

Communication

Multiplex Detection of Antimicrobial Resistance Genes for Rapid Antibiotic Guidance of Urinary Tract Infections

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Abstract: Identification of antimicrobial resistance markers in urinary tract infections could provide a more targeted approach in the diagnosis and treatment of UTIs while reducing overall public health burdens. We describe a molecular assay as a diagnostic tool for antibiotic resistance characterization to promote faster diagnosis of antibiotic regimens compared to standard microbiology techniques. Targeted antibiotic usage for pathogenic infections remains a main goal for effective antibiotic treatment protocols and reducing the overall public health burden. Rapid identification of the pathogen(s) causing the infection and harboring the antibiotic resistance gene is also a main area of exploration for antibiotic appropriation and stewardship. Urinary tract infections are a common clinical disease and reservoir for pathogenic infection and the development of antibiotic resistance, especially in hospital- and community-acquired settings. Standard methods require urine culture, which is time consuming and relies on phenotypic characterization. A genetic diagnostic method is warranted for the rapid molecular characterization of antibiotic resistance genes to reduce inappropriate exposure to antibiotics while improving the overall treatment model for urinary tract infections. The purpose of this study is to demonstrate logical viability for real-time molecular diagnostics for early identification, active surveillance and overall targeted antibiotic stratification that is proposed as an in vitro rapid and comprehensive tool for assessing proper antibiotic stewardship in UTIs. Here, we describe a multiplex real-time fluorescence polymerase chain reaction (PCR) for probe-based detection of the top 24 antibiotic resistance genes with targeted relationships to target molecular drug classes and administered antibiotics. Multiplexed analysis based on molecular features enables rapid testing while shifting the diagnostic detection paradigm from monocentric infections towards polymicrobial infections. We utilized 366 samples from the FDA-CDC Antimicrobial Resistance Isolate Bank to test the efficacy of the assay and propose a model to infer the identity of bacterial isolates. We found that, in addition to a high level of accuracy in predicting bacterial genus classification, the assay was mostly in agreement with CDC-tested genotypic and phenotypic results. This study provides evidence for using genetic diagnostic methods, such as multiplex qPCR, in the rapid identification of antibiotic resistance (ABR) genes for the characterization and treatment of urinary tract infections.

Keywords: antibiotic resistance; urinary tract infections; multiplex PCR; pathogen detection; molecular diagnostics; antibiotic stewardship; pathogen identification; molecular stratification



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1. Introduction, Background and Significance

Urinary tract infections (UTIs) are common bacterial infections that result in billions of dollars in annual health care costs and affect an estimated 150 million people globally [1]. In the United States alone, there were an estimated 10.5 million UTI-related hospital visits in 2007 of which nearly 21% resulted in hospital emergency department visits, as compared to 2018 estimates of approximately 3 million US hospitalization of complicated UTIs alone [2,3]. UTIs are one of the most common clinical diseases for prescribed antibiotics [4–6]. The overuse and prolonged therapeutic duration of antibiotics are associated with antibiotic resistance and poor patient outcomes such as hospital mortality, high readmission rates, *Clostridium difficile* infections, and antimicrobial adverse drug events [7–9]. The Centers for

Disease Control and Prevention (CDC) estimate that up to 50% of all prescribed antibiotics across all infectious diseases are inappropriately administered [10]. According to the CDC, at least 2 million people are infected with antibiotic-resistant bacteria in the US, of which approximately 23,000 people die every year [11]. UTIs are commonly treated with antibiotics, but with the increasing rate of antibiotic resistance, it is important to robustly test UTIs for the presence of UTIs to accurately inform the clinical in prescribing targeted antibiotic therapies.

Current diagnostic methodologies for UTIs entail dipstick tests and urine culture on blood agar plates to isolate a specific pathogen and determine antibiotic susceptibility testing (AST) and minimum inhibitory concentration (MIC) profiles. This process requires 48–72 h of culture-based methods with additional time for AST/MIC profiling, which often takes 5+ days. It is common practice for physicians to prescribe broad-spectrum antibiotic therapy in this time interval to treat the UTI, but such practices may contribute to the overuse or unnecessary use of antibiotics [12]. Culture methods are limited in their ability to detect many microorganisms and may miss fastidious and non-aerobic organisms as well as slow growing microbes and many Gram-positive organisms. While it has been shown that modification of culture methods with enhanced growth conditions can significantly increase the detection rates of uropathogens in symptomatic patients, the process remains time consuming [13]. PCR-based methods have gained momentum in the field as it offers accurate and faster results than traditional culture methods. A recent study evaluated multiplex PCR-based molecular testing relative to standard urine analysis across 582 specimens and found that results between the two methods were in agreement in >90% cases [14].

