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Research Paper

Correcting a Fundamental Flaw in the Paradigm for Antimicrobial Susceptibility Testing

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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.

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 continues to rely on a single bioassay standardized in 1961 by the World Health Organization to determine antibiotic efficacy (World Health Organization, 1961). Although this bioassay has been immensely valuable for several decades, it is fundamentally flawed because it is based largely on in vitro efficacy, and often fails to correlate with patient outcome (Kubicek-Sutherland et al., 2015). Reliance on this bioassay may have inadvertently contributed to the rise in multidrug-resistant bacteria because it disqualifies efficacious compounds (Diene and Rolain, 2014).

A key parameter that guides decisions regarding antimicrobial therapy is the clinical breakpoint: the antimicrobial concentrations that are used to define isolates as susceptible ("S"), intermediate ("I"), or resistant ("R") (Clinical and Laboratory Standards Institute, 2012a; European Committee on Antibiotic Susceptibility Testing, 2014). Clinical breakpoints are established by a sequential procedure. (1) In vitro efficacy is assessed by standard antimicrobial susceptibility testing (AST), which determines the minimum inhibitory concentration ("MIC") of antibiotics to which a pathogen is sensitive. (2) Pharmacokinetic/pharmacodynamic (PK/PD) parameters are measured in animals (dosing, distribution, localization). (3) Efficacy/toxicity is established in animals for a limited number of model pathogens. (4) Dosing protocols are validated with limited patient clinical data. Unfortunately, this testing pipeline is fundamentally unsound because the first step, AST, is performed on Mueller-Hinton Broth (MHB), a rich laboratory medium that fails to recapitulate most aspects of host environments. So, the fact that clinical breakpoints are based on a foundational assay performed in vitro raises questions as to how relevant they are to patient outcome.

Supporting this notion, several reports suggest that the clinical predictive value of AST in the assignment of appropriate therapy is limited. (1) Clinical observations have given rise to the "90–60" rule:

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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 Pfaller, 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 Cottage 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. lugdunensis*, 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 Lane; *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* (YPIII/pIB1; 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 2% yeast extract incubated at 37 °C in a 5% CO_2 incubator. Gram negative bacteria were isolated on Luria-Bertani (LB) agar (Davis et al., 1980) incubated at 37 °C or 28 °C (*Yersinia*) in ambient air. Standard AST broth medium is Mueller–Hinton Broth (MHB) supplemented with $CaCl_2$ and $MgCl_2$ to make cation-adjusted MHB (Ca-MHB) (Clinical and Laboratory Standards Institute, 2012a). AST was also performed in Dulbecco's Modified Eagle Medium (DMEM (Dulbecco and Freeman, 1959); High Glucose [Life Technologies]); Lacks medium (Lacks, 1966); modified Lacks medium (MLM) (Hathaway et al., 2012); or low phosphate, low magnesium medium (LPM) (Coombes et al., 2004). DMEM cultures were incubated in a 5% CO_2 incubator; all other conditions were incubated in ambient air. To facilitate growth, DMEM was supplemented with 5% LB broth for *Staphylococci*, and 5% Lysed 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(hydroxymethyl)aminomethane (Fisher Scientific); and DMEM liquid pH 7.4 (containing 44 mM $NaHCO_3$; Difco/Becton Dickinson). All other media conditions were adjusted to pH 7.4 with 100 mM Tris including: MHB medium w/$NaHCO_3$; and $NaHCO_3$-free powdered DMEM w/wo $NaHCO_3$. Bacteria were grown overnight in specified medium and diluted as described above. For *S. pneumoniae* isolates, $NaHCO_3$ assays were performed in MHB medium in the CO_2 incubator due to viability considerations since *S. pneumoniae* isolates tested did not grow in either MHB medium with $NaHCO_3$ in ambient air; or in DMEM in the absence of $NaHCO_3$ in the CO_2 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 10^2 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 $A_{600} = 0.4$, 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 of 2×10^8 CFU) were grown overnight in TSB and sub-cultured to $A_{600} = 0.4$; and *K. pneumoniae* ATCC 13883 (dose of 2×10^8 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), ceftiofur (40 mg/kg/day), ceftriaxone (50 mg/kg/day), cephalothin (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 4×10^8 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 ceftiofur 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 $^{***}P < 0.001$, $^{**}P < 0.01$, 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 and whose altered MICs crossed clinical breakpoint designations that advise on patient therapy. For example, antibiotics for which MRSA was classified as "R" in MHB medium, but classified as "S" in tissue culture medium (cephalothin); and antibiotics for which MSSA was classified as "I" in MHB medium, but classified as "S" in tissue culture medium (erythromycin) (Supplementary Table 1a). Notably, although many pathogen-antibiotic combinations have significant changes in MIC in host-mimicking media, many do not cross breakpoint designations (R to S; S to R) and would not alter physician making on appropriate therapy. For example, 3/3 MRSA isolates exhibited a 4- to 32-fold increased susceptibility to oxacillin in tissue culture medium, but the "altered MICs" of two MRSA isolates did not cross clinical breakpoints. Thus, they remain "Resistant" to oxacillin as defined by AST standards for clinical use.

