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Real-time PCR for universal antibiotic susceptibility testing

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***Objectives*: Determination of bacterial antimicrobial susceptibility is usually performed using phenotypic methods. In this study, we developed a universal 16S rRNA and *rpoB* quantitative PCR assay for susceptibility testing of bacteria commonly isolated in clinical microbiology laboratories.**

***Methods*: Antibiotic susceptibilities for 24 bacterial strains of various species were tested by real-time quantitative PCR assay and by conventional methods. Quantification of DNA copies of either the 16S RNA genes or *rpoB* were recorded over time in the presence or absence of antibiotics to determine the bacterial growth kinetics and the optimal testing time.**

***Results*: Molecular results for antibiotic susceptibility or resistance were in accordance with those obtained using a standard macrodilution broth assay. The method was reproducible, sensitive and rapid (2 h for Gram-negative bacilli and 4 h for Gram-positive cocci). Moreover, this assay was also able to determine the antibiotic susceptibilities of fastidious bacteria, such as mycobacteria, within 5 days.**

***Conclusions*: These results demonstrate that molecular detection of bacteria could be more rapid than phenotypic methods for antibiotic susceptibility testing.**

Keywords: quantitative PCR, antibiotic resistance, MICs

Introduction

Physicians are encountering increasing difficulties in treating and managing patients with infectious diseases due to the continuous emergence of single and multidrug resistant organisms. The contribution of clinical microbiology laboratories to the effective treatment of patients with bacterial infections depends on accurate identification and rapid susceptibility testing of bacteria.[1] Currently, several conventional or automated antimicrobial susceptibility tests are available. Owing to the inherent time delay imposed by bacterial growth rates, culture-based systems have traditionally provided results several hours to days after initial isolation. Antibacterial activity of antibiotics is determined after various incubation times, and quantification of bacteria may be achieved by enumeration of cfu/mL after subculture on agar plates, turbidimetric measurement of the suspension, fluorometric detection or detection of a bacterial metabolite such as CO_2.[2] However, test methods with even shorter analysis times are needed so that reporting can occur in a more relevant time period.[2]

The mathematical descriptions of PCR and bacterial growth are very similar, with an initial exponential rate of growth. Growth kinetics of bacteria may be determined more accurately by enumeration of DNA copies over time. PCR is faster and more specific than bacterial culture; using short cycle times, and assuming a good PCR efficiency, DNA doubles 40 times faster than bacteria.[3]

Recent advances in molecular biology have led to the development of genotypic assays suitable for antibiotic susceptibility testing.[4,5]

Here, we describe a universal method for measuring the inhibitory effects of antimicrobial agents on common bacterial pathogens using universal primers and quantification of DNA copies using a LightCycler.

Methods

Reference strains of bacteria, antibiotics and critical concentrations are listed in Table 1.

Antibiotic susceptibilities of the 22 reference strains and two clinical isolates of *Mycobacterium tuberculosis* (Table 1) were tested by real-time PCR assay and by conventional methods in accordance with NCCLS guidelines.[6] The tests were performed in sterile tubes using Mueller–Hinton broth (with 5% horse blood for *Streptococcus* spp.) and the initial inoculum size was adjusted to match that of a 0.5 McFarland standard. The inoculum was added to each tube as well as 100 μL of the antimicrobial solution. The remaining tubes without antimicrobial agent served as growth

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Table 1. Results of susceptibility to antibiotics and delay for 24 bacterial strains as determined either by real-time PCR or by macrobroth (MB) dilution

