Correcting a Fundamental Flaw in the Paradigm for Antimicrobial Susceptibility Testing

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1. Introduction

Multidrug-resistant bacteria are a leading cause of death worldwide and undermine advances in medical and surgical management of multiple diseases (Centers for Disease Control and Prevention, 2013; World Health Organization, 2014). Despite this urgent threat (World Health Assembly, 2014; U.S. White House, 2015), the healthcare industry still relies on a single in vitro bioassay to determine antibiotic efficacy. This assay fails to incorporate environmental factors normally present during host-pathogen interactions in vivo that significantly impact antibiotic efficacy. Here we report that standard antimicrobial susceptibility testing (AST) failed to detect antibiotics that are in fact effective in vivo; and frequently identified antibiotics that were instead ineffective as further confirmed in mouse models of infection and sepsis. Notably, AST performed in media mimicking host environments succeeded in identifying specific antibiotics that were effective in bacterial clearance and host survival, even though these same antibiotics failed in results using standard test media. Similarly, our revised media further identified antibiotics that were ineffective in vivo despite passing the AST standard for clinical use. Supplementation of AST medium with sodium bicarbonate, an abundant in vivo molecule that stimulates global changes in bacterial structure and gene expression, was found to be an important factor improving the predictive value of AST in the assignment of appropriate therapy. These findings have the potential to improve the means by which antibiotics are developed, tested, and prescribed.

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Abstract

The emergence and prevalence of antibiotic-resistant bacteria are an increasing cause of death worldwide, resulting in a global ‘call to action’ to avoid receding into an era lacking effective antibiotics. Despite the urgency, the healthcare industry still relies on a single in vitro bioassay to determine antibiotic efficacy. This assay fails to incorporate environmental factors normally present during host-pathogen interactions in vivo that significantly impact antibiotic efficacy. Here we report that standard antimicrobial susceptibility testing (AST) failed to detect antibiotics that are in fact effective in vivo; and frequently identified antibiotics that were instead ineffective as further confirmed in mouse models of infection and sepsis. Notably, AST performed in media mimicking host environments succeeded in identifying specific antibiotics that were effective in bacterial clearance and host survival, even though these same antibiotics failed in results using standard test media. Similarly, our revised media further identified antibiotics that were ineffective in vivo despite passing the AST standard for clinical use. Supplementation of AST medium with sodium bicarbonate, an abundant in vivo molecule that stimulates global changes in bacterial structure and gene expression, was found to be an important factor improving the predictive value of AST in the assignment of appropriate therapy. These findings have the potential to improve the means by which antibiotics are developed, tested, and prescribed.

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“susceptible” infections respond well to appropriate therapy in 90% of cases, whereas “resistant” infections respond well to these antibiotics in 60% of cases (Doern and Brecher, 2011; Rex and Pfaffer, 2002). (2) Pneumococcal patients treated with antibiotics that failed standard tests (discordant therapy) had similar treatment outcomes as those that passed standard tests (concordant therapy) (Victor et al., 2003). (3) AST-recommended antibiotics failed to clear Salmonella enterica Typhimurium and Enterobacter cloacae in murine models of sepsis (Kubicek-Sutherland et al., 2015; Band et al., 2016). (4) An AST-disqualified antibiotic cleared multidrug-resistant Gram-negative pathogens in murine pulmonary models of infection (Lin et al., 2015). Here we propose that the antimicrobial testing assay should be revamped to account for pathogen conditions in the host, and show several circumstances in which susceptibility testing in host-mimicking media is more accurate than standard AST in predicting antibiotic efficacy in vivo. We have termed this behavior in vivo altered susceptibility (IVAS), providing insight into why some patients fail to respond to certain antibiotics despite passing standard tests for clinical use.