3.1.2. S. pneumoniae

Altered MICs were also examined for *S. pneumoniae* clinical isolates tested in host-mimicking media vs. standard MHB medium. Most *S. pneumoniae* strains tested showed increased susceptibility to azithromycin in tissue culture medium and modified Lacks medium relative to MHB medium; and increased resistance to daptomycin and trimethoprim in modified Lacks medium (4 to 32-fold; Fig. 1b). Many *S. pneumoniae* MICs derived in host-mimicking media crossed clinical breakpoint designations (listed in Table 1); e.g., antibiotics for which *S. pneumoniae* was classified as "S" in MHB medium, but classified as "R" in modified Lacks medium (trimethoprim); and those for which *S. pneumoniae* classified was "R" in MHB medium, but classified as "S" in modified Lacks medium (azithromycin) (Supplementary Table 1b).

3.1.3. Gram-negative Bacteria

Antibiotic efficacy was also examined for Gram-negative bacterial isolates tested in host-mimicking media vs. standard MHB medium. A subset of these antibiotics (10 of 20), which were not subject to acute pH and/or media composition effects under LPM pH 5.5 conditions (Kubicek-Sutherland et al., 2015), were also interrogated. Several Gram-negative bacteria were associated with increased resistance to colistin or polymyxin B in tissue culture medium and LPM pH 5.5 conditions relative to MHB medium (4 to 512-fold) (Fig. 1c). Growth of *Yersinia* spp. (4 of 4 isolates) was associated with increased susceptibility to trimethoprim and co-trimoxazole in tissue culture medium relative to MHB medium (8 to 64-fold). Many Gram-negative bacteria MICs derived in host-mimicking media crossed clinical breakpoint designations (listed in Table 1); e.g., *Salmonella* Typhimurium (*ST*) susceptibility to colistin was classified as "S" in MHB medium but "R" in tissue culture medium (Supplementary Table 1c–f).

3.1.4. Comparison Summary of MICs Derived From Host-mimicking Media vs. Standard MHB Medium

We evaluated the percentage of pathogen-antibiotic combinations that resulted in altered MICs when derived from 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