Infections caused by a single organism are usually treatable with an antibiotic regimen based on antibiotic susceptibility tests. However, it has been estimated that as many as 39% of UTIs are polymicrobial, which can result in increased virulence and antibiotic resistance [13,15,16]. Clinical microbiology techniques do not report organisms isolated from urine in mixed cultures unless there is a significant count of a predominant microorganism, so many polymicrobial infections go undetected. Simultaneous detection of a larger number of pathogens may confer benefits for outcome of UTIs. We previously highlighted that UTIs possess polymicrobial characteristics that may be more representative of clinical disease when determining diagnostic efficacies as compared to single isolate urine culture methods [17]. There is increased pathogenic potential in infections with multiple microbes when compared to monomicrobial culture samples [15]. As a result, traditional microbiology culture may lack the sensitivity required to properly diagnose UTIs with antibiotic resistance profiles. Here, we present a multiplexed, nucleic acid detection diagnostic for rapid identification of ABR genes for the characterization of infections to demonstrate increased sensitivity as compared to traditional urine culture.

2. Materials and Methods

Samples: De-identified isolate samples (IRB reviewed and deemed non-human subject research) were acquired from the FDA-CDC Antimicrobial Resistance Isolate Bank (database accessed on 1 August 2022; <https://wwwn.cdc.gov/arisolatebank/>), a publicly available resource to combat antimicrobial resistance [18]. The bank contains almost 1000 curated bacterial isolates with a variety of clinically important resistance mechanisms that have been phenotypically and genotypically characterized, whereby culture data for MIC/AST and whole-genome sequencing data are publicly available [18]. Custom pivot data tables were generated in Microsoft Excel for data analysis based on the original CDC data for comparison to multiplex PCR data. Below is a table of bank isolate panels and the number of samples from each panel.

CDC/FDA AR Bank Isolate Panels	Panel Codes	Number of Samples
<i>Acinetobacter baumannii</i> Panel	ACI	29
Aminoglycoside/Tetracycline Resistance Panel	ATR	30
Ceftazidime/Avibactam Panel	CZA	20
Ceftolozane/Tavibactam Panel	CTV	20
Drug Resistance Candida Panel (CAN)	CAN	23
Enteric Pathogen Diversity Panel	GI	29
<i>Enterobacteriaceae</i> Carbapenem Breakpoint Panel	BIT	22
<i>Enterobacteriaceae</i> Carbapenemase Diversity Panel	CRE	29
Gram-Negative Carbapenemase Detection Panel	CarbaNP	53
Imipenem/Relebactam Panel	IMR	18
<i>Neisseria gonorrhoeae</i> Panel	GC	28
<i>Pseudomonas aeruginosa</i> Panel	PSA	28
<i>Staphylococcus</i> with Borderline Oxacillin Susceptibility Panel	BOR	24
Vancomycin Intermediate <i>Staphylococcus aureus</i> Panel	VISA	13

DNA Extraction: DNA extraction from bacterial isolates was conducted via Qiagen QIAamp DNA Blood Mini Kit for the purification of total DNA. Briefly, silica-membrane-based DNA purification was performed by pipetting 200 µL of the original sample as starting material, and subsequently run through an automated DNA extraction protocol on the QiaCube instrument. Eluted DNA was collected in 1.5 mL microcentrifuge tubes at 100 µL volumes. Each QiaCube run accommodates 12 samples at a time. All DNA extractions of all samples were conducted in multiples of 12 extractions at a time.

Standardized Multiplex Reaction Model: A standardized reaction model is established for multiplexing of four interrogated amplicon targets comprised of 5' nuclease real-time PCR assays such that 12 oligonucleotides (four 5' nuclease probes, four forward primers, four reverse primers) are present within a real-time PCR reaction. Each reaction involves the use of four distinct fluorophores (FAM, HEX, ROX, CY5) for detection of 5' nuclease probes during reaction cycles. A standardized custom multiplex optimized master mix is utilized for the 4-plex reactions. Altogether, a single reaction can identify 4 unique targets within a single well/compartment via fluorescence readings and cycle threshold (Ct) values. Aggregation of this model allows for multiple reactions targeting specific interrogated amplicon sequences in a scaled format such that 24 antimicrobial resistance markers grouped into 6 reactions allow for UTI diagnostics (Table 1).

