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Simultaneous Identification and Susceptibility Determination to Multiple Antibiotics of *Staphylococcus aureus* by Bacteriophage Amplification Detection Combined with Mass Spectrometry

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Abstract

The continued advance of antibiotic resistance in clinically relevant bacterial strains necessitates the development and refinement of assays that can rapidly and cost-effectively identify bacteria and determine their susceptibility to a panel of antibiotics. A methodology is described herein that exploits the specificity and physiology of the Staphylococci bacteriophage K to identify *Staphylococcus aureus* (*S. aureus*) and determine its susceptibility to clindamycin and cefoxitin. The method uses liquid chromatography–mass spectrometry to monitor the replication of bacteriophage after it is used to infect samples thought to contain *S. aureus*. Amplification of bacteriophage K indicates the sample contains *S. aureus*, for it is only in the presence of a suitable host that bacteriophage K can amplify. If bacteriophage amplification is detected in samples containing the antibiotics clindamycin or cefoxitin, the sample is deemed to be resistant to these antibiotics, respectively, for bacteriophage can only amplify in a viable host. Thus, with a single work flow, *S. aureus* can be detected in an unknown sample and susceptibility to clindamycin and cefoxitin can be ascertained. This Article discusses implications for the use of bacteriophage amplification in the clinical laboratory.

Graphical Abstract

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Notes

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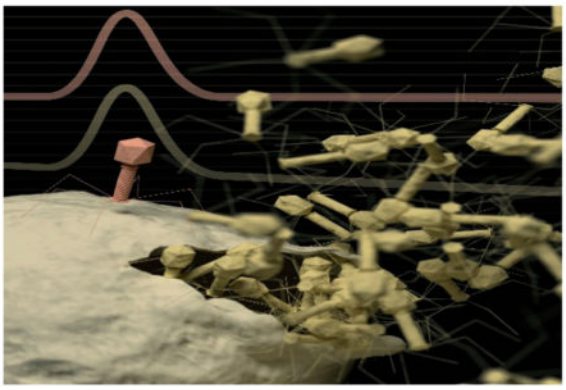
The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

List of proteins identified from the bacteriophage K preparations, signal to noise rations of serial dilutions of bacteriophage K, PAD signal to noise rations for serial dilutions of BAA-1720, calibration curves for *S aureus*, and PAD in the presence of cefoxitin. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00959.



Shortly following the introduction of antibiotics to combat *Staphylococci* infections, evidence arose showing the emergence of multiple *Staphylococcus* strains resistant to the prescribed antibiotics.[1] Today, antibiotic resistance among both Gram-positive bacteria, such as *Staphylococcus aureus*,[2] and Gram-negative bacteria, such as extended-spectrum beta-lactamase *Escherichia coli* and *Klebsiella sp*,[3] is endemic and problematic in nosocomial and community settings. Further, the economic burden of antibiotic resistant bacteria is substantial,[4,5] and the specter of increasing numbers of resistant strains and the lack of adequate antibiotics to ameliorate the impact of these strains is of profound concern.[6] Implicit in the proper treatment regimen of bacterial infections is the rapid and accurate ascertainment of which bacteria are present and a determination of their susceptibility to specific antibiotics.[7]

Bacteriophage amplification detection (PAD) is an emerging technique for bacterial identification. Using PAD, the presence of a specific, viable bacterium is determined by detecting the replication of a selective bacteriophage that has purposely been introduced into the sample.[8,9] The well-documented specificity of a bacteriophage for its host is exploited in PAD, wherein a phage can only replicate in the presence of a suitable host. By manipulating the concentration of the introduced bacteriophage below the detection limit of the chosen detector, or by marking the phage so that introduced bacteriophage can be distinguished by the detector from newly amplified bacteriophage, an increased concentration of bacteriophage in a sample can be readily monitored. Detectors that can specifically distinguish structural components of the bacteriophage, such as mass spectrometry or immuno-diagnostics, allow the implication of the presence of a bacterium in a sample without the need to utilize bacterial colony isolation techniques prior to analysis, a time-consuming process.[10]

Though discussed in a limited fashion in the scientific literature, PAD can also be used to ascertain antibiotic resistance of a bacterial strain in addition to establishing identity.[11,12] The general PAD methodology to determine identification and antibiotic susceptibility of a bacterial strain is to split a sample in two and add bacteriophage to Fraction A while adding antibiotic and bacteriophage to Fraction B. Using this design, if Fraction A shows phage amplification, that implies the presence of the bacterium for which the phage is specific. Subsequently, if Fraction B shows no phage amplification, then the bacterium is susceptible

to the antibiotic (at that concentration), because phage amplification can only occur in viable strains of bacteria. If Fraction B shows amplification of phage, then the bacterial strain is resistant to the derived concentration of antibiotic.

The methods most widely used in clinical microbiology laboratories across the world to identify bacteria and determine antibiotic resistance are based upon culture, where time to report results ranges from 24 to 48 h for rapidly growing bacteria. Routine culture methodologies include isolation and subculture of a strain, incubation, assignment of bacterial identification, and antibiotic susceptibility testing. The recent commercial introduction of MALDI-MS-based instrumentation holds promise to improve time-to-results for bacterial identification,[13,14] and several workflows have been proposed for detecting drug-resistant strains.[15–17] PCR assays are available for rapid antibiotic resistance determination for MRSA/MSSA detection, but these tests are relatively expensive, and cannot distinguish between live and dead bacteria.[18] Further, not all strains of MRSA contain the mecA gene at which PCR probes are targeted.[19,20]

For this study, we use liquid chromatography coupled with multiple reaction monitoring mass spectrometry (LC-MS/MRM) to monitor the progress of PAD experiments designed to detect *S. aureus*. The bacteriophage utilized in this study is *Staphylococcus* phage K, a well-characterized bacteriophage with a wide virulence against *S. aureus* strains and a known genome.[21] PAD experiments were conducted without antibiotic to determine identification of *S. aureus*. Additionally, PAD was implemented in the presence of cefoxitin, a commonly used small molecule for testing susceptibility to beta lactam antibiotics, and clindamycin, a protein synthesis inhibitor of the lincosamide class used to treat MRSA in skin and soft tissue infections.[22] We report conditions necessary for successful determination of antibiotic susceptibility, as well as issues regarding expansion of the methodology for use in clinical laboratories.

