Genotypic antimicrobial resistance assays for use on *E. coli* isolates and stool specimens

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Abstract

Antimicrobial resistance (AMR) is an emerging public health problem and methods for surveillance are needed. We designed 85 sequence-specific PCR reactions to detect 79 genes or mutations associated with resistance across 10 major antimicrobial classes, with a focus on *E. coli*. The 85 qPCR assays demonstrated >99.9% concordance with sequencing. We evaluated the correlation between genotypic resistance markers and phenotypic susceptibility results on 239 *E. coli* isolates. Both sensitivity and specificity exceeded 90% for ampicillin, ceftriaxone, cefepime, imipenem, ciprofloxacin, azithromycin, gentamicin, amikacin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol phenotypic susceptibility results. We then evaluated the assays on direct stool specimens and observed a sensitivity of 97% ± 5 but, as expected, a lower specificity of 75% ± 31 versus the genotype of the *E. coli* cultured from stool. Finally, the assays were incorporated into a convenient TaqMan Array Card (TAC) format. These assays may be useful for tracking AMR in *E. coli* isolates or directly in stool for targeted testing of the fecal antibiotic resistome.

Introduction

Antimicrobial resistance (AMR) is a critical public health issue. Antimicrobial-resistant infections can require prolonged treatments, extend hospital stays, and result in greater disability and death compared with susceptible infections [1]. An objective of the World Health Organization (WHO) global action plan on AMR is to strengthen the evidence base through surveillance [2]. Phenotypic culture-based antimicrobial susceptibility testing (AST) is routinely used, however it requires culture and lacks resistance gene information, such as mutations in chromosomal genes or the presence of mobile genetic elements which harbor AMR genes [3–5]; such genotypic information offers useful resolution for epidemiologic purposes, such as
tracking the spread of CTX-M [6]. Furthermore, assays that can work in direct stool are advantageous because this specimen is readily accessible compared with those of invasive sites.

We designed and developed 85 genotypic assays primarily targeting Enterobacteriaceae since antibiotic resistance in these bacteria is a particularly threat [1, 7, 8]. We focused on E. coli because this was the most frequently reported bacteria in the WHO global antimicrobial resistance surveillance system (GLASS) [9] and has been associated with the greatest mortality and morbidity [10]. The assays covered 10 important antimicrobial classes used in human and veterinary medicine including penicillins, cephalosporins, carbapenems, fluoroquinolones, macrolides, aminoglycosides, polymyxins, folate pathway inhibitors, tetracyclines, and phenicols. Here we demonstrate the performance of these assays versus sequencing, compare genotypic results to phenotypic AST, and evaluate the utility of the assays on direct stool.

Materials and methods

Bacterial isolates

For validation we tested a variety of both retrospectively and prospectively collected bacterial isolates, including 201 isolates from the Food and Drug Administration and Centers for Disease Control and Prevention Antibiotic Resistance Isolate Bank (FDA-CDC AR bank, CDC, Atlanta, GA, USA), 15 isolates from Antibacterial Resistance Leadership Group (ARLG, Durham, NC, USA), and 20 isolates from American Type Culture Collection (ATCC, Manassas, VA, USA), all of which had been previously sequenced. These isolates represented a range of species, mostly from Enterobacteriaceae (S1 Table). The AMR gene accession numbers provided by the resources are summarized in S2 and S3 Tables. Additionally, we used 81 E. coli isolates from human stool from Tanzania (Haydom Lutheran Hospital, Haydom), collected as part of the Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) birth cohort study [11] to yield a distribution of phenotypically resistant isolates. We also used 107 E. coli isolates from swine feces which were prospectively collected starting February 2018 for an AMR monitoring study in Thailand (Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok). This allowed us to obtain at least several resistant bacterial isolates for each antimicrobial agent.

Stool specimens

Two hundred and twenty direct stool specimens were used, including 70 human stool samples from Tanzania (Haydom Lutheran Hospital, Haydom) also collected as a part of the MAL-ED study. The MAL-ED study was reviewed and approved by the National Institute for Medical Research, Tanzania and the University of Virginia Institutional Review Board (IRB), and informed consent was obtained from the parents or legal guardians of all subjects. One hundred and fifty consecutive swine stool samples from Thailand (Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok) were collected in February 2018. Animal specimen collection protocol no. 013/2561 was reviewed and approved by Siriraj Animal Care and Use Committees, Siriraj Hospital, Mahidol University. For culture, stool samples were streaked on MacConkey agar and incubated at 35 ± 2°C for 18–24 hour. Five to ten suspected E. coli colonies were screened by using E. coli specific PCR assay then confirmed. E. coli colonies were pooled and stored in preservative media at -70°C. Prior to AST, bacteria were subcultured on blood agar (TSA w/ 5% sheep blood, Thermo Scientific, NY, USA) at 35 ± 2°C for 18–24 hours.
DNA extraction
Genomic DNA from direct stool was extracted using the QIAamp Fast DNA Stool mini kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. Bacterial DNA was extracted by resuspending bacterial colonies in 200 μl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) or from 500 μl of 0.5 McFarland standard bacterial suspension prepared for phenotypic antimicrobial susceptibility test by centrifugation at 5000x g for 10 min, followed by resuspending the bacterial pellet with 200 μl TE buffer. The bacterial suspensions were incubated at 95˚C for 15 min followed by centrifugation at 5000x g for 10 min. The supernatant was stored at -20˚C to be used as DNA template.

PCR assay development
The primers for amplification of 80–150 bp products and TaqMan probes were designed using Primer Express3 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) and online available tool Primer3 (http://bioinfo.ut.ee/primer3/) or adopted from published sources (S4 Table). For Sanger sequencing confirmation, primers that amplified longer products (400–800 bp) were designed using primer3 (http://bioinfo.ut.ee/primer3) (S5 Table). The in silico specificity of primers and probes were tested by using Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Optimization of conditions and specificity testing of AMR-PCR assays was performed using 384 well plates on the ViiA7 platform (Applied Biosystems, Life Technologies Corporation). Each assay was amplified in duplex (see pairings in S4 Table). Primer/probe sets (final concentrations of 0.9 μM and 0.25 μM for primers and probes, respectively) were assayed in a 5 μl PCR mixture containing 2.5 μl of 2x PCR buffer, 0.2 μl of 25x PCR enzyme of AgPath-ID-PCR kit (Applied Biosystems, Life Technologies Corporation), 0.89 μl nuclease free water, and 1 μl of genomic DNA. Cycling conditions included an initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. The positive control sources included either well-characterized bacterial isolates or synthetic fragment/plasmid controls (Genewiz Inc., South Plainfield, NJ, USA). Synthetic positive control plasmids were constructed (Genewiz Inc.) if neither the genomic material nor the relevant bacterium was available; this included CTX-M8, CMY-1, FOX, GES, gyrA87G-E.coli, gyrA87N-Y-Salmonella spp., and mcr-2. A synthetic positive control plasmid was also constructed (Genewiz Inc.) to contain the primer and probe regions of all 85 targets and used as a positive control for evaluating analytical performance. Genomic DNA of E. coli ATCC 25922 was used as negative control, and nuclease-free water was used as a no-template control.

