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ABSTRACT*:

Background:

Current antibiotic therapy approaches for suspected bacteremic and septic patients regularly take a conservative approach treating broadly. There is a need to identify (or rule out) the specific pathogens known to be important markers of severity of sepsis and patient outcome and assess their antibiotic susceptibility or resistance. Detection of bacteria has been demonstrated using bacteriophages that bind to target cell receptors. Upon infection, input phage direct production of thousands of progeny per cell, culminating in a release of phage into the sample. Here we apply this phenomenon to *Staphylococcus aureus*, a principal bloodstream pathogen strongly linked to patient outcome.

Materials and Methods:

Phage were sourced from clinical samples and existing collections. Phage were isolated from lawns of *Staphylococcus aureus* on agar plates. Standard plaque assay methods were employed to define host ranges and burst characteristics of candidate phage.

Results:

The growth patterns of susceptible and resistant strains of *S. aureus* follow predictable patterns under unchallenged and antibiotic challenged conditions. The replication of bacteriophage in cultures similarly treated cultures closely correlated that of the culture.

Conclusions:

Bacteriophage amplification in cultures with and without exposure to antibiotic closely correlate the growth patterns of the bacteria. When coupled to a rapid test method, such as lateral flow immunoassay, the phage replication can be used as an early detection method for antibiotic susceptibility.

INTRODUCTION:

Current technology takes 1 to 3 days to accurately ID bacteria and determine antibiotic susceptibility. This is often too slow to assist physicians in formulating initial treatment plans. Consequently, physicians prescribe broad-spectrum antibiotics to treat most clinical conditions prophylactically. When infections are bacterial, research shows that targeted, narrow spectrum antibiotic therapy results in better patient outcomes than does broad-spectrum antibiotic therapy. For life threatening conditions such as sepsis, this difference can be critical. The long-term public health consequences of indiscriminate antibiotic usage are also serious because it has the potential to accelerate the development of antibiotic resistance which can ultimately shorten the useful lifetime of important antibiotics. Providing accurate and timely diagnostic data to physicians about bacterial infections will reduce health care costs and save lives.

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

The following discusses a novel approach to antibiotic susceptibility testing using the release of progeny phage as an indicator of bacterial cell growth. In the presence of sufficient antibiotic, susceptible bacteria will fail to grow, limiting phage replication. Resistant bacteria, on the other hand, will continue to grow and allow for phage replication and release.

METHODS:

Bacteria

Bacteria were sourced from Phage Therapeutics (Seattle, WA)

Bacteriophage

The bacteriophage used in this study was derived from a phage sourced from Phage Therapeutics (Seattle, WA).

Media

All bacterial/phage growth was performed in Cation Adjusted Mueller Hinton Broth (CAMHB) with 2% NaCl

Indicator BioAssays and Antibiotic Sensitivity Assays

All bacterial/phage growth was performed in cation adjusted Mueller Hinton Broth (CAMHB) with 2% NaCl. Plaque assays for bacteriophage concentration determination were conducted according to traditional methods using tryptic soy media.

Bacteria were grown to log phase and quantified using optical density. An aliquot of the bacterial sample was added to a known quantity of phage, and allowed to incubate at 37 deg for the duration of the experiment. Concurrently, an equal aliquot of the same bacteria was added to a sample containing an equal concentration of phage and oxacillin added to a final concentration of 4 microgram/ml. At various intervals, a sample was taken from each vial and bacteriophage concentration determined by the traditional plaque assay.