Multiplex PCR Reactions: The ABR multiplex assay was designed using FAM, HEX, ROX and Cy5 fluorophore-labeled 5' hydrolysis PCR probes with quenchers targeting the interrogated amplicon sequences (proprietary sequences from www.locusscience.com, accessed on 13 March 2023), both in independent and multiplexed formats for single reaction wells (Table 2). A multiplex-specific master mix containing thermostable MMLV reverse transcriptase, dNTPs, UNG, and thermostable Fast DNA polymerase was utilized for each reaction. Cycling conditions are as follows: 25 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 3 sec and 60 °C for 30 s with fluorescence capture at every 60 °C cycle. Multiplex PCR data were obtained from the multiplex PCR reactions of the groupings (Tables 1 and 2), whereby quantitative amplification values based on Cq/Ct (Cycle threshold) and RFU (relative fluorescence units) generated a binary qualitative result (presence or absence of ABR gene). Additional information is available within the Supplementary Materials.

Table 1. Categorization of 6 ABR groupings for the detection of antibiotic resistance. Each group contains 4 ABR markers with respective targeted drug classes shown via color coordination. (Example: ABR3 contains KPC, IMP, VIM and NDM—if KPC (green) is detected, then Class A beta-lactamase resistance (green) is identified.)

Antibiotic Resistance	Targeted Drug Classes
ABR1 CTX M Group 1 CTX M Group 2 CTX M Group 8/25 CTX M Group 9	Class A beta-lactamase resistance (cephalosporins, penicillins, aztreonam)
ABR2 OXA-1 OXA-48 qnrA qnrS	Class D oxacillinases resistance (cloxacillin, oxacillin, penicillins, carbapenems, extended spectrum cephalosporins, B-lactamase inhibitors) Fluoroquinolones resistance (ciprofloxacin, gemifloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin)
ABR3 KPC IMP-1 VIM NDM	Class A beta-lactamase resistance (carbapenems, cephalosporins, penicillins, b-lactamase inhibitors, aztreonam) Class B metallo-beta-lactamase resistance (carbapenems, cephalosporins, penicillins, b-lactamase inhibitors)
ABR4 PER VEB GES ermB	Minor ESBL Resistance: (Extended spectrum cephalosporins, penicillins, aztreonam, Carbapenems, cephamycins, extended spectrum cephalosporins, penicillins) Macrolides resistance (erythromycin, clindamycin, azithromycin)
ABR5 FOX mecA vanA CMY-2	AmpC beta- lactamase resistance (cephalosporins, penicillins, b-lactamase inhibitors) Methicillin resistance Vancomycin resistance Cephamycin/cephalosporin resistance
ABR6 OXA-23 OXA-139 OXA-206 OXA-58	Oxacillinases resistance

Model for Predictive Inferencing via Antimicrobial Resistance Markers: The antibiotic resistance genes used in our 24-plex assay are associated with Class A beta-lactamases, Class D oxacillinases, Fluroquinolones, Class B metallo beta-lactamases, Minor ESBLs, Macrolides, AmpC beta-lactamases, Beta-lactams, Erythromycin ribosomal methylases and more. Predictive scoring algorithms can be useful in instances where an antibiotic resistance marker is identified without any of the targeted uropathogens. The probabilistic groupings where antibiotic resistance markers cover multiple genera can aid in predictive assessments of pathogens (Figure 1). The assay further allows for drug class avoidance stratification of patients for drugs including, but not limited to, cephalosporins, penicillins, aztreonam, carbapenems, b-lactamase inhibitors, cloxacillin, oxacillin, extended spectrum cephalosporins, cephamycins, erythromycin, clindamycin, azithromycin, ciprofloxacin, gemifloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin and more (Table 1). The predictive power of the assay will additionally enable indirect screening for other potential pathogens through the panel of antibiotic resistance markers.

Table 2. Multiplexed qPCR ABR targets. Each ABR target is shown with its amplicon length, fluorophore, and excitation and emission spectra.

ABR Gene Target	Amplicon Length (bp)	Fluorophore	Excitation (nm)	Emission (nm)
CTX-M-Group 1	185	FAM	495	520
CTX-M-Group 2	111	HEX	538	555
CTX-M-Group 8/25	145	ROX	588	608
CTX-M-Group 9	85	Cy5	648	668
OXA-1	109	FAM	495	520
OXA-48	100	HEX	538	555
qnrA	107	ROX	588	608
qnrS	100	Cy5	648	668
KPC	115	FAM	495	520
IMP	102	HEX	538	555
VIM	108	ROX	588	608
NDM	130	Cy5	648	668
PER	92	FAM	495	520
VEB	97	HEX	538	555
GES	96	ROX	588	608
ermB	105	Cy5	648	668
FOX	129	FAM	495	520
mecA	128	HEX	538	555
vanA	119	ROX	588	608
CMY-2	124	Cy5	648	668
OXA-23	81	FAM	495	520
OXA-139	106	HEX	538	555
OXA-206	106	ROX	588	608
OXA-58	93	Cy5	648	668

