature, were developed and evaluated for improved kinetics.[53] The Che12 LRP (icI promoter driving *FFlux*), TM4ts LRP (hsp60 promoter driving *FFlux*) and TM4ts LRP (acr promoter driving *FFlux*) exhibited a sensitivity of detection ranging from 8×10^1 to 6×10^5 *M. tuberculosis* H37RV cells per mL; however, the LRP phage sensitivity dropped significantly when detecting a clinical isolate (10^5–10^7 cells/mL).[53] Therefore, the level of sensitivity in laboratory grown cultures was not replicated with clinical isolates. The drop in sensitivity was possibly due to the extensive genetic variation found in many *M. tuberculosis* strains or reflects metabolic differences between laboratory and clinical isolates when grown under standard laboratory conditions.

In a departure from previous reporter phage constructs, Piuri and colleagues recently generated a group of TM4-derived mycobacteriophages that contained the fluorescent reporter genes *gfp* or *ZsYellow*.[54] The fluorophages were able to detect approximately 50% of *M. tuberculosis* cells 16 h post-infection when examined by microscopy. Unlike a luciferase reporter, the GFP reporter does not require an exogenous substrate and GFP-expressing bacilli can be enumerated at the single cell level by fluorescence microscopy or flow cytometry. Thus, fluorophages may offer a potential advantage of detecting phage-infected cells within mixed populations.

Antibiotic susceptibility testing. The emergence of MDR-TB and XDR-TB strains has fueled the search for alternative, more rapid methods to assess antibiotic susceptibility. MDR-TB is defined as resistance to two first-line anti-tuberculosis drugs (rifampin and isoniazid), whereas XDR-TB strains are resistant to isoniazid and rifampin, plus any fluoroquinoline and at least one of three injectable second-line drugs (capreomycin, kanamycin or amikacin). Antimicrobial susceptibility testing (AST) of *M. tuberculosis* has traditionally been performed by the agar proportion method or by macrobroth testing on an instrument such as the BACTEC MGIT 960 (Becton Dickinson) or ESP culture system II (Trek Diagnostic Systems). The agar proportion method, while considered the reference standard, is labor intensive and slow (3 weeks), and cannot directly test microbes in patient specimens. In most cases an indirect method is used, where strains are grown in pure culture from patient samples with subsequent inoculation on drug-containing and drug-free media. TB isolates are considered resistant if the proportion of bacilli resistant to a single concentration of drug exceeds 1%. Automated broth culture detection systems (MGIT 960 and ESP) are cleared by the FDA to provide break point drug concentrations for first line drugs, with an average time to detection of 2 to 4 weeks.[21] Several alternative AST methods are under development, including phenotypic susceptibility tests (MycoTB MIC plate, Trek Diagnostic Systems), molecular methods to detect resistance-associated mutations (INNO-LipARif.TB, Innogenetics; Xpert-MTB/RIF, Genexpert system; GenoType-MTBDR, Hain LifeScience), and phage-based diagnostic assays.[21,55]

Phage-based assays measure the ability of viable mycobacteria to support bacteriophage replication or to synthesize a reporter gene product that is carried in the phage genome. Strains are classified as drug-susceptible when these assays detect *M. tuberculosis* in drug-free media, but fail to detect *M. tuberculosis* in drug-containing samples. A recent meta-analysis examined the diagnostic accuracy and performance characteristics of phage-based assays detecting rifampin resistance in *M. tuberculosis* (31 studies, 3,085 specimens).[55] The FASTPlaqueTB, luciferase-reporter phage, and in-house phage amplification assays were similar in terms of pooled estimates of sensitivity and specificity: 95.5%, 98.5%, and 99.3% sensitivity and 95.0%, 97.9% and 98.6% specificity, respectively. In agreement with this study, El-Sayed Zaki and Goda[56] reported a sensitivity of 100%, a specificity of 97.2%, and an accuracy of 97.6% for the

FASTPlaque TB-MDR system; results were available within 10.5–11.5 d from the time of specimen arrival. Failure rates due to contamination or uninterpretable results varied widely across the studies (0–36%, mean 5.8%), which was largely associated with evaluations using direct patient samples.[55] The high failure rates have been highlighted as a potential limiting factor and may provide a partial explanation for the lack of endorsement in widespread implementation of this technology.

Contamination issues are a common problem associated with many TB diagnostic assays, including automated liquid culture based systems, and are largely due to normal flora escaping the sputum decontamination procedure.[57] Antimicrobial formulations such as PANTA, PACT and NOA are often incorporated into media used for the primary isolation of tubercle bacilli to minimize the proliferation of normal flora.[58-61] Inclusion of the NOA supplement reduced contamination (13.9% to 4.1%), increased the number of interpretable results (69.3% to 79.0%) and did not adversely affect the performance of the FASTPlaque-Response test for determination of rifampin resistance in *M. tuberculosis*.[60] Several research groups are currently pursuing alternative approaches such as the use of phage cocktails and phage lysins for the elimination of non-mycobacterial species that commonly contaminate processed sputum samples.[62-64]

Yersinia Pestis

Y. pestis, the etiological agent of the plague, is a zoonotic disease affecting rats and other rodents. *Y. pestis* is transmitted from animal to animal by flea bites, which is also the most common route of transmission to humans.[65] *Y. pestis*-infected flea bites, results in the migration of the bacterium to the lymph nodes in humans. Bubonic plague, which develops 2–8 d later, is characterized by fever, chills, weakness and the development of swollen lymph nodes, or buboes. In some cases, the flea bites develop into septicemia without a bubo, or occasionally into pneumonic plague.[66] There are 1,000 to 5,000 cases and 100 to 200 deaths each year worldwide,[67] but the disease is relatively rare in the US with an average of 5–15 cases reported each year, mostly in rural areas.

Although the plague occurs infrequently, it is a reemerging disease and has important bioterrorism implications.[68,69] *Y. pestis*, along with *Bacillus anthracis* and *Francisella tularensis* are the three Category A bacterial pathogens listed by the Centers for Disease Control and Prevention (CDC) as the most likely to be used in a bioterrorist attack. These pathogens are considered high priority because of their rapid clinical course and high mortality rate. *Y. pestis*, unlike *B. anthracis* and *F. tularensis*, can be transmitted from person to person.[70] Transmission can occur through infectious respiratory droplets from pneumonic cases of the plague, or even from inhalation via contaminated clothes. The infectious dose is estimated at 100–500 cells.[71] The WHO estimated that an aerosolized release of 50 kg over a populated city could cause 150,000 cases of pneumonic plague and 36,000 fatalities.[72] These estimates did not take into account secondary cases that would inevitably occur through person-to-person contact. Symptoms for pneumonic plague include high fever, chills, and malaise, followed by a cough progressing rapidly to dyspnea (shortness of breath), stridor (high-pitched wheezing), cyanosis (blue coloration of the skin), and respiratory failure. Pneumonic plague is nearly always fatal if not treated within the first 24 h of symptom onset.[66] Therefore, early detection and diagnosis would be vital in order to quickly implement public health measures.