MATERIALS AND METHODS

Phage Propagation

All experiments using bacteriophage and bacterial strains were conducted in a biosafety level 2 laboratory. We purchased bacteriophage K and its host (ATCC-19685) from the American Type Culture Collection (ATCC) (Manassas, VA). Bacteriophage K was propagated by combining 1 mL of host grown to density with 10^4 pfu/mL of bacteriophage and spreading the milieu on top of a TSB agar plate. After overnight incubation, tryptic soy broth (TSB) was poured on the agar plate, the surface agitated with a disposable plastic spreading loop, and the contents aspirated with a pipet and placed in a centrifuge tube. Following centrifugation to remove debris, phage preparations were filtered through a 0.2 μm filter and refrigerated until further use. ^{15}N phage were propagated in like manner, substituting ^{15}N enriched growth media (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) for TSB in all broths and agar plates. To ensure complete incorporation of ^{15}N into the protein structure of bacteriophages, propagation bacteria were subcultured twice in ^{15}N growth media prior to infection with bacteriophage. Concentrations of bacteriophage stocks were determined by traditional plaque assay.

Bacteria Strains and Antibiotic Susceptibility Determination

The bacterial strains ATCC-12598 (*S. aureus*), ATCC-25923 (*S. aureus*), ATCC-29213 (*S. aureus*), ATCC-43300 (*S. aureus*), BAA-1707 (*S. aureus*), BAA-1720 (*S. aureus*), ATCC-35547 (*S. epidermidis*), and ATCC-1706 (*Klebsiella pneumoniae*) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). For all phage amplification experiments described in this paper, bacterial strains were grown overnight in TSB broth, with estimates of concentration determined as described by Pierce et al.[9] Clindamycin and cefoxitin salts were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in deionized water. Antibiotic susceptibility of bacterial strains was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines using guiding document M100-S25. Briefly, colonies of each bacterial strain were suspended in cation-adjusted Mueller-Hinton broth equal to a 0.5 McFarland standard. To aliquots of each suspension were added either cefoxitin or clindamycin at concentrations of 0, 0.5, 1, 2, 4, 8, 16, or 32 μg/mL in a 96-well plate. After incubation for 20–24 h, growth of bacteria in each respective well was determined. Antibiotic resistance was determined if growth was detected in wells with ≥4 μg/mL of cefoxitin relative to quality control strains or ≥2 μg/mL of clindamycin. Table 1 summarizes the bacterial strains used in this experiment and the results of antibiotic resistance determination.

PAD Experimentation

"Parent" phage are defined as bacteriophage that are introduced into a sample at the beginning of a PAD experiment to initiate the phage infection, and "progeny" phage are defined as all bacteriophage generated during the course of the infection by the parent phage. All phage amplification experiments were conducted using ^{15}N-labeled bacteriophage as the parent phage to differentiate signal derived from the parent from the newly generated progeny phage containing ^{14}N-labeled proteins. PAD experiments were carried out in TSB by inoculating bacteria grown overnight into a sample containing parent phage with or without cefoxitin or clindamycin. To identify *S. aureus*, PAD experiments were set up using 1×10^7 cfu/mL of bacteria and 5×10^8 pfu/mL of ^{15}N labeled bacteriophage, and allowed to incubate for 5 h at 37 °C. For clindamycin-resistance determination, experiments were conducted using 1×10^7 cfu/mL bacteria and 5×10^8 pfu/mL ^{15}N labeled bacteriophage combined with 2 μg/mL clindamycin, and allowed to incubate for 5 h at 37 °C. For cefoxitin resistance determination, 1×10^7 cfu/mL of each bacterial strain was incubated in 4 μg/mL cefoxitin for 2.5 h, followed by addition of 5×10^8 pfu/mL ^{15}N-labeled bacteriophage and incubation for an additional 2.5 h at 37 °C. To illustrate the capability of PAD to detect *S. aureus* and determine antibiotic resistance when cocultured with another bacterium, *S. epidermidis* ATCC-35547 and *K. pneumoniae* BAA-1706 were added at equal concentration (1×10^7 pfu/mL) to cultures of cefoxitin-sensitive *S. aureus* ATCC-12598 and cefoxitin-resistant *S. aureus* BAA-1720 prior to phage amplification as described above.

Trypsin Digest

Following amplification, samples were prepared for MRM analysis by pelleting out large debris from each sample vial via centrifugation, and aliquoting 500 μL of the supernatant

into a 100 kDa molecular weight cutoff filter (Amicon Ultra; Millipore, Billerica, MA). Each sample was filtered by centrifugation for 10 min at 14 000 rpm and washed twice with 500 μL of 50 mM ammonium bicarbonate centrifuged through the same filter (10 min at 14 000 rpm). Subsequently, 10 μL of filter retentate was recovered, combined with 10 μL of 0.1% Rapigest (Waters, Inc., Bedford, MA), and boiled at 100 °C for 10 min. Upon cooling, 10 μL of sequence grade trypsin (Promega, Madison, WI) at 0.4 μg/μL was added to the sample and enzymatically digest at 52 °C for 3 min. Following digestion, 2 μL of 2 M HCl was added to lower the pH for degradation of the Rapigest, and the sample was incubated at 37 °C for 30 min. Finally, the sample was centrifuged at 14,000 rpm for 10 min to pellet any remaining debris, and the supernatant was transferred to LC-MS/MS vials for analysis.

Mass Spectrometry Analysis

Discovery experiments to determine which phage K proteins and corresponding tryptic peptides are amenable to mass spectrometric detection were carried out using nanoflow liquid chromatography electrospray tandem mass spectrometry combined with database searching.[23,24] A Waters nano-Aquity HPLC (Waters, Inc., Bedford, MA) was coupled to a Thermo Scientific Velos Orbitrap mass spectrometer (San Jose, CA) fitted with a Michrom CaptiveSpray source (Bruker-Michrom, Auburn, CA). A BEH130 reverse phase C18, 100 mm × 100 μm i.d. analytical column was used for reversed phase liquid chromatography flowing at 600 nL/min prior to introduction into the mass spectrometer. The aqueous mobile phase (A) consisted of 0.1% formic acid with HPLC-grade water (Sigma-Aldrich, St. Louis, MO) while the organic phase (B) was composed of HPLC-grade acetonitrile with 0.1% formic acid (Sigma-Aldrich, St. Louis, MO). The gradient profile consisted of an initial holding at 5% B. After 5 min, the gradient was ramped to 30% B over 90 min, continuing to 95% B for 15 min, and then returning to 5% B for 20 min for column equilibration. The Velos-Orbitrap performed a data-dependent acquisition where the parent ion mass was detected in the orbitrap at a resolution of 60,000 and a scan range from m/z 400–1600 amu. The top 15 most intense ions were selected for MS/MS in the linear ion trap, where the selection window was set at m/z of 2, conventional collision-induced dissociation was performed, and dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 120 s.

