Nanoparticles Functionalized with Ampicillin Destroy Multiple Antibiotic Resistant Isolates of *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and Methicillin Resistant *Staphylococcus aureus*

Ashley Brown[1], Kathryn Smith[1], Tova A. Samuels[2], Jiangrui Lu[2], Sherine Obare[2], and Maria E. Scott[1]

1. Procedure for Antibiotic susceptibility test by disc diffusion analysis

The Sensi-disc^{TM} disc diffusion method was used to verify ampicillin resistant status of the strains, Becton-Dickinson BBL, MD [1]. Manufacturer's instructions are based on procedures adopted by the Clinical and Laboratory Standards Institute [2-4]. Trypticase soy broth at pH 7.0 was inoculated with two colonies of bacteria and incubated for 6 hours at 37°C with aeration. When the culture reached log phase growth OD600 nm of 1.0, the turbidity of the culture was adjusted to the 0.5 McFarland standard. Sample was applied with a sterile cotton swab onto the entire surface of a trypticase soy agar plate to ensure complete coverage of the plate with viable bacteria then the plate was air dried for 5 minutes. The drug-impregnated Sensi-Disc was antiseptically applied to the surface of the plate and samples were incubated at 37°C for 18 hours.

Sensi-disc contained exact concentrations of antibiotic agents based on usual dosage regimens of antibiotics. The susceptibility of the bacterial strains were tested against the following antibiotics: ampicillin (AM-10) 10 μg, penicillin (P-10) 10 units, kanamycin (K-30) 30 μg, erythromycin (E-15) 15 μg, gentamicin (GM-120) 120 μg, tetracycline (Te-30) 30 μg and vancomycin (Va-30) 30 μg per disc. The diameter of the zone of inhibition to the nearest millimeter for each bacterial isolate determined susceptibility of the bacterial strain to the antibiotic. (The zone of inhibition is an area of clearing where bacteria did not grow.) The interpretation of the diameter of the zone of inhibition used criteria published by the manufacturer of the susceptibility disc from the "Zone Diameter Interpretive Chart" authorized by the National Committee for Clinical Laboratory Standards (NCCLS). The diameters of zones of inhibition for the isolates tested are in Table 1S.

2. Detection of β-lactamase by cefinase disc method

The cefinase disc contains a chromogenic cephalosporin called nitrocefin sensitive to all known β-lactamase enzymes including *Staphylococcus* penicillinases. Nitrocefin undergoes a rapid color change from yellow to pinkish-red following cleavage of the amide bond of the β-lactam ring by β-lactamase producing bacteria. Although the nitrocefin reagent detects β-lactamase, it is not predictive of clinical susceptibility of *Enterobacteriaceae* organisms to β-lactam antibiotics because methods used to effect resistance can be quite variable within this group. With aseptic technique, cefinase discs were placed into sterile Petri dishes moistened with sterile purified water and then a portion of an isolated colony from each strain was smeared onto the discs. Discs were incubated at room temperature for up to 1 hour and where applicable color change recorded.

Positive strains produced a rapid color change from yellow to pinkish-red as the β-lactamase hydrolyzed the amide bond in the β-lactam ring. *E. aerogenes*, *P. aeruginosa*, MRSA, and *E. coli* K12 substrain DH5α (pPCRScript AMP SK^{+}) were positive for β-lactamase (data not

shown). *V. cholerae*, STEC O91:H21, STEC O157:H7 and *E. coli* k-12 substrain DH5α were negative for β-lactamase production and produced no detectable color change. The results of the cefinase tests agree with the disk diffusion ampicillin susceptibility tests.