2. Materials and Methods

2.1. Bacterial Strains and Media

Staphylococcal clinical isolates analyzed included USA300, a community-associated methicillin-resistant Staphylococcus aureus (SA) isolate causing the most MRSA infections in the United States (Diekema et al., 2014); and 9 isolates from human sepsis patients (Santa Barbara Hospital, 2016) with various host sites of pathogen origin including blood, wound, urine, sputum (termed MRSA Blood [MT3302]; Wound [MT3315]); MSSA (Blood [MT3305]; Wound [MT3307]; Urine [MT3309]; Sputum [MT3314]); and CoNS (S. epidermidis, blood [MT3320]; S. hydrexinensis, blood [MT3317]; S. warneri, blood [MT3321]). S. pneumoniae (SPN) clinical isolates included D39 (ser. 2) (Lanie et al., 2007), and 5 SPN isolates derived from the nasopharynx of children with sickle cell anemia at risk for invasive pneumococcal disease (Daw 1 [serotype 6]; Daw 2 [serotype 23]; Daw 19 [serotype 6]; Daw 20 [serotype 11]; Daw 25 [serotype 35C]) (Daw et al., 1997; Carter et al., 2014). Gram-negative bacterial isolates included Salmonella spp., Salmonella Typhimurium ATCC 14028, TY1212; and var. 5 (04)-9639; S. Dublin; S. Newport (03)-721; S. Choleraesuis χ3236 (Heithoff et al., 2012; Heithoff et al., 2008); E. coli ATCC 25922; UPEC J96; UPEC ECR12; UPEC ATCC 11775; APEC χ7126; A96 χ7117; EPEC χ2927); RDEC-1 χ2862; EPEC JPN 15; Yersinia pseudotuberculosis (YPii/pB1; IP32953; IP2515; IP2666) (Kubicek-Sutherland et al., 2014); Shigella flexneri ATCC 29903; Providencia stuartii ATCC 29914; Citrobacter freundii ATCC 8090; Klebsiella pneumoniae ATCC 13883; Pseudomonas aeruginosa ATCC 10145. All Staphylococcus strains were isolated on Tryptic Soy Broth Agar (TSA) incubated at 37 °C in ambient air. S. pneumoniae strains were isolated on Columbia Sheep’s Blood Agar (CSBA) and grown in Todd-Hewitt Broth (THB) supplemented with 5% LB broth for Staphylococci, and 5% Lyzed Horse Blood (LHB) for S. pneumoniae; MLM was supplemented with 5% THB for S. pneumoniae D39.

2.2. MIC Assays

The minimum inhibitory concentration (MIC) was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines by either broth or agar dilution (Clinical and Laboratory Standards Institute, 2012a; Wiegand et al., 2008). For determination of MIC in alternative media conditions, bacteria were obtained from overnight culture (Staphylococci and Gram-negative bacteria) or after a 4 h incubation period (S. pneumoniae) in specified medium and diluted into same medium containing 2-fold serial dilutions of antibiotics. To control for the potential effects of pH and media composition for LPM pH 5.5 comparisons, antibiotic resistance and clinical breakpoint designations were calculated by comparing the MIC in LPM medium divided by the MIC in MHB medium at both pH 5.5 and pH 7 (unbuffered) (ratio of LPM pH 5.5/pH 7.0 to MHB pH 5.5/pH 7.2) (Kubicek-Sutherland et al., 2015). MIC values were derived after 20 h incubation, and were the result of at least 6 independent determinations.

2.3. Sodium Bicarbonate Susceptibility Assays

Strains were grown in MHB pH 7.2; unbuffered; MHB adjusted to pH 7.2 with 100 mM Tris/2-hydroxyethylammonium (Fishers Scientific); and DMEM liquid pH 7.4 (containing 44 mM NaHCO3; Difco Becton Dickinson). All other media conditions were adjusted to pH 7.4 with 100 mM Tris including: MHB medium w/NaHCO3; and NaHCO3-free powdered DMEM w/wo NaHCO3. Bacteria were grown overnight in specified medium and diluted as described above. For S. pneumoniae isolates, NaHCO3 assays were performed in MHB medium in the CO2 incubator due to viability considerations since S. pneumoniae isolates tested did not grow in either MHB medium with NaHCO3 in ambient air; or in DMEM in the absence of NaHCO3 in the CO2 incubator. MIC values were the result of at least 6 independent determinations.