MS/MS data were extracted from the resulting instrument files using Mascot Distiller (Matrix Science, London, UK; version 2.4.2). Database searching was conducted using Mascot (Matrix Science, London, UK; version 2.2.0) with the following parameters: peptide tolerance of 200 ppm; MS/MS tolerance of 0.8 Da; 2 allowed missed cleavages, variable modifications of deamidation (NQ) and oxidation (M); and the enzyme of trypsin. The database utilized was the NCBInr (accessed January 14, 2012, www.ncbi.nih.gov) as it has the sequences for multiple *S. aureus* phages, with searches conducted on an in-house, 30-node Beowulf cluster, with each node consisting of an Intel Xeon, quad-core 3.00 GHz processor and 4 GB or RAM. Peptides were validated using Scaffold (version 3.1.2, Proteome Software, Inc., Portland, OR),[25] with peptide identifications accepted if they could be established at a greater than 95.0% probability as specified by the Peptide Prophet algorithm.[26] Protein identifications were considered valid if they could be established at a

greater than 99.0% probability and if they contained at least two peptides with unique amino acid sequences.[27]

Phage K protein sequences identified above were imported into the software program Skyline[28] to generate and optimize suitable MRM transitions. Candidate peptides for detection met the following criteria: length of the peptide was greater than 5 amino acids and less than 15 amino acids; the peptide did not contain methionine or cysteine; the peptide did not contain any missed cleavages; and arginine or lysine was not followed in sequence from the c-terminus by a proline residue. Liquid chromatography was performed using a Waters Aquity HPLC (Waters, Inc., Bedford, MA) flowing at 200 μL/min with an aqueous mobile phase (A) consisting of 0.1% formic acid with HPLC-grade water and an organic phase (B) consisting of HPLC-grade acetonitrile with 0.1% formic acid. A 12 min HPLC method was used with the mobile phase beginning at 98%, ramping down to 70% over 5 min, dropping to 5% and holding for 3 min, and then returning to 98% for the duration of the run. The HPLC flowed into an Applied Biosytems AB SCIEX QTRAP 5500 mass spectrometer with an electrospray interface (Framingham, MA) operating in MRM mode. Optimized transitions for bacteriophage K were incorporated into an LC/MS/MRM method along with calculated transitions for the ^{15}N-labeled bacteriophage. Transitions that emerged following prescreening of all possible transitions were then incorporated into a new MRM method along with the calculated transitions of the ^{15}N-labeled phage.

RESULTS AND DISCUSSION

Characterization of Bacteriophage K Infections

Bacteriophage K-derived peptides were readily detected by LC-MS/MS analysis coupled with database searching, and the inferred proteins, accession numbers, normalized spectral counts and molecular weights are shown in Supporting Information Table 1. The most abundant protein elicited as determined by spectral counts is the 51 kDa capsid protein of phage K. This result is understandable because phage are largely composed of repeating protein capsomere units. It is also in agreement with other mass spectrometry studies wherein the capsid protein is the most readily detected protein.[29] Subsequently, the sequences of proteins described in Supporting Information Table 1 were entered into the software package Skyline, where transitions were investigated and optimized for MRM analysis. Not surprisingly, the transitions yielding the largest peaks as determined by area count corresponded to the capsid protein. The transitions utilized throughout this study based upon this optimization are the capsid-derived peptides VTATVETK^{+2Y6} for quantitation and HLNEAAVR^{+2Y6} for confirmation. We used a calibration curve with parent phage as the internal standard to estimate wild type bacteriophage concentration.

Utilizing these peptides and transitions, the limit of detection for phage K was determined by analyzing serial dilutions of wild type bacteriophage by MRM. Supporting Information Figure 1 shows signal/noise ratios of decreasing concentrations of phage K, with S/N dropping below 3 between the dilutions of 1.56×10^6 pfu/mL and 7.80×10^5 pfu/mL, resulting in an estimated phage K detection limit of ~1×10^6 pfu/mL. Subsequently, an estimation of bacterial limit of detection was investigated using serial dilutions of *S. aureus* BAA-1720 and a starting ^{15}N parent bacteriophage concentration of 5×10^8 pfu/mL.

Supporting Information Figure 2 shows signal/noise ratios derived from amplified ^{14}N progeny phage from decreasing concentrations of *S. aureus* BAA-1720 after 5 h of incubation, with the *S*/*N* ratio dropping below 3 at 2.5×10^4 cfu/mL. Thus, for a PAD assay concentration, the bacterial limit of detection after 5 h of incubation is ~5×10^4 cfu/mL.

Time to results of the PAD assay using ^{15}N-labeled phage K and *S. aureus* BAA-1720 was determined by measuring ^{14}N progeny signal every 30 min from an infection initiated with 5×10^8 pfu/mL of ^{15}N parent phage and 1×10^6 cfu/mL of *S. aureus*. Figure 1 shows a plot of estimated ^{14}N progeny phage for each sampling time point as determined by LC-MS/MRM analysis. The increase of progeny phage in the sample begins in earnest between 1 and 1.5 h, with a plateau in progeny phage concentration occurring at approximately 3.5 h. The data presented in Figure 1 are for a single strain of *S. aureus* BAA-1720, and the time-to-results for *S. aureus* BAA-1720 was experimentally determined to be applicable to the other strains of *S. aureus* utilized throughout this study. However, as PAD assays are developed for the broad range of *S. aureus* strains, time-to-results should be determined for a statistically relevant number of *S. aureus* strains to account for strains that may have a protracted burst time or lower efficiencies of plating. For the purposes of this study, an incubation time of 5 h for all assays was chosen to ensure that for all conditions (various antibiotics versus no antibiotic), sufficient time was allowed to achieve a productive phage amplification event.

S. aureus Detection by PAD

PAD experiments designed to identify multiple strains of *S. aureus* were based on our previously published work[10] in which all bacterial strains at a concentration of 1×10^7 cfu/mL were inoculated with high (5×10^8 pfu/mL) parent ^{15}N phage concentrations. The advantages of using a high initial phage concentration are 3-fold: (1) Provided that multiplicity of infection (ratio of phage particles to bacteria) is a least 3 and that population density of phage and bacteria is high, it is probabilistically likely that all bacteria in the sample are infected with a phage particle immediately after inoculation. (2) Because all bacteria in the sample are infected immediately after inoculation, the time-to-results for the PAD experiment will be minimized. (3) The known, high concentration of isotopically labeled bacteriophage can act as an internal standard for accurate bacterial quantitation, as all bacteria in a sample have been infected simultaneously thereby effectively eliminating secondary amplification events that can skew the accuracy of quantitation.

Figure 2 shows the ^{14}N progeny phage concentration for PAD experiments using a starting bacteria concentration of 1×10^7 cfu/mL and a starting ^{15}N parent concentration of 5×10^8 pfu/mL. For all 4 strains of *S. aureus*, ^{14}N progeny phage signal was detected after 5 h of incubation, signifying a positive phage amplification event and implying the presence of *S. aureus* in each respective sample. Because the starting concentrations of each respective bacterium were similar, the signal derived from phage amplification should be similar if this assay is to be quantitative as it is in our previous work. The average amplification concentration of progeny phage for all 4 strains adjusted to the same starting concentration computes to 6.03×10^8 pfu/mL with a relative standard deviation of 19.1%, an impressive figure-of-merit considering 4 different strains of bacteria are used.