PCR assay evaluation
AMR-PCR assay efficiency and linearity were first determined on the 384 well plate format and subsequently on the TaqMan array card format. For the 384 well plate, the synthetic positive control plasmids (Genewiz Inc.) which contained primer/probe regions of all targets were 10-fold serially diluted in a range of 10^7 to 1 copy/μl then 1 μl of diluted samples was tested in each 5 μl reaction in triplicate. For the array card format, since the volume of DNA used in the array card is 5-fold lower (0.2 μl/reaction), dilutions of positive control plasmids were prepared in a range of 5x10^7 to 5 copy/μl to ensure equivalence on both formats. Twenty microliter of each diluted sample was tested in triplicate by mixing with PCR reagents to a total 100 μl then loaded into an array card. The limit of detection (LOD) and precision (repeatability and reproducibility) were determined by spiking positive control plasmid into donor stool followed by extraction and then amplification on the array card. Repeatability was tested with eight repeats of two samples respectively spiked with a high (10^6 copies/200 mg stool) and a
low ($10^4$ copies/200 mg stool) concentration of positive control plasmid. Reproducibility was tested with 10 identically spiked samples for each concentration ($10^6$ and $10^4$ copies/200 mg stool were interrogated) that were extracted and assayed over 5 days. LOD was defined as the lowest concentration at which the target could be detected in all 10 spiked samples. When comparing the performance of the AMR assays against previously-sequenced bacterial isolates, any discrepancies between PCR and sequence underwent confirmatory repeat PCR and sequencing.

### Evaluation of TaqMan array card

The TaqMan array card was performed as previously described [12]. Briefly, primer and TaqMan probe oligonucleotides were synthesized and spotted into the microfluidic card by Applied Biosystems (Life Technologies Corporation). Twenty microliters of input DNA was mixed with 50 μl of 2x PCR buffer, 4 μl of 25x PCR enzyme of AgPath-ID-PCR kit (Applied Biosystems, Life Technologies Corporation), and 26 μl of nuclease free water to yield a 100 μl final volume. This mixture was loaded into each port of the card and the card was centrifuged twice at 1,200 rpm for 1 min and then sealed. The loading ports were excised and the full card was inserted into a ViiA7 instrument (Life Technologies Corporation) and run under the same cycling conditions as described above.

### Sanger sequencing

The resistance-associated genes were amplified using primers described in S5 Table. The PCR reaction assembly and cycling conditions were described previously [13]. In brief each 25 μl PCR mixture contained 12.5 μl HotStarTaq master mix (Qiagen), 0.25 μl of the 50 μM forward and reverse primers (final concentration of 0.5 μM), 7 μl nuclease free water, and 5 μl of genomic DNA. PCR was performed on a CFX96 (Bio-Rad, Hercules, CA, USA) and included an initial denaturation step at 95˚C for 15 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec, with a final extension step at 72˚C for 10 min. PCR products were analyzed on 2% agarose-gels and verified PCR products were purified using MinElute 96 UF PCR Purification Kit (Qiagen) following the manufacturer’s protocol. The purified PCR products were measured spectrophotometrically, diluted with nuclease free water and mixed with primers then submitted to GeneWiz for DNA sequencing (Genewiz Inc.).

### Phenotypic antimicrobial susceptibility testing

The repository isolates and the isolates from Thailand underwent susceptibility testing by broth microdilution method while isolates from Tanzania were previously tested by disc diffusion for ampicillin (AMP), ampicillin/sulbactam (SAM), cefazolin (CFZ), ceftazidime (CAZ), ceftriaxone (CRO), aztreonam (ATM), cefepime (FEP), cefoxitin (FOX), ertapenem (ETP), ciprofloxacin (CIP), gentamicin (GM), and trimethoprim/sulfamethoxazole (TMP-SMX). All of the isolates were tested by broth microdilution method for imipenem (IPM), azithromycin (AZM), amikacin (AMK), kanamycin (KAN), tetracycline (TET), chloramphenicol (CHL), and colistin (CL) and disc diffusion method was used for streptomycin (STR) on all isolates. All methodologies were performed according to the Clinical and Laboratory Standards Institute (CLSI) protocol [14, 15]. Antimicrobial agents used for broth microdilution were AMP, CFZ, FOX, CRO, CAZ, ETP, CIP, AZM, GM, AMK, KAN, TMP-SMX, TET, CHL, CL (all from Sigma-Aldrich, St. Louis, MO, USA), ATM, IPM, sulbactam (all from AdooQ Bioscience, Irvine, CA, USA) and FEP (Alfa Aesar, Tewksbury, MA, USA). In brief for broth microdilution, antimicrobial agents were 2-fold serially diluted in cation-adjusted Mueller Hinton broth.
CamHb, BBL Mueller Hinton II Broth, Becton Dickinson, Sparks, MD, USA) and 100 μl of each dilution including no-antibiotic control media were dispensed into 96 well round bottom culture plates. Bacterial suspensions were prepared in normal saline and adjusted to 0.5 McFarland standards following diluting at 1:20 in sterile distilled water to obtain 5 x 10^6 cfu/ml. Then 10 μl of bacterial inoculum was inoculated into 96 well round bottom plates and incubated at 35 ± 2 °C for 16–20 hour. Antimicrobial agents used for disc diffusion were AMP (10 μg), SAM (10/10 μg), CFZ (30 μg), FOX (30 μg), CRO (30 μg), CAZ (30 μg), FEP (30 μg), ATM (30 μg), ETP (10 μg), CIP (5 μg), GM (10 μg), STR (10 μg), and TMP-SMX (1.25/23.75 μg) (all from Becton Dickinson). For disc diffusion, the 0.5 McFarland standard bacterial suspensions were dipped by sterile cotton swab and swabs were streaked over the entire Mueller Hinton agar (MHA, BBL Mueller Hinton II Agar, Becton Dickinson) surface. The disc containing antibiotics were placed onto the surface of inoculated agar plate, and incubated at 35 ± 2 °C for 16–18 hour. The E. coli ATCC 25922, and P. aeruginosa ATCC 27853 (for carbapenem) were used as quality control and the minimal inhibitory concentration (MIC) and zone diameter interpretative standard of CLSI-M100 Ed29 [16] were used for interpretation. The results of standard phenotypic AST and genotypic PCR testing were unblinded to the reader. If there were any discrepancies between PCR and AST then both methods were repeated and the repeat results were considered final (540/568 or 95.1% were identical to the original result). The phenotypic AST results of all isolates are shown in S6 Table.

Statistical analysis

The sensitivity, specificity, and accuracy of genotypic test methods were analyzed against phenotypic methods as the gold standard. The kappa coefficient (κ) was calculated with GraphPad QuickCalcs (https://www.graphpad.com/quickcalcs/kappa1.cfm) to measure agreement between methods. Receiver-operating characteristic (ROC) analysis was performed with SPSS Statistics Software to define a Ct (quantification cycle) cut-off that optimized sensitivity and specificity.

