

Plasmid-mediated β -Lactamases: Nomadic Microbial Assassins Lurking in Multidrug Resistant Gram-negative Strains

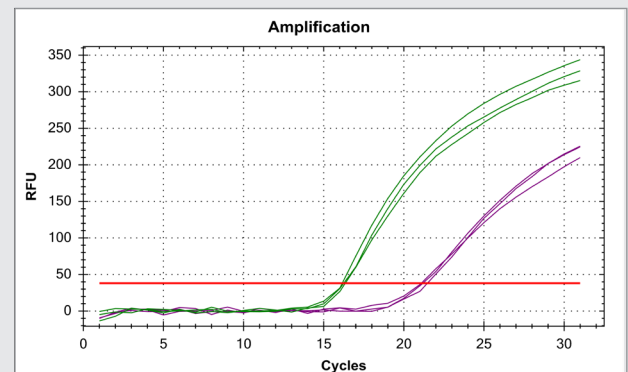
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Introduction

Alexander Fleming's discovery of penicillin was one the greatest advances in therapeutic medicine of our time. The discovery of other antibiotics with various modes of action would follow in the coming years. Unfortunately, the inappropriate use of antibiotics has coincided with increases in antibiotic resistance spurring bacteria to evolve and, consequently, develop different mechanisms to evade the activity of these antibacterial agents. Combined with a slowing pace of new antibiotic discovery, the emergence and spread of resistant bacteria has led to untreatable infections. The paradoxical nature of such a monumental medical advance evolving into one of the most serious public health threats of our time has necessitated a global effort to identify new platforms for resistance detection, a comprehensive understanding of the molecular basis of resistance evolution, and development of novel antibiotic therapies.

Towards the development of new antibiotic resistance detection strategies, much of that effort has focused on the incorporation of molecular tests that more specifically characterize the underlying genetic mechanisms responsible for resistance to antibiotics. Additionally, during the development of new detection methods, it's imperative that we understand the different types of mechanisms by which bacteria become resistant. These can be both intrinsic and extrinsic to the bacteria, with defined clinical criteria for their identification and susceptibility to antibiotics. This is important, especially if we are to understand the strengths and limitations of the diagnostic methods utilized for identification of these resistant organisms. There are 4 primary modes of action by which bacteria become intrinsically resistant: (1) prevent the antibiotic from reaching its target by reducing its ability to permeate the cell through porins, (2) utilize efflux pumps to remove the antibacterial from the cell, (3) enzymatic degradation of the antibiotic, such as Gram-negative bacteria that produce β -lactamases, or (4) modify the antibiotic within the bacteria to render it inactive. These modes of

A. ARM-D® Kit, β -Lactamase – Mix 2
VIM TEX615



B. ARM-D Kit, β -Lactamase – Mix 3
NDM TEX615

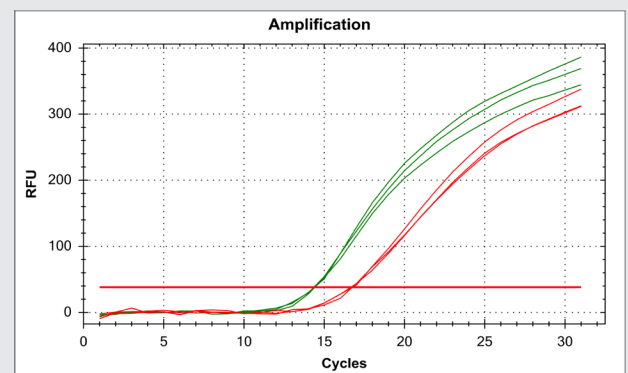
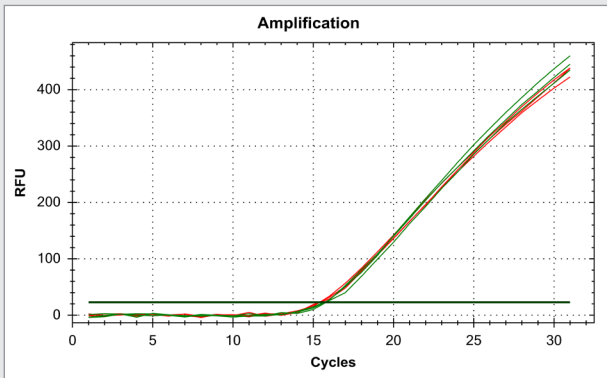


Figure 1. Streck ARM-D Kit Real-Time PCR Amplification Plots Verifying Prior Sample Characterization. Real-time PCR detection of a VIM-like positive gene target (A), and NDM-like gene target (B). No template controls are indicated by the black lines demonstrating no amplification for those groups.