Supporting Information Figure 3 shows the calibration curves for strains BAA-1720 and ATCC-25923, plotting $^{14}N/^{15}N$ ratio versus bacterium concentration. For both strains of bacteria, a linear relationship is observed across the concentration range that would be detectable in a PAD assay, from 5×10^4 to 5×10^7 cfu/mL for each respective bacterium. Furthermore, the value of the slope for both calibration curves is similar, indicating that a calibration curve for one strain of *S. aureus* may be used to calculate the concentration of another strain of *S. aureus*.

Determining the concentration of *S. aureus* in various samples with the calibration curve of a single strain of *S. aureus* hinges on all strains of phage-susceptible bacteria generating a similar number of progeny phage following a phage amplification event for a given concentration of bacteria. Experimentally, this can be evaluated using the concept of efficiency of plating (EOP) which can be defined as the relative number of plaques a phage stock can produce on various strains of susceptible bacteria. For example, a phage stock may produce an average of 80, 100, 110, and 120 plaques on 4 different strains of susceptible bacteria, respectively. By normalizing to the strain producing the lowest number of plaques (an arbitrary assignment), the EOP would be 1.0, 1.25, 1.375, and 1.5, respectively for this example. Table 2 shows the EOP results of plating the same titer of bacteriophage on the 4 strains of *S. aureus* evaluated in this study normalized so that the EOP of BAA-1720 equals one. EOP values show relative similarity among the 4 *S. aureus* strains, indicating that a calibration curve from a single strain of *S. aureus* may be used to quantify all 4 strains in this study with a precision and accuracy acceptable in a clinical microbiology laboratory. However, EOP values are known to vary considerably,[30] a factor that may render quantification of a bacterial species from a single strain of that species ineffective. Thus, if quantification is desired in a PAD assay covering all strains of a specie, then host range analyses testing for phage susceptibility should also ascertain EOP to validate the possibility of a quantitative assay from a single-strain calibration curve.

Clindamycin Resistance Determination by PAD

Clindamycin is a lincosamide antibiotic used to treat skin and soft tissue infections and pneumonia caused by *S. aureus*.[22] Clindamycin is a protein synthesis inhibitor, binding to the 50S ribosomal subunit preventing ribosomal translocation resulting in the stoppage of protein production. Phage are reliant upon the protein synthesis machinery of the host bacterium to generate phage proteins. Therefore, the effect of clindamycin susceptibility on phage replication would be realized in the inability of the host to synthesize progeny phage protein. For a PAD experiment using ^{15}N bacteria as the parent phage, the result of clindamycin susceptibility would be seen in absence of ^{14}N progeny signal when compared with a positive identification for that same strain without the presence of clindamycin.

Figure 3 shows the magnitude of progeny phage amplification for clindamycin susceptibility testing on the 4 strains of *S. aureus* used in this study along with the results of positive identification testing with no antibiotic. Inoculations were carried out with starting bacterial concentrations of 1×10^7 cfu/mL of *S. aureus* bacteria and 5×10^8 pfu/mL of ^{15}N bacteriophage K, and allowed to proceed for 5 h. Strains BAA-1720 and BAA-1750 show a clear, unambiguous PAD signal for the sample containing clindamycin as well as the sample

containing no antibiotic. These results are consistent with antibiotic resistance testing using traditional methods (Table 1). Further, the strains ATCC-12598 and ATCC-25923 produce no detectable signal in the samples containing clindamycin, indicating susceptibility to the antibiotic. These results are also consistent with the susceptibility results obtained by traditional antibiotic susceptibility testing.

We investigated the effects of increasing clindamycin concentration on progeny phage production using the clindamycin resistant strain BAA-1720. Figure 4 shows the results of PAD experimentation as clindamycin concentration increased by 2-fold starting with 0.5 μg/mL and ending with 32.0 μg/mL. While ^{14}N phage amplification is substantial across all concentrations of clindamycin, we observed a trend of decreasing output phage concentration as antibiotic concentration increased. Output ^{14}N bacteriophage was reduced by 50% at 32.0 μg/mL of clindamycin as compared to the no-antibiotic control. This reduction in output ^{14}N phage concentration can be explained by clindamycin directly hampering the ability of the infected host to produce necessary progeny phage proteins despite viability or by a slow-down in overall bacterial doubling time as the bacterial cells combat the high concentration of antibiotic. Indeed, Rabinovitch et al. argued that the eclipse period, latent period, and “rate of ripening” during the rise period of a bacteriophage infection, all independent parameters of bacteriophage development, could be described by their dependence solely on the doubling times of the bacterium prior to initiation of an infection.[31] If doubling times of the bacterium are slowed because an antibiotic is present, the magnitude of a bacteriophage amplification event will likely be depressed. This depression could become important in cases where the minimum inhibitory concentrations of antibiotic are near the defined antibiotic breakthrough point.

Cefoxitin (MRSA) Resistance Determination by PAD

Differing from clindamycin in its mode of action, cefoxitin is a beta-lactam antibiotic that exerts its effect by inhibiting cell wall synthesis, thereby blocking the ability of a susceptible bacterium to divide. However, until bacteria begin the division process initiating the pathway to cell death caused by the action of beta-lactam antibiotics, the protein synthesis pathways remain intact and should be able to generate new proteins. Thus, until all bacteria in a sample are completely dead because of the effects of cefoxitin, measuring a successful phage amplification event in the presence of cefoxitin may be possible, even though the bacterial strain may be susceptible to the antibiotic. Indeed, our experimentation demonstrates that when a PAD infection is started with initial concentrations (T = 0 h) of 5 × 10^8 pfu/mL ^{15}N parent phage and 1 × 10^7 cfu/mL bacteria in the presence of cefoxitin, a sizable production of ^{14}N progeny phage was detected in strains that are clearly sensitive to cefoxitin at 4 μg/mL using traditional antibiotic susceptibility testing (Supporting Information Figure 4).