Results

Antimicrobial resistance associated gene targets

We sought to develop assays to detect resistance to the antimicrobial classes commonly used in both human and veterinary medicine, namely penicillins, cephalosporins, carbapenems, fluoroquinolones, macrolides, aminoglycosides, folate pathway inhibitors, tetracyclines, phenicols, and polymyxins. The gene targets were chosen based on previously reported genes or mutations and we prioritized candidates based on global prevalence (S7 Table). Because there are many subgroups of genes (e.g., CTX-M), most assays were designed in conserved regions as group-specific assays (S8 Table). In addition to AMR targets, since a goal was to later evaluate these assays directly on stool specimens we also included E. coli/Shigella spp., Salmonella spp., and Campylobacter spp. specific assays for fluoroquinolone (in gyrA and parC) and macrolide resistance (in 23S rRNA), as well as previously published detection assays for these genera [17–20]. Additionally, internal and external controls were included (bacterial 16S rRNA and phocine herpesvirus, respectively). This amounted to PCR assays that included 69 primer pairs and 85 specific probes (S4 Table).

PCR assay performance versus sequencing

We organized the assays into 42 duplex reactions and 1 singleplex reaction on a 384 well plate using dilutions of positive control plasmid. The overall linearity of the 85 assays was
| Targets            | No of positive\(^a\) tested | No of negative\(^a\) tested | PCR assay result | Concordance (%) | Targets            | No of positive\(^a\) tested | No of negative\(^a\) tested | PCR assay result | Concordance (%) |
|--------------------|------------------------------|------------------------------|------------------|----------------|--------------------|------------------------------|------------------------------|------------------|-----------------|
| **Beta lactam genes** |                              |                              |                  |                | **Beta lactam genes** |                              |                              |                  |                |
| TEM 104E           | 105                          | 131                          | 105              | 131            | TEM 104K          | 3                            | 233                          | 3                | 233            | 100            |
| TEM 164R           | 106                          | 130                          | 106              | 130            | TEM 164SC         | 2                            | 234                          | 2                | 234            | 100            |
| DHA                | 3                            | 233                          | 3                | 233            | TEM 238S         | 5                            | 231                          | 5                | 231            | 100            |
| SHV                | 68                            | 168                          | 68               | 167/168        | SHV 238-240SE-SK | 28                           | 208                          | 28               | 208            | 100            |
| CTX-M1             | 43                            | 193                          | 43               | 193            | CTX-M8-M25        | 0                            | 236                          | 0                | 236            | 100            |
| CTX-M2-M74         | 5                            | 231                          | 5                | 231            | CTX-M9           | 6                            | 230                          | 6                | 229/230        | 99             |
| PER                | 3                            | 233                          | 3                | 233            | VEB               | 1                            | 235                          | 1                | 235            | 100            |
| CMY1-MOX           | 0                            | 236                          | 0                | 236            | FOX               | 0                            | 236                          | 0                | 236            | 100            |
| CMY2-LAT           | 37                           | 199                          | 37               | 199            | ACT-MIR          | 14                           | 222                          | 14               | 222            | 100            |
| KPC                | 37                           | 199                          | 37               | 199            | GES               | 0                            | 236                          | 0                | 236            | 100            |
| NDM                | 37                           | 199                          | 37               | 199            | VIM               | 10                           | 226                          | 10               | 226            | 100            |
| IMP                | 5                            | 231                          | 5                | 231            | OXA-48           | 12                           | 224                          | 12               | 224            | 100            |
| OXA-1              | 37                           | 199                          | 37               | 199            | OXA-9            | 30                           | 206                          | 30               | 205/206        | 99             |
| **Fluoroquinolone genes** |                              |                              |                  |                | **Fluoroquinolone genes** |                              |                              |                  |                |
| QnrA               | 3                            | 233                          | 3                | 233            | QnrS             | 8                            | 228                          | 8                | 228            | 100            |
| QnrB1              | 17                           | 219                          | 17               | 218/219        | QnrB4            | 20                           | 216                          | 20               | 216            | 100            |
| aac(6')-Ib-104W    | 68                           | 168                          | 68               | 168            | aac(6')-Ib-104R  | 38                           | 198                          | 38               | 198            | 100            |
| gyrA87G-Esh\(^b\)  | 0                            | 236                          | 0                | 236            | aac(6')-Ib-181Y  | 38                           | 198                          | 38               | 198            | 100            |
| QepA               | 1                            | 235                          | 1                | 235            | gyrA87G-Sal\(^b\) | 2                            | 234                          | 2                | 234            | 100            |
| gyrA83S-Sal\(^c\)  | 8                            | 228                          | 8                | 226/228        | gyrA83FY-Sal\(^f\) | 3                            | 233                          | 3                | 233            | 100            |
| gyrA87D-Sal\(^c\)  | 9                            | 227                          | 9                | 227            | gyrA87NY-Sal\(^f\) | 0                            | 236                          | 0                | 236            | 100            |
| gyrA83S-Esh\(^b\)  | 22                           | 214                          | 22               | 21             | gyrA83L-Esh\(^b\) | 40                           | 196                          | 40               | 195/196        | 99             |
| gyrA87D-Esh\(^b\)  | 23                           | 213                          | 23               | 213            | gyrA87NY-Esh\(^b\) | 39                           | 197                          | 39               | 197            | 100            |
| parC80S-Sal\(^b\)  | 9                            | 227                          | 9                | 226/227        | parC80I-Sal\(^f\) | 2                            | 234                          | 2                | 234            | 100            |
| parC80S-Esh\(^b\)  | 25                           | 211                          | 25               | 211            | parC80I-Esh\(^b\) | 37                           | 199                          | 37               | 199            | 100            |
| gyrA86T-Cj\(^d\)   | 3                            | 233                          | 3                | 233            | gyrA86I-Cj\(^d\) | 2                            | 234                          | 2                | 234            | 100            |
| gyrA86T-Cc\(^e\)   | 3                            | 233                          | 3                | 233            | gyrA86I-Cc\(^e\) | 2                            | 234                          | 2                | 234            | 100            |
| **Macrolide genes** |                              |                              |                  |                | **Tetracycline genes** |                              |                              |                  |                |
| 23S-2075A-Cp\(^f\) | 5                            | 231                          | 5                | 231            | 23S-2075G-Cp\(^f\) | 5                            | 231                          | 5                | 231            | 100            |
| ErmB               | 6                            | 230                          | 6                | 230            | mphA             | 50                           | 186                          | 50               | 186            | 100            |
| **Aminoglycoside genes** |                              |                              |                  |                | **Folate pathway inhibitor genes** |                              |                              |                  |                |
| armA               | 17                           | 219                          | 17               | 219            | rmtB             | 3                            | 233                          | 3                | 233            | 100            |
| aacC1              | 4                            | 232                          | 4                | 232            | aacC2            | 52                           | 184                          | 52               | 184            | 100            |
| aacC4              | 7                            | 229                          | 7                | 229            | aadB             | 21                           | 215                          | 21               | 215            | 100            |
| aphA1              | 38                           | 198                          | 38               | 197/198        | aadA1-2-17       | 93                           | 143                          | 93               | 143            | 100            |
| **Tetracycline genes** |                              |                              |                  |                |                   |                              |                              |                  |                |

(Continued)
0.999 ± 0.002 and PCR efficiencies were 96.2% ± 3.9 (S9 Table). The specificity of the assays was tested against 15 other commonly found enteropathogens including Aeromonas hydrophila, Adenovirus, Bacteroides fragilis, Blastocystis hominis, Clostridium difficile, Cryptosporidium hominis, Entamoeba histolytica, Encephalitozoon intestinalis, Giardia lamblia, Helicobacter pylori, Schistosoma mansoni, Vibrio cholerae, Vibrio parahaemolyticus, Yersinia enterocolitica, and Yersinia pseudotuberculosis and no false positives were observed. Assay performance was then tested against 236 previously-sequenced bacterial isolates consisting of several genera and species (S1 Table). The genotypic PCR assays showed 100% sensitivity and >99.9% overall concordance against sequencing, with 11/20060 discrepancies (Table 1).