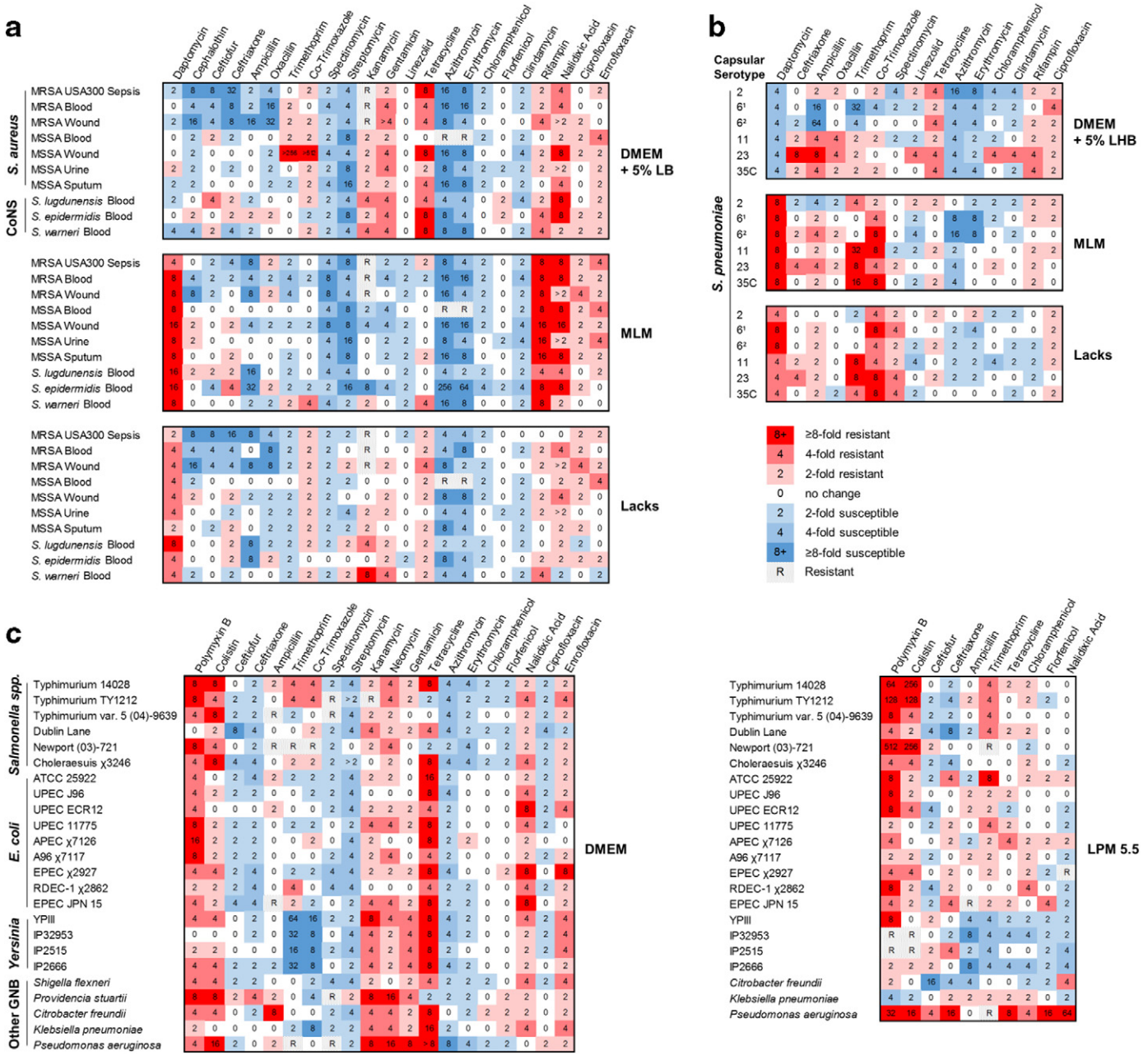

Fig. 1. Comparison of pathogen-antibiotic combinations that exhibited altered MICs derived from host-mimicking media relative to standard MHB medium. A panel of antibiotics was screened for altered MICs against (a) *Staphylococcus* spp., (b) *S. pneumoniae*, and (c) Gram-negative bacteria when tested in Dulbecco's Modified Eagle Medium (DMEM), Lacks medium, modified Lacks medium (MLM), low-phosphate, low-magnesium medium (LPM pH 5.5) relative to standard MHB medium, according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2012a; Wiegand et al., 2008). Values depict the fold-change in MICs when derived in host-mimicking media relative to standard MHB medium (test/standard condition). Increased susceptibility depicted in blue; increased resistance depicted in red. MIC values were obtained from at least 6 independent determinations.

1a). Similarly, nearly all mice (8/10) survived MSSA (MT3307) infection following treatment with erythromycin ($P < 0.001$), 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; $P < 0.001$; $P < 0.01$). 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; $P < 0.001$), 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

Table 1
AST in host-mimicking media identifies MICs that cross clinical breakpoint designations which advise on patient therapy.

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[3]	DMEM	R to S
Ampicillin	Cell wall	CoNS[1]; *SPN*[1,2]	MLM/DMEM	R, I to S
Trimethoprim	Folate	*SPN*[2]	DMEM	R to S
Azithromycin	Protein	CoNS[1,3]; *SPN*[1,2]	MLM/MHB + $NaHCO_3$	R to S, I
Erythromycin	Protein	MSSA[1,2]; CoNS[1]	DMEM/MLM/MHB + $NaHCO_3$	R, I to I, S
Streptomycin	Protein	MRSA[1]; MSSA[1–4]	DMEM/MLM	R, I to S
Decreased susceptibility				
Colistin	Membrane	*ST*; *PA*	DMEM	S to R
Daptomycin	Membrane	MRSA[2,3]; MSSA[1–4]; CoNS[1–3]	MLM	S to R
Ceftriaxone	Cell wall	*SPN*[3]	DMEM	S to R
Ampicillin	Cell wall	*SPN*[3]; CF	DMEM/MHB + $NaHCO_3$	S, I to R, I
Trimethoprim	Folate	MSSA[1]; *SPN*[4, 5]	DMEM/MLM	S to R
Co-Trimoxazole	Folate	MSSA[1]; *SPN*[4, 5]	DMEM/MLM	S to R, I
Gentamicin	Protein	*PA*	DMEM	S to R
Tetracycline	Protein	*KPN*; CF; *ST*; *SC*; *EC*[1–3]; *YP*[1–4]	DMEM/MHB + $NaHCO_3$	S to R, I
Enrofloxacin	DNA	*EC*[4]	DMEM	S to R

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*[1–5] (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 J96; UPEC ATCC 11775; EPEC χ2927); *YP*[1–4] (YPIII; 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).