Modern techniques such as flow cytometry, real-time PCR, magnetic beads, enzyme-linked immunosorbent assay, and immunofluorescence are being developed for the presumptive identification of the pathogen directly in clinical specimens.[73-77] Commercially available, and portable identification methodologies include the Joint Biological Agent Identification and Diagnostic System (JBAIDS, Idaho Technology Inc.) plague detection kits, the Plague BioThreat Alert test strips (BTA; Tetracore) and the ABICAP columns (Senova, Jena, Germany). The latter two methodologies are based on the detection of the fraction 1 (F1) capsular antigen which can be detected as a soluble molecule in clinical specimens during the early course of disease. Expression of the F1 antigen is also the basis for the CDC's immunofluorescence and agglutination testing for presumptive and confirmed diagnosis of the plague. Although, it was previously thought that the F1 antigen was an essential virulence factor, strains lacking the F1 antigen exist and are fully virulent in nature and in animal models of infection;[78] therefore, virulent F1-negative strains may not be detected using assays based on the F1 antigen leading to false negatives. Moreover, the sensitivity limits of detection of the Plague BioThreat Alert strips and the ABICAP columns are between 6×10^3 to 7×10^3 CFU/mL.[73] Since 10^2 CFU/mL in the blood of septicemic patients can be fatal,[79] the sensitivity of the assay may be insufficient without additional laboratory culturing which can take another 24–48 h to complete.

***Y. pestis* phage lysis assay.** One of the first indications of the potential of *Y. pestis* phage occurred in the 1920s when d'Herelle used *Y. pestis* phages as a therapy to treat four plague-infected patients.[80] He injected phages directly into the buboes of the patients; all four patients recorded a two-degree drop in temperature, and subsequently recovered. Unfortunately, further attempts to confirm the efficacy of the phage in clinical trials and animal models were unsuccessful and their potential therapeutic value was largely ignored following the therapeutic success of antibiotics; however, *Y. pestis* phage, and in particular, the CDC "plague diagnostic" phage ϕA1122 has been used over the past 40 years as a means of identifying *Y. pestis* and plague diagnosis.

Y. pestis phages have been placed into four serovars based on their immunogenicity: (1) serovar 1 consists of lytic phages such as the CDC plague diagnostic phage ϕA1122,[81] H[82] and Y;[83] (2) serovar 2 includes the temperate phage such as L-413C;[84,85] (3) serovar 3 consists only of the temperate phage, termed P; and (4) serovar 4 consists of phages such as Tal and 513. The lytic phages of serovar 1, and in particular, the plague diagnostic phage ϕA1122 has received much attention due to their broad strain range and species specificity. This group of phages all have isometric hexagonal heads and short (13–42 nm) non-contractile tails. They belong to the family Podoviridae and are closely related

to the *E. coli* phages T3 and T7. The ϕA1122 genome was sequenced and consists of 37,555 bp, encoding 51 predicted gene products, and a nucleotide identity of 89% to the *E. coli* phage T7.[81] ϕA1122 is particularly suited as a diagnostic phage since it has an unusual ability to infect most *Y. pestis* isolates. According to the CDC, ϕA1122 can grow and lyse on all but two of thousands of natural isolates of *Y. pestis* within the CDC collection (M.C. Chu of the CDC, unpublished observations noted in Garcia et al.[81]). Advier[86] and Gunnison et al.[87] also demonstrated that ϕA1122 lysed all *Y. pestis* strains tested (47 and 52 strains, respectively). Furthermore, ϕA1122 exhibits species specificity to *Y. pestis* with the exception of some strains from the closely related species *Yersinia pseudotuberculosis*;[87-89] however, temperature may be used to differentiate the two species since the phage does not grow on *Y. pseudotuberculosis* at 20°C. Moreover, the *Y. pseudotuberculosis* strains that were deemed phage-susceptible by Gunnison et al.[87] differed markedly in their susceptibility to the phage, with many of the strains showing lysis only when 'spot-tested' using undiluted, or 10-fold serially diluted phage suggesting that a very high multiplicity of infection (MOI) was needed; this susceptibility is in contrast to *Y. pestis* which was susceptible to highly dilute (10^{-6}) phage preparations.[90] Consequently, due to its specific and broad strain infectivity, ϕA1122 is used by the CDC, WHO and US Army Research Institute of Infectious Diseases (USAMRIID) as a diagnostic standard (lysis assay) for the confirmed identification of *Y. pestis*.[65,91]

Two other phages are reportedly used in plague diagnosis, namely the Pokrovskaya phage and the L-413C phage.[84] Like ϕA1122, L-413C phage has a very broad strain range for *Y. pestis*. For example, L-413C phage was able to lyse all but 10 of 7,000 *Y. pestis* strains tested.[92] L-413C also displayed species specificity for *Y. pestis*, with the exception of a few restriction-deficient *E. coli* strains and some *E. coli* K-12 derivatives.[84] Importantly, L-413C has a unique specificity for *Y. pestis* within the *Yersinia* genus; for example, while L-413C has been shown to lyse 99.9% of *Y. pestis* isolates, none of 1,200 *Y. pseudotuberculosis* strains were phage susceptible;[92] therefore, this phage has a unique ability to discriminate between the two closely related species.

***Y. pestis* reporter phage.** As the plaque assays require pure bacterial cultures, culture isolation and the ensuing phage lysis assay takes approximately 4 days from clinical specimen (blood, bubo aspirates and sputum) to confirmed identification. Thus, this lengthy timeframe is somewhat at odds with the rapid and fulminant course of the disease. Nevertheless, the classical phage lysis assay has been used as a "mainstay" by the CDC for over 40 years. The reason for its longevity may be due to the relatively low number of plague infections per year in the US, and thus there has not been a push for new and improved diagnostics. This impetus has changed over the past 10 years following the anthrax letters of 2001 and federal support for bioterrorism preparedness.