### Correlation between genotypic and phenotypic antimicrobial susceptibility testing

We then evaluated the correlation between genotypic and phenotypic AST on 239 E.coli isolates. This included a range of susceptible and resistant isolates from FDA-CDC-AR bank (n = 42), ARLG (n = 4), ATCC (n = 5), clinical human isolates (n = 81) and swine isolates (n = 107). This evaluation is based on the necessary but oversimplified assumption that if a resistance-associated gene or mutation was present, at any quantity (Ct cutoff 30), then that isolate would be resistant to that antimicrobial agent, while if such a gene or mutation was absent then the isolate would be susceptible. This comparison showed that the sensitivity for detecting phenotypic resistance ranged between 86% - 100% for 15 antimicrobial agents (i.e., the very major error rates were 0–14%), whereas sensitivity for resistance to cefoxitin, kanamycin, streptomycin, colistin, and ampicillin/sulbactam was lower at 76%, 75%, 72%, 67%, and 43% respectively (Table 2). The specificity of the assays for detecting phenotypic susceptibility

| Targets                        | No of positive* tested | No of negative* tested | PCR assay result | Concordance (%) | Targets                        | No of positive* tested | No of negative* tested | PCR assay result | Concordance (%) |
|--------------------------------|------------------------|------------------------|------------------|----------------|--------------------------------|------------------------|------------------------|------------------|-----------------|
| tetA                           | 58                     | 178                    | 58               | 100            | tetB                           | 25                     | 211                    | 25               | 100             |
| Phenicol genes                 |                         |                        |                  |                | catB3                          | 8                      | 228                    | 8                | 100             |
| cmlA                           | 27                     | 209                    | 27               | 100            | floR                           | 18                     | 218                    | 18               | 100             |
| Polymyxin genes                |                         |                        |                  |                | mcr-2                          | 0                      | 236                    | 0                | 100             |
| Bacterial genera and controls  |                         |                        |                  |                | E.coli-Shigella                | 61                     | 175                    | 61               | 100             |
| Salmonella spp.                | 11                     | 225                    | 11               | 100            | C. jejuni-coli                | 10                     | 226                    | 10               | 100             |
| PhHV                           | 0                      | 236                    | 0                | 100            | Bacterial 16S                 | 236                    | 0                      | 236              | 100             |
| Total                          | 2171                   | 17889                  | 2171             | 99.9           |                                |                        |                        |                  |                 |

* Whole genome sequencing or Sanger sequencing

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Table 2. Correlation between genotypic (AMR-PCR assay) and phenotypic AST of *E. coli* isolates (N = 239).

| Antibiotic        | Resistant genes                                                                 | PCR assay | Phenotypic AST$^a$ | Sens. (%) | Spec. (%) | Categorical agreement (%) | Kappa$^b$ (κ) |
|-------------------|---------------------------------------------------------------------------------|-----------|--------------------|-----------|-----------|--------------------------|---------------|
|                   |                                                                                  |           | R                  | I         | S         |                          |               |
| Amoxicillin       | Class A β-lactamase; TEM, SHV, CTX-M1, CTX-M8, CTX-M9, KPC                     | Positive  | 202                | 1         | 1         | 99                       | 0.97          |
|                   | Class B β-lactamase; NDM                                                        | Negative  | 1                   | 1         | 33        |                          |               |
|                   | Class C β-lactamase; CMY2-LAT, ACT-MIR, DHA                                    | Negative  | 2                   | 0         | 33        |                          |               |
|                   | Class D β-lactamase; OXA-1, OXA-9, OXA-48                                      | Negative  | 45                  | 8         | 0         | 43                       | 0.38          |
| Ampicillin/       | Class B β-lactamase; NDM                                                        | Negative  | 59                  | 55        | 72        |                          |               |
| Sulbactam         | Class C β-lactamase; CMY2-LAT, ACT-MIR, DHA                                    | Negative  | 5                     | 13       | 183       |                          |               |
| Ceftriazone Class B β-lactamase; NDM                                      | Positive  | 31                  | 2         | 0         | 76       | 100                       | 0.84          |
|                    | Class C β-lactamase; CMY2-LAT, ACT-MIR, DHA                                    | Negative  | 10                  | 13       | 183       |                          |               |
| Cefazolin Class A β-lactamase; TEM, SHV, CTX-M1, CTX-M8, CTX-M9, KPC        | Positive  | 71                  | 6         | 19        | 100      | 88                       | 0.82          |
|                    | Class B β-lactamase; NDM                                                        | Negative  | 0                   | 0         | 143       |                          |               |
|                    | Class C β-lactamase; CMY2-LAT, ACT-MIR, DHA                                    | Negative  | 1                   | 0         | 144       |                          |               |
| Cefepime Class A β-lactamase; CTX-M1, CTX-M8, CTX-M9, KPC                   | Positive  | 58                  | 9         | 8         | 95       | 95                       | 0.88          |
|                    | Class B β-lactamase; NDM                                                        | Negative  | 3                   | 1         | 160       |                          |               |
| Aztreonam Class A β-lactamase; TEM-ESBL, SHV-ESBL, CTX-M1, CTX-M8, CTX-M9, KPC | Positive  | 69                  | 5         | 20        | 100      | 88                       | 0.81          |
|                    | Class B β-lactamase; CMY2-LAT, ACT-MIR, DHA                                    | Negative  | 0                   | 1         | 144       |                          |               |
| Ertapenem Class A β-lactamase; KPC                                          | Positive  | 18                  | 0         | 0         | 86       | 100                      | 0.92          |
|                    | Class B β-lactamase; NDM                                                        | Negative  | 3                   | 2         | 216       |                          |               |
|                    | Class D β-lactamase; OXA-48                                                     | Negative  | 18                  | 0         | 0         | 90                       | 0.94          |
| Imipenem Class A β-lactamase; KPC                                           | Positive  | 18                  | 0         | 0         | 90       | 100                      | 0.92          |
|                    | Class B β-lactamase; NDM                                                        | Negative  | 2                   | 0         | 219       |                          |               |
| Ciprofloxacin$^c$ gyrA, parC                                                | Mutant    | 61                  | 0         | 7         | 97       | 94                       | 0.89          |
|                    | Mt + Wt                                                                          | 17       | 6         | 12        |            |                          |               |
|                    | Wild-type                                                                        | 2         | 17       | 117       |            |                          |               |
| Azithromycin$^d$ ermB, mphA                                                 | Positive  | 78                  | 0         | 3         | 95       | 98                       | 0.93          |
|                    | Negative                                                                         | 4         | 0         | 154       |            |                          |               |
| Gentamicin aacC2, aacC4, aac(6')-lb, aadB, rmtB                               | Positive  | 79                  | 1         | 4         | 96       | 97                       | 0.93          |
|                    | Negative                                                                         | 3         | 0         | 152       |            |                          |               |
| Amikacin aac(6')-lb, rmtB                                                   | Positive  | 12                  | 0         | 6         | 100      | 97                       | 0.79          |
|                    | Negative                                                                         | 0         | 0         | 221       |            |                          |               |
| Kanamycin aphA1                                                            | Positive  | 51                  | 1         | 0         | 75       | 100                      | 0.81          |
|                    | Negative                                                                         | 17        | 2         | 168       |            |                          |               |
| Streptomycin aadA1-2-17                                                    | Positive  | 107                 | 9         | 18        | 72       | 78                       | 0.47          |
|                    | Negative                                                                         | 41        | 1         | 63        |            |                          |               |