(S to R; 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 (*ST*14028) (Fig. 3c; $P = 1.0$), 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 $\leq 2 \times 10^3$ CFU/ml in the blood at day 10 (closed boxes; $P < 0.001$). These data suggest that

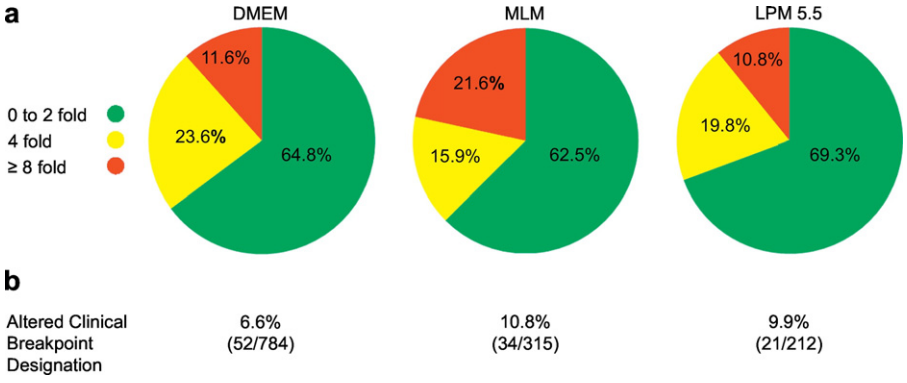

Fig. 2. Comparison summary of MICs derived from host-mimicking media versus standard MHB medium. (a) Colored regions depict the fraction of pathogen-antibiotic combinations tested that exhibited a fold-change in MICs (increased susceptibility or resistance) when derived in host-mimicking media (DMEM, 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 Antibiotic Susceptibility Testing, 2014).

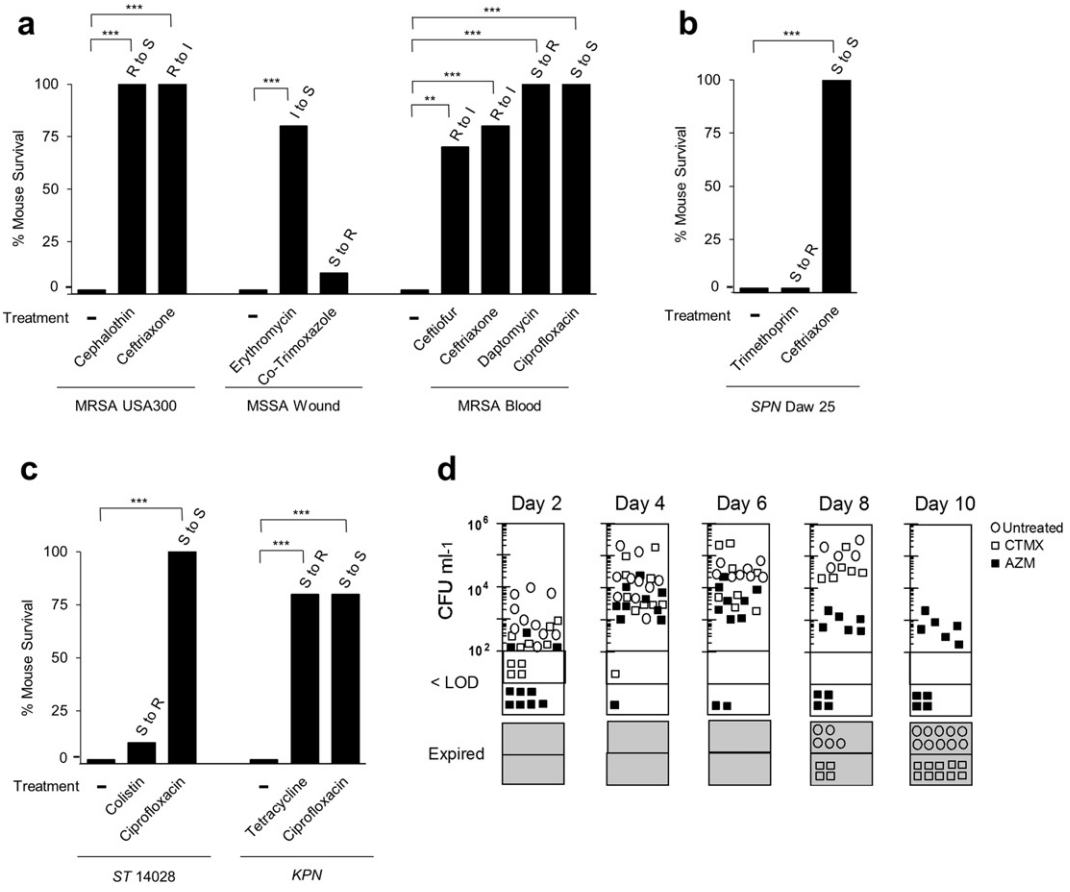

Fig. 3. Antibiotic susceptibility testing in host-mimicking media improves the predictive value of AST in the assignment of appropriate antibiotic therapy in murine models of sepsis. Pathogen-antibiotic combinations that exhibited altered MICs in host-mimicking media relative to standard MHB medium and whose MICs crossed clinical breakpoint designations were evaluated in murine sepsis models of (a) *S. aureus* (MRSA [USA300]; MSSA Wound [MT3307]; MRSA Blood [MT3302]); (b) *S. pneumoniae* (*SPN* Daw25); and (c) *S.* Typhimurium (*ST* 14028) and *K. pneumoniae (KPN* ATCC13883). (d) 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 $^{*}P < 0.05$.

drug testing in host-mimicking media improves the predictive value of standard AST in the assignment of appropriate therapy.