To account for the cefoxitin mode-of-action and the presence of still-viable *S. aureus* cells, 1 × 10^7 cfu/mL of each respective *S. aureus* strain was incubated with no antibiotic (as a positive control) and in 4 μg/mL cefoxitin for 2.5 h prior to addition of 5 × 10^8 pfu/mL of ^{15}N parent phage. Following the addition of parent phage, all samples were incubated an additional 2.5 h to allow for phage amplification, followed by sample processing and LC-

MS/MRM analysis as described. Figure 5 displays the results of each respective strain incubated in cefoxitin versus a control with no antibiotic. The two MRSA strains (BAA-1720 and BAA-1750) used in this study show significant phage amplification in cefoxitin versus a control, while the two MSSA strains (ATCC-12598 and ATCC-25923) show no significant amplification. The increased magnitude of the phage amplification with the cefoxitin resistant strains relative to the no-cefoxitin controls can be explained by the significant growth of bacteria that must have occurred in the presence of the antibiotic prior to addition of the ^{15}N phage. In the positive controls (no antibiotic, phage added at time= 0 h), initial phage:bacteria ratios are 50:1, meaning probabilistically every bacterium in the sample (1×10^7 cfu/mL) will immediately become infected with a bacteriophage, arresting bacterial growth. Thus, initial bacteria concentrations are responsible for the entire production of progeny phage observed in the MS signal, and no secondary infections by newly generated progeny phage can occur, as all the bacteria have been killed by the phage. Conversely, in the samples with cefoxitin, resistant bacteria will experience 2.5 h of growth prior to addition of the bacteriophage, resulting in significantly more bacteria responsible for generating progeny phage signal relative to the no-cefoxitin control. Further, as the ratio of phage:bacteria at time-of-infection is altered due to bacterial growth, the probability of each bacterium initially receiving an infectious phage is less. As a result newly released progeny phage may find bacteria to infect in a secondary infection, which may also contribute to the MS signal

Figure 6 shows the effects of increasing cefoxitin concentration on progeny phage production after incubatin-gATCC-1720 in cefoxitin for 2.5 h followed by adding^{15}N parent bacteriophage and incubating for an additional 2.5 h. Highest progeny phage production is seen in the sample without cefoxitin, resulting in an observable trend. As cefoxitin concentration increases, total output phage decreases significantly, until at 128 μg/mL, progeny phage production becomes negligible. Clearly, the presence of cefoxitin significantly below the MIC attenuates phage production as cellular resources that can potentially maximize phage production are diverted to stave off cell death. Phage production can also be attenuated, as a cell loses fitness and cellular metabolism, including protein synthesis, is compromised. For example, exposure of MRSA strains to subinhibitory concentrations of beta-lactam antibiotics induces within minutes the production of penicillin binding protein 2A,[32] thus plausibly decreasing cellular resources required for progeny phage production. Additionally, Qoronfleh and Wilkinson have shown that although a strain of *S. aureus* is resistant to a beta-lactam antibiotic (methicillin), incubation of the strain in the presence of subinhibitory methicillin concentrations results in cruder cell walls with peptidoglycan containing markedly decreased cross-linking and O-acetylation.[33] These deficient cell walls lead to increased rates of autolysis and susceptibility to lysing reagents. Thus, conceivably, a resistant strain with decreased fitness in the presence of cefoxitin is incapable of producing as much progeny phage due to premature lysis caused by diminished cell wall integrity.

PAD in Bacterial Mixtures

As demonstrated previously, PAD can be utilized to detect the presence of a bacterial strain when cocultured with another bacterial specie.[34] To demonstrate this capacity with phage K

and *S. aureus*, we cultured two Gram-positive species of bacteria, the closely related *Staphylococcus epidermidis* and a *Klebsiella pneumoniae* strain, with a cefoxitin sensitive *S. aureus* (ATCC-12598) and a cefoxitin resistant *S. aureus* (ATCC-1720), and performed PAD experiments as described above. Figure 7 shows the already-demonstrated phage amplification of the *S. aureus* strains when PAD is used for identification in a pure culture, while the *S. epidermidis* and *K. pneumoniae* show negligible phage amplification. When the *S. aureus* strains are cocultured with the *S. epidermidis* and *K. pneumoniae* strains, measured phage amplification is similar in magnitude to the PAD experiments using pure cultures. Further, when the mixed cultures are subjected to PAD in the presence of 4 μg/mL cefoxitin, the cefoxitin sensitive *S. aureus* shows negligible amplification compared to the no cefoxitin control of the mixtures, while the cefoxitin resistant strain shows the significant phage amplification as observed above when pure cultures are subjected to the cefoxitin testing protocol as described. The ability to detect the presence of *S. aureus* and determine antibiotic sensitivity in bacterial mixtures has profound implications in that an acquired sample need not be subcultured for colony isolation prior to analysis. A time-consuming part of many bacterial identification work flows is the need to work with isolated colonies of bacteria, which can only be obtained by subculture and incubation for a considerable amount of time. The ability to detect phage amplification in cocultures of bacteria obviates the need for this subculture step. Eliminating this step means that results of bacterial identification and antibiotic resistance determination can be obtained during a typical (8 h) work shift, including sample prep and incubation time.

Final Assay

Figure 8 shows a schematic of the construct of a complete assay to detect the presence of *S. aureus* in a sample and determine its antibiotic susceptibility to clindamycin and cefoxitin. First, a sample is split into 3 aliquots, one containing no antibiotic, one containing 2 μg/mL of clindamycin, and one containing 4 μg/mL of cefoxitin. To the aliquots containing no antibiotic and clindamycin, ^{15}N-labeled bacteriophage is immediately added and allowed to incubate for 5 h. The sample aliquot containing cefoxitin is allowed to incubate for 2.5 h, after which ^{15}N-labeled bacteriophage is added, followed by incubation for an additional 2.5 h. All 3 sample aliquots can be processed for MRM analysis at the 5 h mark, with identification and quantitation of bacteria (if desired) occurring in the aliquot with no antibiotic, and antibiotic resistance to each respective antibiotic occurring in the other two aliquots.

CONSIDERATIONS

The ability of this assay to identify *S. aureus* and simultaneously determine the phenotypic antibiotic resistance patterns for multiple antibiotics in a rapid fashion is significant. Real-time PCR methods exist for the identification of strains containing the mecA cassette, and have been shown to be in agreement with the gold standard of culture.[35] However, RT-PCR assays can be cost-prohibitive for many laboratories and institutions, and only detect those strains containing the mecA cassette thereby ceding the detection of non-mecA resistant *S. aureus*.[19] Chromagenic agars for MRSA detection are far more cost-effective than RT-PCR assays,[36] yet require longer incubation periods for time-to-results. While comparatively

mass spectrometry coupled with liquid chromatography as a detection platform for PAD assays involves a large initial capital investment, it is not necessarily the detector of choice after dissemination of the PAD assay into laboratories. With the advent of MALDI as a bacteria detector in many clinical laboratories[14,37] the PAD scheme could foreseeably be adapted to existing MALDI platforms to ascertain antibiotic resistance, which is currently an inherent weakness of MALDI bacterial identification algorithms. Future work on PAD techniques will be focused on harnessing the parallel capacity of MALDI to analyze numerous phage amplification samples on a platform that is being widely accepted in clinical settings.