(Continued)
ranged between 88% - 100% for all antimicrobial agents (i.e., major error rates 0–12%) except streptomycin and cefazolin (78% and 70%, respectively). Overall, sensitivity and specificity exceeded 90% for ampicillin, ceftriaxone, cefepime, imipenem, ciprofloxacin, azithromycin, gentamicin, amikacin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol phenotypic susceptibility results, with substantial or better kappa ($\kappa$) agreement between the two methods ($\kappa = 0.79–0.97$). Categorical agreement of the genotypic versus phenotypic method, ignoring intermediate results which cannot be categorized, was greater than 90% for all antimicrobial agents except for ampicillin/sulbactam (66%) and streptomycin (74%).

**AMR detection in direct stool specimens**

We then sought to evaluate the sensitivity of these AMR-PCR assays on direct stool specimens versus the genotypic pattern of the *E. coli* cultured from the stool. The focus on *E. coli* was based on its importance as an indicator organism, a member of the stool microbiome, and a reservoir of AMR genes. Comparing results from 220 stool DNA (70 human, 150 swine) versus the paired *E. coli* isolates cultured from those stools, using a Ct cut-off of 32 (S1 Fig for ROC analysis), direct genotypic testing of stool predicted the cultured *E. coli* genotype with an overall sensitivity of 97% ± 5 across all genes, an overall specificity of 75% ± 31, and an overall accuracy 85% ± 17 (Table 3).

**Performance of AMR-TAC**

The AMR-PCR assays were then compartmentalized into a TaqMan array card (TAC) format and the analytical PCR performance of each assay was determined (Fig 1). The overall linearity of the 85 targets was 0.999 ± 0.001 and PCR efficiencies were 95.1% ± 2.5 (S10 Table). The limit of detection, defined as lowest copy number that was detected in all 10 extractions/amplification, was $10^4$ copies per 200 mg stool (10 copies per PCR reaction). The coefficient of variant (CV) of Ct values was 3.6% ± 2.0 and 4.7% ± 2.1 for repeatability and reproducibility, respectively. The performance of AMR-TAC was then determined against 122 DNA samples

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**Table 2. (Continued)**

| Antibiotic | Resistant genes | PCR assay | Phenotypic ASTa | Sens. (%) | Spec. (%) | Categorical agreement (%) | Kappab ($\kappa$) |
|------------|----------------|-----------|-----------------|-----------|-----------|--------------------------|-----------------|
| Trimethoprim/ sulmefhoxazole | dfrA1, dfrA5-14, dfrA12, dfrA17, sul1, sul2, sul3 | Positive | 168 0 2         | 92        | 96        | 93                       | 0.83            |
| Tetracycline | tetA, tetB | Positive | 172 0 4         | 99        | 94        | 97                       | 0.94            |
| Chloramphenicol | catA1, catB3, cmlA, floR | Positive | 112 6 9         | 99        | 92        | 96                       | 0.91            |
| Colistin* | mcr-1 | Positive | 29 0 14         | 67        | 99        | 94                       | 0.76            |

a Excluded intermediate (I) from analysis  
b Strength of the kappa ($\kappa$) coefficients: 0.01–0.20 slight; 0.21–0.40 fair; 0.41–0.60 moderate; 0.61–0.80 substantial; 0.81–1.0 almost perfect agreement  
c Excluded 35 mixed mutant and wild-type from analysis  
d Used interpretative criteria of CLSI M100 29Ed [16] for *Salmonella enterica* Typhi where MIC ≤16 is susceptible and ≥32 is resistant  
*e Used interpretative criteria of CLSI M100 29Ed [16] for *Pseudomonas aeruginosa* where MIC ≤2 is susceptible and ≥4 is resistant

Sens.; sensitivity, Spec.; specificity

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Table 3. Comparison of AMR-PCR assay detection in direct stool and paired *E. coli* isolates (N = 220).