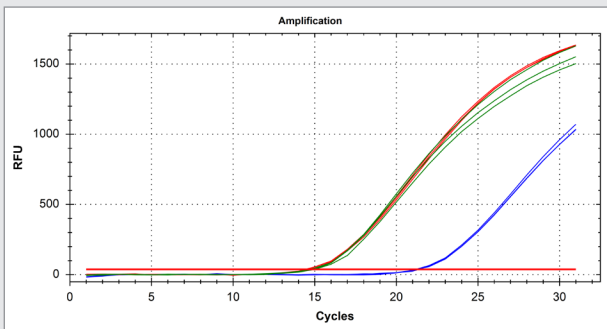
Streck ARM-D Kits are for Research Use Only. Not for use in diagnostic procedures. A CE version of these kits is also available.

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A. ARM-D Kit, β -Lactamase – Mix 1 CTX-M-15 HEX-Positive



B. ARM-D Kit, *ampC* PCR – Mix 2 CMY-2 TEX615-Positive



C. ARM-D Kit, β -Lactamase PCR – Mix 1 CMY-2 FAM-Positive

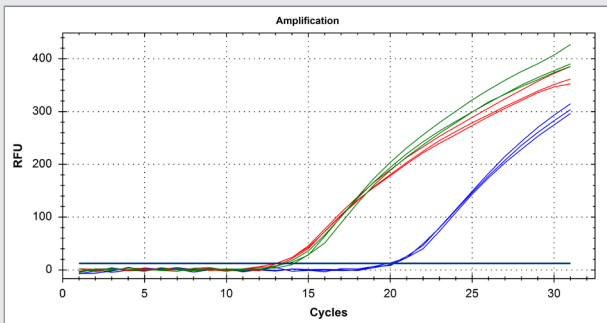


Figure 2. Detection of Additional Resistance Genes using the Streck ARM-D Kits: A CTX-M-15-like gene (red line) family member was detected in the ATCC BAA-2146 sample, as compared to the ARM-D CTX-M-15 Control (green line) using Mix 1 of the Streck ARM-D Kit, β -Lactamase (A). ATCC BAA-2146 (red lines) and CAP GNBR-01 (blue lines) both tested positive for a CMY-2-like family member, compared to the ARM-D Kit positive controls (green lines) for both the ARM-D Kits, *ampC* Mix 2 (B) and β -Lactamase Mix 1 (C).

resistance can be enhanced extrinsically by selective pressure and mutation or by horizontal gene transfer. The latter is especially concerning because it permits bacteria to disseminate these traits to other bacteria, providing a mechanism to rapidly enhance the spread of resistance and/or to improve survival in a host organism. Plasmids contain all necessary genetic elements to express these genes and, through transposons and integrons, can readily transfer and/or accumulate new virulence and resistance factors. In total, this creates a competitive advantage for bacterial survival. Furthermore, as these plasmids reside within bacteria and/or move to other bacteria, they can be extremely difficult to detect phenotypically as compared to their chromosomally-located counterparts.

The most common plasmid-mediated β -lactam resistance mechanism in Gram-negative organisms is β -lactamase production. These enzymes target the largest class of antibiotics. Hydrolysis of the β -lactam ring renders the β -lactam ineffective allowing the infection to persist. Following a CDC report outlining the top drug-resistant Gram-negative threats, the primary focus was given to the carbapenem-resistant Enterobacteriaceae (CRE) and the extended spectrum β -lactamase (ESBL)-producing Enterobacteriaceae. Organisms that produce ESBLs can hydrolyze the 2nd and 3rd generation cephalosporins whereas carbapenemases can also hydrolyze the carbapenems, leaving few β -lactam therapeutic options. Interestingly, plasmid-mediated *ampC* β -lactamases were not specifically addressed. This adds further complexity during detection because the plasmid-mediated *AmpC* (*pAmpC*) β -lactamases are often constitutively overexpressed and can either mask the phenotypic detection of an ESBL or present as an ESBL phenotype in the absence of an ESBL. As a result, these pathogens are typically resistant to most β -lactam classes of antibiotics presenting a challenge for clinical laboratories to detect these serious mechanisms using phenotypic detection methods. Standard susceptibility testing does not distinguish plasmid-mediated resistance from the chromosomally expressed *AmpC* enzyme in *E. coli*. Therapeutic failure has been reported when patients infected with a *pAmpC*-producing isolate were not correctly identified resulting in the use of an inappropriate β -lactam antibiotic. Genetic tests that can rapidly and accurately identify plasmid-mediated resistance can complement standard susceptibility tests and are imperative for improved surveillance and epidemiologic tracking, which translates into better infection control and can increase the effectiveness of antibiotic stewardship programs.

