The correlation of the genetic antibiotic resistance markers to antibiotic susceptibility testing (AST)

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Introduction

Abstract

Antibiotic resistant infections pose a risk for increased patient mortality due to long time-to-results for current diagnostic methods. Results from a ~1,500 patient trial demonstrated bacterial blood culture required 51 hours on average for a positive result[1]. Additionally current antibiotic susceptibility testing (AST) requires two additional days for phenotypic results. Studies show delays in effective treatment increases mortality rates in septic patients by 7.6% every hour[2]. The CDC classifies carbapenem-resistant Enterobacterales (13,000 annual deaths) as urgent threats, and vancomycin-resistant *Enterococci* and methicillin-resistant *S. aureus* (~15,000 annual deaths combined) as serious threats[3]. Identifying resistance markers direct from patient samples may aid clinical decisions, reduce antibiotic use, reduce time to effective therapy, and improve patient outcomes. 184 antibiotic resistant bacterial strains were genotyped for 13 clinically-relevant resistance genes and screened for antibiotic resistance phenotypes. Results identified correlations between resistance markers and phenotypic resistance and potential aid for clinical treatment. High resistance to vancomycin and piperacillin-tazobactam, first-line antibiotics, were seen in 31 Gram-positive and 98 Gram-negative strains. Presence of Gram-positive markers *vanA* or *vanB* correlated to 100% (31/31) vancomycin resistance and 86% (25/29) ampicillin resistance. In 125 sequenced Gram-negative strains, 57 harbored metallo-β-lactamase (bla_{IMP}, bla_{NDM}, bla_{VIM}) 58 contained extended spectrum β-lactamase ($bla_{CTX-M-14}$, $bla_{CTX-M-15}$), 36 contained AmpC β-lactamase (bla_{DHA}, bla_{CMY}), and 47 contained carbapenemase (bla_{KPC}, bla_{OXA-48}). These markers correlated to ≥99% resistance to ampicillin, cefazolin, and cefuroxime in all strains. Together, these data suggest that molecular diagnostics that identify genetic markers may provide clinicians with needed information during a window when therapeutic intervention can improve patient outcomes.

Importance of Early Targeted Therapy

Current standard of care for a patient suspected of sepsis is blood culture and subsequent antimicrobial susceptibility testing, which can take 2-5 days. During this time no clinical data is available to direct targeted patient treatment, therefore, patients receive empiric therapy. A meta-analysis of 70 studies found that empiric antibiotic therapy was inappropriate in 46.5% of patients with blood stream infections[4]. It has also been demonstrated that for every hour of delay in appropriate therapy there is a 7.6% decrease in survival for septic shock patients[2]. A study of patients with blood stream infections caused by carbapenem resistant *Enterobacteriaceae* (CRE) demonstrated that the median time to appropriate therapy was 47 hours and that 49% of infected individuals died within 30 days[5]. Additionally, blood stream infections treated appropriately within the first 24 hours had significant reduction in hospital length of stay, mortality and cost[6]. The ability to determine antibiotic resistance using genetic markers could help to get patients on the appropriate therapy faster then the current standard.

Methods

Bacterial Isolates

Strains were obtained from the CDC & FDA Antibiotic Resistance (AR) Isolate Bank, the American Tissue Culture Collection (ATCC), BEI Resources, National Collection of Type Cultures (NCTC), and JMI Laboratories. Bacterial strains were cultured with optimized growth conditions for each species.

Molecular Detection

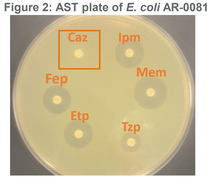
All bacterial isolates were PCR screened for bla_{OXA}, bla_{KPC}, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, bla_{DHA}, bla_{CMY}, bla_{NDM}, bla_{IMP}, bla_{VIM}, *mecA*, *mecC*, *vanA*, and *vanB*. 16S and *rpoB* were used to confirm species. PCR products (Figure 1) were sent for Sanger sequencing at Genewiz (Cambridge, MA). Resulting sequences were aligned to the respective keystone sequence using Benchling. 16S and *rpoB* sequences were analyzed using NCBI BLASTn. Sequences with a Genewiz quality score of at least 40 or that had majority sequence alignment were considered to have that marker. Strains with amplicons that did not align with any marker sequences or had off target chromosomal amplification were not considered to have that marker present. Figure 1 is an example of a molecular marker screen, performed for *E. coli* AR-0081 and demonstrates presence of bla_{cmy} marker

Figure 1: Gel image of *Escherichia coli* AR-0081

Antibiotic Susceptibility Testing

184 strains were examined for phenotypic resistance profiles to clinically relevant antibiotics. Isolates were grown in optimal media then plated to generate a lawn of growth. Antibiotic diffusion disks were selected for each isolate based on clinical relevance and placed on the plate prior to incubation. Zones of inhibition (ZOI) were measured, and resistant, intermediate, and sensitive profiles were interpreted based on established CLSI breakpoint guidelines found in CLSI EP07-A2[7]. Figure 2 is an example of an AST result, performed on *E. coli* AR-0081 and demonstrates ceftazidime resistance corresponding to bla_{CMY} marker.