| Targets                | N positive by culture<sup>a</sup> | N negative by culture<sup>a</sup> | Direct stool result | Sens. (%) | Spec. (%) | Targets                | N positive by culture<sup>a</sup> | N negative by culture<sup>a</sup> | Direct stool result | Sens. (%) | Spec. (%) |
|------------------------|-----------------------------------|-----------------------------------|---------------------|-----------|-----------|------------------------|-----------------------------------|-----------------------------------|---------------------|-----------|-----------|
| **Beta lactam genes**  |                                   |                                   |                     |           |           | **Beta lactam genes**  |                                   |                                   |                     |           |           |
| TEM 104E               | 190                               | 30                                | 0/30                | 100       | 0         | TEM 104K               | 0                                 | 220                               | 0                   | 220       | NA        |
| TEM 164R               | 190                               | 30                                | 0/30                | 100       | 0         | TEM 164SC              | 0                                 | 220                               | 0                   | 220       | NA        |
| DHA                   | 2                                 | 218                               | 2                   | 183/218   | 100       | TEM 238S               | 0                                 | 220                               | 0                   | 220       | NA        |
| SHV                   | 2                                 | 218                               | 2                   | 181/218   | 100       | SHV 238-240SE-SK       | 0                                 | 220                               | 0                   | 205/220   | NA        |
| **CTX-M1**            | 42                                | 178                               | 40/42               | 143/178   | 95        | CTX-M8-M25             | 1                                 | 219                               | 1                   | 211/219   | 100       |
| **CTX-M2-M74**        | 0                                 | 220                               | 0                   | 218/220   | NA        | CTX-M9                 | 29                                | 191                               | 27/29               | 139/191   | 93        |
| **PER**               | 0                                 | 220                               | 0                   | 218/220   | NA        | VEB                    | 2                                 | 218                               | 2                   | 151/218   | 100       |
| **CMY1-MOX**          | 0                                 | 220                               | 0                   | 217/220   | NA        | FOX                    | 0                                 | 220                               | 0                   | 220/220   | NA        |
| **CMY2-LAT**          | 8                                 | 212                               | 7/8                 | 170/212   | 87        | ACT-MIR                | 1                                 | 216                               | 1                   | 178/216   | 100       |
| **KPC**               | 0                                 | 220                               | 0                   | 220/220   | NA        | GES                    | 0                                 | 220                               | 0                   | 196/220   | NA        |
| **NDM**               | 0                                 | 220                               | 0                   | 220/220   | NA        | VIM                    | 0                                 | 220                               | 0                   | 220       | NA        |
| **IMP**               | 0                                 | 220                               | 0                   | 216/220   | NA        | OXA-48                 | 0                                 | 220                               | 0                   | 220       | NA        |
| **OXA-1**             | 14                                | 206                               | 11/14               | 138/206   | 79        | OXA-1                  | 1                                 | 219                               | 1                   | 220       | NA        |
| **QnrB**              | 0                                 | 220                               | 0                   | 218/220   | NA        | QnrA                   | 126                               | 94                                | 126                  | 54/94     | 100       |
| **aac(6')-lb-104W**   | 3                                 | 217                               | 3                   | 141/217   | 100       | aac(6')-lb-104R        | 4                                 | 216                               | 4                   | 197/216   | 100       |
| **gyrA87G-Esh<sup>b</sup>** | 2                         | 218                               | 2                   | 217/218   | 100       | gyrA87G-Esh<sup>b</sup> | 4                                 | 216                               | 4                   | 192/216   | 100       |
| **gyrA835-Esh<sup>b</sup>** | 196                      | 24                                | 195/196             | 20/24     | 99        | gyrA835-Esh<sup>b</sup> | 86                                | 134                               | 77/86               | 124/134   | 89        |
| **gyrA87D-Esh<sup>b</sup>** | 206                   | 14                                | 206                  | 12/14     | 100       | gyrA87D-Esh<sup>b</sup> | 37                                | 183                               | 33/37               | 180/183   | 89        |
| **parC805-Esh<sup>b</sup>** | 199                 | 21                                | 197/199             | 19/21     | 99        | parC805-Esh<sup>b</sup> | 30                                | 190                               | 28/30               | 188/190   | 93        |
| **QepA**              | 1                                 | 219                               | 1                   | 214/219   | 100       | QepA                   | 1                                 | 219                               | 1                   | 220       | NA        |
| **ermB**              | 39                                | 181                               | 39                  | 57/181    | 100       | mphA                   | 70                                 | 150                               | 67/70               | 92/150     | 96        |
| **Aminoglycoside genes** |                                   |                                   |                     |           |           | **Aminoglycoside genes** |                                   |                                   |                     |           |           |
| **armA**              | 0                                 | 220                               | 0                   | 220       | NA        | rmtB                   | 5                                 | 215                               | 5                   | 205/215   | 100       |
| **aacC1**             | 0                                 | 220                               | 0                   | 220/220   | NA        | aacC2                  | 95                                | 125                               | 93/95               | 81/125     | 98        |
| **aacC4**             | 7                                 | 213                               | 7                   | 72/213    | 100       | aadB                   | 5                                 | 215                               | 5                   | 87/215     | 100       |
| **aphA1**             | 80                                | 140                               | 80                  | 43/140    | 100       | aadA1-2-17             | 172                               | 48                                | 171/172             | 7/48       | 99        |
| **Folate pathway inhibitor genes** |                             |                                   |                     |           |           | **Folate pathway inhibitor genes** |                             |                                   |                     |           |           |
| **dfrA1**             | 43                                | 177                               | 43                  | 23/177    | 100       | dfrA12                 | 137                               | 83                                | 136/137             | 52/83      | 99        |
| **dfrA5-14**          | 82                                | 138                               | 78/82               | 80/138    | 95        | dfrA17                 | 46                                | 174                               | 36/46               | 138/174   | 78        |
| **sul1**              | 73                                | 147                               | 73                  | 14/147    | 100       | sul2                   | 154                               | 66                                | 154                  | 66/160     | 100       |
| **sul3**              | 145                               | 75                                | 142/145             | 65/75     | 98        | sul3                   | 100                               | NA                                |                     |           |           |
| **Tetracycline genes** |                                   |                                   |                     |           |           | **Tetracycline genes** |                                   |                                   |                     |           |           |
| **tetA**              | 177                               | 43                                | 176/177             | 1/43      | 99        | tetB                   | 108                               | 112                               | 108                  | 15/112     | 100       |
| **Phenicol genes**    |                                   |                                   |                     |           |           | **Phenicol genes**    |                                   |                                   |                     |           |           |
| **catA1**             | 44                                | 176                               | 41/44               | 148/176   | 93        | catB3                  | 3                                 | 217                               | 3                   | 173/217   | 100       |
| **cmlA**              | 143                               | 77                                | 141/143             | 68/77     | 99        | floR                   | 72                                 | 148                               | 71/72               | 123/148    | 99        |
| **Polymyxin genes**   |                                   |                                   |                     |           |           | **Polymyxin genes**   |                                   |                                   |                     |           |           |
| **mcr-1**             | 26                                | 194                               | 26                  | 176/194   | 100       | mcr-2                  | 0                                 | 220                               | 0                   | 192/220   | NA        |

(Continued)
including direct stools (n = 56) and cultured isolates (n = 66). TAC yielded nearly perfect concordance with the plate results: 100% concordance on cultured isolates and 99.6% ± 1.5 sensitivity and 99.2% ± 3.5 specificity on direct stools (Table 4).

**Discussion**

In this work we developed an extensive menu of qPCR assays to detect AMR-associated genes or mutations for 10 antimicrobial classes that can be used for epidemiologic purposes. The accuracy was almost perfect compared to direct sequencing, with only 0.05% discrepancy. When used on direct stool samples, the PCR assays were sensitive at detecting the AMR genes carried by resident *E. coli*. As expected the specificity was lower, presumably because AMR genes in stool derive from any member of bacteria besides *E. coli*. Such a high sensitivity assay could be useful as a screening test of the resistome in surveillance specimens such as human and livestock stool or environmental materials for epidemiologic purposes [21, 22]. Of course,

![Fig 1. Antimicrobial resistance TaqMan array card (AMR-TAC) layout. The TaqMan array card includes 8 sample ports. Each well was configured and grouped according to antimicrobial resistance associated with those gene targets.](https://doi.org/10.1371/journal.pone.0216747.g001)

| Targets                  | N positive by culturea | N negative by culturea | Direct stool result Positive | Sens. (%) | Spec. (%) | Targets                  | N positive by culturea | N negative by culturea | Direct stool result Positive | Sens. (%) | Spec. (%) |
|--------------------------|------------------------|------------------------|------------------------------|-----------|-----------|--------------------------|------------------------|------------------------|------------------------------|-----------|-----------|
| Bacterial genera and control |                        |                        |                              |           |           | *E. coli-Shigella*        | 220                    | 0                      | 220                          | 0         | 100       |
| Bacterial 16S            |                        |                        |                              |           |           | *E. coli isolated from paired stool samples* | 220                    | 0                      | 220                          | 0         | 100       |

a *E. coli* isolated from paired stool samples

NA; not applicable, Sens.; sensitivity, Spec.; specificity

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Table 4. Performance of TaqMan array card (TAC) compared with 384 well PCR plate for AMR detection in direct stool and cultured isolates (N = 122).