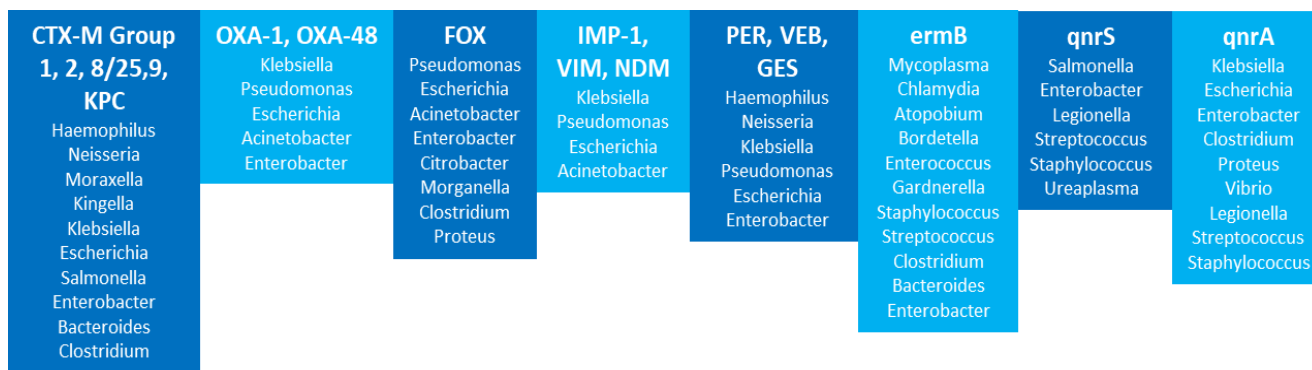


Figure 1. If one or more antibiotic resistance markers are identified without a uropathogen, then the probability of possible genus classifications for that marker is presented. This enables additional predictive value for potential organismal pathogenic targets.

Principal components analysis: Using XLSTAT, we performed a Pearson correlation-based PCA using a filter factor maximum number = 5. Input values for PCA were based on relative fluorescent units (RFUs) derived from qPCR that were translated and log-transformed. Summary statistics, Eigenvalues, and correlations between factors and variables are provided in Supplementary Materials.

Correlating MIC values and multiplex qPCR: MIC assay results were obtained from the CDC and FDA AR Isolate Bank website for all samples used in this study. Multiplex PCR results were filtered on samples with detectable ABR markers and MIC assays for all filtered samples were collated. Only instances in which >10 samples were represented after filtering were considered to prevent errors associated with low sampling. Resistance to antibiotics was determined based on MIC values; values ≥ 32 were included in the analysis to ensure robust resistance. Percent values of “likelihood of drug resistance with ABR

marker” are based on number of samples with MIC values ≥ 32 out of total samples that were tested.

3. Results

3.1. Classification and Analysis of Antibiotic Resistance Markers

We obtained 366 samples from 14 different curated isolate panels of CDC and FDA AR Isolate Bank for this study. The breakdown of samples from each panel is shown in Figure 2A. The most frequently occurring microorganisms were *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *A. baumannii*, and *E. coli* (Figure 2B), which are among the most common microorganisms that cause UTIs. Based on multiplex qPCR results, *ermB* was the most frequently occurring antibiotic resistance gene, followed by *CMY-2*, *CTX-M-G1*, and *OXA-1* (Figure 2C). Approximately one-quarter of samples did not have any detectable ABRs, ~40% had one or two ABR markers, and the remaining samples had ≥ 3 ABR markers (Figure 2D). Across the six ABR groupings (Table 1), there are a similar number of antibiotic resistance genes represented (Figure 2E).

