Multiplex Real-Time PCR Detection of Klebsiella pneumoniae Carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM-1)

Background

This procedure provides instructions for Taqman-based real-time PCR detection of *bla*$_{KPC}$ and *bla*$_{NDM-1}$ in a single reaction from gram-negative bacteria. The universal 16S rRNA gene is used as a control for DNA extraction and amplification for each reaction. If desired, either KPC or NDM-1 can be assayed independently by excluding the other set of primers and probe. Although KPC and NDM appear to be the most common carbapenemases in the United States, it is important to note that there are other less common carbapenemases, as well as other mechanisms of carbapenem resistance.

Use of trade names is for identification only and does not constitute endorsement by the Centers for Disease Control and Prevention.

Validation

This assay was validated on the ABI 7500 Fast real-time PCR instrument (Applied Biosystems, Inc., Foster City, CA) using a collection of NDM-1-positive and KPC-positive isolates, as well as isolates containing other resistance mechanisms (i.e. AmpC, CTX-M, OXA, SME, VIM, IMP, GIM, SIM, SPM). This assay demonstrated 100% sensitivity & specificity for detection of NDM-1 and KPC.

Materials needed

Equipment	Reagents & Media	Supplies
• Real-time PCR system and analysis software	• 2x QuantiFast Probe PCR Kit (Qiagen) • Primers and probes (below) • Sterile reagent grade type I H_2O	• Micropipettes and aerosol-free pipette tips • Sterile 1.5 ml microcentrifuge tubes • 96-well reaction plate appropriate for PCR system • Optical adhesive film • Ice bucket and ice

Primers & Probes

Oligonucleotide	Nucleotide sequence, 5'–3'
KPC-F Primer	GGC CGC CGT GCA ATA C
KPC-R Primer	GCC GCC CAA CTC CTT CA
KPC-Probe (FAM)	FAM-TG ATA ACG CCG CCG CCA ATT TGT-BHQ
NDM-F Primer	GAC CGC CCA GAT CCT CAA
NDM-R Primer	CGC GAC CGG CAG GTT
NDM-Probe (HEX)	HEX-TG GAT CAA GCA GGA GAT-BHQ
16S rRNA-F	TGG AGC ATG TGG TTT AAT TCG A
16S rRNA-R	TGC GGG ACT TAA CCC AAC A
16S rRNA-Probe (CY5)	CY5-CA CGA GCT GAC GAC AR*C CAT GCA-BHQ

* R = A or G

Special Precautions

- Probes are light-sensitive and should be shielded from light with foil.
- Use aerosol-free pipette tips at all stages of testing to prevent contamination.
- Use powder-free gloves, as powder can cause unwanted fluorescence in this assay.

Control Strains

Run DNA lysates from the following control strains with each run:

- KPC- and NDM-negative control - *K. pneumoniae* ATCC strain BAA-1706
- KPC-positive control - *K. pneumoniae* ATCC strain BAA-1705
- NDM-positive control - *K. pneumoniae* ATCC strain BAA-2146
- No Template control (NTC) - sterile reagent grade type I water

Preparing Sample Lysates

Note: Select several colonies of pure overnight growth from a trypticase soy agar plate with 5% sheep's blood.	
1.	Resuspend a 1 µl loopful of bacterial growth in 25 µl of sterile reagent-grade water in a labeled 1.5 ml centrifuge tube
2.	Add 25 µl of 0.1 N NaOH to each sample and mix by inverting
3.	Heat at 95-99˚C for 10 minutes
4.	Cool 3-5 minutes on ice, then neutralize by adding 18 µl of 0.5 M Tris-HCl, pH8.0
5.	Add 400 µl of cold, sterile reagent grade water to each tube
6.	Mix tube by inversion and flicking with your finger. Centrifuge at 16.1 rcf for 3 min.

7.	Transfer lysate (approx. 400 µl) into new, appropriately labeled centrifuge tube
8.	Store lysate at -20˚C to -30˚C until needed

Performing the KPC and/or NDM real-time Screening Assay

1.	Thaw all reagents on ice.
2.	Prepare primer-probe mix containing a final concentration of 20 uM each primer and 10 uM each probe listed above. Store on ice, covered to protect from light. (Remainder can be frozen for subsequent use.)
3.	Prepare Master Mix below to yield one 20 µl reaction for each test sample and control, and one additional reaction (to account for pipetting loss). Mix by flicking the tube; do not vortex. <u>Each reaction contains:</u> 2x QuantiFast Probe PCR Master Mix - 10 µl Primer-probe mix - 5 µl* Sterile reagent grade H_20 - 3 µl Total volume = 18 µl Master Mix (+ 2 µl of template = 20 µl reaction) *Final concentrations: 500nM each primer and 250nM each probe
4.	Pipet 18 µl of the Final Master Mix into each appropriate well in the 96-well plate.
5.	Add 2 µl of template to respective wells. Ensure that the no template control is added last. Pipet up and down to mix.
6.	Cover 96-well plate securely with optical adhesive film.

Real-time PCR setup and run

1.	Ensure that the instrument is set up to detect the following reporter signals: 16s Universal: CY5 KPC: FAM NDM 1 (HEX): VIC
2.	Thermal cycling conditions are as follows: 1) Enzymy activation step: 95°C for 3 minutes 2) 40 PCR cycles of : Melt: 95°C for 3 seconds Anneal/ extend: 60°C for 30 seconds

Interpretation of Results

When analyzing the results, it is important to only consider amplification between 10-30 cycles as positive. Amplification prior to 10 cycles means the template should be diluted before repeating. Amplification after 30 cycles can indicate trace contamination. The no template control (water) control should not yield a product (Ct >40) but may produce a trace 16S result in the 31-40 cycle range; both are acceptable. Amplification of any target other than 16s, or amplification of the 16s target below 30 cycles, in the no template control well indicates cross-contamination, resulting in an invalid run. Positive and negative controls should yield results described below.

16S (CY5) Result	**KPC (FAM) Result**	**NDM (HEX) Result**	**Report**	
			KPC	**NDM**
Ct 10-30	Ct 10-30	Undetected	Positive	Negative
Ct 10-30	Undetected	Ct 10-30	Negative	Positive
Ct 10-30	Ct 10-30	Ct 10-30	Positive	Positive
Ct 10-30	Undetected	Undetected	Negative	Negative
Ct 10-30	Ct <10 (in either)		Dilute template 1:100, repeat	
Ct <10 or Ct >40	anything		Consult supervisor	

References

Applied Biosystems 7500 Fast Real-Time PCR System Guide
Clinical Microbiology Procedures Handbook, 3rd Ed., 2010. Garcia, L., editor. Detection of the *bla*$_{KPC}$ Gene Encoding *Klebsiella pneumoniae* Carbapenemase by Real-Time PCR. 12.5.4.1−12.5.4.10.
Yong, D., M.A. Toleman, C.G. Giske *et al*. 2009. Characterization of a new metallo-β-lactamase gene, *bla*$_{NDM-1}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *K. pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother. 43:5046-54.