Capillary PCR and Chip Electrophoresis for Rapid Detection of Carbapenamese genes in *Klebsiella pneumoniae*

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Background

Klebsiella pneumoniae and other Gram-negative bacteria with genes encoding carbapenemases such as KPC or New Delhi metallo-β-lactamase (NDM-1) are potentially a grave concern for public health. Carbapenems (imipenem, *etc*) are drugs of last resort for Gram-negative pathogens. Besides coding for resistance to these drugs, carbapenemase genes frequently reside on highly transmissible plasmids simultaneously coding for resistance to numerous other classes of antibiotics, leading to infections that are highly intractable to treatment.

Carbapenamases can be difficult to detect by susceptibility testing methods, and we are developing rapid genetic (PCR) tests for these resistance genes, using novel instrumentation designed for limited-resource settings. Genetic tests also have drawbacks: they may miss novel sequence variants, and may pick up non-functional or non-expressed genes, and are usually limited to detecting a few common genes per test.

Real-time PCR tests are popular in clinical settings due to the relative simplicity of amplification + detection in a single tube, although real-time tests have more limited multiplexing ability. Multiplex PCR with amplicon sizing can detect more targets per reaction, but require extra work to transfer samples to a gel for analysis. We are developing instrumentation automating sample prep, amplification, and analysis allowing rapid, high-sensitivity analysis of amplicons. In this work, we test components of this system for a small panel of carbapenemase genes, with the ultimate goal of performing rapid, multiplexed detection of a large panel of drug resistance genes directly in a clinical setting..

Methods

As a first step toward applying our instrumentation to detecting carbapenemase genes, we have tested a small panel of strains with a low-level multiplex PCR for $bla_{\text{NDM-1}}$, bla_{KPC}, and Enterobacteriaceae 16S gene (internal control). The degree of multiplexing was limited primarily by the strains we had available for testing, rather than an inherent limitation of the technology.

Primer name	Sequence	Amplicon size (bp)	Reference
NDM-GBM-F	CCCGGCCACACCAGTGACA	129	Voets, *Int. J. Antimicrob. Agents* 2011
NDM-GBM-R	GTAGTGCTCAGTGTCGGCAT		
MultiKPC_for	CATTCAAGGGCTTTCTTGCTGC	538	Dallene, *J. Antimicrob. Chemother.* 2010
MultiKPC_rev	ACGACGGCATAGTCATTTGC		
ENT-F (16S)	GTTGTAAAGCACTTTCAGTGGTGAGGAAGG	424	Nakano, *J. Food Protection* 2003
ENT-R (16S)	GCCTCAAGGGCACAACCTCCAAG		
DG74 (16S)	AGGAGGTGATCCAACCGCA	370	Griesen, *J. Clin. Microbiol.* 1994
RW01 (16S)	AACTGGAGGAAGGTGGGGAT		

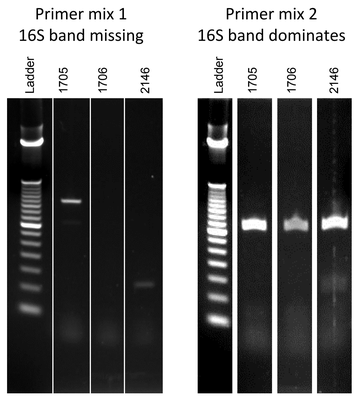
We obtained purified DNA from the following ATCC control strains:

ATCC Strain	Comments
K. Pneumoniae BAA-1705	KPC positive control for modifiied Hodge test (MHT)
K. Pneumoniae BAA-1706	Negative control strain for modified Hodge test
K. Pneumoniae BAA-2146	Multi-drug resistant; bla_{KPC} negative, $bla_{\text{NDM-1}}$ positive

We optimized a "conventional" multiplex PCR reaction for these primers and these strains. Final conditions were [pmol/µL] each primer, 57 C annealing temperature, 1 ng template DNA in a 25 µL reaction. As a positive control for amplification, we used either a "universal" 16S primer set (DG74 + RW01), or the "ENT" 16S primer set. This set targets most Enterobacteriaceae and Vibrioaceae but is less sensitive to contamination than "universal" 16S primers. We obtained positive amplification in 30 cycles from 50-500 pg of bacterial genomic DNA in a 25 µL reaction.

Rapid-cycle capillary PCR

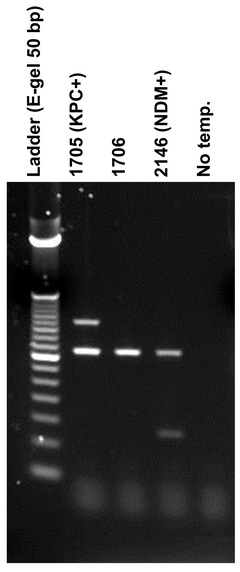
We have developed a patent-pending low-power, rapid thermal cycler, which reduces ramp times between temperatures to <1 second, with automated processing of few-microliter volume samples in a capillary format. Optimization and integration with nucleic acid extraction (on the front end) and chip electrophoresis (on the back end) is ongoing. The high sensitivity of the chip electrophoresis will enable sensitive detection from small volume samples with reduced number of cycles (<30 minutes total run time).