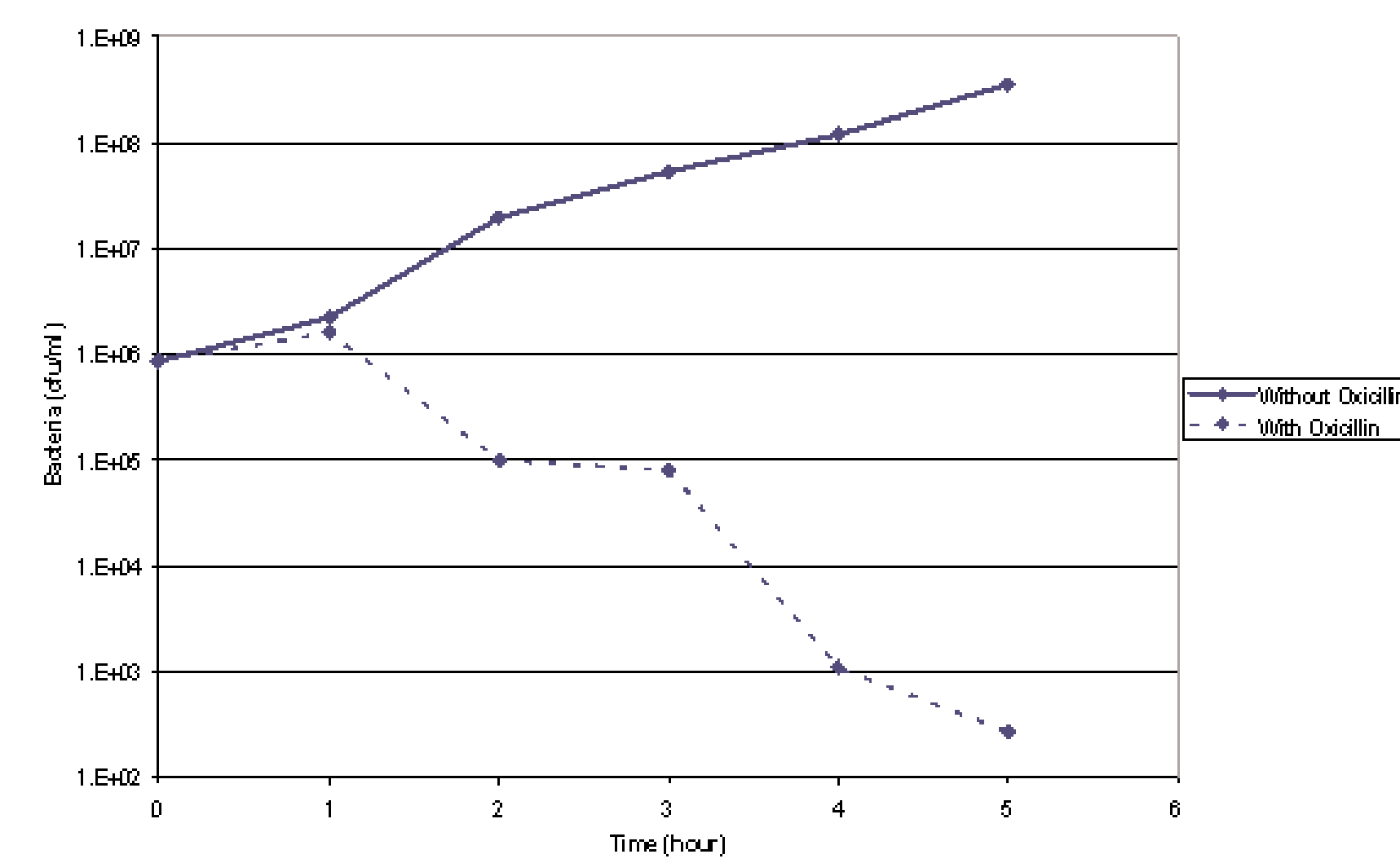
RESULTS:

The following graphs reflect the results of incubating strains of Methicillin susceptible and resistant *Staphylococcus aureus* with bacteriophage in the presence and absence of oxacillin. The graphs on the left represent sampling of cells and determination of cell growth in the presence or absence of 4 ug/ml oxacillin. The graphs on the right reflect the production of bacteriophage under the same conditions.