2.4. Virulence Assays

2.4.1. Intraperitoneal (i.p.) Infection

S. Typhimurium 14028 (dose of 10^6 CFU) and S. pneumoniae Daw 25 (dose of 9 × 10^7 CFU) were grown overnight in LB or Todd-Hewitt medium with 2% yeast extract, respectively, and sub-cultured to A600 = 0.4, resuspended in 0.15 M NaCl, and administered to mice via the i.p. route of infection.

2.4.2. Intravenous (i.v.) Infection

MRSA USA300 (dose 1 × 10^8 CFU), MRSA Blood (MT3302; dose 1.5 × 10^8 CFU) and MSSA Wound (MT3307; dose 2 × 10^8 CFU) were grown overnight in TSB and sub-cultured to A600 = 0.4; and K. pneumoniae ATCC 13883 (dose of 2 × 10^6 CFU) were grown overnight in LB medium. Strains were resuspended in 0.15 M NaCl and administered i.v. to mice by retro-orbital injection.

2.4.3. Antibiotic Treatment

Infected mice were treated (or mock-treated) with the following dosing regimens beginning 2 h post-infection: azithromycin (100 mg/kg/day), ceftriaxone (40 mg/kg/day), ceftriaxone (50 mg/kg/day), cefalothin (200 mg/kg/day), ciprofloxacin (30 mg/kg/day), colistin (30 mg/kg/day), co-trimoxazole (15 mg/kg/day), daptomycin (10 mg/kg/day), erythromycin (100 mg/kg/day), tetracycline (100 mg/kg/day), or trimethoprim (30 mg/kg/day).

2.4.4. Bacterial Clearance

Mice infected with MSSA Wound (MT3307; dose of 4 × 10^6 CFU) were treated with azithromycin or co-trimoxazole. All drug doses were delivered once every 24 h except cephalothin, ciprofloxacin, colistin, and co-trimoxazole, which were delivered once every 12 h; ceftriaxone and cefotiofur were given every 12 h for MRSA Blood (MT3302) experiments. All drugs were delivered by the i.p. route with the
exception of cephalothin (subcutaneous). Mouse survival was assessed for 10 days post-infection. Equal numbers of male and female 10- to 12-week-old litter-mate C57BL/6j mice were used in all virulence studies. Institutional Animal Care and Use Committee of the University of California, Santa Barbara approved all mouse research protocols undertaken herein.

2.5. Statistical Analysis

Statistical significance for difference in proportions of animal survival was calculated using Chi-square (Epi Info 7, CDC). For all statistical analyses, a significance level (P) of <0.05 was considered to be statistically significant. Degrees of statistical significance are presented as \( **p < 0.001, *p < 0.01, \text{or } P < 0.05 \).

3. Results

3.1. Antibiotic MICs Are Markedly Different When Derived From Host-mimicking Media vs. Standard MHB Medium

A collection of human and veterinary clinical isolates was subjected to antimicrobial susceptibility testing in host-mimicking media vs. standard MHB medium. Four host-mimicking media were examined including (i) Dulbecco’s Modified Eagle Medium (DMEM), a tissue culture medium supporting mammalian cell growth (Dulbecco and Freeman, 1959); (ii) Lacks medium, supporting pneumococcal growth (Lacks, 1966; Trombe et al., 1992); (iii) modified Lacks medium (MLM), simulating the nasopharynx for invasive pneumococcal carriage (Hathaway et al., 2012); and (iv) low-phosphate, low-magnesium medium (LPM pH 5.5) simulating the macrophage phagosome in which many intracellular pathogens reside/replicate (Coombes et al., 2004; Steele-Mortimer, 2008). Emphasis was placed on the identification of pathogen-antibiotic combinations that exhibited altered MICs from host-mimicking media relative to standard MHB medium; and whose MICs crossed clinical breakpoint designations that are used to define isolates as susceptible (“S”), intermediate (“I”), or resistant (“R”), and can impact clinical decision making on appropriate antibiotic therapy. Thus, we sought to identify antibiotics for which a given pathogen is classified as “S” in MHB medium but “R” in host-mimicking media (S to R); and antibiotics for which a given pathogen is classified as “R” in MHB medium but “S” in host-mimicking media (R to S).