Figure 2: AST plate of *E. coli* AR-0081



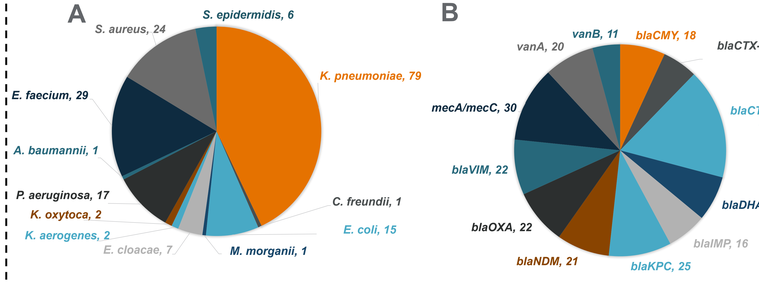
	Species	Tested Antibiotics
Gram –	*Citrobacter freundii, Escherichia coli, Enterobacter cloacae, Klebsiella aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii*	Ampicillin (Amp), Ampicillin-sulbactam (Sam), Piperacillin-tazobactam (Tzp), Aztreonam (Atm), Cefazolin (Cz), Cefuroxime (Cxm), Cefotaxime (Ctx), Ceftazidime (Caz), Cefepime (Fep), Ertapenem (Etp), Imipenem (Ipm), Meropenem (Mem)
	Pseudomonas aeruginosa, Acinetobacter baumannii	Meropenem, Cefotaxime, Ceftazidime, Cefepime
Gram +	*Enterococcus faecium*	Ampicillin, Vancomycin (Van)
	Staphylococcus aureus, Staphylococcus epidermidis	Cefoxitin (Fox), Vancomycin

Results

Characterization of strains included in study

184 strains across 12 species were tested for phenotypic antibiotic resistance profile and were sequenced to identify the presence of 13 clinically significant genetic markers.

Figure 3: Total number of bacterial isolates by species marker and total of each genetic marker. (A) Total number of bacterial isolates, value indicates number of strains tested per species. **(B)** Total number of genetic markers, value indicates number of each marker identified



Majority of resistant bacterial isolates have multiple genetic resistance markers

59 of 184 strains sequenced (32%) were found to contain multiple genetic markers that influence the phenotypic resistance profile (Table 1). The screened Gram-positive species tended to have low prevalence of multiple markers, with *mecA* and *vanA* being paired twice. The screened Gram-negative species had a higher prevalence of multiple markers with $bla_{CTX-M-15}$ being associated with multiple other resistance markers.

Table 1: Total number of each marker identified and number of strains with a multiple marker identified

Resistance Marker	Number of strains with marker	Number of strains with multiple resistance markers	Percentage of strains with multiple resistance markers	Most common additional resistance marker identified
vanB	11	0	0%	N/A
mecA/mecC	30	2	7%	*vanA*
bla_{KPC}	25	6	24%	N/A*
vanA	20	2	10%	*mecA*
bla_{VIM}	22	5	23%	N/A*
bla_{IMP}	16	7	44%	$bla_{CTX-M-15}$
$bla_{CTX-M-14}$	14	7	50%	bla_{DHA} and bla_{OXA}
bla_{DHA}	18	13	72%	$bla_{CTX-M-15}$ and $bla_{CTX-M-14}$
bla_{CMY}	18	15	83%	$bla_{CTX-M-15}$
bla_{NDM}	21	19	90%	$bla_{CTX-M-15}$
bla_{OXA48}	22	20	91%	$bla_{CTX-M-15}$
$bla_{CTX-M-15}$	44	41	93%	bla_{OXA}

*No common 2nd marker

Antibiotic resistance profiles can be provided based on gene class

Antibiotic resistance genes can be grouped into classes (Table 2 and 3) based on the resistances conferred and protein structure. The identification of a class of antibiotic resistance genes can provide insight on the resistance profile, due to similar function and action on bacterial cells. The resistance profile of the different gene classes were examined by comparing the presence of all occurrences of the group of genes to the occurrence of the genes alone in a bacterial isolate (Table 4). For the purpose of calculating correlation between the presence of a resistance marker and phenotype, strains that demonstrated an "intermediate" phenotype in AST based on CLSI guidelines were defined as resistant due to the clinical significance of an intermediate reading.