In order to expedite the detection process, a genetically engineered "bioluminescent" reporter phage-based on the CDC plague diagnostic phage ϕA1122 was developed.[93,94] This reporter methodology is based on integrating the genes encoding the bacterial *Vibrio harveyi* luciferase (encoded by *luxA* and *luxB*) into the phage genome to create a "*luxAB*-tagged" phage. In the absence of the host, the reporter phage by itself, is not able to express the *luxAB* reporter genes. If viable *Y. pestis* is present the reporter phage infects the cell, uses the host's transcriptional and translational machinery and produces the luciferase enzyme; following the addition of an aldehyde substrate such as n-decanal the luciferase enzyme, in the presence of oxygen and a flavin mononucleotide, catalyzes a complex reaction of which one of the products is "light" (**Fig. 1**). For the *Y. pestis* reporter phage, the *luxAB* genes were targeted for integration by homologous recombination into a non-coding region of the ϕA1122 genome downstream of the A1, A2, A3 promoters and upstream of gene *0.3*. In doing so, 6 bp of phage DNA was replaced with 2,117 bp of reporter DNA and thereby placed *luxAB* expression under A1-A3 transcriptional control. This strategy was chosen for several reasons: (1) insertion of reporter DNA (~2 kb) into the phage genome was not expected to compromise phage packaging since the homologous T7 capsid is able to accommodate a similarly sized genome; (2) the use of endogenous phage promoters (as opposed to the introduction of a heterologous promoter) was less likely to disrupt/interfere with phage function, and (3) in *E. coli*, the homologous T7 A1, A2 and A3 promoters are among the strongest unregulated promoters known and are strongly expressed during the early stages of phage infection.[95,96] Phage-infection of the target cells and soft-agar overlays may be used to identify "bioluminescing" plaques using a standard camera based gel documentation system with a drop of n-decanal on the surface of the lid. For the recombinant ϕA1122::*luxAB* reporter phage, bioluminescence at the plaque-cell periphery was readily detected using this technique.

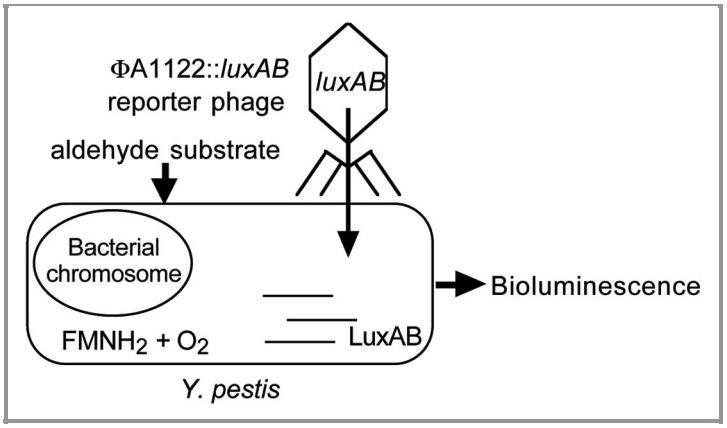

Figure 1. The bacterial *luxAB* reporter genes were integrated into the CDC plague diagnostic phage to create the ϕA1122::*luxAB* reporter phage. In the presence of *Y. pestis*, the reporter phage specifically infects the cell and then uses the host transcriptional and translational machinery to express the luciferase enzyme (LuxAB). In the presence of the reduced flavin mononucleotide ($FMNH_2$), oxygen and exogenously added aldehyde, luciferase catalyzes a complex reaction of which one of the products is light (maximum emission at ~490 nm). The light signal can be detected by a variety of photomultiplier tube and charge-coupled device instruments.

One of the major advantages of luciferase reporter phage technology is that there is no inherent background associated with bioluminescence, and thus, it offers the potential for direct detection in complex clinical samples without the need for isolation of pure bacterial cultures. However, the method does not permit absolute quantification of the signal, and hence number of target bacteria. In addition, the bioluminescent signal response may be transient due to the rapid depletion of the co-factor flavin mononucleotide within the cell.[97] Consequently, "flash" bioluminescence must be measured soon after the addition of the aldehyde substrate. The ability of the ϕA1122::*luxAB* reporter phage to transduce a bioluminescent signal response to *Y. pestis* A1122 has been evaluated. In growth media, the reporter phage detected ~700 CFU/mL within 40 min (Schofield et al., unpublished results). Since viable, metabolically active cells are required for phage infection and reporter expression, it is important to perform assays in growth media and at temperatures conducive for optimal growth. Different forms of the plague can be caused by *Y. pestis* through direct contact with the skin, inhalation, digestion or a flea or rodent bite.[98] The most common clinical samples taken from plague suspected patients for culture identification are blood samples (pneumonic, bubonic and septicemic plague), sputum/oral samples (pneumonic and pharyngeal plague) and bubo aspirates (bubonic plague). The ability of the detection system to function directly with human serum that was spiked with cultured *Y. pestis* cells was assessed. Following a 60 min outgrowth period, reporter phages were subsequently added and the samples measured for bioluminescence 60 min later. Although serum partially quenched the bioluminescent signal, a limit of detection (LOD) of ~900 CFU/mL was observed (Schofield et al., unpublished results). If the reporter phage can function similarly in whole blood or sputum samples, this may circumvent the need for culture isolation and enrichment which is time consuming and can delay definitive identification. This is particularly important for *Y. pestis* because this species grows very slowly in comparison to other Enterobacteriaceae.[66,99]

Y. pestis isolates are not typically drug resistant; however, a streptomycin-resistant strain, and a multidrug resistant (MDR) strain were isolated from bubonic plague patients in Madagascar.[68,100] The MDR strain was resistant to antibiotics (e.g., streptomycin, tetracycline and chloramphenicol) that are frequently used in therapy or prophylactic antibiotic regimes for the plague. Since the phage infection process and signal generation is strictly dependent on the host, the ability of the *Y. pestis* reporter phage to determine an antibiotic susceptibility profile was determined and compared with the standard Clinical Laboratories Standard Institute (CLSI) microdilution method. In the presence of non-inhibitory or inhibitory antibiotic concentrations (chloramphenicol, tetracycline and streptomycin), the bacterial growth profile and phage-mediated bioluminescent signal response, as mediated by the "fitness" of the host, were similar (Schofield et al., unpublished results). However, the reporter phage generated susceptibility data within 2–3 h compared with the standard CLSI method, which requires up to 48 h.[101] Since plague is an infectious disease, and is usually fatal if not treated within the first 24–48 h after symptom onset, reporter phage that can rapidly diagnose and simultaneously gather antibiotic susceptibility information, should improve patient prognosis.

Detection of amplified phage DNA. As an alternative to directly detecting the presence of *Y. pestis* DNA, Sergueev et al.[89] developed a real-time PCR methodology for the detection of amplified phage DNA. This method relies on the premise that if lytic *Y. pestis* specific phage are added to a sample containing *Y. pestis*, following infection and phage amplification, the amount of phage DNA after a given time point will be significantly greater than the original amount of input phage DNA. As the burst size of ϕA1122 and L-413C are 57 and 115 plaque forming units, and the lengths of the lytic cycles are 30 and 90 min, respectively, then there will be a significant increase in phage DNA after a relatively short time period. Using ϕA1122, ~10^3 CFU/mL was detectable within 4 h from cells cultured in growth media, or ~10^6 CFU/mL in 5 h from mock-infected blood (**Table 1**). L-413C mediated detection was less sensitive but afforded greater specificity as this phage is strictly specific for *Y. pestis* within the Yersinia genus. The advantages of this indirect PCR technique compared with conventional PCR are that (1) it will only detect viable or metabolically active cells which is important in environmental applications; (2) it does not require the extraction of purified DNA, and (3) simplex real-time PCR applications that target a single plasmid or chromosomal gene may provide false-negative results due to 'plasmid-less' strains or deletion mutants. The limitation of this technique is that an increase in phages must be evident over the amount of input phages; this may be problematic at low bacterial concentrations when it is vital to a have a high amount of input phages in order to increase the likelihood of a phage-cell interaction.