3.1.1. Staphylococcus (MRSA; MSSA; CoNS)

A panel of antibiotics used in human and veterinary medicine was tested for efficacy against clinical isolates of methicillin-resistant and -sensitive S. aureus (MRSA/MSSA), and coagulase negative Staphylococcus (CoNS) (Fig. 1). Growth of Staphylococcus in tissue culture medium and modified Lacks medium conferred increased susceptibility to azithromycin, erythromycin, and streptomycin relative to MHB medium (4 to 256-fold; Fig. 1a). Conversely, Staphylococcus exhibited increased resistance to daptomycin and rifampin in modified Lacks medium, and to tetracycline in tissue culture medium, relative to MHB medium (4 to 16-fold). Table 1 lists pathogen-antibiotic combinations that exhibited at least an 8-fold change in MIC when derived in host-mimicking media vs. standard MHB medium (Fig. 2a). Although the MICs obtained from host-mimicking media were comparable to those from MHB medium for approximately two-thirds of cases tested (852/1311), one third of these cases exhibited at least a 4-fold change in MIC, which may signal altered antibiotic susceptibility in vivo. Further, 8.2% (107/1311) of altered MICs derived from the host-mimicking media tested resulted in a change in clinical breakpoint designation, which may impact physician decision making (Fig. 2b). Taken together, these data suggest that inclusion of environmental factors normally present during host-pathogen interactions may improve the predictive value of standard AST in identifying effective antibiotics to treat microbial infections.

3.2. Drug Testing in Host-mimicking Media Improves the Assignment of Appropriate Antibiotic Therapy

Several pathogen-antibiotic combinations that exhibited altered MICs in host-mimicking media were tested for efficacy in murine models of sepsis. We focused on antibiotics whose MICs exhibited at least an 8-fold altered susceptibility in host-mimicking media relative to standard MHB medium, and whose MICs crossed clinical breakpoint designations. This analysis was limited to human and veterinary clinical isolates that also infect mice.

3.2.1. MRSA, MSSA

All mice (10/10) survived infection with MRSA (USA300) following treatment with cephalothin or ceftriaxone (Fig. 3a; \( P = 0.001 \)), identified as efficacious in tissue culture medium even though these agents failed standard testing in MHB medium (R to S; R to I; Supplementary Table...
Similarly, nearly all mice (8/10) survived MSSA (MT3307) infection following treatment with erythromycin ($P_{b} \leq 0.001$), identified as bioactive in tissue culture medium but relatively ineffective by standard testing (I to S). Treatment with co-trimoxazole, often used clinically (Holland et al., 2014), failed to improve survivorship (1/10; $P = 1.0$), as predicted by testing in tissue culture medium but not MHB medium (S to R).

Further analysis was done using a MRSA isolate (MT3302) linked to a fatal case of human sepsis. AST in host-mimicking media was evaluated in an effort to retroactively identify alternative therapeutic options. Treatment with cephalosporins (ceftriaxone or ceftiofur) resulted in high efficacy in murine models of MRSA sepsis (8/10; 7/10; $P_{b} \leq 0.001$; $P_{b} \leq 0.01$). Both of these antibiotics were identified as efficacious in tissue culture medium even though they were rejected by standard testing (R to I). Further, all mice (10/10) survived treatment with daptomycin and ciprofloxacin (Fig. 3a; $P_{b} \leq 0.001$), as predicted by testing of daptomycin in standard MHB medium and tissue culture medium; and of ciprofloxacin in all media examined (Supplementary Table 1a). Notably, testing of daptomycin in modified Lacks medium predicted resistance (S to R), indicating that this drug may be effective against certain types of infections but not others (e.g., systemic vs. localized).

3.2.2. *S. pneumoniae*

Despite passing standard testing in MHB medium, trimethoprim failed to protect mice (0/10) from SPN infection (strain Daw 25) (Fig. 3b; $P = 1.0$), as predicted by testing in modified Lacks medium.
susceptibility in MHB medium but an activity against intracellular pathogens (Carryn et al., 2003). Notably, K. pneumoniae ATCC 25922; UPEC J96; UPEC ATCC 11775; EPEC to treatment with ciprofloxacin (8/10; Supplementary Table 1b). Further, all mice (10/10) survived following treatment with ceftriaxone (P < 0.001), for which susceptibility was indicated in all media tested.