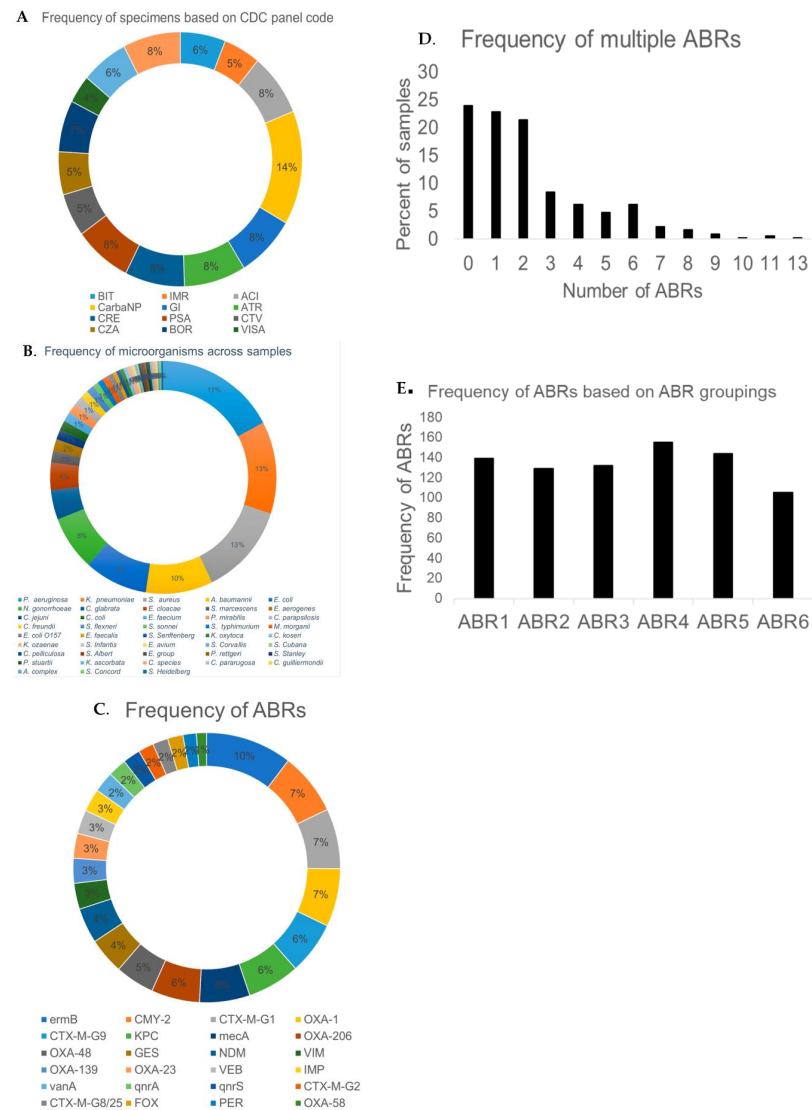


Figure 2. Classification and analysis of antibiotic resistance markers. (A). Frequency of samples from CDC panel codes (see Section 2 for panel code abbreviations) (B). Frequency of microorganisms across samples. (C). Frequency of antibiotic resistance genes based on multiplex qPCR results. (D). Prevalence of multiple antibiotic resistance genes from a sample based on multiplexed qPCR. (E). Frequency of antibiotic resistance genes based on ABR groupings as described in Figure 1.

3.2. Correlation between Antibiotic Resistance Markers

Using the multiplex qPCR data, we performed a principal component analysis (PCA) to reduce the high dimensionality of the dataset, emphasize variation, and extract trends and/or features of the data. Though the first two principal components had relatively low variance (F1 = 16.64, F2 = 8.80%), there were few observable outliers among the 366 samples based on these two components (Figure 3A). To determine whether there were any positive or negative correlations between ABR markers, we performed a Pearson correlation analysis. As shown in Figure 3B, there are potential “hotspots” of correlation; for example, a high correlation exists among members of the CTX-M group, as noted between CTX-M-G1 and CTX-M-G9 and, to a lesser extent, between CTX-M-G1 and CTX-M-G2. Further, a positive correlation between OXA family members is readily observable between OXA-206 and OXA-139 and OXA-206 and OXA-23. Another potential positive correlation may exist between OXA-48 and CTX-M family members, but this association is less robust. Among possible negative correlations, that between CTX-M-G8/25 and OXA-206 is the most robust.