***Bacillus anthracis*.** *B. anthracis* is a Gram-positive, spore-forming, pathogen of animals and humans that can exist in two states; either as a vegetative cell or as a spore. The vegetative cell, which is the replicative form, survives poorly outside the host. In contrast, spores, which are formed under starvation conditions and are the infectious form, can survive in a dormant state for years. Spores germinate under conditions rich in amino acids and nutrients[102] such as in blood and tissues. Once the spores have germinated, replicating bacilli release three toxin components that combine to form the lethal and edema toxin (encoded by genes on the pX01 plasmid).[103] In addition to toxins, bacilli have a capsule (encoded by genes on the pX02 plasmid), which is induced during infection and inhibits phagocytosis.[104] In combination, these virulence factors enable the bacilli to multiply and cause cell damage which can result in edema, necrosis, and septicemia, and if untreated, organ failure and death.[105,106]

"Naturally occurring" anthrax, which is caused by inhalation, cutaneous, or gastrointestinal (GI) exposure to *B. anthracis*, is extremely rare in the US. For example, there have been very few (approximately 20) cases of inhalation anthrax in the US in the 20th Century, of which most occurred in specialized risks groups such as goat hair mill or wool mill employees, or by accidental contamination of laboratory personnel.[107] Similarly, only isolated cases of GI anthrax, caused by the ingestion of *B. anthracis* contaminated meat, have been reported in the US.[108,109] However,

in the autumn of 2001, bioterrorists released *B. anthracis* spores via the US postal system which caused 18 confirmed cases of cutaneous and inhalation anthrax.[110] Although the number of cases was relatively small, mortality rates associated with inhalation anthrax was 45% and all patients exhibiting symptoms of toxemia died, despite receiving appropriate antimicrobial therapy.[110]

Rapid and early diagnosis of anthrax is critical for a positive prognosis because the onset of disease is fairly rapid and inhalational anthrax is usually always fatal if not treated within the first 24 h following symptom onset.[66] Diagnosis is difficult as both GI and inhalational anthrax presents non-specific symptoms.[66,107,111] *B. anthracis* is a large rod (1–1.5 × 3–10 μm) and is presumptively identified by microbiological and morphological methods. The organism is sensitive to penicillin, non-motile (which is an unusual characteristic among other Bacillus species), selectively grows on PLET agar, and is not β-hemolytic on sheep- or horse-blood agar plates. Capsule formation is verified by staining clinical specimens (e.g., blood smears and cerebrospinal fluid [CSF]) with India Ink and visualization by light microscopy; surface capsule on the vegetative cells occludes the ink particles resulting in the appearance of a clear zone surrounding the vegetative cells.[112,113] M'Fadyean stain and the direct fluorescence assay (DFA) for capsular antigen may also be used for the detection of encapsulated Bacilli. Spores will be absent from clinical samples unless exposed to atmospheric levels of carbon dioxide. Molecular assays such as the JBAIDS anthrax detection kit and instrument is a PCR-based system and is FDA cleared for testing blood and culture samples for presumptive *B. anthracis* identification. The LOD of the PCR assay in mock-infected whole blood was 1,000 CFU/mL. Bacteremia in anthrax-infected rhesus monkeys at levels of 10–10^5 CFU/mL are evident during the course of the disease, and rise to as high as 10^9 CFU/mL in moribund animals.[114,115] Therefore, the LOD of JBAIDS should be within the sensitivity range for disease diagnosis.

FDA-approved gamma (γ) phage assay. The **γ** phage assay gained FDA approval in 2005 for identifying *B. anthracis*, and remains the only lysis assay to have achieved this status. Originally developed by the CDC in the mid-1950s, John Ezzell and colleagues at USAMRIID further refined the assay for use with clinical isolates. The Laboratory Response Network (LRN) currently employs the **γ** phage assay concomitantly with capsule detection for the confirmed identification of *B. anthracis*.

The story behind the isolation and identification of the **γ** phage is interesting. A temperate *B. anthracis* phage called phage W was identified by McCloy in 1951 (subsequently renamed Wβ).[116] Wβ infected all 171 strains of *B. anthracis* analyzed but could not "attack" encapsulated (smooth) forms.[116,117] Capsule formation occurs only during vegetative growth in vivo (during infection) or under specific environmental conditions that mimic the mammalian host[117-120] and is one of the principle virulence factors during infection due to its ability to fight off host defenses by inhibiting phagocytosis.[104] The Wβ phage displayed species specificity to *B. anthracis* since the phage did not lyse 242 out of 244 strains analyzed from 17 different non-*anthracis Bacillus* species.[116] However, two "unusual" *Bacillus cereus* strains that manifest phenotypes of both *B. anthracis* and *B. cereus* were identified which were susceptible to phage infection [NCTC 1651 and NCTC 6222 (ATCC 4342)]. In 1955, Brown and Cherry isolated a lytic variant of Wβ, designated γ;[117] γ was isolated as a Wβ variant by reinfecting a Wβ lysogenic strain with the Wβ phage. Unlike Wβ, γ was able to lyse encapsulated *B. anthracis* strains. Wβ and γ are morphologically identical: they are similar to the Siphoviridae family of tailed phages (double stranded DNA viruses) consisting of an icosahedral head and a long contractile tail.[121] The Wβ and γ (d'Herelle isolate) genomes were recently sequenced (40,867 bp and 37,373 bp, respectively) (GenBank accession numbers DQ289555 and DQ289556, respectively) and were found to encode for 53 open reading frames each.[121] Comparison of Wβ and γ indicated that the γ variant evolved from temperate Wβ by deletions and modification at the lysogenic locus and by key mutations in the tail fiber gene, *wp14*. The major genetic differences between Wβ and γ are: (1) a 25 bp deletion between *wp25* and *wp26* (in the lysogenic locus); (2) a 2,003 bp deletion in *wp28* and *wp29*, which encodes for a C1 repressor homolog (controls lysogenic functions), and (3) 69 point mutations in the tail fiber gene *wp14*. These differences have been attributed to the lysogenic vs. lytic lifestyle, and the ability to infect encapsulated or non-encapsulated strains. A key difference between Wβ and γ, which is unrelated to lifestyle and host range, is that γ has also acquired a 1,360 bp island of which one of the gene products, Gp41, is highly related to a family of fosfomycin resistance proteins. Heterologous expression of Gp41 in a sensitive background led to the acquisition of resistance to fosfomycin (MIC of 500 μg/mL) indicating that γ acquired a fosfomycin resistance module in a likely recombination event with a *B. anthracis* prophage.