| Targets               | N positive tested | N negative tested | TAC result | Sens. (%) | Spec. (%) | Targets               | N positive tested | N negative tested | TAC result | Sens. (%) | Spec. (%) |
|-----------------------|-------------------|-------------------|------------|-----------|-----------|-----------------------|-------------------|-------------------|------------|-----------|-----------|
| Beta lactam genes     |                   |                   |            |           |           | Beta lactam genes     |                   |                   |            |           |           |
| TEM 104E              | 97                | 25                | 97         | 100       | 100       | TEM 104K             | 3                 | 119               | 3          | 119       | 100       |
| TEM 164R              | 98                | 24                | 98         | 100       | 100       | TEM 164SC            | 3                 | 119               | 3          | 119       | 100       |
| DHA                   | 18                | 104               | 17/18      | 94        | 99        | TEM 238S             | 3                 | 119               | 3          | 119       | 100       |
| SHV                   | 44                | 78                | 44         | 100       | 100       | SHV238-240SE-SK      | 21                | 101               | 20/21     | 101       | 95        |
|CTX-M1                 | 51                | 71                | 50/51      | 98        | 99        | CTX-M8-M25           | 15                | 107               | 15        | 106/107   | 100       |
|CTX-M2-M74             | 6                 | 116               | 6          | 116       | 100       | CTX-M9               | 39                | 83                | 39        | 83        | 100       |
| PER                   | 5                 | 117               | 5          | 117       | 100       | VEB                  | 28                | 94                | 28        | 94        | 100       |
| CMY1-MOX              | 3                 | 119               | 3          | 119       | 100       | FOX                  | 0                 | 122               | 0         | 122       | NA        |
| CMY2-LAT              | 39                | 83                | 39         | 83        | 100       | ACT-MIR             | 26                | 96                | 26        | 96        | 100       |
| KPC                   | 5                 | 117               | 5          | 117       | 100       | GES                  | 19                | 103               | 19        | 103       | 100       |
| NDM                   | 11                | 111               | 11         | 111       | 100       | VIM                  | 3                 | 119               | 3         | 119       | 100       |
| IMP                   | 9                 | 113               | 9          | 112/113   | 100       | OXA-48               | 3                 | 119               | 3         | 119       | 100       |
| OXA-1                 | 42                | 80                | 42         | 80        | 100       | OXA-9               | 10                | 112               | 10        | 112       | 100       |
| Fluoroquinolone genes |                   |                   |            |           |           | Fluoroquinolone genes |                   |                   |            |           |           |
| QnrA                  | 5                 | 117               | 5          | 117       | 100       | QnrS                | 52                | 70                | 52        | 70        | 100       |
| QnrB1                 | 32                | 90                | 32         | 90        | 100       | QnrB4               | 19                | 103               | 19        | 103       | 100       |
| aac(6')-Ib-104W       | 52                | 70                | 52         | 69/70     | 100       | aac(6')-Ib-104R      | 30                | 92                | 30        | 92        | 100       |
| gyrA87G-ESh<sup>b</sup> | 1                  | 121               | 1          | 121       | 100       | gyrA87G-ESh<sup>b</sup> | 2                 | 120               | 2         | 120       | 100       |
| QepA                  | 8                 | 114               | 8          | 114       | 100       | gyrA83Fy-Sal<sup>b</sup> | 3                | 119               | 3         | 119       | 100       |
| gyrA83S-Sal<sup>a</sup> | 4                 | 118               | 4          | 118       | 100       | gyrA87Ny-Sal<sup>a</sup> | 0                | 122               | 0         | 122       | NA        |
| gyrA87D-Sal<sup>a</sup> | 4                 | 118               | 4          | 118       | 100       | gyrA87Ny-Sal<sup>a</sup> | 0                | 122               | 0         | 122       | NA        |
| gyrA83S-ESh<sup>b</sup> | 58                | 64                | 58         | 64        | 100       | gyrA83L-ESh<sup>b</sup> | 57                | 65                | 57        | 65        | 100       |
| gyrA87D-ESh<sup>b</sup> | 61                | 61                | 61         | 61        | 100       | gyrA87Ny-ESh<sup>b</sup> | 33               | 89                | 33        | 89        | 100       |
| parC80S-Sal<sup>a</sup> | 4                 | 118               | 4          | 118       | 100       | parC80I-Sal<sup>a</sup> | 2                | 120               | 2         | 120       | 100       |
| parC80S-ESh<sup>b</sup> | 61                | 61                | 61         | 61        | 100       | parC80I-ESh<sup>b</sup> | 27               | 95                | 27        | 95        | 100       |
| gyrA86T-CJ<sup>h</sup> | 5                 | 117               | 5          | 117       | 100       | gyrA86I-CJ<sup>h</sup> | 2                | 120               | 2         | 120       | 100       |
| gyrA86T-Cc<sup>e</sup> | 2                 | 120               | 2          | 120       | 100       | gyrA86I-Cc<sup>e</sup> | 15               | 107               | 15        | 107       | 100       |
| Macrolide genes       |                   |                   |            |           |           | Macrolide genes       |                   |                   |            |           |           |
| 23S-2075A-Cp<sup>f</sup> | 22                | 100               | 21/22      | 100       | 95        | 23S-2075G-Cp<sup>f</sup> | 33               | 89                | 33        | 89        | 100       |
| ermB                  | 50                | 72                | 50         | 72        | 100       | mphA                | 65                | 57                | 65        | 57        | 100       |
| Aminoglycoside genes  |                   |                   |            |           |           | Aminoglycoside genes  |                   |                   |            |           |           |
| armA                  | 6                 | 116               | 6          | 116       | 100       | rmtB                | 13                | 109               | 13        | 109       | 100       |
| aacCl                 | 15                | 107               | 15         | 107       | 100       | aacC2               | 68                | 54                | 68        | 54        | 100       |
| aacC4                 | 41                | 81                | 41         | 80/81     | 100       | aadB                | 44                | 78                | 44        | 78        | 100       |
| aphA1                 | 62                | 60                | 62         | 60        | 100       | aadA1-2-17           | 84                | 38                | 83/84     | 38        | 99        |
| Folate pathway inhibitor genes |           |                   |            |           |           | Folate pathway inhibitor genes |           |                   |            |           |           |
| dfrA1                 | 65                | 57                | 65         | 57        | 100       | dfrA12              | 61                | 61                | 61        | 61        | 100       |
| dfrA5-14              | 60                | 62                | 60         | 60/62     | 100       | dfrA17              | 41                | 81                | 41        | 77/81     | 100       |
| sulI                  | 95                | 27                | 95         | 27        | 100       | sul2                | 83                | 39                | 83        | 39        | 100       |
| sul3                  | 50                | 72                | 50         | 72        | 100       |                       |                   |                   |            |           |           |

(Continued)
further evaluation in this area is needed, as mechanisms of resistance may differ geographically, and we only assessed stool specimens from two countries.