3.2.3. Gram-negative Bacteria

Colistin, a drug of last resort (Yahav et al., 2012), failed to protect mice (1/10) from infection with S. Typhimurium (ST74028) (Fig. 3c; P = 0.01), as predicted by testing in tissue culture medium (S to R; Supplementary Table 1c). Conversely, all mice (10/10) survived treatment with ciprofloxacin (P < 0.001), for which susceptibility was indicated in all media tested. Additionally, most mice (8/10) survived infection with K. pneumoniae following treatment with tetracycline (P < 0.001). Such efficacy was predicted by standard testing in MHB and LPM pH 5.5 media (S to S), which mimics the macrophage phagosome wherein K. pneumoniae resides and replicates during infection (Cano et al., 2015) (Supplementary Table 1f). Such efficacy was comparable to treatment with ciprofloxacin (8/10; P < 0.001) that has established activity against intracellular pathogens (Carryn et al., 2003). Notably, testing of tetracycline in tissue culture medium predicted resistance (S to R), suggesting that testing in media that reflect the intracellular lifestyle of K. pneumoniae is a more accurate predictor of treatment outcome for this pathogen.

3.2.4. Bacterial Clearance

Bacterial clearance from circulation in the blood was investigated following treatment with antibiotics predicted as highly efficacious by testing in standard MHB medium (co-trimoxazole) or tissue culture medium (azithromycin), respectively (Supplementary Table 1a). Treatment with the AST-recommended antibiotic, co-trimoxazole, was ineffective in MSSA (MT3307) clearance as predicted by testing in host-mimicking media (S to R) (Fig. 3d). This treated cohort exhibited a progressive bacteremia (up to 2.5 × 10^5 colony forming units (CFU)/ml blood by day 6), with all mice (10/10) succumbing to infection by day 10 (open boxes). Such efficacy was comparable to that of untreated animals (open circles). Conversely, as predicted by testing in tissue culture medium, azithromycin was able to clear MSSA from circulation, with all mice (10/10) surviving the infection and harboring ≤ 2 × 10^5 CFU/ml in the blood at day 10 (closed boxes; P < 0.001). These data suggest that

### Table 1

| Drug                  | Target      | Pathogen         | Host-mimicking media                        | Clinical breakpoint |
|-----------------------|-------------|------------------|----------------------------------------------|---------------------|
| **Increased susceptibility** |             |                  |                                              |                     |
| Cephalothin           | Cell wall   | MRSA^1            | DMEM/MHB + NaHCO_3                          | R to S              |
| Ceftriaxone           | Cell wall   | MRSA^1-3          | DMEM/MHB + NaHCO_3                          | R to S, I           |
| Oxacillin             | Cell wall   | MRSA^2            | DMEM                                         | R to S              |
| Ampicillin            | Cell wall   | CoNS; SPN^2-2     | MLM/DMEM                                     | R to S, I           |
| Trimethoprim          | Folate      | SPN^2             | DMEM                                         | R to S, I           |
| Azithromycin          | Protein     | CoNS^1-3; SPN^2-2 | MLM/MBH + NaHCO_3                          | R to S, I           |
| Erythromycin          | Protein     | MSSA^2-3, CoNS^1  | DMEM/MLM/MHB + NaHCO_3                      | R to I, S           |
| Streptomycin          | Protein     | MRSA^1-2, MSSA^1-4| DMEM/MLM                                     | R, I to S           |
| **Decreased susceptibility** |             |                  |                                              |                     |
| Colistin              | Membrane    | ST; PA            | DMEM                                         | S to R              |
| Daptomycin            | Membrane    | MRSA^1-3, MSSA^1-4; CoNS^1-3 | DMEM/MHB + NaHCO_3                          | S to R, I           |
| Ceftriaxone           | Cell wall   | SPN^2             | DMEM                                         | S to R, I           |
| Ampicillin            | Cell wall   | SPN^2; CF         | DMEM/MHB + NaHCO_3                          | S, I to R, I        |
| Trimethoprim          | Folate      | MSSA^1; SPN^2-4   | DMEM/MLM                                     | S to R              |
| Co-Trimoxazole        | Folate      | MSSA^1; SPN^2-4   | DMEM/MLM                                     | S to R, I           |
| Gentamicin            | Protein     | PA                | DMEM/MHL                                     | S to R              |
| Tetracycline          | Protein     | KPN; CF; ST; SC; EC^1-3; YPF^1-8 | DMEM/MHB + NaHCO_3 | S to R, I           |
| Enrofloxacin          | DNA         | DNA              | DMEM/MBL                                     | S to R, I           |