To this end, we developed the Streck ARM-D® Kits, β -Lactamase and *ampC*, as multiplex real-time PCR kits that target a comprehensive list of β -lactamase genes found in Gram-negative organisms conferring resistance to β -lactams. To illustrate the need for improved detection

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Table 1 - DNA Sequencing results for β -lactamases not previously characterized in the indicated samples.

Sample	Target/ARM-D Kit	β -Lactamase Gene Variants (Sequences Alignment 98-100%)
ATCC BAA-2146	CMY-2-like / <i>ampC</i> Kit	CMY-2, 4-7, 12, 14-18, 20-25, 27-34, 36-38, 42-45, 53-62, 69, 94, 95, 99, 102, 107, 111, 121, 125, 130-134, 138-141
ATCC BAA-2146	CMY-2-like / β -Lactamase Kit	CMY-2, 4-7, 12, 14-18, 20-25, 27-36, 38, 42-45, 53-62, 69, 94, 95, 99, 102, 107, 111, 121, 125, 130-134, 138-141
GN-01	CMY-2-like / <i>ampC</i> Kit	CMY-2, 41, 47, 48, 50, 51, 65-68, 72, 75, 76, 78, 79, 81, 84, 85, 87, 89, 90, 97, 103, 105, 110, 112, 113, 114, 116-118, 128, 135
GN-01	CMY-2-like / β -Lactamase Kit	CMY-2, 41, 48, 51, 65, 72, 75, 76, 78, 79, 81, 84, 85, 87, 89, 90, 92, 105, 109, 110, 112, 114-118, 135
ATCC BAA-2146	CTX-M-15 / β -Lactamase Kit	CTX-M-1, 15, 32, 55, 64, 88, 188-190

methodologies that can identify genes associated with plasmid-mediated antibiotic resistance, we demonstrate the retrospective characterization of four different commercially available control strains using the ARM-D assays. These control strains were generated from past patient samples and were either characterized as Gram-negative organisms that tested negative for antibiotic-resistance genes or, in the case of two samples, as positive controls for VIM-1 or NDM β -lactamase resistance. Following analysis of each sample, the Streck ARM-D Kits correctly verified the presence of the indicated resistance mechanisms for each control or confirmed the samples were negative for a resistance gene. However, when tested for plasmid-mediated *ampC* β -lactamases, some of these control strains unexpectedly tested positive for plasmid-mediated CMY-2-like genes and in one case, also for CTX-M-15. These data clearly demonstrate that plasmid-mediated resistance is present in the bacterial population infecting patients but escapes characterization during routine testing. Because laboratories may lack the tools to adequately identify plasmid-mediated resistance, it continues to spread through the population. Incorporation of the Streck ARM-D Kits combined with standard susceptibility testing is a low-cost rapid screening option to improve antimicrobial stewardship and infection control.

Materials and Methods

Two sets of control samples were used in this study: (1) *Klebsiella pneumoniae* (Schroeter) Trevisan (ATCC BAA-2146), isolated from human urine and characterized as NDM-1-positive and KPC negative, and (2) a Gram-negative Blood Culture Panel provided by a proficiency organization, which included 3 blinded samples, labeled here as GN-01 through GN-03. These samples were identified as

follows: GN-01 and 03 were negative for VIM, IMP, KPC, OXA-48, and NDM. GN-02 was characterized as VIM positive. No other resistance classifications were provided. *K. pneumoniae* DNA was purified from an overnight bacterial culture of this strain using the QIAGEN DNeasy Blood and Tissue Kit per the manufacturer's instructions. The Gram-negative blood culture samples were received and processed within 48 hours. DNA was extracted from a 200 μ L volume of each control sample using the AutoGen QuickGene DNA tissue kit as instructed in AutoGen Application Guide 37 using the AutoGen QuickGene-810 automated DNA extraction system. DNA was eluted with 50 μ L of molecular biology grade water. For accurate identification of clinical samples, the amplification with respect to positive controls was monitored for each gene.