Similar to issues regarding the use of phage as therapeutics, the use of PAD for identification of bacteria and determination of antibiotic resistance hinges on the ability of a phage or phage cocktail to infect an acceptable number of strains. Some lytic bacteriophages possess a broad host range for a bacterial specie and are currently utilized in laboratory tests for identification purposes, such a gamma phage for *Bacillus anthracis*[38,39] and phiA122 for *Yersinia pestis*.[40] Many phages have a narrower host range, allowing a defined set of phages to be used for phage typing and epidemiological purposes.[41–44] Particular to this study, bacteriophage K has a broad host range against both coagulase positive and coagulase negative *Staphylococci*, infecting over 70% of *S. aureus* strains.[45] While bacteriophage K alone may not be sufficient for a suitable PAD assay, a recent publication using an FDA approved phage diagnostic with positive predictive values of 100% for MRSA and MSSA shows the viability of a phage based diagnostic for *S. aureus*.[11] Refinement of this assay would almost certainly require a cocktail of *S. aureus* phages.

CONCLUSION

We have shown the ability to identify the presence of *S. aureus* in a sample and determine antibiotic susceptibility to clindamycin and cefoxitin with 5 h of incubation followed by mass spectrometric analysis. The unique affinity of bacteriophages for their host combined with the sequence-based detection methodology of LC-MS/MRM results in a highly specific assay that could plausibly be adapted for use in a clinical lab setting. Furthermore, while two antibiotics have been tested for *S. aureus* susceptibility in this study other antibiotics could likely be incorporated into this assay with minimal effort. While the proteinaceous nature of bacteriophages makes them ideally suited for detection by mass spectrometry, any method capable of detecting changes in protein concentration may be adapted as a detector for phage amplification detection, provided an ability to distinguish parent from progeny phage is present. This assay could also be developed for other bacteriophage-host bacterial species to identify bacteria and determine antibiotic susceptibility. As the specter of antibiotic resistance continues to proliferate and with the rise of personalized medicine, the high specificity of a bacteriophage assay combined with the ability to rapidly test an infecting strain against multiple antibiotics may play a definitive role in achieving positive patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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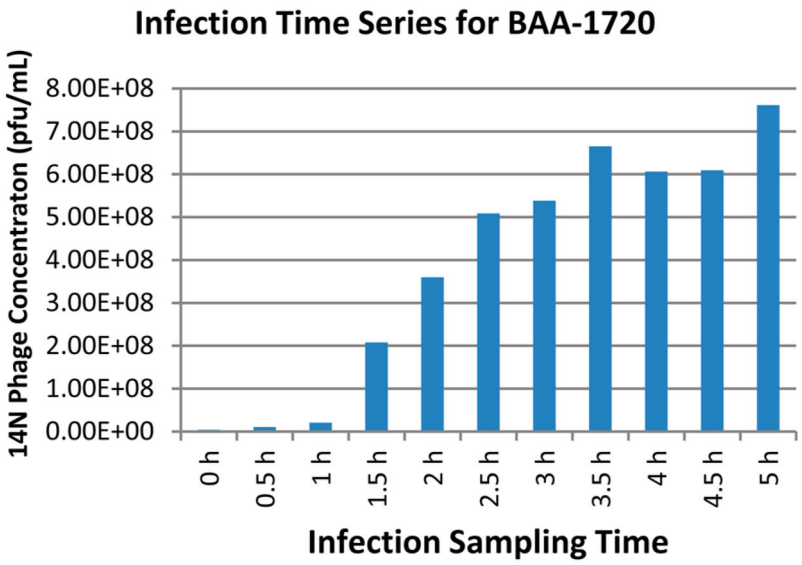

Figure 1.

Time series for phage amplification using 5×10^6 cfu/mL of *S. aureus* strain BAA-1720 and 5×10^8 pfu/mL of ^{15}N-labeled bacteriophage. Amplification begins in earnest at 1.5 h, and plateaus at 3.5 h.

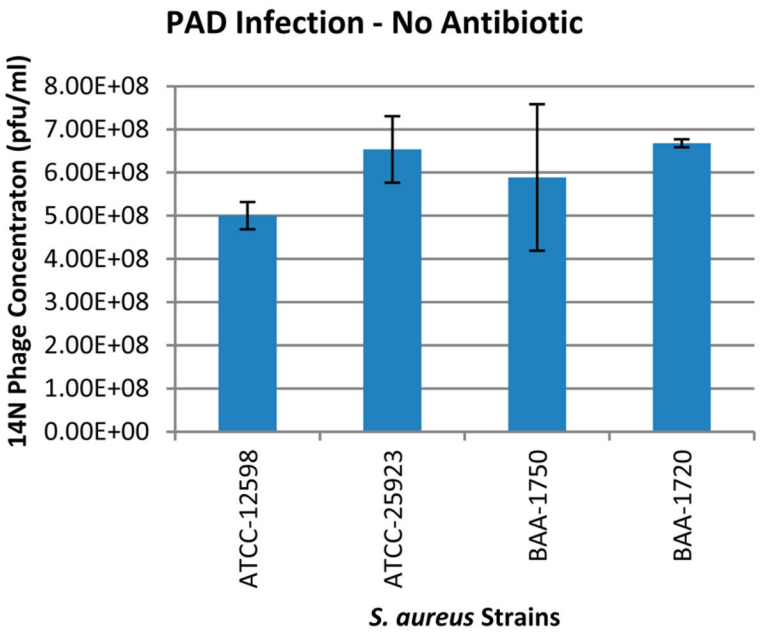

Figure 2.

Phage amplification detection ($n = 2$) at $t = 5$ h for 4 strains of *S. aureus* without the addition of antibiotic. Significant amplification of wild-type phage is seen for all 4 strains.

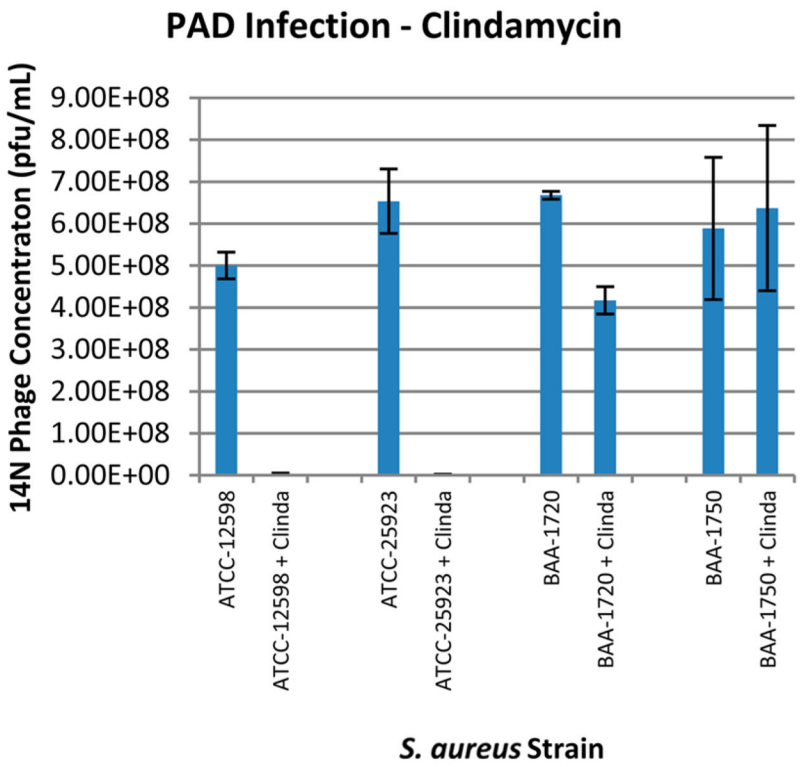

Figure 3.
Phage amplification ($n = 2$) to determine susceptibility of 4 strains of *S. aureus* to 2 μg/mL clindamycin.