Table 1S. Results of Disk Diffusion Test: Zone Diameter of Growth Inhibition

	Antibiotics Tested					
Bacterial Species Tested	**Pen**[1]	**AM**[2]	**Kan**[3]	**Gen**[4]	**Van**[5]	**Tet**[6]
E. coli DH5α	(0) R[10]	(23) S	(24) S	(28) S	(0) R	(27) S
E. coli DH5α (pPCRscript AMP SK$^+$)[8]	(0) R	(0) R	(25) S	(28) S	(0) R	(29) S
E. coli O91:H21 strain B2F1 (STEC)[7]	(0) R	(19) S	(18) S	(18) S	(0) R	(22) S
E. coli O157:H7 strain 86-24 (STEC)[7]	(0) R	(20) S	(18) S	(15) S	(0) R	(23) S
Vibrio cholerae strain TRH7000	(2) R	(18) S	(20) S	(23) S	(0) R	(29) S
Pseudomonas aeruginosa	(0) R	(0) R	(0) R	(16) S	(0) R	(10) R
Enterobacter aerogenes	(0) R	(10) R	(28) S	(22) S	(0) R	(25) R
Staphylococcus aureus strain ST398 MRSA[9]	(0) R	(12)R	(8) R	(20) S	(5) R	(24) S

[1]Pen--penicillin. [P10] Virtually all Gram-negative bacteria are resistant.
[2]AM--ampicillin. [AM10] resistant (≤13 mm); intermediate (14-16 mm); sensitive (≥17 mm).
[3]Kan--kanamycin. [K30] resistant (≤13 mm); intermediate (14-17 mm); sensitive (≥18 mm).
[4]Gen--gentamicin. [GM10] resistant (≤12 mm); intermediate (13-14 mm); sensitive (≥15 mm).
[5]Van--vancomycin. [Va30] Gram-negative bacteria are resistant by virtue of its outer membrane.
[6]Tet--tetracycline. [Te30] resistant (≤11 mm); intermediate (12-14 mm); sensitive (≥15 mm).
[7]STEC are pathogenic diarrheal *E. coli* that produce Shiga Toxin.
[8]pPCRScript-AMP SK$^+$ is a cloning vector that carries the β-lactamase gene.
[9]MRSA stands for methicillin resistant *S. aureus*.
[10]number in parenthesis is the diameter of the zone of inhibition; R-resistant; S-sensitive.

3. Ampicillin Antibiotic and Nanoparticle Scheme

Ampicillin is a broad-spectrum antibiotic active against Gram-negative and Gram-positive bacteria. Ampicillin is an aminobenzyl penicillin derivative of the original β-lactam antibiotic, penicillin, and penetrates the outer membrane of Gram-negative bacteria. The mode of action of ampicillin is to inhibit peptidoglycan cross-linkage by competitive binding to transpeptidase enzyme blocking the final crucial steps of cell wall synthesis. The thioether group is indicated which was used to bind ampicillin to Ag and Au nanoparticles. The β-lactam group is shown, the functional group of the antibiotic and the target of β-lactamases produced by some ampicillin resistant bacteria.

The structure of ampicillin is shown in Figure 1S Panel A below. A diagrammatic representation of our scheme is shown in Figure 1S Panel B as well.