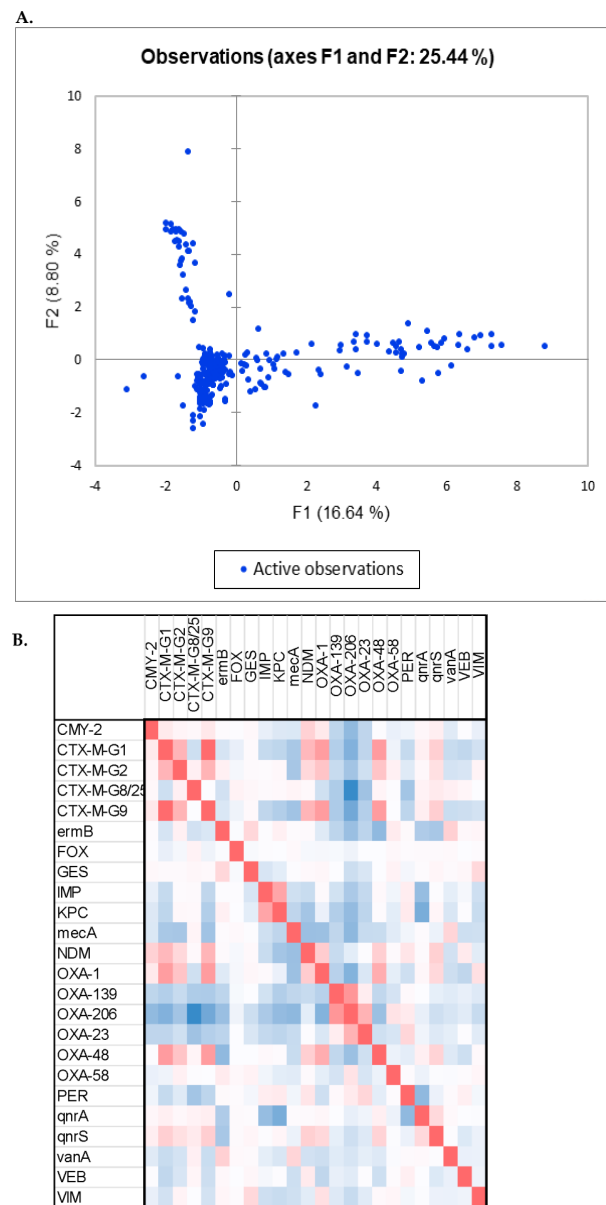


Figure 3. Correlation between antibiotic resistance markers. (A) Correlations between observations based on top two principal components (F1 and F2). (B) Heatmap showing correlations between ABR genes, whereby color shading shows strength of correlation (red = positive; blue = negative).

3.3. Accuracy of Model for Predictive Inferencing via Antimicrobial Resistance Markers

Based on the multiplex qPCR readouts, we determined the accuracy of the model for predictive inferencing via ABR markers as outlined in Figure 1. As shown in Figure 4A, the genera of 86% of bacterial samples were correctly predicted, 8.5% were incorrectly predicted, and 5.5% were not predictable based on the model. It is important to note that though neither *mecA* nor *OXA-23/139/206* are presented in the model, the genus classification was predicted to be *Staphylococcus* and *Acinetobacter*, respectively. The *mecA* gene is strongly associated with *Staphylococcus aureus*, which confers high-level resistance to methicillin and is part of a 20- to 60-kb staphylococcal chromosome cassette *mec* (*SCCmec*) [19,20]. Thus, we presumed that samples in which *mecA* was detected would be *Staphylococcus*. Several *OXA* family members, such as *OXA-51/23/206* have been identified in many clinical isolates of *Acinetobacter baumannii* and it has been shown that the genes encoding *blaOXA-51-like* beta-lactamases are chromosomally located in most *A. baumannii* isolates studied to date [21]. Therefore, we predicted that samples in which *OXA-23/139/206* were detected would be *Acinetobacter*, which was the case in this study. We recognize, however, that this model must be verified using clinical isolates as well.