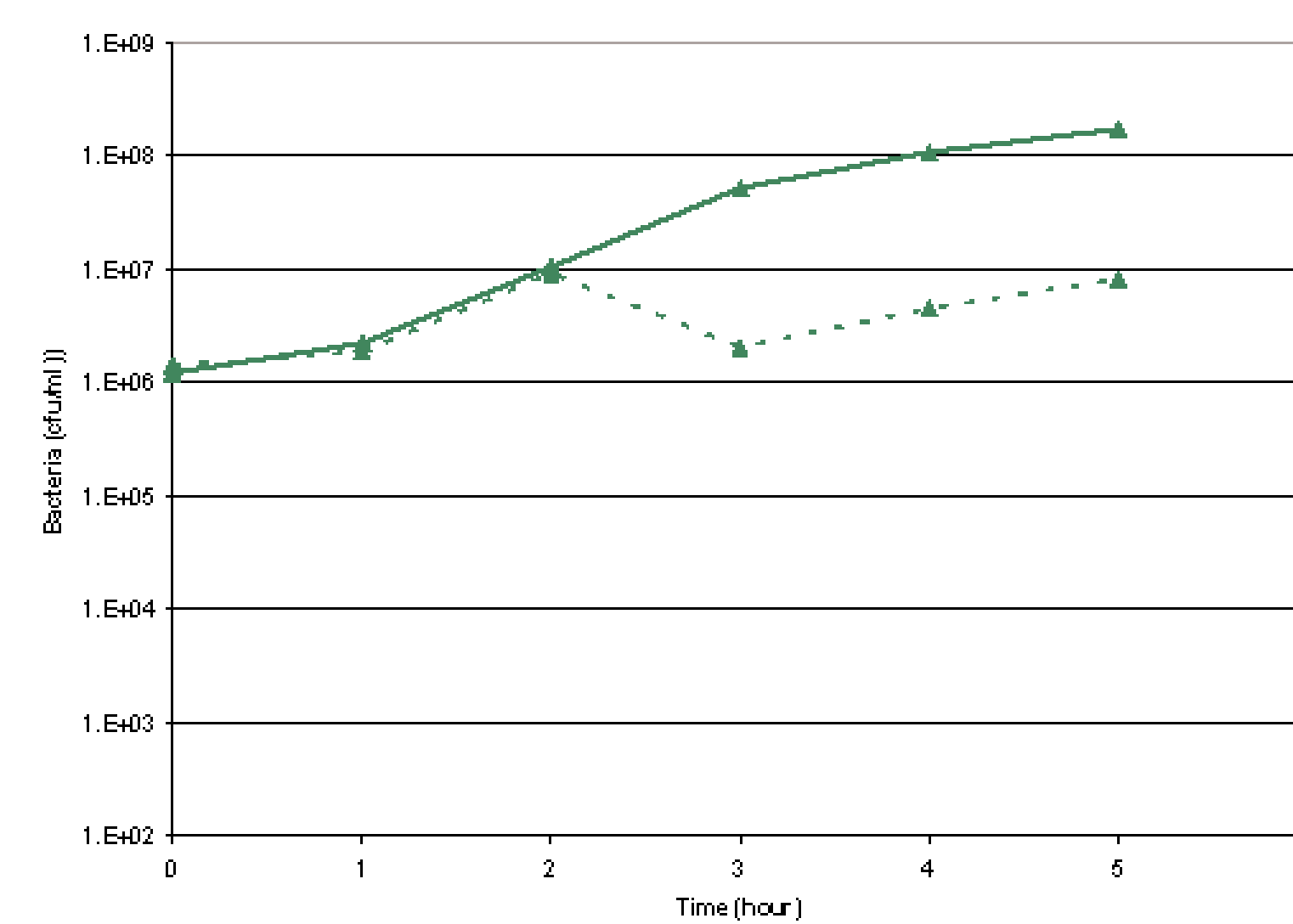
Culture conditions for both sets were CAMHB at 37C. The mixtures were sampled at intervals and viable cell counts performed by performing serial 10-fold dilutions and plating the dilutions. The concentration of phage determined using standard soft-agar plaque count methods from similar dilutions. For both studies, bacteria were targeted at 1×10^6 cfu/ml initial concentration; phage at 1×10^5 pfu/ml.