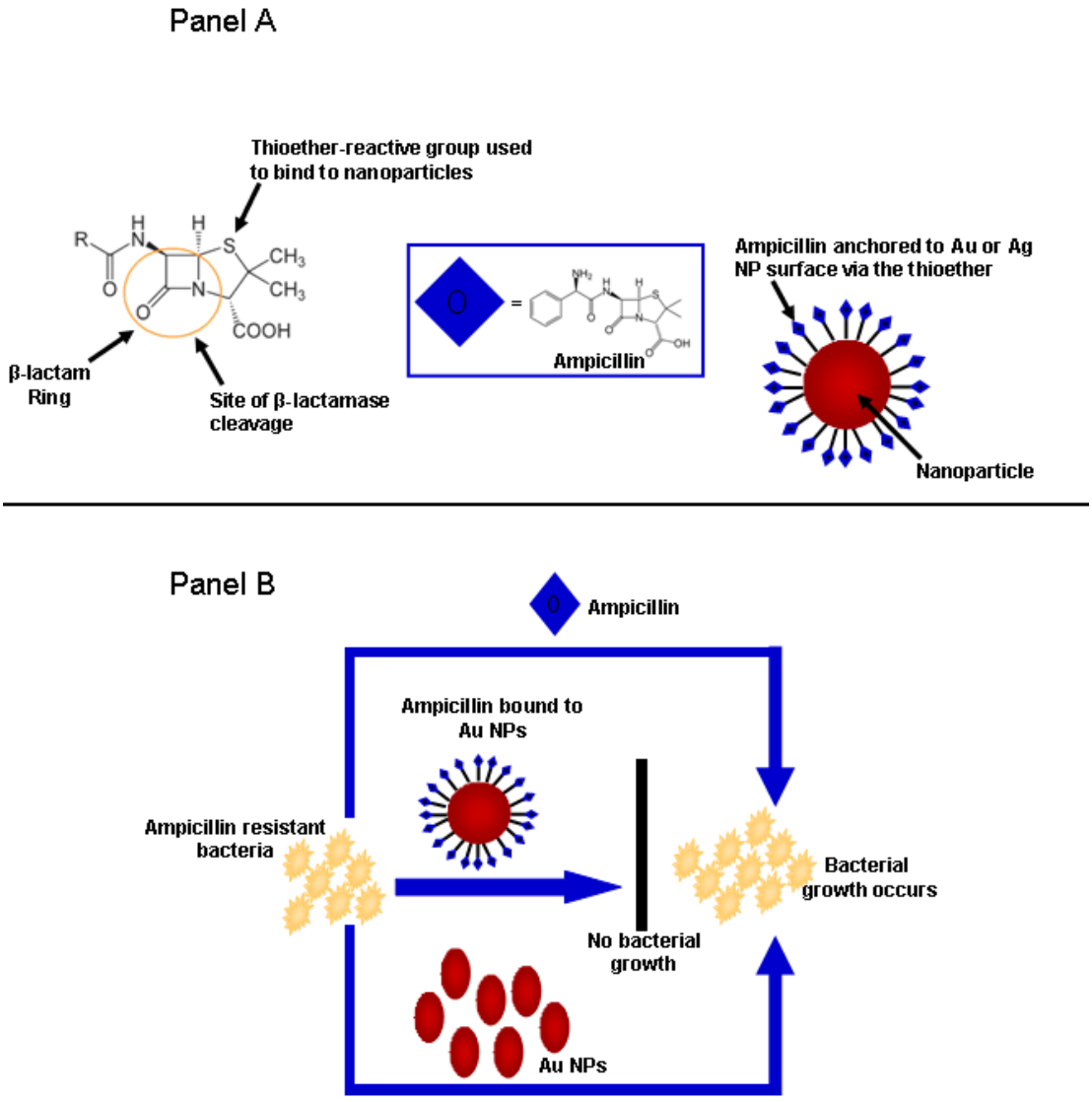


Figure 1S: Structure of ampicillin and nanoparticle scheme

4.Transmission electron microscopy of nanoparticles dispersed into MOPS or Bacto M Staphylococcus growth media

Dilutions of nanoparticles were made in MOPS or Bacto M Staphylococcus media, tests were done, and the original sample was stored at 4 °C in the dark for up to 4 weeks. TEM were captured and the size of the nanoparticles assessed. Nanoparticles did not aggregate in growth media so the size was comparable to those present in the original stock. Histograms and TEM are shown in Figure 2S Panels A and B located on the next two pages.