Depicted are pathogen-antibiotic combinations that exhibited altered MICs derived from host-mimicking media relative to standard MHB medium; and whose MICs crossed clinical breakpoint designations that are used to define isolates as susceptible (“S”), intermediate (“I”), or resistant (“R”), and advise on patient therapy. R to S refers to an “R” classification when tested for susceptibility in MHB medium but an “S” classification in host-mimicking media. MRSA^1-3 (USA 300; Blood; Wound); CoNS^1-3 (S. epidermidis; S. lugdunensis; S. warneri); SPN^1-3 (serotype 6; 6; 23; 11; 35C); MSSA^1-4 (Wound; Sputum; Urine; Blood; ST (S. Typhimurium); PA (P. aeruginosa); CF (C. freundii); KPN (K. pneumoniae); SC (S. Choleraesuis); EC^1-4 (E. coli ATCC 25922; UPEC J06; UPEC ATCC 11775; EPEC y2927); YPF^1-8 (YPB; IP32953; IP2515; IP2666). Clinical breakpoint concentrations for listed drugs (Societe Francaise de Microbiologie, 2012, Clinical and Laboratory Standards Institute, 2012b, Clinical and Laboratory Standards Institute, 2013, Clinical and Laboratory Standards Institute, 2014, European Committee on Antimicrobial Susceptibility Testing, 2016, Fuchs et al, 1997, Landman et al, 2008).

Fig. 2. Comparison summary of MICs derived from host-mimicking media versus standard MHB medium. (a) Colored regions depict the fraction of pathogen-antibiotic combinations tested that exhibited a fold-change in MICs (increased susceptibility or resistance) when derived in host-mimicking media (DMEM, MLM, LPM pH 5.5) relative to standard MHB medium (test/standard condition); ≤2-fold (green), 4-fold (yellow), >8-fold (red). (b) Depicted are percentages of pathogen-antibiotic combinations that resulted in altered MICs that crossed clinical breakpoint designations, used to define isolates as susceptible (“S”), intermediate (“I”), or resistant (“R”), that can impact clinical decision making on appropriate antibiotic therapy (Clinical and Laboratory Standards Institute, 2012a; European Committee on Antimicrobial Susceptibility Testing, 2014).
drug testing in host-mimicking media improves the predictive value of standard AST in the assignment of appropriate therapy.

3.3. Addition of NaHCO3 to Standard MHB Medium Improves the Accuracy of Antibiotic Efficacy In Vivo

We suspected that sodium bicarbonate (NaHCO3) may be a key in vivo molecule contributing to antibiotic susceptibility for a number of pathogens for the following reasons. NaHCO3 serves as an abundant ionic factor present in mammalian tissues that stimulates global changes in bacterial structure, gene expression, and membrane permeability that correspond to increased susceptibility to human cationic antimicrobial peptides (Dorschner et al., 2006). NaHCO3 is present in nearly all host-mimicking media examined that resulted in altered antibiotic susceptibility relative to MHB medium. Thus, we evaluated whether supplementation of standard MHB medium with physiological levels of NaHCO3 improved the predictive value of the AST standard for clinical use. This analysis was initially focused on Staphylococcus-antibiotic combinations that exhibited at least an 8-fold change in MIC in tissue culture medium vs. MHB medium, representing 13.5% (31/230) of combinations examined (Fig. 1a, top panel).