Viable Cell Count

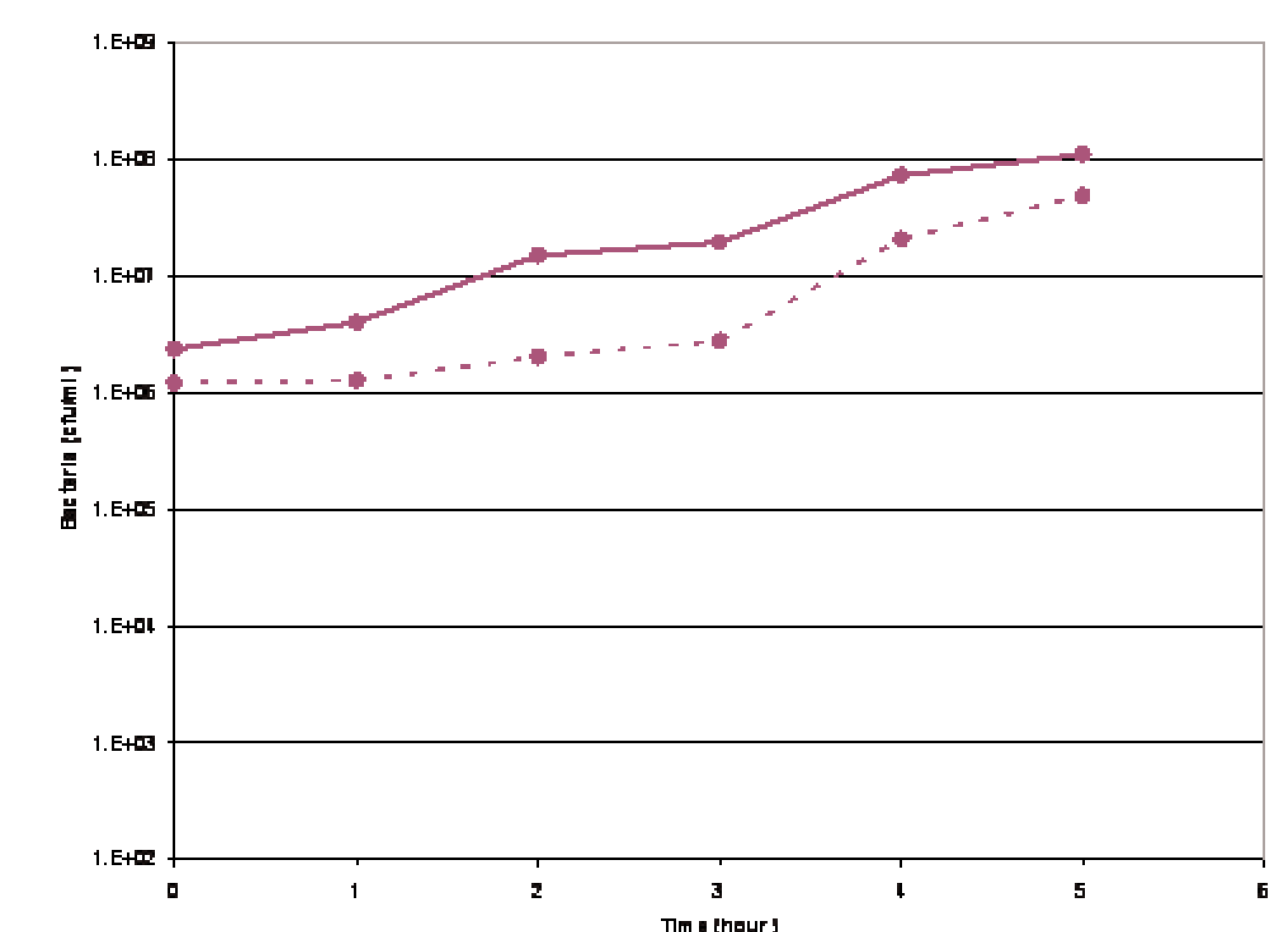
MSSA strain 106