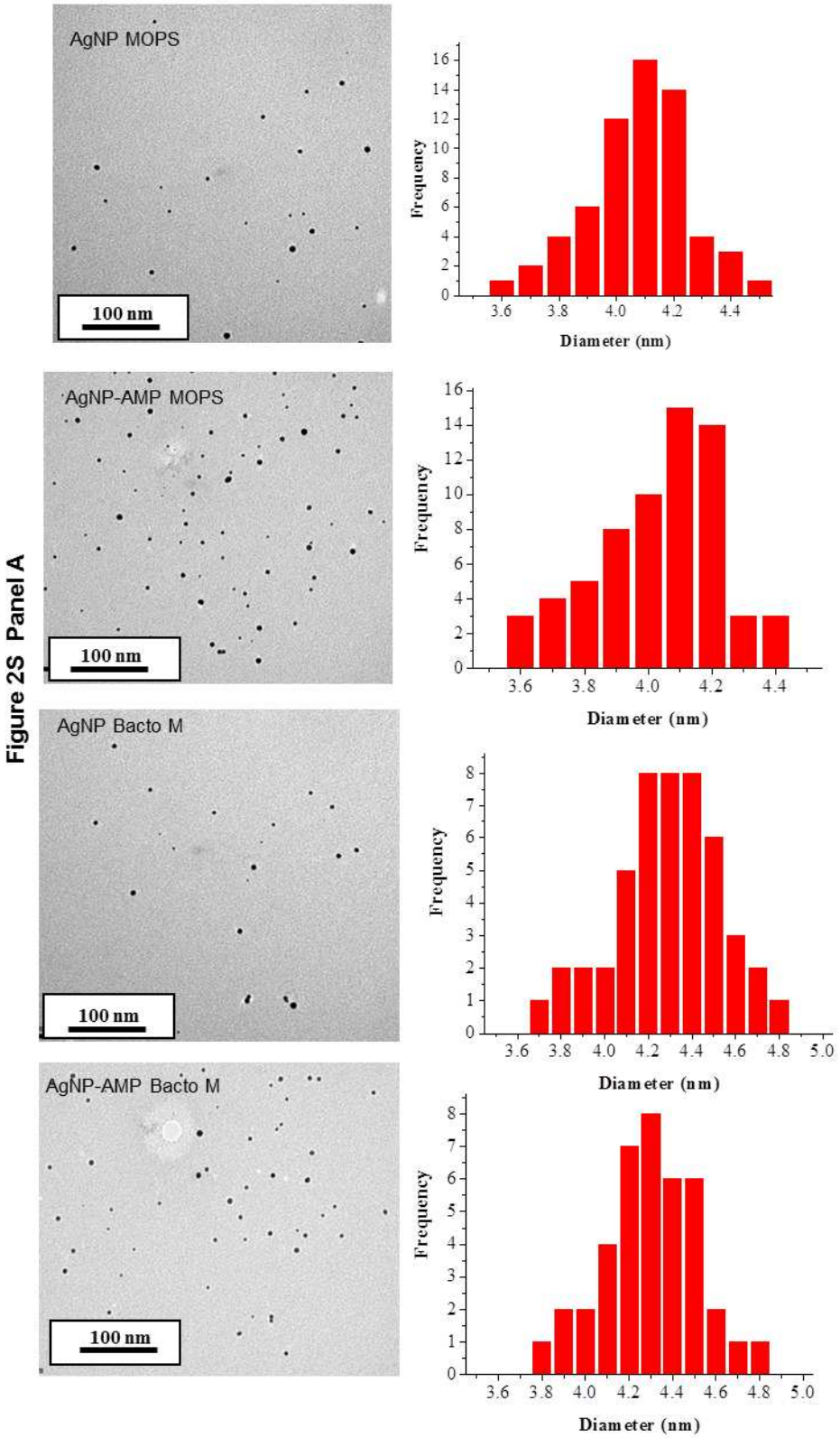