Abshire et al.[122] validated the use of γ phage for the identification of *B. anthracis* in terms of phage production, analytical specificity, and repeatability. Clear or primarily clear circular zones of lysis on *B. anthracis* lawns were evident with 49 out of 51 *B. anthracis* strains collected from diverse geographical locations such as Pakistan, Canada, Argentina, England, US and South Africa.[122] Specificity testing against non-*anthracis Bacillus* species proved negative for 48 out of 49 strains analyzed (98% specificity). The 1 non-*anthracis Bacillus* strain (out of 49 tested) which was susceptible was an 'unusual' *B. cereus*. Consequently, due to its species specificity and broad strain susceptibility, the γ phage assay is used as a standard clinical diagnostic tool by the CDC and various public health laboratories for the identification of *B. anthracis* isolates. Further studies have found additional non-*anthracis Bacillus* strains that are γ phage susceptible (3 *B. cereus*, 1 *B. megaterium*, and 1 *B. mycoides* strains).[121,123,124] In addition, Sozhamannan et al.[125] found 10 out of 115 *B. anthracis* strains that were γ resistant. It is interesting to note however that among different labs and stock collections, there are genetically distinct versions of γ including isolates d'Herelle, Louisiana State University, USAMRIID and Cherry.[121,126] These differences may have arisen due to different propagating procedures (e.g., different host strains and temperatures) between the various labs, and the genetic instability that is common to phages. It is not known if these small, but distinct genetic differences translate to

differences in phenotype and host range susceptibilities between the different γ isolates.

Other phage-based *B. anthracis* assays. The γ phage assay is a standard for the confirmed identification of *B. anthracis*. However, the assay requires bacterial isolation and cultivation followed by a 20 h incubation to observe the clear lysis zones.[122] Consequently, in an effort to improve the traditional lysis assay, the unique characteristics of the *B. anthracis* phages are being utilized in a variety of phage-based techniques for the detection of *B. anthracis* (**Table 1**). A real-time PCR system, which relied on the detection of amplified γ phage DNA, enabled the detection of ~10^2 CFU/mL; the 5 h time to detection included 4 h of phage amplification within the bacterial host, followed by 1 h of real-time PCR.[127] In contrast to conventional PCR methodologies which directly detect the presence of bacterial DNA, and is independent on host viability, phage amplification will only occur if metabolically active cells are present. Thus, this method will have specific advantages over conventional PCR, especially for the detection of environmental isolates and in remediation strategies when it is essential to know if the specimen is viable and potentially infectious. The detection of viable *B. anthracis* cells is also mediated using reporter phage technology. A *luxAB*-tagged reporter phage was generated using the *B. anthracis* Wβ temperate phage by replacing genes which were predicted to be non-essential (*wp40* and *wp41*;[121]) with the *luxAB* genes encoding bacterial luciferase.[128] The 'light-tagged' Wβ:: *luxAB* reporter phage was able to detect ~10^3 CFU/mL vegetative cells 80 min after infection of the attenuated *B. anthracis* Sterne strain. Similar results were obtained with fully virulent wild-type strains (Schofield et al., unpublished results). One of the advantages of using a temperate phage (as opposed to a lytic phage) for the reporter phage technology is that the prophage may exist in harmony with the host, and consequently luciferase has the potential to accumulate within the cell (without cell lysis); however, for signal accumulation to occur effectively, it is important to express the reporter from a heterologous promoter. The disadvantage of using Wβ is its inability to infect encapsulated vegetative cells[116] which are found during infection. However, encapsulated *B. anthracis* cells, when incubated with reporter phage under non-encapsulating conditions in vitro (brain heart infusion media with atmospheric levels of carbon dioxide), become phage susceptible rapidly as indicated by the acquisition of a bioluminescent phenotype within 30 min (Schofield et al., unpublished results). Therefore, the potential exists for using the reporter phage to rapidly and directly detect *B. anthracis* in infected clinical specimens (e.g., blood, CSF, stool and sputum).

B. anthracis phage components are also being exploited as a means for detection. The γ PlyG lysin is used by the phage to hydrolyze peptidoglycan components, resulting in cell wall lysis and the concomitant release of progeny phages. When purified PlyG is added externally to the cell, rapid lysis ensues resulting in the release of ATP.[129] If the enzyme luciferase and luciferin are added, an enzymatic reaction occurs resulting in light emission. Because PlyG displays *B. anthracis* specificity, light should be produced only if *B. anthracis* cells are present, and the lysin is able to cause cell lysis resulting in ATP release. Using this method, approximately 100 cells were detected with a hand-held luminometer within 60 min after the addition of PlyG. Alternatively, the binding domain of PlyG has been used as a capture element for *B. anthracis*.[112,130] PlyG is composed of two functional domains; the N-terminal catalytic domain and the C-terminal binding domain which specifically binds to the cell wall of *B. anthracis*. The binding domain of PlyG was fused to glutathione *S*-tranferase to create a recombinant bioprobe capable of binding and detecting *B. anthracis*. When used in combination with the horseradish peroxidase system, approximately 10^3 CFU were detectable within 3 h. Importantly, the probe was able to bind both non-encapsulated and encapsulated cells indicating it may have value for use with clinical specimens.

Staphylococcus Aureus

S. aureus is a Gram-positive, coagulase positive, non-motile, non-sporulating bacterium that has been a prominent cause of human infections throughout history.[131] In the pre-antibiotic era, mortality rates for *S. aureus* bacteremia exceeded 80%.[132] The introduction of penicillin in the 1940s significantly decreased mortality rates but strains rapidly became resistant due to the acquisition of a penicillin-hydrolyzing enzyme, penicillinase.[133] Semi-synthetic antibiotics that were resistant to the actions of this enzyme were introduced into clinical practice in 1959; however, within two years resistant isolates began to appear. These isolates are commonly referred to as methicillin-resistant *S. aureus* (MRSA).[132,134,135]

MRSA is a major cause of both health care- and community-associated infections that are increasingly difficult to treat because of resistance to commonly prescribed antibiotics such as penicillin and oxacillin. Clinical syndromes associated with MRSA disease include skin and soft tissue infections, bacteremia, endocarditis, pneumonia, bone and joint infections and central nervous system infections.[136-139] Studies have demonstrated that delays in *S. aureus* diagnosis and treatment, results in increased hospitalization and mortality; MRSA patients receiving appropriate therapy within 48 h of blood draw exhibit a mortality rate of 6%, compared with 50% for those not receiving the appropriate treatment until after 48 h.[140] This points to a critical need for the development of diagnostic techniques which are not only capable of rapidly identifying, but also rapidly differentiating between MRSA and methicillin-susceptible *S. aureus* (MSSA).