3.3. Addition of $NaHCO_3$ to Standard MHB Medium Improves the Accuracy of Antibiotic Efficacy In Vivo

We suspected that sodium bicarbonate ($NaHCO_3$) may be a key in vivo molecule contributing to antibiotic susceptibility for a number of pathogens for the following reasons. $NaHCO_3$ 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). $NaHCO_3$ 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 $NaHCO_3$ 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 $NaHCO_3$ (test/standard condition; left of slash); and in tissue culture medium in the absence/presence of $NaHCO_3$ (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 $NaHCO_3$ 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 $NaHCO_3$ 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 $NaHCO_3$ 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 $NaHCO_3$ had no effect on MICs in MHB medium or tissue culture medium (trimethoprim). These data indicate that addition of $NaHCO_3$ 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 $NaHCO_3$ 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) strains revealed that physiological levels of $NaHCO_3$ (~25 mM) (Mayo Clinic, 2017) were necessary to induce altered antibiotic susceptibility in MHB medium (Fig. 4b). These data

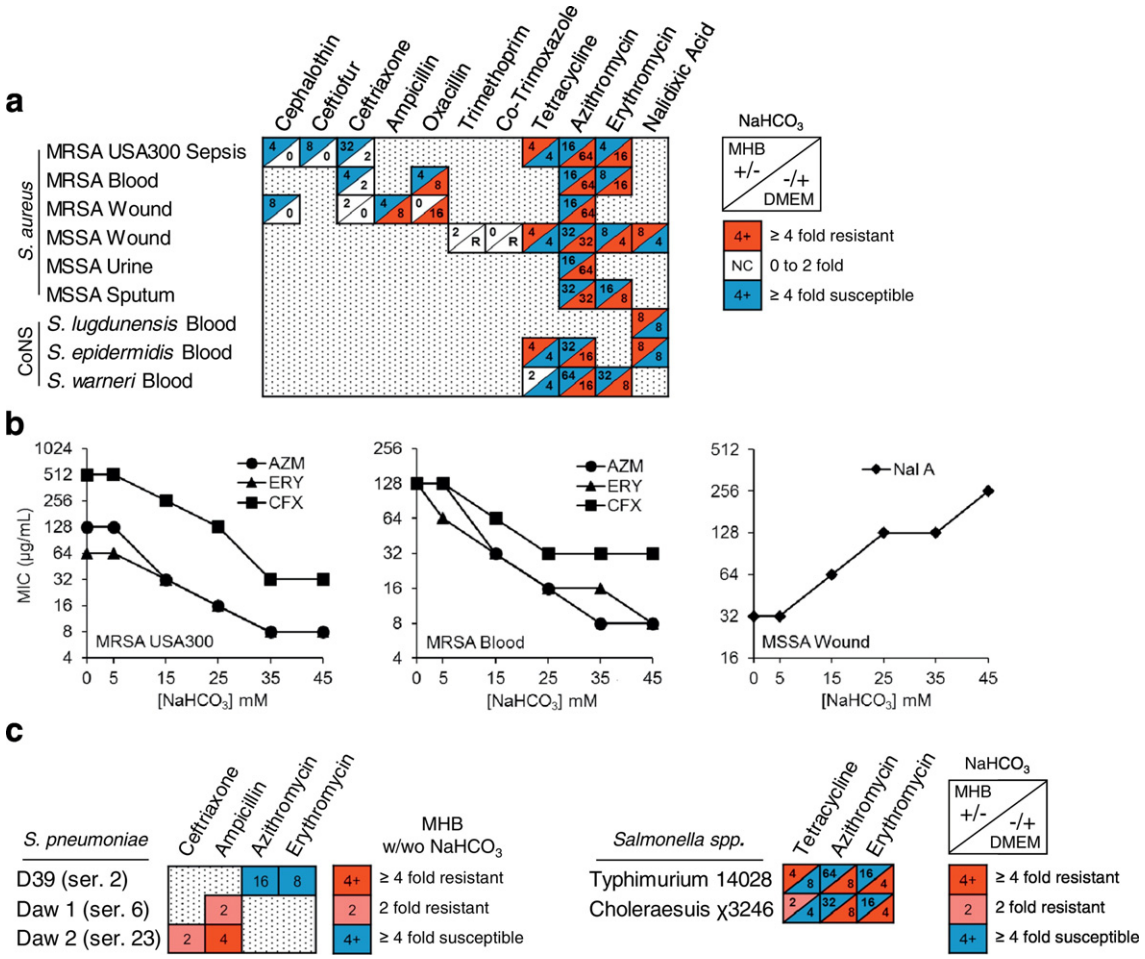

Fig. 4. Supplementation of standard MHB medium with physiological levels of $NaHCO_3$ improves the predictive value of AST in the assignment of appropriate antibiotics for therapeutic intervention. (a) *S. aureus* exhibiting at least an 8-fold change in MIC in tissue culture medium (DMEM) vs. MHB medium were subjected to susceptibility tests in the presence and absence of physiological levels of $NaHCO_3$. Values represent MIC fold-change when derived in MHB medium in the presence/absence of $NaHCO_3$ (test/standard condition; left of slash); and in DMEM medium in the absence/presence of $NaHCO_3$ (test/standard condition; right of slash). Increased susceptibility is depicted in blue; increased resistance is depicted in red. Stippled boxes represent those that exhibited <8-fold altered susceptibility between MHB and DMEM media. To control for pH and buffer considerations, strains were grown in MHB pH 7.2; MHB adjusted to pH 7.2 w/100 mM Tris; and DMEM liquid pH 7.4 (containing 44 mM $NaHCO_3$); all other media conditions were adjusted to pH 7.4 with 100 mM Tris including: MHB w/$NaHCO_3$; and $NaHCO_3$-free powdered DMEM w/wo $NaHCO_3$ (Supplementary Table 2a). (b) Dose response analysis of MRSA (USA300; MT3302) and MSSA (MT3307) antibiotic susceptibility following exposure to increasing concentrations of $NaHCO_3$ in standard MHB medium. AZM (azithromycin); ERY (erythromycin); CFX (ceftriaxone). (c) Susceptibility of *S. pneumoniae* and *Salmonella* spp. in the presence/absence of physiological levels of $NaHCO_3$ in MHB and/or DMEM media. For *S. pneumoniae*, values represent fold-change between MICs derived in MHB medium in the presence/absence of $NaHCO_3$ (test/standard condition). For *Salmonella* spp. values represent fold-change between MICs derived in MHB medium in the presence/absence of $NaHCO_3$ (test/standard condition; left of slash); and DMEM in the absence/presence of $NaHCO_3$ (test/standard condition; right of slash). No change (NC), Resistant (R). MICs were a consensus of at least 6 independent isolates.