Table 2. Gene classes for identified genetic markers

Gene Class	Genetic Marker(s)
Metallo- β-lactamases (MBLs)	bla_{IMP}, bla_{VIM}, bla_{NDM}
Extended-Spectrum β-lactamases (ESBLs)	$bla_{CTX-M-14}$, $bla_{CTX-M-15}$
AmpC β-lactamases (AmpC)	bla_{CMY}, bla_{DHA}
Carbapenemases (CR)	bla_{KPC}, bla_{OXA}
Methicillin Resistance (Mec)	*mecA, mecC*
Glycopeptide Resistance (Van)	*vanA, vanB*

Table 3: Antibiotic classes of clinically-relevant tested antibiotics

Antibiotic Class	Antibiotic(s)
Penicillin	Ampicillin
Penicillin-β-lactamase Inhibitor (PBLI)	Ampicillin-sulbactam, Piperacillin-tazobactam
Monobactam	Aztreonam
Cephalosporin	Cefazolin, Cefuroxime, Cefotaxime, Ceftazidime, Cefepime
Carbapenem	Ertapenem, Imipenem, Meropenem
Cephamycin	Cefoxitin
Glycopeptide	Vancomycin

Table 4: Percent resistance of tested antibiotic classes based on gene class for Gram-negative isolates

	Penicillin	PBLI		Monobactam	Cephalosporin					Carbapenems		
All markers total percent	99%	96%	92%	80%	100%	100%	98%	91%	85%	81%	68%	70%
	Amp	Sam	Tzp	Atm	Cz	Cxm	Ctx	Caz	Fep	Etp	Ipm	Mem
MBL with multiple markers	98%	100%	98%	74%	100%	100%	100%	100%	96%	98%	95%	91%
Single MBL	100%	100%	100%	31%	100%	100%	100%	100%	97%	100%	94%	90%
ESBL with multiple markers	100%	93%	88%	95%	100%	100%	98%	86%	88%	71%	66%	64%
Single ESBL	100%	60%	50%	70%	100%	100%	90%	30%	60%	10%	10%	10%
AmpC with multiple markers	100%	100%	89%	86%	100%	100%	97%	94%	69%	69%	44%	39%
Single AmpC	100%	100%	88%	63%	100%	100%	100%	100%	25%	75%	0%	0%
CR with multiple markers	100%	100%	98%	93%	100%	100%	98%	87%	89%	98%	84%	87%
Single CR	100%	100%	100%	88%	100%	100%	100%	89%	84%	100%	94%	95%

Molecular marker of resistance correlates to antibiotic resistance phenotype

The resistance profiles and percent resistance of relevant antibiotics was examined based on the presence of individual genes. Percent resistance was calculated for all strains with the same genetic marker and compared to the percentage of strains that contained only that marker by itself. An intermediate classification was categorized as resistant due to the clinical significance of an intermediate AST read. Values above 75% were considered significant and indicated a correlation between resistance marker and phenotypic resistance for corresponding antibiotics (Table 5 and Table 6).

In strains that were tested multiple times, 27% (12/44) had variation in their antibiotic resistance profiles, demonstrating that there can be growth-to-growth variance with the same isolate. Additionally, variation can be seen between gene types within a genetic marker classification[8].

Table 5: Resistance profiles of genetic markers tested in Gram-positive species

Genetic Marker	*vanB*	*vanA*	*mecA/mecC*
Antibiotic Resistance (% Resistant Strains)	Amp (100%), Van (100%)	Van (100%) Amp (78%), Fox (100%)	Fox (80%)
Gram-positive Species Containing Marker	*E. faecium*	*E. faecium, S. aureus*	*S. aureus, S. epidermidis*

