



Genotypic Susceptibility Testing of Bacteria

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Conventional methods for testing the susceptibility of bacteria to antibiotics are based on exposure of cultured organisms to different concentrations of antibiotics. These methods may require three or more days for common bacteria and up to several weeks for slow-growing bacteria, like *Mycobacteria* spp. Conventional susceptibility testing methods include disk diffusion, broth dilution, agar dilution and gradient diffusion (epsilometer test). Commercial platforms have been developed by manufacturers that use broth dilution in a microtiter format (microbroth dilution) and are automated or semi-automated (e.g., Vitek™; bioMérieux, St. Louis, MO or MicroScan™, Dade International, West Sacramento, CA). These platforms accommodate higher volumes of tests and require less hands-on time than the manual methods, disk diffusion, agar dilution and gradient diffusion.

Genetic methods for assessing the antibiotic susceptibilities of bacteria offer several advantages over conventional culture-based (phenotypic) methods.¹ Genetic tests can be performed directly from clinical specimens, and therefore isolation of an organism by culture becomes unnecessary.² Genetic methods assess the “genotype” of the organism, whereas conventional susceptibility techniques assess the “phenotype” or expression of the genotype under artificial or laboratory conditions. Although debate exists among authorities about which of these assessments is more clinically relevant, it seems reasonable that the lowest-risk approach for the patient is to determine the genotype. This strategy may be especially important if one is dealing with serious life-threatening infections such as endocarditis or osteomyelitis which require prolonged courses of antimicrobial therapy.³ In some cases, genotypes may be discerned long before phenotypes can be determined because of the slow growth of an organism. With the recent availability of rapid-cycle real-time polymerase chain reaction (PCR), or rapid probe amplification techniques, antibiotic resistance can be determined in less than one hour directly from patient samples or for bacteria isolated by culture from these specimens.⁴ Some organisms are not easily cultured or cannot be cultured; therefore, only genotypes can be determined.⁵ Genetic methods may lessen the biohazard risk that may occur with the propagation by culture of an organism that is required for conventional test methods.

In some instances, genetic testing methods may have less utility than conventional susceptibility test methods.¹ They may lack sensitivity when few organisms are present in a sample.² Different assays are required for each antimicrobial agent tested (individual antimicrobial agents may also be associated with multiple target genes or a large array of mutations).³ A genetic method for resistance for some antimicrobial agents may not have been defined.⁴ False-positive results may occur because of contamination of the sample with extraneous nucleic acid. This last-mentioned problem is of particular concern when nucleic acid amplification techniques such as PCR are used. The specificity, however, has been considerably enhanced with the development of enzymatic and chemical sterilization techniques for amplified nucleic acid and the manufacture of closed systems for performing both PCR and detection of amplified product.

Over the last decade, a broad range of innovative molecular techniques have been developed for detecting

antimicrobial resistance.^{1,2,3,4} Because of the technical difficulties encountered in performing many of these assays, few have gained widespread use. However, two test platforms, real-time PCR and probe amplification, have recently gained popularity due to ease of performance, rapid turn-around time (TAT) for results and low risk for contamination.

Cycling probe technology was available for a short time in the United States as an FDA-approved test for detecting the *mecA* gene in isolated colonies of staphylococci (Velogene, ID Biomedical, Vancouver, BC, Canada).^{5,6} This probe amplification method had excellent performance characteristics and was relatively easy to perform. However, the test was removed from the market by the manufacturer for unknown reasons. Cycling probe technology is an isothermal process; the end product of the reaction is a modified (“amplified”) version of the original probe used to detect the target DNA.

Rapid-cycle real-time PCR, more frequently referred to as “real-time PCR” combines rapid thermocycling with real-time fluorescent detection of target DNA in the same closed vessel. We have determined that rapid-cycle, real-time PCR using the LightCycler instrument (Roche Applied Science, Indianapolis, IN) and fluorescent energy transfer probes (FRET, Roche Applied Science), is an effective method for detection of the *mecA* gene and van genes (*van A*, *van B*, *van B 2/3*) in isolates of staphylococci and enterococci respectively. Additionally, we have demonstrated the utility of LightCycler PCR for detection of van genes directly from anal swabs or stool specimens.

In prospective clinical studies for qualitative detection of microbial pathogens, we have consistently noted increases in sensitivities for assays that use the LightCycler real-time PCR technology compared to the sensitivities for standard culture-based phenotypic methods. These include assays for *Bordetella pertussis* (219% increase),⁷ herpes simplex virus (23% increase),⁸ varicella-zoster virus (91% increase),⁹ and cytomegalovirus (88% increase) (M.J. Espy and T. F. Smith, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. C-62, 2000) and Group A Streptococcus (7% increase).¹⁰ As a result of these clinical studies we have replaced standard culture-based methods with LightCycler PCR assays for direct detection of these organisms from clinical samples.

In ongoing studies at Mayo, we have also noted enhanced sensitivities for detecting van genes directly from perianal swabs (30% increase for *van A* and >100% increase for *van B* genotypes) compared with sensitivities for detecting vancomycin resistance using cultured-based phenotypic methods. These results are not surprising considering the difficulties encountered in isolating vancomycin-resistant enterococci (VRE) from stool culture screening plates. Importantly, the time required to complete the LightCycler van assay, including DNA extraction and analysis, is approximately two hours. This time requirement is considerably shorter than the time required in our laboratory to identify VRE from stools by culture based methods (≥ 72 hours).