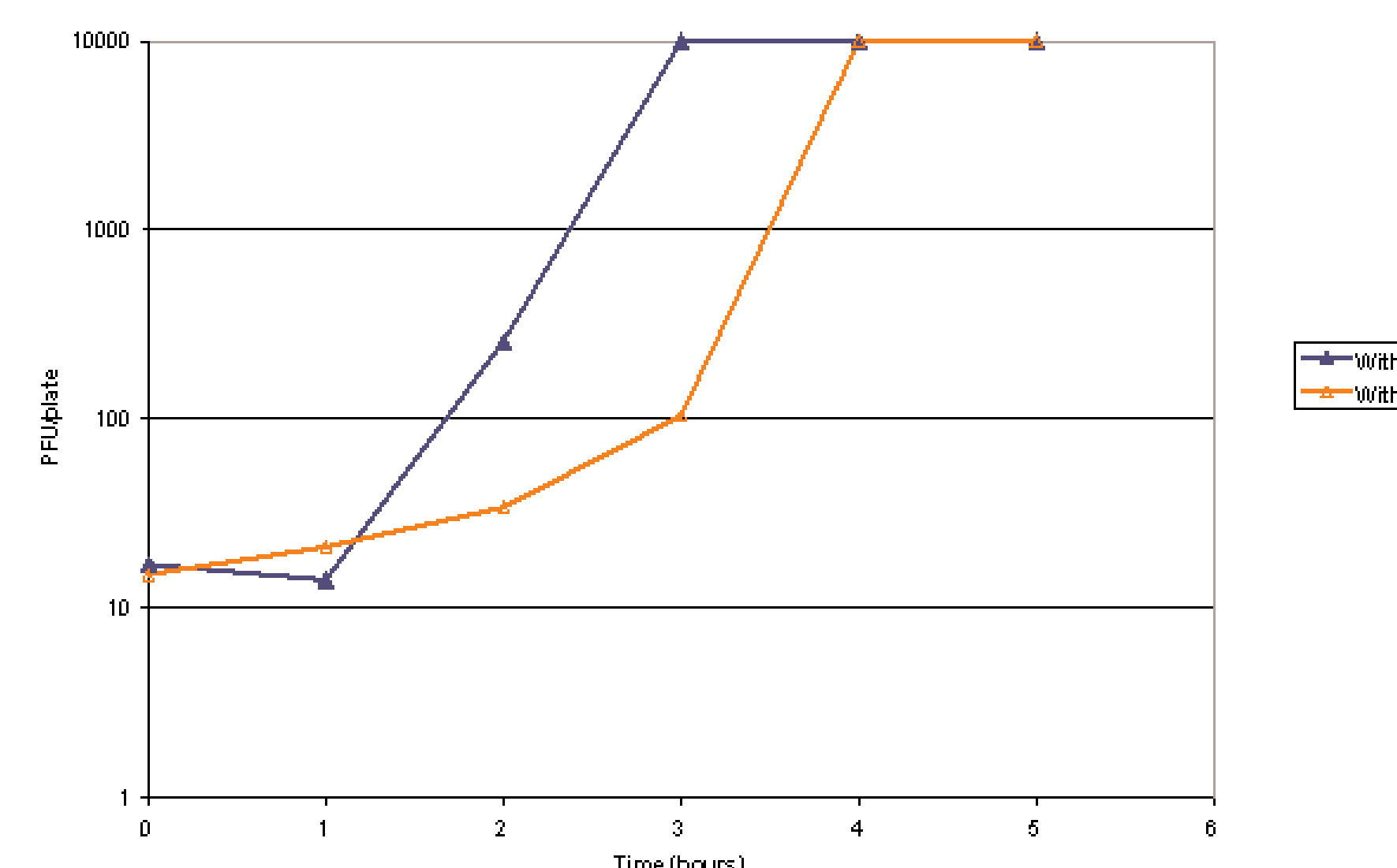
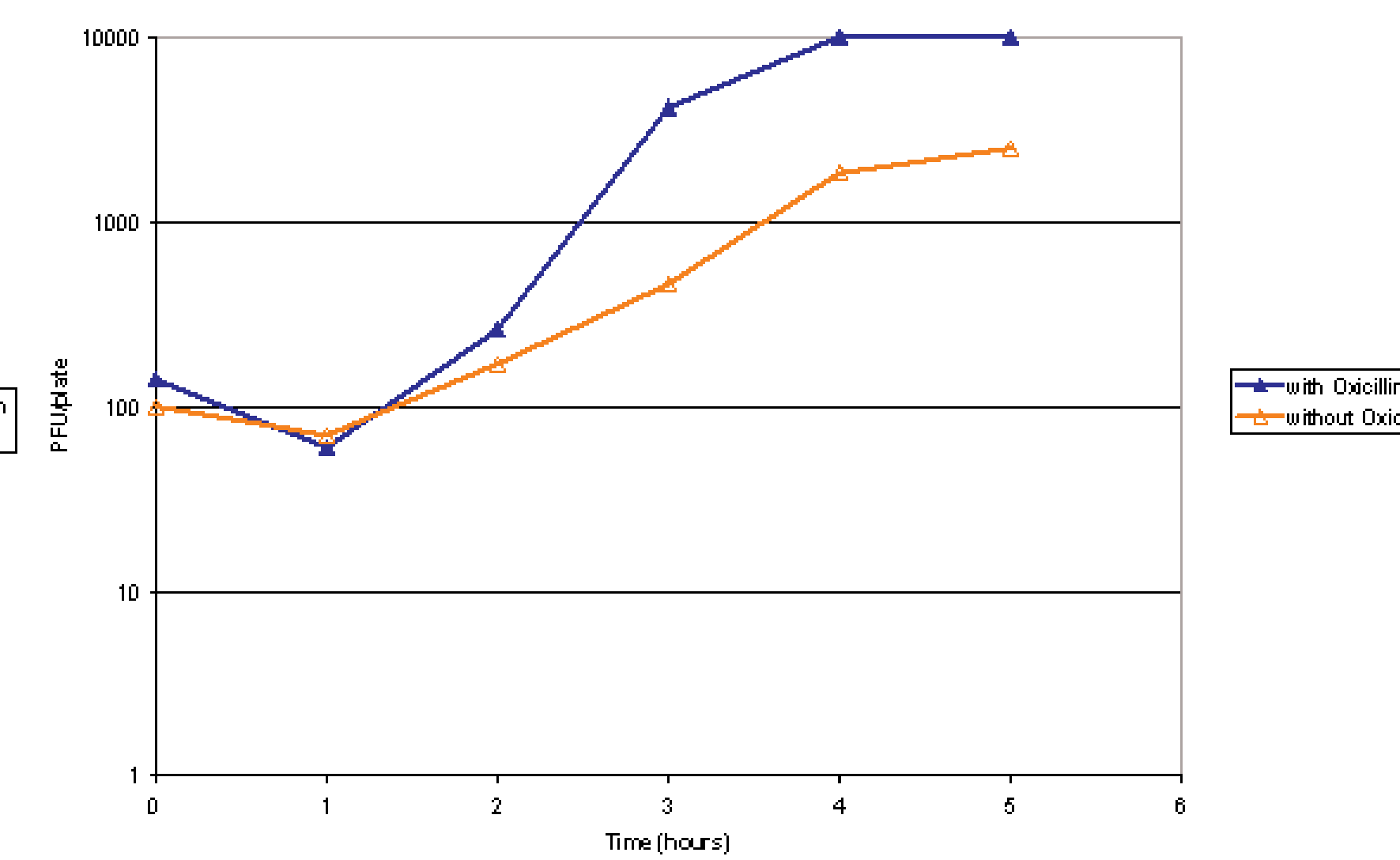
