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Detection of Mobilized Colistin Resistance (*mcr*) Genes by Multiplex Real-Time PCR: Improving Surveillance of an Emerging Threat

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ABSTRACT

Background Recent increases in colistin-resistant infections led the CDC to launch an urgent public health response for *mcr* surveillance. Colistin is a last resort antibiotic that is more utilized due to increases in carbapenem-resistant infections. Since *mcr*-1 was first reported, more *mcr* gene variants have been identified, but few screening tools have been developed to rapidly detect *mcr*-positive samples. To improve surveillance for *mcr* genes, we describe a multiplex real-time PCR assay that detects *mcr* gene families 1 through 5 in less than 45 minutes.

Materials/ methods This study utilizes sequence-specific primers and probes for the real-time PCR-based detection of mobilized colistin resistance *mcr* variants. An internal control (IC), targeting a conserved region in Gram-negative bacteria, is also included in the multiplex mix to discriminate false negatives samples. Positive DNA controls are included with the multiplex assay. Data was generated using the Bio-Rad CFX96 Touch™ Real-time PCR Detection System.

Results The *mcr* real-time PCR multiplex assay is optimized to amplify *mcr* families 1 through 5. Amplification of serial dilutions of target controls generated PCR efficiencies ≥ 99% and correlation coefficients ≥ 0.998. The sensitivity and specificity of the assay was evaluated using 90 clinical isolates and determined to be 100%. Internal control DNA were detected in 100% of samples and within 20 PCR cycles.

Conclusions Streck ARM-D Kit, MCR provides a rapid amplification and detection strategy to monitor plasmid-mediated colistin resistance genes. The data demonstrate a sensitive and specific assay, with no observed cross-reactivity with previously characterized clinical isolates from Gram-negative organisms. The results demonstrate this assay can serve as a screening tool for surveillance of *mcr*-mediated colistin resistance, thereby improving antimicrobial stewardship practices to minimize *mcr* gene dissemination into the community.

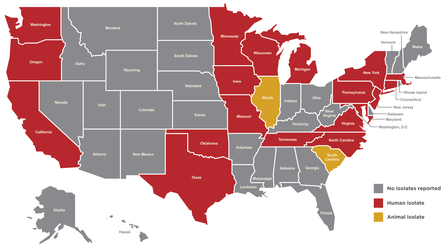
BACKGROUND

- Global increase in carbapenemase-producing Enterobacteriaceae has resulted in increased use of colistin.
- Colistin is considered a last resort antibiotic for multi-drug resistant Gram-negative bacteria.
- Mobilized colistin resistance gene *mcr*-1 was first reported in 2015 in *Escherichia coli* strain isolated from pigs and humans in China.
- During the last 4 years *mcr* genes have been detected in over 30 countries and other types of bacteria such as *Klebsiella pneumoniae* and *Salmonella enterica*.
- According to the CDC, in the U.S. alone, over 50 human isolates have been reported across 19 states.

Figure 1: **Tracking *mcr* in the U.S.**

The following map shows where the *mcr* gene has been reported in U.S. human and food animal sources as of Nov. 2, 2018.

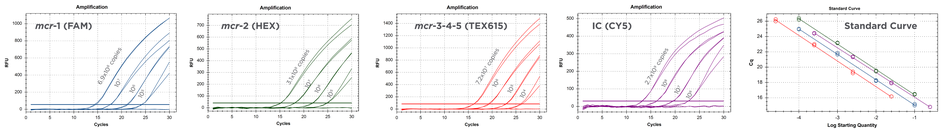
Map source from the CDC website: https://www.cdc.gov/drugresistance/biggest-threats/tracking/mcr.html



RESULTS

The results in **Figure 2** show the amplification of serial dilutions of ARM-D Kit, MCR Control Mix targets. Standard curves are shown for each target control. PCR efficiencies were over 99% for all the target controls and correlation coefficients were over 0.998. The amplification of the Multiplex Control Mix is shown in **Figure 3** and respective C_q values for target controls are shown in **Table 1**.

Out of the 90 isolates evaluated, 12 expressed *mcr* gene families. Representative amplification data of *mcr*-positive isolates with respect to positive controls is shown in **Figure 4**. The *mcr* positive isolates amplified within 20 cycles of the PCR run (**Table 2**). The clinical isolates were correctly identified by the *mcr* assay and the sensitivity and specificity were 100%.



mcr-1: E=100.5%, R^2=0.999 ***mcr-2***: E=100.3%, R^2=0.998 ***mcr-3-4-5***: E=99.0%, R^2=0.999 **IC:** E=106.2%, R^2=0.999

Figure 2

Real-time PCR amplification of serially-diluted Multiplex Control Mix of Streck ARM-D Kit, MCR. Standard curves show corresponding efficiencies and correlation coefficients for each target control, respectively.