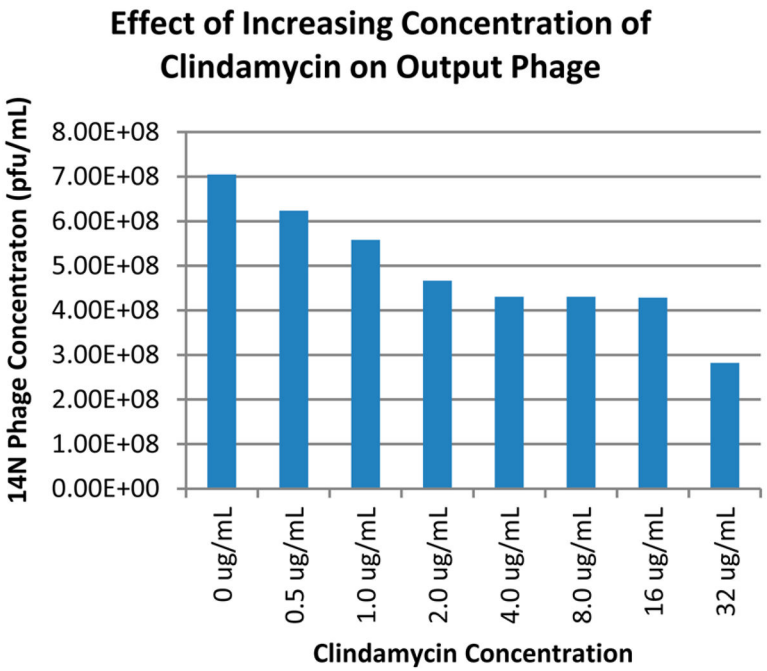

Figure 4.

Effect on phage amplification with increasing concentrations of clindamycin on the clindamycin resistant strain BAA-1720.

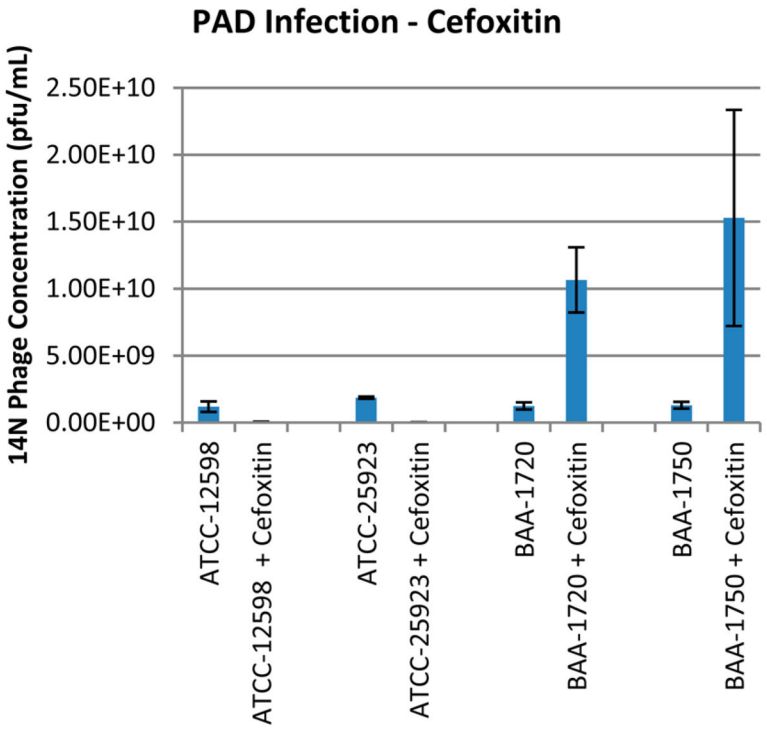

Figure 5.

Phage amplification detection ($n = 2$) to determine susceptibility of 4 strains of *S. aureus* to cefoxitin and subsequent MRSA\MSSA designation.

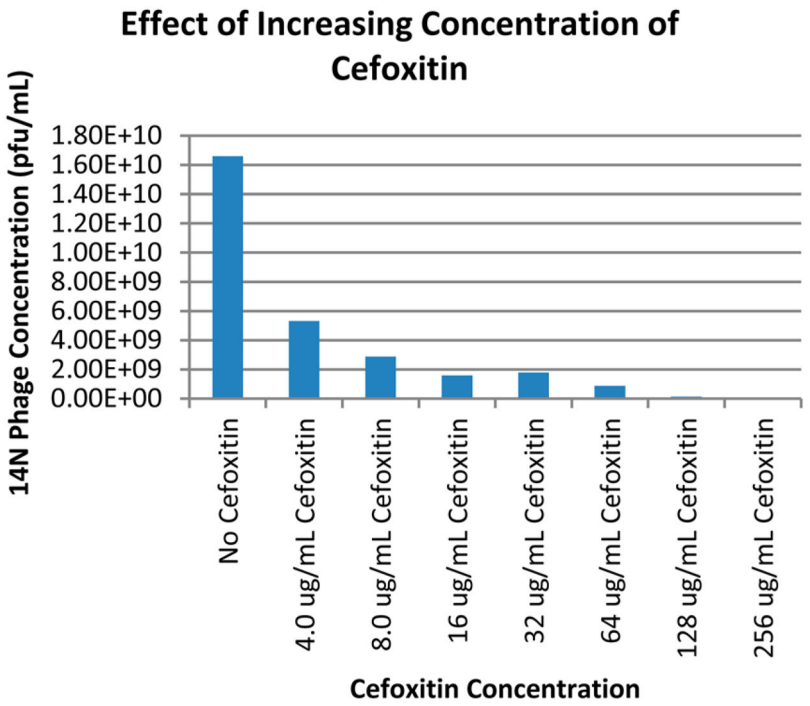

Figure 6.

Effects of increasing cefoxitin concentration on phage amplification measured at 5 h using *S. aureus* strain BAA-1720.