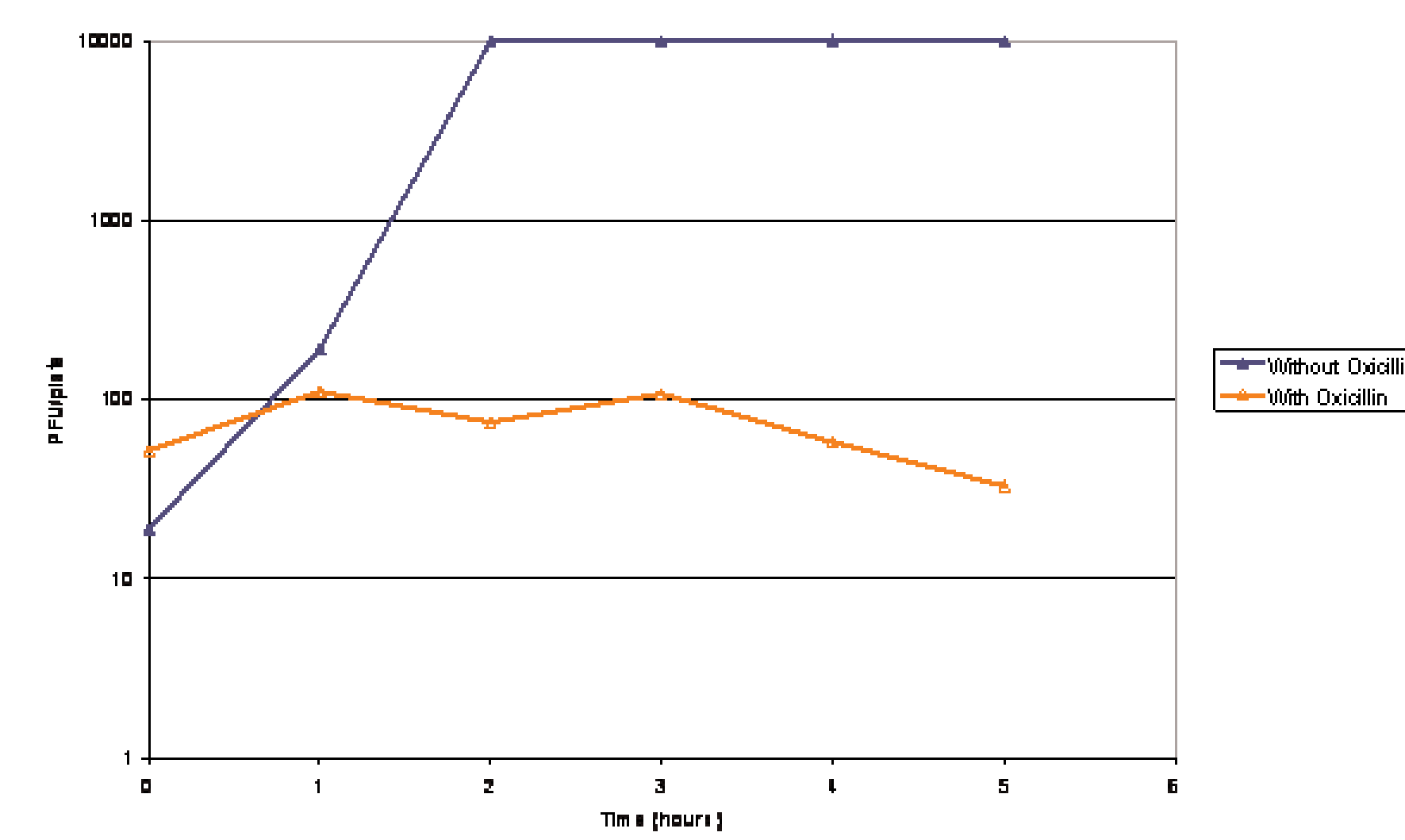
MRSA strain 276



