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Development and application of real-time PCR assay for detection of mutations associated with macrolide resistance in Mycoplasma pneumoniae directly in clinical specimens

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Background: *Mycoplasma pneumoniae* (*MPN*) is a common bacterial cause of respiratory tract infections and is commonly treated with macrolides. However, lately macrolide resistance in *MPN* has been reported with increasing frequency and has been associated with treatment failures. The mechanism of macrolide resistance in *MPN* involves mutations at specific positions of 23S rRNA gene. Here we report on the development and application of a new multiplex real-time PCR assay for detection of ML resistance mutations in *MPN* directly in clinical specimens.

Material/methods: A new assay was developed based on real-time PCR chemistry that utilises the effect of fluorescence energy transfer between an oligonucleotide probe and one of the amplification primers. Two fragments of 23S rDNA spanning the hotspots of macrolide resistance mutations (positions 2063-2064 and 2617 by *MPN* numbering) were simultaneously amplified using the two pairs of primers with one primer in each pair designed to anneal close to the mutation site and internally labelled with fluorescence quencher. The probes targeting the corresponding mutation sites were 3'-

end labelled with fluorophores (FAM and R6G). A post-PCR probe melting curve analysis was used to detect any mutations at the target sites. The assay was first validated on genomic DNA samples of macrolide-susceptible reference strain *MPN* (ATCC 15531) and four resistant strains with previously characterized mutations in the 23S rDNA: A2064G, A2063G, A2064C, and C2617G, and then applied to analysis of 194 *MPN*-positive sputum and throat swab specimens (one per patient/case of infections) obtained from 95 sporadic cases and 3 unrelated outbreaks of pneumonia in three cities of Russia in 2014-2016. Specificity of the assay was assessed using genomic DNA of various bacteria found in human respiratory tract as well as 100 *MPN*-negative sputum/throat swab specimens.

Results: The developed assay allowed unambiguous discrimination of the wild-type and all macrolide-resistant genotypes of *MPN*. When applied to clinical specimens, positive for *MPN* by commercial PCR assay (AmpliSens *MPN*-FL), our assay detected the signal and identified the status of macrolide-resistance genotype in 178/194 samples (relative sensitivity 91.7%), while no false-positive results were obtained for *MPN*-negative samples (specificity 100%). In 164 positive samples, melting temperatures (Tm) of the probes were consistent with the presence of wild-type *MPN*; fourteen samples (7.8%) revealed changes in Tm of the probe for positions 2063-2064 characteristic for macrolide resistance mutations which were independently confirmed by sequencing as A2063G in twelve cases and A2064G in the other two. No samples were found to contain mutations at position 2617.

Conclusions: We have developed a simple, rapid and cost-effective real-time PCR assay for detection of ML-resistance mutations in MPN that can be applied for epidemiological studies and further adopted for clinical use to assist in directing appropriate treatment for MPN infections.