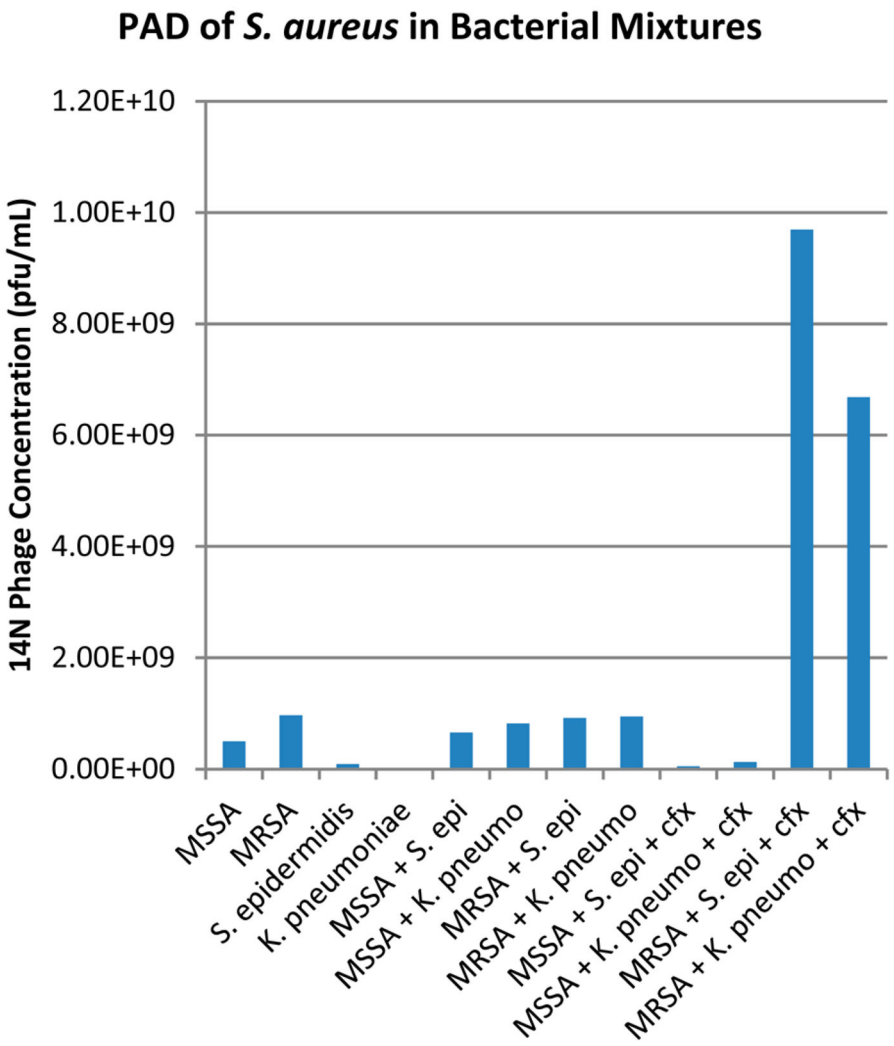

Figure 7.
Phage amplification detection results of a MSSA (ATCC-12598) and MRSA (BAA-1720) strain cultured individually and in the presence of other bacterial species.

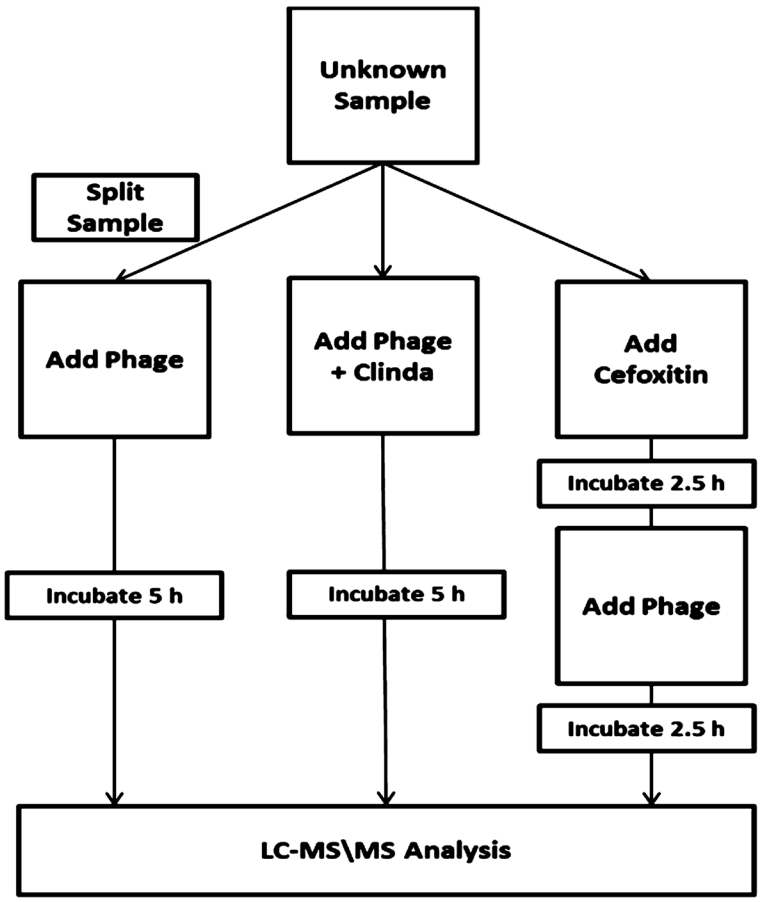

Figure 8.
Construct of final assay to determine presence of *S. aureus* in an unknown sample and to determine its susceptibility to clindamycin and cefoxitin.

Table 1

Results of Antibiotic Resistance Testing of Bacterial Strains to Clindamycin and Cefoxitin Using Traditional Microbiology Testing Methodology

strain	clindamycin resistant	cefoxitin resistant
ATCC-12598	no (MIC < 0.5 μg/mL)	no (MIC < 0.5 μg/mL)
ATCC-25923	no (MIC < 2 μg/mL)	no (MIC < 0.5 μg/mL)
BAA-1720	yes (MIC > 32 μg/mL)	yes (MIC > 32 μg/mL)
BAA-1750	yes (MIC > 32 μg/mL)	yes (MIC > 32 μg/mL)
ATCC-29213	no (MIC < 0.5 μg/mL)	no (MIC < 2 μg/mL)
ATCC-43300	yes (MIC > 32 μg/mL)	yes (MIC > 32 μg/mL)

Table 2

Efficiency of Plating for 4 Strains of *S. aureus* Normalized to Strain BAA-1720

strain	efficiency of plating (EOP)
ATCC-12598	1.4
ATCC-25923	1.6
BAA-1720	1
BAA-1750	1.4