suggest that $NaHCO_3$ 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 $NaHCO_3$ also resulted in altered drug susceptibilities in *S. pneumoniae* and *Salmonella* spp. isolates (Fig. 4c; Supplementary Table 2b, c). Further, 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 $NaHCO_3$ 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. Susceptibility 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 $NaHCO_3$ 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 drug failure in a particular host-mimicking media may indicate that certain drugs may be effective against certain types of infections but not others (e.g., systemic vs. localized). Supporting this suggestion, MRSA inactivates daptomycin by releasing membrane phospholipids under certain experimental conditions (Pader et al., 2016); and herein we show that a MRSA isolate was susceptible to daptomycin in tissue culture medium and in a murine model of sepsis, but displayed resistance in other host-mimicking media examined (minimal Lacks medium).

Future considerations must be given to host-pathogen interactions that can also influence drug susceptibility. (1) Animals, including primates, often tolerate drugs differently than humans (pharmacokinetic parameters such as drug clearance, volume of distribution, and half-life 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, underlying 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 L.B. 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., L.B., and M.J.M. 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

References

Ambrose, P., Bhavnani, S., Rubino, C., Louie, A., Gumbo, T., Forrest, A., Drusano, G., 2007. Pharmacokinetics-pharmacodynamics of antimicrobial therapy: it's not just for mice anymore. Clin. Infect. Dis. 44, 79–86.

Band, V., Crispell, E., Napier, B., Herrera, C., Tharp, G., Vavikolanu, K., Pohl, J., Read, T., Bosinger, S., Trent, M., Burd, E., Weiss, D., 2016. Antibiotic failure mediated by a resistant subpopulation in *Enterobacter cloacae*. Nat Microbiol 1, 16053.

Cano, V., March, C., Insua, J., Aguiló, N., Llobet, E., Moranta, D., Regueiro, V., Brennan, G., Millán-Lou, M., Martín, C., 2015. *Klebsiella pneumoniae* survives within macrophages by avoiding delivery to lysosomes. Cell. Microbiol. 17, 1537–1560.

Carlsson, S., Wiklund, N., Engstrand, L., Weitzberg, E., Lundberg, J., 2001. Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. Nitric Oxide 5, 580–586.

Carryn, S., Chanteux, H., Seral, C., Mingeot-Leclercq, M.-P., van Bambeke, F., TULKENS, P., 2003. Intracellular pharmacodynamics of antibiotics. Infect. Dis. Clin. N. Am. 17, 615–634.

Carter, R., Wolf, J., van Opijnen, T., Muller, M., Obert, C., Burnham, C., Mann, B., Li, Y., Hayden, R., Pestina, T., 2014. Genomic analyses of pneumococci from children with sickle cell disease expose host-specific bacterial adaptations and deficits in current interventions. Cell Host Microbe 15, 587–599.