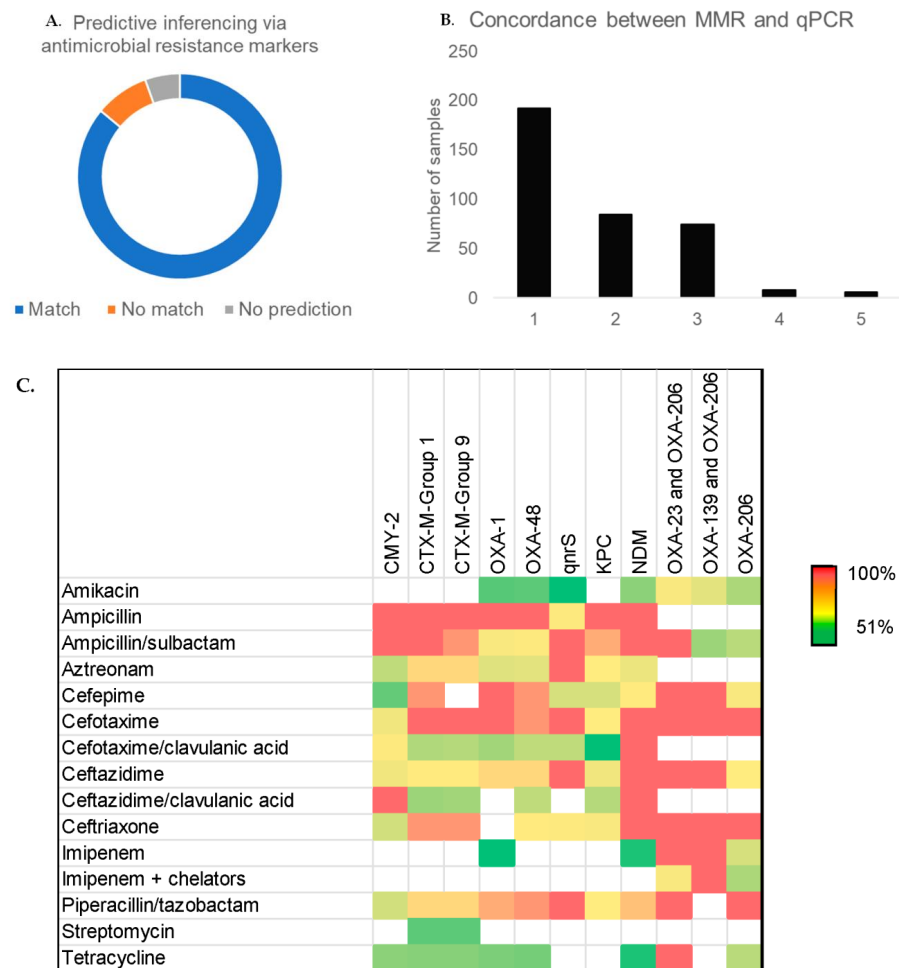


Figure 4. Concordance between CDC-tested diagnostic methods and multiplex qPCR. (A) Predictive inferencing of samples via ABR markers based on model in Figure 2. (B) Association between molecular method of resistance (MMR) assay and multiplex qPCR; 1 = MMR and qPCR are in agreement, 2 = qPCR did not test for MMR-detected gene(s), 3 = no detected ABR genes in either MMR or qPCR, 4 = ABR genes undetected in qPCR, detected in MMR, 5 = discordant results between MMR and qPCR. (C) Correlation between minimum inhibitory concentration (MIC) assay and multiplex qPCR. Percentages reflect likelihood of drug resistance with ABR markers based on results collected from >10 samples with qPCR-detectable resistance genes.

3.4. Concordance between CDC-Tested Methods and Multiplex qPCR

Many of the bacterial isolates included in this study have been phenotypically and genotypically characterized by the CDC. Thus, we compared the results obtained from whole-genome sequencing by the CDC, denoted as “molecular mechanisms of resistance” (MMR), to the results from the multiplex qPCR. Specifically, we compared whether the ABR genes identified by the two methods were in accordance. Of 366 samples, 192 (52.5%) had concordant results between MMR and multiplex qPCR. Eighty-five (23.2%) samples had detectable ABR genes that were not tested by the multiplex qPCR and seventy-five (20.5%) samples were not assayed by MMR and did not have detectable ABR genes by qPCR. Finally, eight (2.2%) samples were not assayed by MMR but did have detectable ABR genes by qPCR and six (1.6%) samples had discordant results between MMR and multiplex qPCR (Figure 4B).

Next, we evaluated whether the results obtained from the multiplex qPCR can be predictive of the results obtained from minimum inhibitory concentration (MIC) assays. MIC assays are used to determine the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism. In clinical settings, they are used to identify whether microorganisms are resistant or sensitive to antibiotics to determine the optimal drug regimen for an individual. However, MIC assays take 16–20 h of incubation, which is not ideal in situations where appropriate antibiotics should be immediately prescribed. Identification of ABR genes based on multiplex qPCR is a faster alternative to MIC assays, but whether these results can be used to determine the optimal antibiotic regimen is unclear. Thus, we filtered samples based on ABR markers identified by multiplex qPCR and correlated these results to those of the MIC assay. As shown in Figure 4C, several ABR markers were potentially correlated with resistance to some antibiotics. For example, 100% of samples in which CMY-2 was detected were resistant to ampicillin (MIC value > 32). In some cases, the likelihood of drug resistance was lower based on presence of the ABR marker, but a larger sample size should be utilized to confirm this.

4. Discussion

Monocentric uropathogen isolation via urine culture is the common diagnostic method for the identification of UTIs. UTIs exhibit polymicrobial infections coupled to heterogeneity in antibiotic resistance genes that enable persistent infections, which may be missed by culture due to restrictive growth mediums and fastidious microorganisms. Persistent infections can be observed over time and following therapy, suggesting an active bacterial evolutionary process where polymicrobial infections enable antibiotic resistance gene transfer. While urine culture can identify a limited set of pathogens, culture is likely to miss polymicrobial infections and cannot identify antibiotic resistance genes within a critical diagnostic window when compared to multiplex molecular panels [14,22,23]. Such UTI-related bacterial evolution can lead to antimicrobial resistance and relapse, thereby presenting challenges to current diagnostic standards of UTI treatment.

Here, we describe a rapid, molecular multiplexed characterization of uropathogens with antibiotic resistance gene identification that is integral to understanding the polymicrobial nature of UTIs and shifting the diagnostic paradigm towards molecular methods. While culture methods with antibiotic sensitivity testing (AST)/minimal inhibitory concentration (MIC) have traditionally provided single pathogenic infectious states for a limited number of organisms, urine culture does not provide the multivariable resolution that is critical to understanding the polymicrobial interactions in UTIs that promote persistent infection, acquisition of antibiotic resistance and disease progression.