MRSA strain 277



Plaque Count



DISCUSSION and CONCLUSIONS:

Methicillin and Oxacillin are members to the β -lactam class of antibiotics which act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms. β -lactam antibiotics are analogues of D-alanyl-D-alanine; the structural similarity between β -lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of penicillin binding proteins (PBPs). The irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the peptidoglycan layer, disrupting cell wall synthesis. Inhibition requires rapidly growing and dividing cells and is time dependent.

The requirement for rapidly growing cells both favors and challenges the phage approach to β -lactam susceptibility testing. Phage replication requires metabolically active host cells. However, phage replication is very rapid; a round of replication can be completed in as little as 30 minutes. β -lactams inhibit cell wall synthesis but do not interfere with other metabolic pathways leaving the cell capable of supporting phage replication.

The results presented here support the balance between cell growth and phage production. In the first case, strain 106, the untreated bacteria replicate quickly resulting in nearly 2.5 log increase in viable cells in 5 hours. However, in the presence of oxacillin, cell growth is stopped and viable cell counts rapidly decrease in a similar time. Consistent with that pattern is the strong production of phage in untreated cells while there is no increase in antibiotic treated cells.

In contrast, strains 276 and 277, which are MRSA, demonstrate both different growth curves as well as phage production. In strain 276, untreated bacteria grow almost as well as seen with strain 106 and phage production, while somewhat slower, reaches similar levels to that seen in the MSSA strain. In the presence of oxacillin, however, cell growth rate is reduced. Phage production, in the presence of antibiotic, is also reduced but not eliminated as in the case of the MSSA strain.

Strain 277 shows stronger resistance, both in growth rates and in phage production. Viable cell counts follow similar patterns; in the presence of oxacillin, the growth rate mirrors that of the untreated cells with a delay in the initiation of growth. In phage production, both the treated and untreated bacteria ultimately produce greater than 10^5 pfu/plate; growth of phage in oxacillin treated cells was delayed by approximately one hour.

These results demonstrate that bacteriophage replication can be used as a method to monitor host cell growth. While the results presented here utilize plaque counts to measure this response, a similar approach can be used with detection measured using an immunological method, such as an enzyme immunoassay or lateral flow immunoassay, or via quantitative nucleic acid detection, reducing the time to results.

OPPORTUNITIES:

Rapid direct detection and identification of bacteria from samples from septic patients has the potential to influence antibiotic therapy choices and improve clinical outcomes. Phage replication has been demonstrated to follow patterns of bacterial growth both in this study and elsewhere in the literature. Monitoring of phage replication by incorporating phage amplification into immunoassays appears to provide rapid simplified method which can determine antibiotic susceptibility and guide therapeutic decisions.

In addition to work on blood culture positive bacteria detection, the method holds promise for applications in wound care management, aiding respiratory diagnosis, and reducing repeat urinary tract infections with targeted treatment. The method also holds promise for additional bacterial targets, such as *S. pneumoniae*, *Enterococcus sp.*, and *Pseudomonas sp.*, all with lytic phage candidates available for development.