Unfortunately, many laboratories do not have the funding capability and/or the necessary expertise to perform the often time consuming and technically demanding diagnostic assays for detecting MRSA isolates in patient samples. Immunoassays and molecular-based assays for MRSA diagnosis include pulse-field gel electrophoresis, real-time PCR/multiplexing, PVL gene typing, SCC*mec* typing, multilocus sequence typing, and/or *spa* typing. Despite the fact that culture based MRSA techniques are comparatively easy, cheap and sensitive, definitive identification of test results often requires 24–48 h and thus allows for potential MRSA cross-transmission if patients are not preemptively placed under contact precautions.[141,142] Classical methods for determining antibiotic susceptibility (disc diffusion, Etest and or

broth dilution), latex agglutination and screening techniques with solid culture medium containing oxacillin can be insufficiently sensitive and often yield false-positive results which may lead to inappropriate treatment regimes.[143]

MicroPhage. MicroPhage recently released the FDA-cleared KeyPath™ MRSA/MSSA Blood Culture Test (herein after called KeyPath™) as a means of simultaneously identifying *S. aureus* and differentiating MSSA and MRSA. The test is simple to perform, requires no instrumentation, and uses a "dipstick-like" detector for bacterial identification and antibiotic susceptibility testing. The KeyPath™ system is based on a lateral flow immunoassay that utilizes phage amplification and antibody capture technology to detect *S. aureus* (**Fig. 2**). Designed to work in conjunction with BD BACTEC™ blood culture bottles (Plus Aerobic/F and Plus Anaerobic/F), the KeyPath™ test must be performed ≤ 24 h after a positive blood culture alarm; for *S. aureus*, this typically occurs within 12–16 h (~5 × 10^7 CFU/mL). Small volumes of the positive blood cultures (10 µL) are added directly to two test bottles labeled ID and RS. The ID and RS test bottles contain a proprietary phage cocktail diluted in a growth medium that is designed to sustain the growth of *S. aureus*; 1.4 × 10^8 PFU/mL is required to ensure a 95% probability of *S. aureus* detection by phage. In addition, the RS test bottle also contains cefoxitin, a bactericidal antibiotic, which is active against *S. aureus* isolates including penicillinase-producing strains. MSSA strains are unable to grow in the presence of cefoxitin and therefore cannot support phage amplification. Phage amplification is determined by dispensing the contents of the ID and RS bottles into two lateral flow chambers that contain anti-phage antibodies. A positive reaction in the ID chamber indicates the presence of *S. aureus*, while a positive reaction in the RS chamber indicates MRSA, while the absence of a reaction in the RS chamber represents MSSA.[144,146,147] The KeyPath™ test was able to differentiate between MRSA and MSSA within 5.5 h[148,149] and could reliably detect *S. aureus* in a positive blood culture, even when diluted up to 100-fold.[150]

The KeyPath™ test was compared against currently FDA-cleared rapid *S. aureus*/MRSA detection kits (501K clearance number K102342). Six tests were included in the analysis: (1) the BD GeneOhm StaphSR assay; (2) the Coagulase tube test; (3) the catalase slide test; (4) the Remel Staphaurex assay; (5) the Oxoid PBP2' latex agglutination test, and (6) the BD BBL cefoxitin Sensi-disc. The KeyPath™ test was determined to be substantially equivalent to all currently marketed "gold standard" detection kits and was granted FDA clearance in May 2011. Clinical sensitivity and specificity for detection of *S. aureus* was 91.8% and 98.3%, respectively. Accuracy of methicillin resistance and susceptibility determination was 99%, illustrating the ability of the KeyPath™ test to correctly detect and identify a wide range of *S. aureus* strains.[149] The limitation of KeyPath™ technology, as with other currently available clinical diagnostics, is that it requires a positive BD BACTEC™ blood culture before MSSA/MRSA testing can proceed. However, the advantage of KeyPath™ is that the test does not require specialized instrumentation or technically trained personnel to obtain results. The assay is designed as a practical, low cost screening procedure for patients arriving at care facilities