In this study, we used samples from the CDC and FDA AR Isolate Bank, containing bacterial isolates which are frequently used to understand more about known and novel mechanisms of resistance to design innovative diagnostic methods and therapeutics. An advantage to using these samples is the availability of their genetic and phenotypic characteristics to compare with the results of the proposed multiplex qPCR in order to determine concordance between standard microbiological techniques and rapid molecular diagnostics.

The samples covered a broad range of uropathogens, including those that are common causative agents of UTIs, such as *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*. Across 366 samples, the most frequently occurring ABR gene was *ermB*, which confers resistance to macrolides, followed by *CMY-2*, *CTX-M-G1*, and *OXA-1*, which all confer resistance to cephalosporins as well as other drug classes.

We identified a potential positive correlation between families of ABR markers, including one between *CTX-M-G1* and *CTX-M-G2*, *OXA-23* and *OXA-206*, and *OXA-139* and *OXA-206*. Indeed, all samples with detectable *OXA-23* also had *OXA-206* and all samples with detectable *OXA-139* also had *OXA-206*. Co-infections with microorganisms with ABR markers of the same group are common and can enhance resistance to certain antibiotics. Our results provide evidence for this in Figure 4C, which shows that the likelihood of resistance increased for multiple drugs when both *OXA-139* or *OXA-23* was detected with *OXA-206* compared to detection of *OXA-206* alone. Some families of the OXA-beta lactamases are most commonly found in *A. baumannii*, such as *OXA-23*, *OXA-139*, and *OXA-206*. In this dataset, all samples with any of these three family members or any combination of the three OXA enzymes were from *A. baumannii*. Identification of these enzymes is important as the genus classification of uropathogens can be inferred based on detection of ABR markers. Based on our proposed model to infer bacterial genus classification via ABR markers (Figure 1), we found that almost 90% of the identity of bacterial isolates were correctly inferred (Figure 4A). While these results must be verified in a larger clinical sample size, our model is a promising tool to identify uropathogen genera, which will ultimately aid in the appropriate antibiotic regimen for treatment of UTIs.

Across 366 samples, we found that there was a high level of concordance (>50%) between results obtained from whole-genome sequencing/molecular mechanism of resistance and multiplex qPCR. In many instances of disagreement between the two methods, the multiplex qPCR did not assay for the ABR marker that was reported from the MMR or MMR results were not available and the multiplex qPCR found detectable ABR markers. These results suggest that the multiplex qPCR is a reliable and accurate genetic method to identify ABR markers and aid in the diagnosis of UTI treatment regimens. We plan to expand our analyses to clinical specimens to confirm that the assay works as well as it does in known samples of bacterial isolates. The correlation matrix between detectable ABR markers and MIC values (Figure 4C) offers insight into clinical suggestions for UTI treatment regimens. For example, identification of *CMY-2* is highly likely to confer resistance to ampicillin, cefoxitamine, and ceftazidime, which is unsurprising as *CMY-2* is known to target cephalosporins (Table 1). Previous studies have reported similar findings of *CMY-2*-producing *E. coli* isolates being resistant to ampicillin, cefotaxime, ceftazidime and cefoxitin, but susceptible to imipenem and meropenem [24]. Consistent with this, only 17% of samples with detectable *CMY-2* were resistant to imipenem in the present study. We utilize a novel molecular method that employs real-time, multiplexed qPCR for functional analysis in resolving UTIs and enabling targeted antibiotic efficiency and stewardship overall.

Finally, cost efficiencies are attainable when comparing culture vs. multiplex molecular methods. Urine cultures entail the cost of a trained and certified laboratory technician, culturing reagents and materials, including agar plates, bacterial broth, glassware/plasticware, incubators and other machines. Based on the Healthcare blue book (accessed on 1 September 2022; <https://healthcarebluebook.com/>), the cost of urine culture is estimated to be 14 USD. Costs associated with a multiplex PCR based assay include a technician, PCR reagents, including primers and probes, PCR plates, and a well-maintained PCR machine. Based on costs of PCR-based rapid HIV tests as a comparator, the estimated cost for the proposed multiplex molecular ABR assay is approximately 8–50 USD [25]. Overall, molecular detection methods can enable high-throughput cost efficiencies in the proposed multiplex model.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microbiolres14020041/s1>.

Author Contributions: Conceptualization, M.H. and T.F.; methodology, M.H.; software, M.H.; validation, M.H.; formal analysis, M.H., T.F., D.I. and N.J.D.; investigation, M.H.; resources, M.H. and N.B.; data curation, M.H.; writing—original draft preparation, M.H.; writing—review and editing, M.H., T.F., D.I. and N.J.D.; visualization, M.H.; supervision, T.F.; project administration, M.H. and T.F. All authors have read and agreed to the published version of the manuscript.

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