We investigated the fold-change between MICs derived in MHB medium in the presence/absence of NaHCO3 (test/standard condition; left of slash); and in tissue culture medium in the absence/presence of NaHCO3 (test/standard condition; right of slash) (Fig. 4a; Supplementary Table 2a). Increased susceptibility is depicted in blue; increased resistance is depicted in red. Four phenotypic classes were identified.

Class 1 (21/31). Addition of NaHCO3 to MHB medium resulted in MICs similar to tissue culture medium; its removal from tissue culture medium resulted in MICs similar to MHB medium (azithromycin, erythromycin, tetracycline).

Class 2 (5/31). Addition of NaHCO3 to MHB medium resulted in MICs similar to tissue culture medium; its removal from tissue culture medium had no effect on the MIC (ceftriaxone, ceftiofur).

Class 3 (2/31). Addition of NaHCO3 addition to MHB medium had no MIC effect; its removal from tissue culture medium resulted in MICs similar to MHB medium (oxacillin).

Class 4 (3/31). Addition/removal of NaHCO3 had no effect on MICs in MHB medium or tissue culture medium (trimethoprim). These data indicate that addition of NaHCO3 to MHB medium restored the altered susceptibility observed in tissue culture medium in 83.9% (26 of 31) of cases tested.

Next, we examined whether physiological levels of NaHCO3 in MHB medium were required to stimulate the altered susceptibility observed in tissue culture medium. A dose response analysis of MRSA (USA300; MT3302) and MSSA (MT3307) clearance from blood circulation was examined following treatment with antibiotics predicted as highly effective via testing in standard MHB medium (co-trimoxazole, open boxes) or tissue culture medium (DMEM) (azithromycin, closed boxes), respectively. Untreated mice (open circles); expired mice (gray region); Colony Forming Units (CFU); Limit of Detection (LOD) = 100 CFU/ml (Patterson et al., 2013). Ten mice were evaluated per cohort. **P < 0.001, *P < 0.01, or *P < 0.05.
suggest that NaHCO₃ may be a key in vivo component contributing to antibiotic susceptibility for a number of pathogens. Supporting this suggestion, supplementation of MHB medium with physiological levels of NaHCO₃ also resulted in altered drug susceptibilities in *S. pneumoniae* and *Salmonella* spp. isolates (Fig. 4c; Supplementary Table 2b, c). Furthermore, many altered MICs crossed clinical breakpoint designations (listed in Table 1), and such predicted changes in antibiotic efficacy were confirmed in mouse models of infection and sepsis (Fig. 2a); e.g., MRSA (cephalothin [R to S]; ceftriaxone [R to I]); and MSSA (erythromycin [I to S]); (Supplementary Table 2a). These findings suggest that supplementation of standard MHB medium with physiological levels of NaHCO₃ improved the predictive value of AST in the assignment of appropriate antibiotics for therapeutic intervention.

### 4. Discussion

Multidrug-resistant bacteria are a significant cause of sepsis, the most common cause of death in hospitalized patients, with an annual incidence of 1 million cases and 200,000 deaths in the U.S. alone (Deutschman and Tracey, 2014). This dire perspective reflects the failed efforts to fully contain bacteria with the misuse of antibiotics, and the legal, financial, and scientific hurdles to discovering new ones. We demonstrate that one viable approach to address this alarming threat is to incorporate host-mimicking media in standard AST methods for clinical use. Validation of the improved predictive value of AST in the assignment of appropriate antibiotic therapy was provided in several Gram-positive and -negative animal models of infection and sepsis. Our findings suggest that standard AST may be hindering optimal patient treatment, and slowing the process of discovery of new, effective, and safe antibiotics because it disqualifies efficacious compounds. Sensitivity testing that accounts for the biology of a pathogen in the context of its host may enable the re-purposing of omitted antibiotics while aiding the discovery of new ones by screening compounds under conditions that more accurately reflect the host milieu.