The genotypic-phenotypic correlation on bacterial isolates was good, yielding >90% sensitivity and specificity versus the phenotypic results for ampicillin, ceftriaxone, cefepime, imipenem, ciprofloxacin, azithromycin, gentamicin, amikacin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol across a range of genera. Antimicrobial agents whose genotypic-phenotypic correlation was suboptimal included ampicillin/sulbactam, potentially because we included a limited number of class D \( \beta \)-lactamase (OXA-type) targets or because of other mechanisms of resistance, such as penicillinase hyperproduction, overproduction of constitutive AmpC cephalosporinase, and inhibitor-resistant TEM (IRT) \( \beta \)-lactamase [23]. Cefoxitin resistance was also difficult to detect genotypically (76% sensitivity), perhaps because we only included plasmid mediated AmpC \( \beta \)-lactamase, not chromosomal AmpC, or because we did not test for outer membrane porins [24, 25]. Similarly, for colistin (67% sensitivity) we only included the plasmid-mediated \( mcr \) gene, while resistance may be due to several other mechanisms [17]. Detecting kanamycin and streptomycin resistance was also of lower sensitivity, perhaps because other targets such as \( \text{aph}(3')-\text{IIa}, \text{strA}, \text{and strB} \) [26] were not included. As for the specificity to detect susceptibility, cefazolin was the lowest (70%) and mostly due to \( \text{bla}_{\text{TEM}} \) positive but phenotypically susceptible isolates, likely due to low expression. Results for streptomycin and \( \text{aadA} \) were similar. Therefore, if a phenotypic susceptibility result for these drugs is desired, further assay optimization is needed. These drugs aside, however, the assays worked are usable for surveillance purposes for the 11 drugs with >90% sensitivity and specificity: ampicillin, ceftriaxone, cefepime, imipenem, ciprofloxacin, azithromycin, gentamicin, amikacin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol. Certainly, use for clinical care would require

| Targets | N positive\(^a\) tested | N negative\(^a\) tested | TAC result | Sens. (%) | Spec. (%) | Targets | N positive\(^a\) tested | N negative\(^a\) tested | TAC result | Sens. (%) | Spec. (%) |
|---------|------------------------|------------------------|------------|-----------|-----------|---------|------------------------|------------------------|------------|-----------|-----------|
| tetA    | 76                     | 46                     | 76         | 46        | 100       | tetB    | 70                     | 52                     | 70         | 52        | 100       |
| catA\(^1\) | 45                     | 77                     | 45         | 77        | 100       | catB\(^3\) | 28                     | 94                     | 28         | 93/94     | 100       |
| cmlA    | 58                     | 64                     | 58         | 64        | 100       | floR    | 52                     | 70                     | 52         | 70        | 100       |
| mcr-1   | 30                     | 92                     | 30         | 92        | 100       | mcr-2   | 9                      | 113                    | 9          | 113       | 100       |
| E.coli-Shigella | 81                 | 41                     | 81         | 41        | 100       | Shigella spp. | 5                      | 117                    | 5          | 117       | 100       |
| Salmonella spp. | 7                  | 115                    | 7          | 115       | 100       | C. jejuni-coli | 24                     | 98                     | 24         | 98        | 100       |
| PhHV    | 56                     | 66                     | 54/56      | 66        | 96        | Bacterial 16S | 122                    | 0                      | 122        | 0         | 100       | 100 | 100 |

\(^a\) Results on 384 well PCR plate
\(^b\) ESh; E. coli-Shigella spp.
\(^c\) Sa; Salmonella spp.
\(^d\) Cj; C. jejuni
\(^e\) Cc; C. coli
\(^f\) Cp; Campylobacter spp.
NA: not applicable, Sens.; sensitivity, Spec.; specificity

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commercial development, and major error and very major error rates should be below 3% and 1.5% per CLSI.

If desired, the PCR assays can be used on the TaqMan Array Card format. We have found this to be an easy to perform, rapid, and high-throughput tool. Cost of the TAC reagents (~$50 per specimen) and platform remain a substantial limitation. However the alternatives are costly as well. Sanger sequencing is costly, as are whole genome sequencing technologies, which also requires extensive bioinformatic interpretation [27–29].

In sum, we present a menu of AMR qPCR assays that can be used for tracking AMR in bacterial isolates, primarily Enterobacteriaceae and E. coli, and also in direct stool specimens for epidemiologic purposes.

**Supporting information**

S1 Table. Previously-sequenced bacterial isolates.

S2 Table. Summary of AMR genes of 236 bacterial isolates.

S3 Table. Glossary of AMR genes.

S4 Table. Primer and probe sequences of the 42 duplex and 1 singleplex PCR reactions.

S5 Table. Sequencing primers.

S6 Table. Phenotypic AST results of 239 E. coli isolates.

S7 Table. Antimicrobial agent classes and gene targets included in AMR-TAC.

S8 Table. Subgroups or members of group assays.

S9 Table. Analytical PCR performance of each assay on 384 well plate format.

S10 Table. Analytical performance of antimicrobial resistance TaqMan array card (AMR-TAC).

S1 Fig. Scatter plot of difference Ct values. Scatter plot of difference Ct values of 220 direct stools against paired E. coli isolates results of each target gene associated resistance to β-lactam (A), fluoroquinolone (B and C), Macrolide (D), aminoglycoside (E), trimethoprim/sulfamethoxazole (F), tetracycline (G), chloramphenicol (H), and colistin (I). Receiver Operating Curves (ROC) identified cut-off for optimized positive/negative categorization of direct stool against E. coli culture isolates for E. coli specific gene gyrA and parC, then the same cut-off was applied to all other gene targets which non-E. coli specific.

S1 ARRIVE guidelines checklist.

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S1 Table. Previously-sequenced bacterial isolates.

S2 Table. Summary of AMR genes of 236 bacterial isolates.

S3 Table. Glossary of AMR genes.

S4 Table. Primer and probe sequences of the 42 duplex and 1 singleplex PCR reactions.

S5 Table. Sequencing primers.

S6 Table. Phenotypic AST results of 239 E. coli isolates.

S7 Table. Antimicrobial agent classes and gene targets included in AMR-TAC.

S8 Table. Subgroups or members of group assays.

S9 Table. Analytical PCR performance of each assay on 384 well plate format.

S10 Table. Analytical performance of antimicrobial resistance TaqMan array card (AMR-TAC).

S1 Fig. Scatter plot of difference Ct values. Scatter plot of difference Ct values of 220 direct stools against paired E. coli isolates results of each target gene associated resistance to β-lactam (A), fluoroquinolone (B and C), Macrolide (D), aminoglycoside (E), trimethoprim/sulfamethoxazole (F), tetracycline (G), chloramphenicol (H), and colistin (I). Receiver Operating Curves (ROC) identified cut-off for optimized positive/negative categorization of direct stool against E. coli culture isolates for E. coli specific gene gyrA and parC, then the same cut-off was applied to all other gene targets which non-E. coli specific.
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