Centers for Disease Control and Prevention, 2013. Antibiotic Resistance Threats in the United States. p. 2013.

Clinical and Laboratory Standards Institute, 2012a. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically (Approved standard-ninth edition).

Clinical and Laboratory Standards Institute, 2012b. Performance Standards for Antimicrobial Resistance Testing (twenty-second informational supplement).

Clinical and Laboratory Standards Institute, 2013. Minimum Inhibitory Concentration (MIC) Breakpoints for Veterinary Pathogens.

Clinical and Laboratory Standards Institute, 2014. Performance Standards for Antimicrobial Resistance Testing (twenty-fourth informational supplement).

Coombes, B., Brown, N., Valdez, Y., Brumell, J., Finlay, B., 2004. Expression and secretion of Salmonella pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. J. Biol. Chem. 279, 49804.

Davis, R., Botstein, D., Roth, J., 1980. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory Press, Plainview, N. Y.

Daw, N., Wilimas, J., Wang, W., Presbury, G., Joyner, R., Harris, S., Davis, Y., Chen, G., Chesney, P., 1997. Nasopharyngeal carriage of penicillin-resistant Streptococcus pneumoniae in children with sickle cell disease. Pediatrics 99 (e7), 1–7.
Deutschman, C., Tracey, K., 2014. Sepsis: current dogma and new perspectives. Immunity 40, 463–475.
Deziel, M., Heine, H., Louie, A., Kao, M., Byrne, W., Basset, J., Miller, L., Bush, K., Kelly, M., Drusano, G., 2005. Effective antimicrobial regimens for use in humans for therapy of Bacillus anthracis infections and postexposure prophylaxis. Antimicrob. Agents Chemother. 49, 5099–5106.
Diekema, D., Richter, S., Heilmann, K., Dohrn, C., Riahi, F., Tendolkar, S., McDanel, J., Doern, G., 2014. Continued emergence of USA300 methicillin-resistant *Staphylococcus aureus* in the United States: results from a nationwide surveillance study. Infect. Control Hosp. Epidemiol. 35, 285–292.
Diene, S., Rolain, J.M., 2014. Carbapenemase genes and genetic platforms in Gram-negative bacilli: Enterobacteriaceae, Pseudomonas and Acinetobacter species. Clin. Microbiol. Infect. 20, 831–838.
Doern, G., Brecher, S., 2011. The clinical predictive value (or lack thereof) of the results of in vitro antimicrobial susceptibility tests. J. Clin. Microbiol. 49, S11–S14.
Dorschner, R., Lopez-Garcia, B., Peschel, A., Kraus, D., Morikawa, K., Nizet, V., Gallo, R., 2006. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. FASEB J. 20, 35–42.
Dulbecco, R., Freeman, G., 1959. Plaque production by the polyoma virus. Virology 8, 396–397.
European Committee on Antibiotic Susceptibility Testing, 2014. EUCAST Definitions of Clinical Breakpoints and Epidemiological Cut-off Values. http://www.srga.org/Eucastwt/eucastdefinitions.htm.
European Committee on Antimicrobial Susceptibility Testing, 2016. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 6.0.
Fuchs, P., Barry, A., Brown, S., 1997. Interpretive criteria and quality control parameters for testing of susceptibilities of Haemophilus influenzae and Streptococcus pneumoniae to trimethoprim and trimethoprim-sulfamethoxazole. The antimicrobial susceptibility testing OC group. J. Clin. Microbiol. 35, 125–131.
Hathaway, L.J., Brugger, S.D., Morand, B., Bangert, M., Rotzetter, J.U., Hauser, C., Graber, W.A., Gore, S., Kadioglu, A., Mühlemann, K., 2012. Capsule type of Streptococcus pneumoniae determines growth phenotype. PLoS Pathog. 8, e1002574.
Heithoff, D.M., Shimp, W.R., House, J.K., Xie, Y., Weimer, B.C., Sinsheimer, R.L., Mahan, M.J., 2012. Intraspecies variation in the emergence of hyperinfectious bacterial strains in nature. PLoS Pathog. 8, e1002647.
Heithoff, D.M., Shimp, W.R., Lau, P.W., Badie, G., Enioutina, E.Y., Daynes, R.A., Byrne, B.A., House, J.K., Mahan, M.J., 2008. Human Salmonella clinical isolates distinct from those of animal origin. Appl. Environ. Microbiol. 74, 1757–1766.
Hermansen, L., Osnes, J., 1972. Blood and muscle pH after maximal exercise in man. J. Appl. Physiol. 32, 304–308.
Holland, T., Arnold, C., Fowler, V., 2014. Clinical management of *Staphylococcus aureus* bacteremia: a review. JAMA 312, 1330–1341.