Using real-time PCR, direct detection of methicillin-resistant *Staphylococcus aureus* (MRSA) from nasal swabs is more challenging than direct detection of VRE from stools. This is because coagulase-negative staphylococci (CNS), like MRSA, can be methicillin resistant (carry the *mecA* gene) and be present in the anterior nares. In a recent survey of nasal swabs submitted for MRSA screening in our laboratory we determined that the frequency of co-colonization of *S. aureus* and CNS in the nares is 23%. In the same survey we also observed that *S. aureus* is present alone in the nares 10% of the time, CNS alone 63% of the time and neither are present 7% of the time. In our patient population approximately 60% of CNS are methicillin-resistant and carry the *mecA* gene. Therefore, one has to develop methods to distinguish whether a *mecA* gene detected in a nasal swab sample originates from *S. aureus* or CNS. Theoretically, one could accomplish this distinction if DNA sequence variation was present within the *mecA* gene or the cassette of genes that flank the *mecA* gene and these sequence variations were unique for *S. aureus* versus CNS. Alternatively the distinction could be made if one could separate CNS bacterial cells from *S. aureus* cells in nasal samples before genetic analysis is performed for the *mecA* gene. If neither of these approaches are possible, one may overcall methicillin resistance for *S. aureus* cells that colonize the nasal mucosa.

DNA sequence variation has not been reported within *mecA* genes for *S. aureus* compared with CNS. However,

unique structural diversity of the mobile genetic element which carries the *mecA* gene, designated the staphylococcal cassette chromosome *mec* (SCC*mec*) has been reported for *S. aureus*.¹¹ At the time of this writing at least four different SCC*mec* types have been described for *S. aureus*.¹² Theoretically, one could develop molecular assays that would specifically identify these SCC*mec* types; however, the question remains as to whether other SCC*mec* types may exist and therefore would not be identified using such an assay. Recently, we evaluated 198 *S. aureus* isolates (148 *mecA*-positive, 50 *mecA*-negative) and 100 CNS (50 *mecA*-positive, 50 *mecA*-negative) for the presence of these SCC*mec* types. PCR primers and probes were designed to anneal with the published sequences for these four SCC*mec* types. None of these SCC*mec* types were found in the 100 CNS isolates and none were found in the 50 *mecA*-negative *S. aureus* isolates. 137 of 148 (93%) *mecA*-positive *S. aureus* isolates carried one of these SCC*mec* types; however, for 11 of 148 (7%) *mecA*-positive *S. aureus* isolates no PCR amplicons were generated. These results suggest that other SCC*mec* types exist for these *S. aureus* strains.

Recently, an immunocapture method has been described which appears to effectively separate *S. aureus* cells from CNS cells in mixed culture or clinical samples.¹³ This method captures *S. aureus* cells using a monoclonal antibody directed against the *S. aureus* cell envelope protein, protein A. The monoclonal antibody - *S. aureus* complex is separated from solution using streptavidin-coated paramagnetic beads. This separation method can be coupled with a real-time PCR platform to detect the *mecA* gene in *S. aureus* and provide results directly from clinical samples in less than 6 hours.

References

1. Cockerill FR III. Genetic methods for assessing antimicrobial resistance. *Antimicrob Agents Chemother.* 1999;43:199-212.
2. Cockerill FR III. Conventional and genetic laboratory tests used to guide antimicrobial therapy. *Mayo Clin Proc.* 1998;73:1007-21.
3. Wolk D, Mitchell PS, Patel R. Principles of molecular microbiology testing methods. In Cockerill FR III (ed): *Infectious Disease Clinics of North America*. Philadelphia. W. B. Saunders Company 2001;1157-204.
4. Louie M, Cockerill FR III. Susceptibility testing: phenotypic and genotypic tests for bacteria and mycobacteria. In Cockerill FR III (ed): *Infectious Disease Clinics of North America*. Philadelphia, W. B. Saunders Company 2001;1205-26.
5. Fong WK, Modrusan Z, McNevin JP, *et al.* Rapid solid-phase immunoassay for detection of methicillin-resistant *Staphylococcus aureus* using cycling probe technology. *J Clin Microbiol.* 2000;38:2525-9.
6. Bekkaoui F, McNevin JP, Leung CH, *et al.* Rapid detection of the *mecA* gene in methicillin resistant staphylococci using a colorimetric cycling probe technology. *Diagn Microbiol Infect Dis.* 1999;34:83-90.
7. Sloan LM, Hopkins MK, Mitchell PS, *et al.* Multiplex LightCycler PCR assay for detection and differentiation of *Bordetella pertussis* and *Bordetella parapertussis* in nasopharyngeal specimens. *J Clin Microbiol.* 2002;40:96-100.
8. Espy MJ, Teo R, Ross K, *et al.* Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. *J Clin Microbiol* 2000;38:795-9.
9. Espy, MJ, Teo R, Ross K, *et al.* Diagnosis of varicella-zoster virus infection in the clinical laboratory by LightCycler PCR. *J Clin Microbiol* 2000;38:3187-9.
10. Uhl JR, Adamson SC, Vetter EA, *et al.* Comparison of LightCycler PCR, rapid antigen immunoassay, and culture for detection of group a streptococci from throat swabs. *J Clin Microbiol* 2003;41:242-9.
11. Ito T, Katayama Y, Asada K, *et al.* Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents and Chemother.* 2001;45:1323-36.

12. Ma XX, Ito T, Tiensasitorn C, *et al.* Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob Agents and Chemother.* 2002;46:1147-52.
13. Francoi P, Pittet D, Bento M, *et al.* Rapid detection of methicillin-resistant *Staphylococcus aureus* directly from sterile or nonsterile clinical samples by a new molecular assay. *J Clin Microbiol.* 2003;41:254-60.