Altered drug susceptibility in vivo provides insight as to why some patients fail to respond to certain antibiotics despite passing standard susceptibility tests. Our findings with a MRSA isolate from a deceased patient provide a clear example as antibiotics omitted by standard AST...
were highly efficacious in bacterial clearance. If these alternative therapeutic options had been made available to clinicians managing this case, it may have changed the patient outcome. Additionally, we show that supplementation of standard MHB medium with physiological levels of sodium bicarbonate improved the predictive value of AST in the assignment of appropriate therapy. The molecular basis likely involves the role of NaHCO₃ as an abundant ionic factor that stimulates global changes in bacterial structure and gene expression, leading to alterations in bacterial cell wall thickness and membrane permeability that correspond with increased susceptibility to human cationic antimicrobial peptides (Dorschner et al., 2006). Two potential alternative mechanisms include the role of bicarbonate in the maintenance of blood pH (Hermansen and Osnes, 1972; Rosenthal, 1948); and/or the inhibition of growth and viability of periodontal pathogens (Newbrun et al., 1984). However, these mechanisms are unlikely to play a role in the improved predictive value of AST due to the inclusion of Tris buffer in the test media to preclude bicarbonate-mediated pH fluctuations that can affect antibiotic potency and bacterial cell viability.

Standard AST in clinical use has likely contributed to the alarming rise of multidrug-resistant bacteria in hospitals because high doses of ineffective antibiotics are given to infected patients without the knowledge that the host environment may render bacteria inherently resistant to the antibiotics prescribed to kill them. Based on the findings of this study, rather than extending the dose/duration of an antibiotic that is not effective, physicians might consider that the more appropriate approach is to prescribe a totally different antibiotic. Standard AST in combination with host-mimicking media may serve as a valuable tool in advising clinicians on appropriate antibiotic therapy. Antibiotics identified by both approaches were efficacious in every animal model examined; thus, such cases should bestow high confidence in clinical decision making on appropriate therapy. Conversely, physicians should exercise caution in cases where marked MIC disparities occur between testing in host-mimicking media vs. standard MHB medium. Further, predicted effects of bicarbonate in the maintenance of blood pH can result in unanticipated changes in antimicrobial efficacy (Ambrose et al., 2007; Deziel et al., 2005). (2) Bacterial community composition can compromise antibiotic efficacy (antibiotic deactivation or biofilm production provides passive resistance for all microbes within a polymicrobial environment) (Vega and Gore, 2014; Sorg et al., 2016). (3) Antimicrobial selection is based on drug concentrations achieved in plasma, but concentrations achieved in different tissues and sites of infection may be greater or less depending on the drug’s properties (pH at the infection site or within an organelle can dictate lipid solubility of the drug or its distribution in cells and tissues) (Logan et al., 2012). (4) Antibiotic resistance may be inadvertently triggered by diet, underlining conditions in the patient, or by clinical interventions that may disrupt drug efficacy (ascorbic acid treatment of urinary tract infections to lower urine pH) (Carlsson et al., 2001). (5) Many patients that develop multidrug-resistant infections have comorbidities, immunosuppressive therapy and/or the presence of invasive medical devices that impact susceptibility to indicated pathogens (Paterson and Bonomo, 2005).

Our findings suggest that the susceptibility testing in media that reflect the host milieu will not only improve the predictive value of AST in the assignment of appropriate antibiotic therapy, but also provides a new paradigm for drug discovery and therapeutic intervention for infectious diseases. However, such testing will always be open to further improvement, especially as we learn more about the subtle nuances of host-pathogen interactions in natural environments that influence the impact of antibiotics on bacterial clearance (e.g., virulence factors, ecological factors, and cell physiological parameters).

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Conflicts of Interests

The authors declare no competing financial interests.

Author Contributions

Experiments were conducted by S.C.E., D.M.H., G.T. and LB. Data was analyzed by S.C.E., D.M.H., LB, J.K.H., J.D.M., J.W.S., and M.J.M. The manuscript was prepared by S.C.E., D.M.H., LB., and M.J.M. and the study was planned and directed by M.J.M.

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Appendix A. Supplementary data

Supplementary data to this article can be found online. Reprints and permissions information is available online at http://dx.doi.org/10.1016/j.ebiom.2017.05.026

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