Table 6: Resistance profiles of genetic markers tested in Gram-negative species

Genetic Marker	bla_{CMY}	$bla_{CTX-M-14}$	$bla_{CTX-M-15}$	bla_{DHA}	bla_{IMP}	bla_{KPC}	bla_{NDM}	bla_{OXA}	bla_{VIM}
Antibiotic Resistance (Percent Resistant Strains)	Amp (94%), Sam (100%), Cz (100%), Cxm (100%), Ctx (100%), Caz (94%), Etp (83%), Tzp (100%)	Amp (100%), Cz (100%), Cxm (100%), Ctx (93%)	Amp (100%), Sam (100%), Atm (100%), Cz (100%), Cxm (100%), Ctx (100%), Caz (98%), Fep (98%), Tzp (95%)	Amp (100%), Sam (100%), Cz (100%), Cxm (100%), Ctx (94%), Caz (94%)	Amp (100%), Sam (100%), Cz (100%), Cxm (100%), Ctx (100%), Caz (100%), Fep (100%), Ipm (85%), Mem (88%), Tzp (100%)	Amp (100%), Sam (100%), Atm (91%), Cz (100%), Cxm (100%), Ctx (96%), Caz (96%), Fep (88%), Etp (95%), Ipm (86%), Mem (86%), Tzp (95%)	Amp (94%), Sam (100%), Atm (100%), Cz (100%), Cxm (100%), Ctx (100%), Caz (100%), Fep (95%), Etp (94%), Ipm (100%), Mem (95%), Tzp (95%)	Amp (100%), Sam (100%), Cz (100%), Cxm (100%), Ctx (100%), Etp (100%), Ipm (82%), Mem (86%), Tzp (100%)	Amp (100%), Sam (100%), Cz (100%), Cxm (100%), Ctx (100%), Caz (100%), Fep (95%), Etp (100%), Ipm (100%), Mem (91%), Tzp (100%)
Gram-negative Species Containing Marker	*K. pneumoniae, E. coli*	*K. pneumoniae*	*K. pneumoniae, E. coli, C. freundii,*	*K. pneumoniae, E. coli, E. cloacae, M. morganii, C. freundii*	*K. pneumoniae, K. aerogenes, K. oxytoca, P. aeruginosa*	*K. pneumoniae, E. coli, E. cloacae, P. aeruginosa*	*K. pneumoniae, E. coli, M. morganii, E. cloacae, P. aeruginosa, A. baumannii*	*K. pneumoniae, E. coli, K. aerogenes*	*K. pneumoniae, P. aeruginosa, E. cloacae, E.coli*

Sequencing and AST analysis of bacterial isolate indicates that the presence of genetic resistance markers can provide insight of the resistance profile of bacterial isolates. Through examination of AST results on an individual marker level, and a gene class level, clear trends in antibiotic resistance are present. Correlations between the molecular marker and antibiotics with phenotypic antibiotic resistance were made through these comparisons (Table 6).

- **100% vancomycin resistance** in Gram-positive species tested
- **100% cefazolin and cefuroxime resistance** in all Gram-negative species tested

Molecular marker testing is more consistent than antibiotic susceptibility testing

- 27% (12/44 strains) of strains tested multiple times had variation in phenotypic profile
- 70% (16/23) of discrepancies were minor errors: the presence of an intermediate zone which can indicate either resistance or susceptibility definition (Table 6)
- 30% (7/23) of discrepancies were major errors: differences between a resistant and susceptible definition (Table 7)
- Variability in resistance definitions may be result of varied daily growth conditions, or operator interpretation of ZOI.
- **Resistant markers are consistent, regardless of AST, indicating that there is less variability in the use of a genetic marker for clinical antibiotic decisions.**

Total strains tested	184
Total strains tested multiple times	44
Total number of strains with AST discrepancies	12
Total number of AST discrepancies	23
Total minor errors	16
Total major errors	7

Table 7: Minor Error: Resistance profile of *K. pneumoniae* AR-0076 varied between intermediate and resistance or intermediate and susceptible

Species	Resistance Markers Identified	Strain ID	Amp	Sam	Atm	Cz	Cxm	Ctx	Caz	Fep	Etp	Ipm	Mem	Tzp
K. pneumoniae	VIM	AR-0076	R	R	S	R	R	R	R	R	I	R	R	R
K. pneumoniae	VIM	AR-0076	R	R	S	R	R	R	R	R	I	I	S	R
K. pneumoniae	VIM	AR-0076	R	R	S	R	R	R	R	R	S	I	S	R

Table 8: Major Error: Resistance profiles of *E. faecium* AR-0809 varied between resistant and susceptible

Species	Resistance Markers Identified	Strain ID	Amp	Van
E. faecium	*vanA*	AR-0809	S	R
E. faecium	*vanA*	AR-0809	S	S

Tables 7 and 8:
S indicates susceptible,
R indicates resistant,
I indicates intermediate.

Conclusions

- **184 strains** across 12 clinically relevant species were tested for antibiotic susceptibility and were sequenced for the presence of **13 antibiotic resistance markers** to determine if there is a correlation between phenotypic and genotypic antibiotic resistance.
- **32% of bacterial isolates contained multiple genetic markers** that contribute to antibiotic resistance phenotype
- **Genetic markers** present in bacteria **correlate to antibiotic resistance phenotypes**
- Genetic profile is consistent while phenotypic resistance profiles demonstrated variation.

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