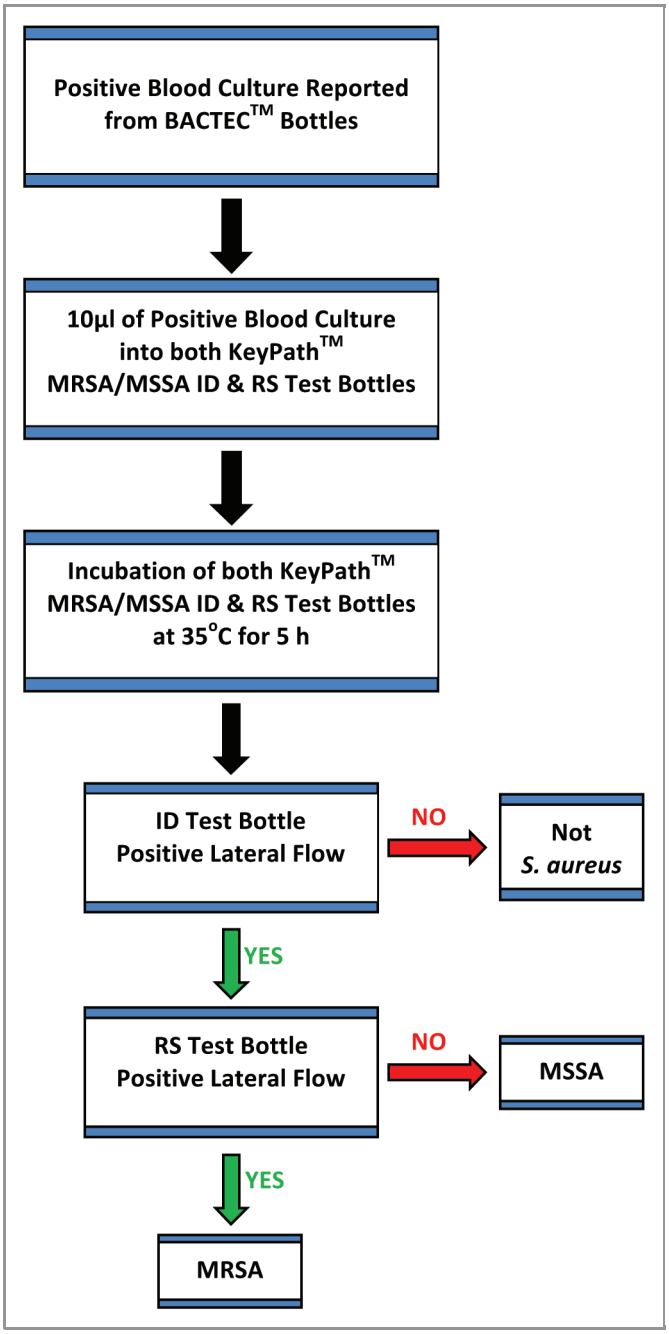

Figure 2. Blood drawn from a patient suspected of MRSA infection/carriage is incubated in BD BACTEC™ blood culture bottles (Plus Aerobic/F and Plus Anaerobic/F). If cultures are positive for infection, 10 µl of the positive culture is added to each KeyPath™ test bottle (ID and RS) and incubated at 35°C for 5 h. Both test bottles contain MicroPhage's proprietary phage cocktail and culture broth; however, RS also contains cefoxitin; a methicillin analog which inhibits bacteriophage amplification in MSSA, but allows amplification to continue if the organism is resistant to methicillin (MRSA). This 5 h incubation period allows phage amplification to proceed only if the target species is present. Six drops of each incubated inoculum is dispensed into the correspondingly labeled lateral flow chamber. Phage amplification is detected via visualization of anti-phage antibodies conjugated to colloidal gold particles present in the lateral flow device. A positive result in ID chamber represents detection of a *S. aureus* strain, positive result in RS chamber represents MRSA; no visualization in the RS chamber represents MSSA. Adapted from references 144 and 145.

for treatment.[148] MicroPhage has also recently developed a nasal screening test, and is currently developing a skin and soft tissue infection test to identify *S. aureus* and differentiate MRSA/MSSA isolates. Future products include the detection of vancomycin and clindamycin resistance/susceptibility and *MicroPh*AST, a Gram-positive blood culture panel test for the detection and antibiotic susceptibility profiles of *S. aureus*, streptococcal and enterococcal species.

Other phage derived detectors. Balasubramanian et al.[151] developed a highly specific, selective, and label-free detection method for *S. aureus* using the lytic phage 12600. In this system, phage 12600 functions as a bio-recognition element when immobilized onto the surface of a plasmon resonance-based SPREETA™ sensor. The detection platform avoids the need for complex and expensive surface chemistry or phage modification because the phage is attached to the sensor by direct physical absorption. *S. aureus* cells, which are pumped across the Spectra channel, adhere and become immobilized; the subsequent phage-cell interaction results in a refractive index change, which is detected by surface plasmon resonance (SPR). The systems moderate limit of detection (10^4 CFU/mL) is thought to be due to blocking of phage receptors via: (1) phage-to-phage interactions and, (2) non-favorable orientation of the phages; ideally the phage head should be immobilized while cell recognition receptors, located in the tail of tailed-phages, must face outward.[15] Further experiments are underway to improve sensitivity using a phage amplification assay and to test the biosensor in "real-life" samples.

Pierce et al.[152] recently developed a phage amplification detection (PAD) method that uses a "heavy"-labeled ^{15}N phage to specifically infect host bacteria and produce a mass spectrometric signal that is indicative of phage amplification. In this proof-of-concept study, an input preparation of ^{15}N isotopically-labeled Staphylococcal phage (phage 53) was incubated in the presence of ^{14}N-labeled *S. aureus* and the resulting output ^{14}N progeny phages were detected by monitoring a phage biomarker protein via matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). At 90 min post-infection a signal indicative of progeny phages (^{14}N-labeled phage capsid protein) was clearly visible within the MALDI-TOF mass spectra, providing a positive identification for *S. aureus*. The detection limit of the isotopically labeled PAD MS-based method was established at 6.7×10^6 CFU/mL at 2 h and 6.7×10^5 CFU/mL at 5 h.

Guntupalli et al.[153] reported a real-time optical detection method to identify MRSA using lytic phages (12600 and ATCC27690-B1) specific for MRSA 2 and 5 strains of *S. aureus.* Combining two complementary technologies (phage for recognition of *S. aureus* and monoclonal antibody for recognition of PBP2a), substrate bound phage monolayers successfully enabled the visualization of MRSA. The development of this bioselective-probe provides a labeling-free diagnostic tool with which to quantify cell-phage interactions in real time. This technique could be of value for clinical diagnostics in situations where time is of the essence, technically trained personnel is limited or funding is restricted.

Other Bacterial Targets

While no other phage products appear to be currently used in clinical diagnostic laboratories, wild-type phages, genetically engineered phages, and phage components are being evaluated as potential bacterial detectors in research and development laboratories. Most of this research is directed toward the detection of food-borne pathogens in various food matrices.[15,17] Although the complexity of food matrices is entirely different to clinical specimens, there is no reason why these same technologies cannot be adapted to the clinical arena. Different versions of reporter phages based on luciferase (bacterial and firefly), green fluorescent protein (GFP), β-galactosidase, and ice nucleation protein have been developed for a wide variety of pathogens such as *L. monocytogenes*,[97,154] Salmonella species,[155-158] and *E. coli* O157:H7.[159,160] For the latter pathogen, the authors developed a bioreporter quorum sensing system in which the *E. coli* phage was engineered with the gene encoding acyl homoserine lactone (AHL) as opposed to luciferase. Upon infection of the target *E. coli*, AHL is produced and diffuses out of the cell. In the presence of a bioreporter harboring the *luxRCDABE* gene cassette, AHL interacts with the LuxR protein which stimulates transcription of *luxRCDABE*, resulting in bioluminescence, without the need for exogenously added aldehyde substrate. Other types of detection schemes based upon the binding of fluorescently labeled phages have been developed for *E. coli* O157:H7 and Salmonella by directly labeling phage DNA with fluorescent binding dyes (e.g., YOYO-1, DAPI and SYBR gold fluorescent dyes) or expression of a phage capsid protein-GFP fusion construct.[161-163] Binding of the fluorescently labeled phage to specific receptors on the cell wall can be detected and measured using fluorescence microscopy, flow cytometry or fluorescent plate readers.

Because of the natural specificity of phages for their hosts, phage or phage components are also being used as capture elements, thus eliminating the need of producing costly antibodies or developing synthetic recognition molecules. Phages O1, P1, and T4 have been used as the recognition agents in an ELISA plate assay for the detection of *S. enterica*, and *E. coli* with a sensitivity of 10^6 CFU/mL.[164] Genetically engineered tailspike proteins derived from P22, immobilized onto gold-coated surfaces via cysteine tags at their N or C termini, were able to effectively function as molecular capture probes.[165] Using surface plasmon resonance, the sensitivity of real-time detection of *S. enterica* serovar Typhimurium was 10^3 CFU/mL.