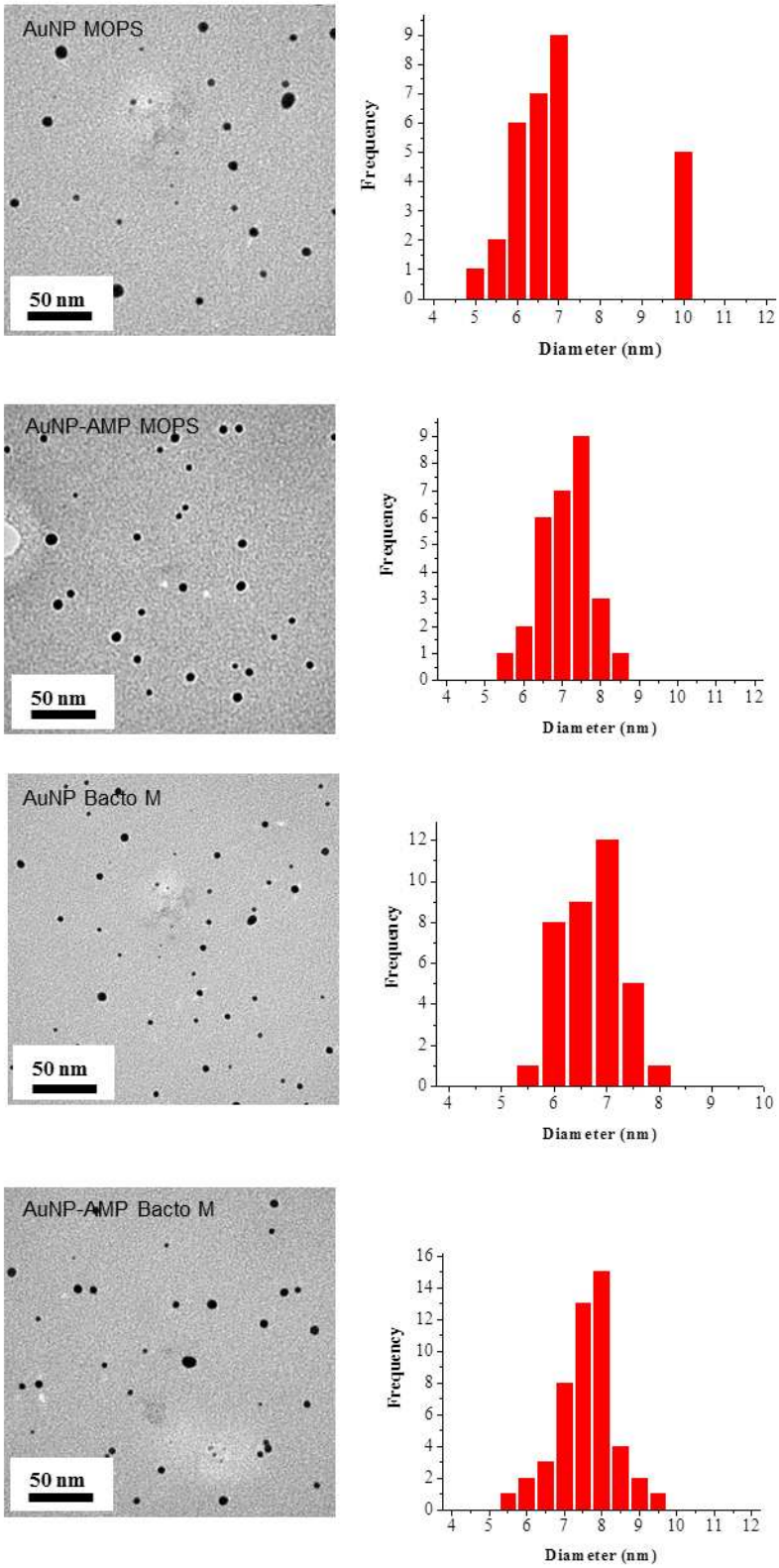