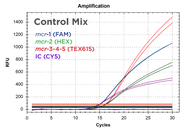


Figure 3

Real-time PCR amplification of Streck ARM-D Kit, MCR Multiplex Control Mix

Table 1: **C_q Values Streck ARM-D Kit, MCR Control Mix**

mcr-like gene family	Fluorophore	Average C_q value of positive pontrol (n=2)
mcr-1	FAM	15.10 ± 0.13
mcr-2	HEX	16.47 ± 0.05
mcr-3-4-5	TEX615	16.18 ± 0
IC	CY5	14.64 ± 0.02

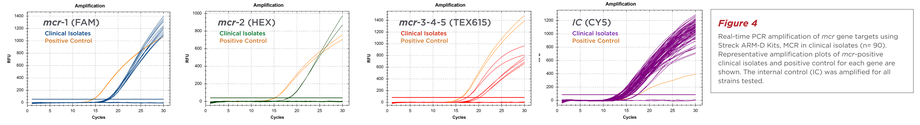


Figure 4

Real-time PCR amplification of *mcr* gene targets using Streck ARM-D Kits, MCR in clinical isolates (n= 90). Representative amplification plots of *mcr*-positive clinical isolates and positive control for each gene are shown. The internal control (IC) was amplified for all strains tested.

Table 2: **Streck ARM-D Kt, MCR-Clinical Isolate Testing**

mcr-like Gene Family	Average C_q Clinical Isolates	Positive Isolates (n)	Negative Isolates (n)	Sensitivity	Specificity
mcr-1	18.42 ± 0.34	8	82	100%	100%
mcr-2	19.36 ± 0.16	1	89	100%	100%
mcr-3	19.65 ± 0.4	2	88	100%	100%
mcr-4	17.5 ± 0.16	1	89	100%	100%
mcr-5*	19.27 ± 0.3	1	89	100%	100%
IC	14.56 ± 1.95	NA	NA	NA	NA

**All the DNA samples evaluated were extracted from clinical isolates except for mcr-5.*
A contrived sample in gram negative bacteria matrix was used to evaluate sensitivity for this variant.

MATERIALS & METHODS

Table 3: **Gene targets covered by the Streck ARM-D Kit, MCR**

Gene Family	Gene Targets
mcr-1	*mcr*-1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 1.11, 1.12, 1.13, 1.14, 1.15
mcr-2	*mcr*-2.1
mcr-3	*mcr*-3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 3.10, 3.11, 3.12, 3.13, 3.14, 3.15, 3.16, 3.18, 3.19, 3.20, 3.21, 3.22, 3.23, 3.24, 3.25
mcr-4	*mcr*-4.1, 4.2, 4.3, 4.4, 4.5, 4.6
mcr-5*	*mcr*-5.1, 5.2, 5.3
IC	16S rRNA

The genes covered in Streck ARM-D Kit, MCR were differentiated using target-specific hydrolysis probes, chemically linked to different fluorescent dyes. The gene variants covered by the assay are shown in **Table 3**. PCR master mix preparation and PCR cycling protocol are shown in **Tables 4** and **5**, respectively. The efficiency and correlation coefficients were determined for each target by the amplification of serial dilutions of the Control Mix. The sensitivity and specificity of the assay was evaluated with DNA purified from *mcr*-negative and *mcr*-positive overnight bacterial cultures (n=90). Positive isolates for *mcr* gene targets were obtained from the CDC & FDA Antibiotic Resistance (AR) isolate bank panel with new or novel antibiotic resistance (AR Bank # 0346, 0349, 0350, 0493, 0494, 0495, 0496, 0497, 0538, 0539, 05400, 0635). DNA was isolated using the Qiagen® DNeasy® Blood and Tissue Kit or Exiprep Dx Bacteria Genomic DNA kit per the manufacturer's instructions. Duplicate reactions were run for all clinical isolates and controls.

Table 4: **Master Mix Preparation**

Source	Component	25 µL Reaction	Final Concentration
Lab supplied	Nuclease free water	9 µL	-
Streck 2X Supermix	-	12.5 µL	1X
Streck ARM-D Kit	10X PCR Mix	2.5 µL	-
Distribute Master Mix into PCR wells or tubes as appropriate before sample			
Lab supplied or Streck ARM-D Kit	Template Unknown or NTC -or- Template-Control	1 µL	-

Table 5: **PCR Cycling Conditions**

Step	Temp (°C)	Duration (sec)	Cycles
Enzyme Activation	98	30	Hold
Denature	98	5	30
Anneal	60	10	
Extension/Capture	72	10	

CONCLUSIONS

The Streck ARM-D Kit, MCR:

- Identifies and differentiates *mcr*-1, *mcr*-2 and *mcr*-3-4-5 gene families with 100% sensitivity and 100% specificity.
- Can detect up to 48 out of 51 *mcr* allelic variants within the described gene families.
- Represents a surveillance tool to track the spread of mobilized colistin resistance.

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