Streck Antibiotic Resistance Monitoring - Detection (ARM-D) Kits, β -Lactamase and *ampC* (Streck, La Vista, NE) were used to detect the following β -lactamases: CMY-2, CTX-M-14, CTX-M-15, OXA-48, IMP, VIM, DHA, KPC, and NDM; and plasmid-mediated *ampCs*: MOX, ACC, FOX, DHA, EBC, and CMY-2. An internal control (IC) that targets a conserved region in Gram-negative bacteria was included in each mix to discriminate false negative results. Genes within a given control mix were differentiated using target-specific probes. Real-time PCR was done on the Bio-Rad CFX96 Touch Real-Time PCR Detection System as per the manufacturer's instructions for use provided with the kit (See streck.com for details on each kit).

PCR amplification products resolved by agarose gel purification were purified from the gel and both the forward and reverse strands were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed with an ABI 3500 Genetic Analyzer.

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Results and Discussion

Four Gram-negative strains, previously characterized by species type and/or the presence or absence of antibiotic resistance gene markers, were tested by real-time PCR using the Streck ARM-D Kits, *ampC* and β -Lactamase. Real-time PCR analysis of each strain using the ARM-D Kit, β -Lactamase, corroborated the characterization of the β -lactamase gene(s) previously reported for all test samples (i.e., VIM for sample GN-02, and NDM for sample BAA-2146, Figure 1A-B). When samples were screened for other β -lactamase genes detected with the ARM-D Kits, the ATCC sample also tested positive for a CTX-M-15-like gene when using Mix 1 of the ARM-D Kit, β -Lactamase (Figure 2A). Additionally, the ARM-D Kits, *ampC* and β -Lactamase identified the presence of an unreported plasmid-mediated *ampC* from the CMY-2-like gene family in both the BAA-2146 and GN-01 samples (Figure 2B and C, respectively). The amplification of CMY-2-like and CTX-M-15-like gene families was verified by performing DNA sequencing (Table 1). Sequencing results confirmed CMY-2-like positive samples but did not produce identical CMY-2 variants indicating each control sample may harbor a different CMY-2 family member.

Conclusions

Molecular methods, such as these, are being utilized as complementary tests to standard phenotypic culture-based testing for the identification of resistant Gram-negative organisms. Therefore, it is important to both retrospectively analyze samples that have only been characterized by antibiotic susceptibility tests, and prospectively incorporate this testing into testing schemes to update epidemiologic data and improve surveillance, antimicrobial stewardship, and infection control programs. The data presented here provide evidence that genetic mechanisms of antibiotic resistance can be missed or mischaracterized during phenotypic testing, especially when present in multidrug resistant isolates. For two of the four control strains tested in this study, a previously uncharacterized CMY-2-like variant was detected by real-time PCR with each of the Streck ARM-D Kits. Additionally, for sample BAA-2146, a CTX-M-15-like gene was identified with the CMY-2-like gene, indicating this patient sample contained multiple resistance mechanisms (NDM, CMY-2, and CTX-M-15). It is a concern that these bacterial strains, provided as controls for research laboratories, may contain additional antibiotic-resistance mechanisms that are unidentified due to the lack of appropriate screening tools that detect a broader range of these target genes. In any case, the ability of plasmids to move between bacteria confers a competitive advantage to both their survival and ability to spread through different bacterial populations. Real-time PCR assays such as the Streck ARM-D Kits, β -Lactamase and *ampC*, are valuable tools that

can identify the most clinically-relevant types of β -lactamase-based antibiotic resistance but also differentiates plasmid-mediated *ampC* β -lactamase resistance from chromosomal, provided the *ampC* gene identified is not identified from the organism of its chromosomal origin. These tests have a clinical sensitivity and diagnostic specificity of $\geq 95\%$ and real-time PCR protocols are completed in less than 1 hour.

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