Figure 2S. Transmission electron microscopy results of AgNP with and without ampicillin functionalization stored at 4°C in MOPS and Bacto M Staphylococcus growth media at pH 7.0. Histograms next to TEM results show the predominant size of the nanoparticle present in solution. Panel A contains AgNP and Panel B contains AuNP

Figure 2S Panel B



6. Gold Nanoparticles are nontoxic to Gram-positive and Gram-negative cells. The results of treatment of bacteria with AuNP is shown below in Figure 3S.

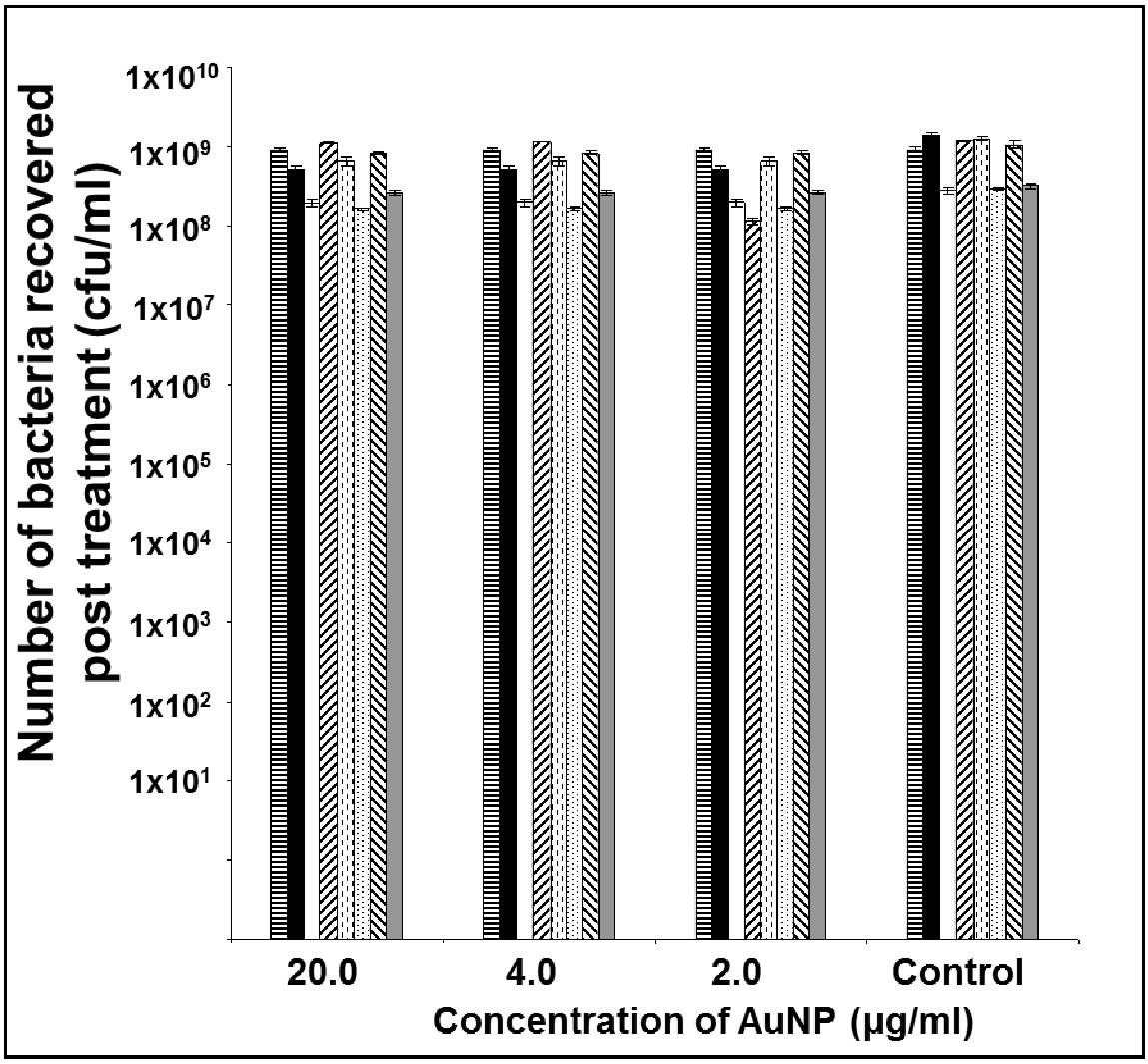


Figure 3S: AuNP have no apparent antimicrobial effect on Gram-positive or Gram-negative bacteria. Organisms tested include: (1) *E. coli* K-12 substrain DH5α (pPCR-Script Amp SK^+) that produces β-lactamase ▤, (2) *E. coli* serotype 0157:H7 strain 86-24 ■, (3) *E. coli* K-12 substrain DH5α□, and (4) *E. coli* serotype 091:H21 strain B2F1▨, (5) *P. aeruginosa* ⁘, (6) *S. aureus*[MRSA] ⁛, *E. aerogenes* ▧, and *V. cholerae* strain TRH7000 ▩. The input inoculum for each test was 5 x 10^9 bacteria. The results are representative of 3 independent experiments done in triplicate. Control samples do not contain AuNP. The error bars denote standard error of the mean (SEM).

REFERENCES

1. **Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck**. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. **45**:493-496.

2. **Clinical and Laboratory Standard Institute**. 2003. Approved standard M2-A8. Performance standards for antimicrobial disk susceptibility tests., *In* . Clinical and Laboratory Standards Institute, Wayne, Pennsylavania 19087 USA.

3. **Clinical and Laboratory Standards Institute**. 2003. M100-S13 (M2). Disk Diffusion Supplemental Tables, *In* . Clinical and Laboratory Standards Institute, Wayne, Pennsylvania 19087 USA.

4. **Clinical and Laboratory Standards Institute.** 2011. Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement., *In* . Clinical and Laboratory Standards Institute, Wayne, Pennsylvania 19087 USA.