Optimization of rapid capillary cycling conditions is underway! The "optimal" mix of primers for uniform intensity of all bands appears to be different from the conventional PCR. The smeared bands in the right-hand set of gels are from not diluting the product sufficiently for gel electrophoresis.

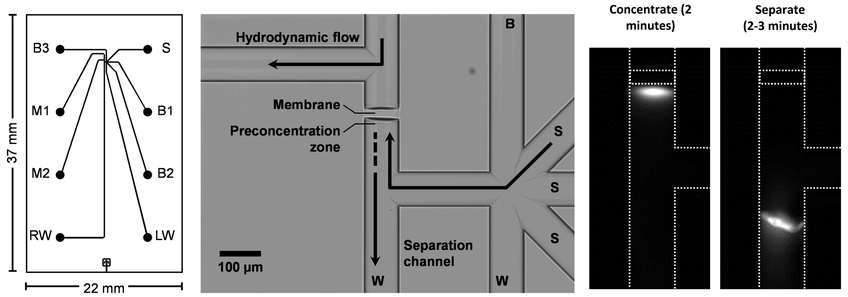
Microchip electrophoresis for amplicon sizing

We recently described a novel microchip electrophoresis platform for ultrasensitive sizing of DNA amplicons, using a photopatterned membrane to accomplish online preconcentration of DNA (Meagher & Thaitrong, *Electrophoresis* 2012). With this device, we can achieve similar speed, resolution and quantitative precision as the Agilent Bioanalyzer, but with >100-fold better sensitivity. The sensitivity approaches that for highly optimized capillary electrophoresis (fg/µL), in a fraction of the run time.

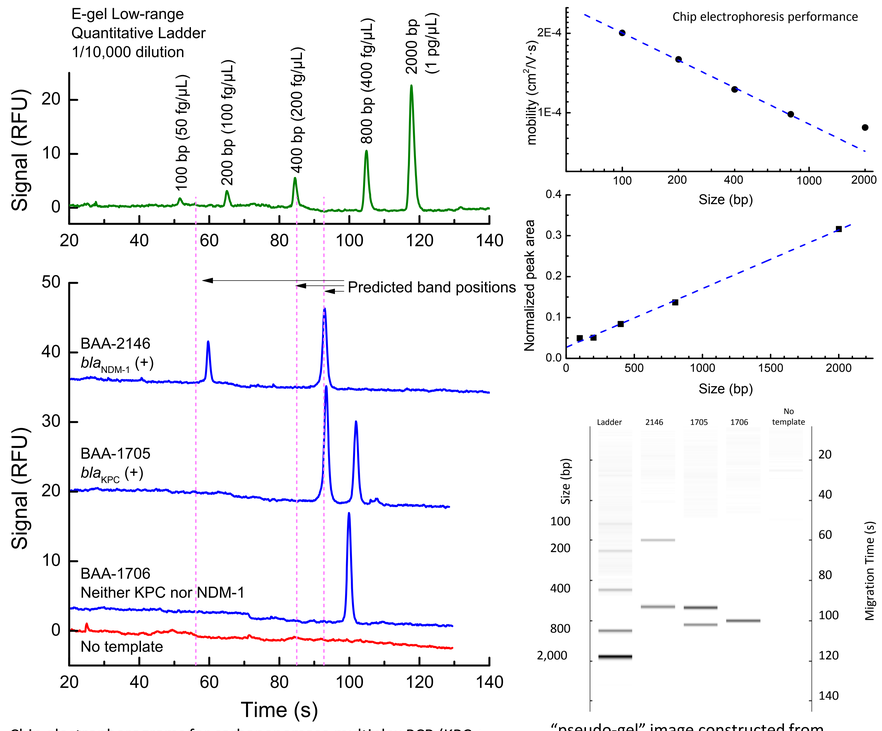


2% Agarose + EtBr gel image of carbapenemase multiplex with "universal" 16S primer control, showing expected bands at 129, 370, and 538 bp.

Microchip electrophoresis (continued)



Schematic illustration & micrographs of the device for electrophoretic preconcentration & amplicon sizing



Chip electropherograms for carbapenemase multiplex PCR (KPC + NDM-1 + ENT 16S primers). Products were diluted 1/5000 and labeled on-chip with SYTOX Orange DNA stain.

"pseudo-gel" image constructed from electropherograms, showing banding pattern.

Chip electrophoresis gives the same banding pattern as the traditional gel, but the migration times are shifted from the expected position, because many runs (>20) were performed sequentially without replacing buffers or sieving polymer, causing a gradual slowing of migration. One solution is to include leading & trailing "reference bands" to tie each run to the size standard.

Conclusions & Future Work

- Ultra-sensitive chip electrophoresis should allow PCR detection with fewer samples with lower reaction volume, but we need to tighten up the migration times.
- The low power rapid thermal cycler with integrated sample prep will enable operations in limited resource settings (hospital/point-of-care)
- The drug resistance multiplex will be expanded, and validated against more isolates.

Acknowledgments

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