Kubicek-Sutherland, J., Heithoff, D., Ersoy, S., Shimp, W., House, J., Marth, J., Smith, J., Mahan, M., 2015. Host-dependent induction of transient antibiotic resistance: a prelude to treatment failure. EBioMedicine 2, 1169–1178.
Kubicek-Sutherland, J., Heithoff, D., Ersoy, S., Shimp, W., Mahan, M., 2014. Immunization with a DNA adenine methylase over-producing Yersinia pseudotuberculosis vaccine confers robust cross-protection against heterologous pathogenic serotypes. Vaccine 32, 1451–1459.
Lacks, S., 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics 53, 207.
Landman, D., Georgescu, C., Martin, D., Quale, J., 2008. Polymyxins revisited. Clin. Microbiol. Rev. 21, 449–465.
Lanie, J., Ng, W.-L., Kazmierczak, K., Andrzejewski, T., Davidsen, T., Wayne, K., Tettelin, H., Glass, J., Winkler, M., 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of Streptococcus pneumoniae and comparison with that of unencapsulated laboratory strain R6. J. Bacteriol. 189, 38–51.
Lin, L., Nonejuie, P., Munguia, J., Hollands, A., Olson, J., Dam, Q., Kumaraswamy, M., Rivera, H., Corriden, R., Rohde, M., 2015. Azithromycin synergizes with cationic antimicrobial peptides to exert bactericidal and therapeutic activity against highly multidrug-resistant Gram-negative bacterial pathogens. EBioMedicine 2, 690–698.
Logan, R., Funk, R., Axcell, E., Krise, J., 2012. Drug-drug interactions involving lysosomes: mechanisms and potential clinical implications. Expert Opin. Drug Metab. Toxicol. 8, 943–958.
Mayo Clinic, 2017. Bicarbonate. Serum Online. http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/876 (Accessed).
Newbrun, E., Hoover, C., Ryder, M., 1984. Bactericidal action of bicarbonate ion on selected periodontal pathogenic microorganisms. J. Periodontol. 55, 658–667.
Pader, V., Hakim, S., Painter, K., Wigneshweraraj, S., Clarke, T., Edwards, A., 2016. *Staphylococcus aureus* inactivates daptomycin by releasing membrane phospholipids. Nat Microbiol 2, 16194.
Paterson, D., Bonomo, R., 2005. Extended-spectrum β-lactamases: a clinical update. Clin. Microbiol. Rev. 18, 657–686.
Patterson, A., Heithoff, D., Ferguson, B., Soh, H., Mahan, M., Plaxco, K., 2013. Microfluidic chip-based detection and intraspecies strain discrimination of Salmonella serovars derived from whole blood of septic mice. Appl. Environ. Microbiol. 79, 2302–2311.
Rex, J., Pfaller, M., 2002. Has antifungal susceptibility testing come of age? Clin. Infect. Dis. 35, 982–989.
Rosenthal, T., 1948. The effect of temperature on the pH of blood and plasma in vitro. J. Biol. Chem. 173, 25–30.
Societe Francaise de Microbiologie, 2012. Comite de l'Antibiogramme de la Societe Francaise de Microbiologie (CASFM).
Sorg, R., Lin, L., van Doorn, G., Sorg, M., Olson, J., Nizet, V., Veening, J.-W., 2016. Collective resistance in microbial communities by intracellular antibiotic deactivation. PLoS Biol. 14, e2000631.
Steele-Mortimer, O., 2008. The Salmonella-containing vacuole—moving with the times. Curr. Opin. Microbiol. 11, 38–45.
Trombe, M.-C., Clavé, C., Manias, J.-M., 1992. Calcium regulation of growth and differentiation in Streptococcus pneumoniae. Microbiology 138, 77–84.
U.S. White House, 2015. The National Action Plan for Combating Antibiotic-resistant Bacteria.
Vega, N., Gore, J., 2014. Collective antibiotic resistance: mechanisms and implications. Curr. Opin. Microbiol. 21, 28–34.
Victor, L., Chiou, C., Feldman, C., Ortqvist, A., Rello, J., Morris, A., Baddour, L., Luna, C., Snydman, D., Ip, M., 2003. An international prospective study of pneumococcal bacteremia: correlation with in vitro resistance, antibiotics administered, and clinical outcome. Clin. Infect. Dis. 37, 230–237.
Wiegand, I., Hilpert, K., Hancock, R.E., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3, 163–175.
World Health Assembly, 2014. Antimicrobial resistance. In: 67.25, R (Ed.), Sixty-seventh World Health Assembly.
World Health Organization, 1961. Standardization of methods for conducting antimicrobial sensitivity tests. Second Report of the Expert Committtee on Antibiotics. WHO Technical Report Series, WHO, Geneva.
World Health Organization, 2014. Antimicrobial Resistance: Global Report on Surveillance.
Yahav, D., Farbman, L., Leibovici, L., Paul, M., 2012. Colistin: new lessons on an old antibiotic. Clin. Microbiol. Infect. 18, 18–29.