Phage Diagnostics: Issues and Considerations

Although phage typing is a mainstay for species and strain identification in bacterial reference laboratories, there are surprisingly few phage diagnostic devices in the clinical arena that are commercially available, that have gained FDA-approval, or are used to clinically diagnose the cause of infection. Bacterial culture isolation and traditional microbiological methods remain the standard for species identification and confirmation. Thus, there continues to be a niche for the classical phage lysis assay in this

regard. However, as new modern molecular and immunological techniques have reduced identification times from days to hours, there is a greater emphasis to be able to directly detect the pathogen from clinical specimens, without the need to isolate pure bacterial cultures. In order to address these needs, different phage-based assays such as phage amplification and genetically engineered reporter phages are being investigated. However, one of the inherent issues of using clinical specimens such as blood for example, is that even though patients may be symptomatic of disease, the bacterial load in the specimen may be below an assays sensitivity limit of detection (e.g., $< 10^2$ CFU/mL). Most phage-based assays, irrespective of the detection method or reporter (PCR, luciferase, immunoassays amplification, GFP, β-galactosidase) require a period of bacterial outgrowth and amplification for the bacteria to reach a minimal threshold to enable detection. This threshold detection level is not because of an inability to detect a single bioluminescing or fluorescing cell, or a single gene copy. It is simply that at low bacterial concentrations, the likelihood of the phage randomly interacting with, and subsequently infecting a cell in any given space is significantly diminished. This bacterial threshold limitation has been described in depth and put into context by Hagens and Loessner[166] and Abedon.[167] To quote the former authors: "it will take on the order of 1,000 years for 1 phage and 1 bacterium to meet within 1 mL of fluid." Although the number of input phages can be maximized in order to increase the probability of a phage/bacterium hit, the initial bacterial concentration is a major controlling factor governing the likelihood of a positive response.

The type and complexity of the clinical specimen must also be taken into account, as for each specimen used (e.g., blood, sputum, fecal and urine), inhibitory factors are present which may interfere with phage infection, stability, and signal detection. Blood components such as hemoglobin, lactoferrin, and immunoglobulin G are PCR inhibitors[168,169] and may inhibit detection methods based on the PCR detection of amplified phage DNA. Hemoglobin also quenches bioluminescence and to a lesser extent, fluorescence based signals[170] which has important implications for reporter phage assays. The standard practice for blood cultures is dilution in complex media, followed by a period of bacterial growth and amplification. Thus, these inhibitory factors may be diluted and their inhibitory effects partially mitigated. Nevertheless, PCR detection of amplified phage DNA can be approximately 1000-fold less sensitive in spiked blood cultures compared with assays performed in the absence of blood components.[89] The complexity of sputum samples also present unique challenges due to: (1) high molecular weight glycoproteins which bind to the target and prevent phage infection; (2) a commensal bacterial and fungal population which contaminates downstream applications, and sequester the phage; (3) proteins from lysed cells which bind to the phage, and (4) proteases which degrade the phage. In order to reduce commensal bacteria, sputum samples may be treated with antibacterial agents that inhibit bacterial and fungal contaminants. For example, incubation with broad spectrum antimicrobial agents such as nystatin, oxacillin and aztreonam or 1% NaOH have been used for processing sputum samples for *M. tuberculosis* identification;[29,61] however, a fine balance exists between inhibiting bacterial/fungal contaminants and maintaining the presence of viable target bacteria. Sputum samples may also be processed with anti-mucolytic agents such as *N*-acetyl-L-cysteine in order to reduce the complexity of the clinical matrix and aid downstream processing.

The value of a phage diagnostic is largely dependent on the ability of the phage to specifically target the host species, and the ability to infect as many strains as possible within that species. While phage cocktails may be used to overcome strains that are phage resistant and ensure a wider coverage within a given species, it comes at the expense of species specificity with increased likelihood of false positives. In contrast to phage therapy, development of phage-resistant bacteria is not a huge concern; the bacterial specimens will be tested for the presence of the target species, and then destroyed. Thus, there is no continuous selective pressure to which the bacteria are exposed. Interestingly, attempts to generate spontaneous *Y. pestis* mutants resistant to the CDC plague diagnostic phage ϕA1122 were unsuccessful.[171] The ϕA1122 receptor was mapped to the lipopolysaccharide and deliberately engineered strains lacking the receptor were avirulent in mouse models of the plague indicating the importance of the LPS for infection. However, one of the problems that may arise is that because phage are genetically unstable, the phage may evolve during continuous passaging leading to small but distinct changes in the host range. For example, Fouts et al.[126] upon sequencing and analysis of the *B. anthracis* diagnostic γ phage from different stocks from Louisiana State University, the USAMRIID, or the d'Herelle collection (Université Laval), found sequence heterogeneity at three different loci. This provides an indication of how γ phage evolved when passaged and maintained under different conditions at different laboratories.

One of the considerations of using reporter phages (or phage components for detection) is that it is possible for the host range of the wild-type phage and the modified phage to be different. This is because wild-type phage specificity is determined by the ability of the phage to "attack" the host which encompasses phage infection, phage replication, and cell lysis; the presence of plaques or "host clearing" being indicative of host-susceptibility. In contrast, host susceptibility to reporter phage, for example, is measured by a signal response which requires fewer steps i.e., phage infection and reporter expression (not multiplication and lysis). Therefore, the sequence of events that is required to generate a signal, are not analogous to those that mediate cell lysis. In addition, there may be host inhibitory factors which can prevent phage replication and lysis, but may not inhibit reporter expression, such as the presence of a prophage in the cell, DNA restriction-modification, and lysis resistant mutants.[172] Thus, it is possible that the reporter phage can have a detection range that is wider that the parental phage cell lysis host range.[158,173] This can be a significant impediment for developing reporter phages as it takes considerable time and effort to genetically engineer and perform the subsequent host range studies.

Concluding Remarks

Phages are the most abundant entities on earth with estimates ranging from 10^{30} to 10^{32}.[174] Within this vast population, exists phage that are uniquely specific for a particular strain, and others that are more promiscuous between species. In order to survive and propagate for millions of years, they have adapted and evolved to be able withstand environmental harsh conditions, and be able to infect and target its host in complex conditions. If you consider for arguments sake, that each bacterial cell can release 100 progeny phages, a 100 L fermenter harboring 10^9 CFU/mL of host bacteria, can produce 10^{16} PFU; assuming 10^9 PFU is required per diagnostic test, this equates to 10^7 separate tests from a small scale fermentation run. These attributes in terms of wide phage selection, host specificity, robustness and extremely low reagent cost, seem to make them ideal candidates for which to be exploited as bacterial detectors. It is therefore perplexing that despite extensive research, there has been minimal impact of phage diagnostics neither in the clinical field, nor for that matter in agricultural, environmental and food applications. As research efforts move into optimizing the transition from culture based detection to detection within complex matrices, it is likely that phage-based diagnostics will mature and start to fulfill their enormous potential.

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