Species	Reference strain (CIP)	Susceptibility (phenotype)	Tested antibiotic (concentration tested mg/L)	Susceptibility assay			
				real-time PCR	peak fusion (°C)	delay (h)	MB assay (18 h)
Staphylococcus aureus	103429	MET S	OXA (4)	S	87.2	4	S
	106415	MET R	OXA (4) VAN (4)	OXA R VAN S	86.1	4	OXA R VAN S
Staphylococcus epidermidis	68.21	MET S	OXA (4)	S	86.4	4	S
	105810	MET R	OXA (4) VAN (4)	OXA R VAN S	86.5	4	OXA R VAN S
Haemophilus influenzae	102514T	wild-type	AMP (8)	S	87.2	4	S
	103777	β-lactamase	AMP (8)	R	87.3	4	R
Streptococcus agalactiae	103227T	wild-type	AMP (8)	S	87.0	4	S
Streptococcus pyogenes	56.41	wild-type	AMP (8)	S	87.1	4	S
Streptococcus pneumoniae	102911	PEN S	PEN (0.1)	S	85.5	4	S
	104470	PEN R	PEN (0.1)	R	85.9	4	R
Pseudomonas aeruginosa	76.110	wild-type	TIC (64)	S	87.7	2	S
	105519	TIC CAZ IPM R	TIC (64) CAZ (8) IPM (4)	R	87.4	2	R
Proteus mirabilis	103181	AMP S	AMP (8)	S	88.7	2	S
	103800	β-lactamase	AMC (8/4)	R	88.9	2	R
Escherichia coli	76.24	AMP S	AMP (8)	S	88.8	2	S
	102181	β-lactamase	AMC (8/4)	R	89.1	2	R
Klebsiella pneumoniae	103623	AMC S	AMC (8/4)	S	88.3	2	S
	106818	ESBL	AMC (8/4)	R	88.6	2	R
Enterococcus faecalis	103214	AMX S	AMX (8)	S	86.8	4	S
	103907	GEN R	GEN (4)	R	86.6	4	R
Enterococcus faecium	103014	AMP S	AMP (8)	S	86.6	4	S
	104106	VAN R	VAN (4)	R	86.4	4	R
Mycobacterium tuberculosis	clinical	RIF S	RIF (1)	S	88.5	5 days	S (10 days)[a]
Mycobacterium tuberculosis	clinical	RIF R	RIF (1)	R	88.3	5 days	R (10 days)[a]

[a]Results of susceptibility to mycobacteria as determined using the non-radiometric Bactec 9000 MB system.

AMX, amoxicillin; AMC, co-amoxiclav; AMP, ampicillin; CAZ, ceftazidime; CIP, Collection Institut Pasteur; ESBL, extended-spectrum β-lactamase; GEN, gentamicin; IPM, imipenem; MET, methicillin; OXA, oxacillin; PEN, penicillin G; RIF, rifampicin; TIC, ticarcillin; VAN, vancomycin; S, susceptible strain; R, resistant strain.

controls. All the control tubes were incubated at 35°C for 8 h. The critical concentration tested for all antibiotics was equivalent to the MIC breakpoint for susceptibility, except for oxacillin for which we tested only *Staphylococcus* spp. using the resistant MIC breakpoint (Table 1). Each experiment was performed in triplicate and repeated twice to confirm results. Samples were collected into aliquots at 0, 0.5, 1, 2, 4, 6 and 8 h intervals. One part of each aliquot was subcultured onto trypticase soy agar or blood agar plates and incubated at 37°C for 18 h for the enumeration of colonies, and the second part was stored at −70°C for the real-time PCR assay. For mycobacteria, susceptibility testing was performed using the non-radiometric Bactec 9000 MB system.[7]

LightCycler PCR assay

Total genomic DNA was extracted from aliquots using a MagnaPure LC instrument (Roche Molecular Biochemicals, Mannheim, Germany) as described by the manufacturer. Genomic DNAs were stored at 4°C until their use as templates in PCR assays. PCR was performed with a LightCycler (Roche Biochemicals, Mannheim, Germany) using primers for 16S rDNA or *rpoB*. Those for 16S rDNA were: for *Pseudomonas aeruginosa*, 5′-TCAGTCACACTGGAACTGAG-3′ and 5′-GTAATTCCGAGGAACGCTTG-3′; for staphylococci, 5′-CGGTACCTAATCAGAAAG-3′ and 5′-TTTCCAGTTTCCAATGAC-3′; for streptococci, 5′-CTCTAGAGATAGAGTTTTAC-3′ and 5′-CGACTCGTTGTACCAACCA-3′; and for mycobacteria, 5′-GAATTACTGGGCGTAAAGAG-3′ and 5′-GCCGTAGCTAACGCATTAAG-3′. Primers for *rpoB* were: for Enterobacteriaceae, 5′-GCCAGCTGTCTCAGTTTATG-3′ and 5′-ACATACGCGACCGTAGTG-3′; and for *Haemophilus influenzae*, 5′-ACAAGTGGTTGTGCCTTCTG-3′ and 5′-TGTCATAAGTTGGATCGACAC-3′.

The PCR mixture had a final volume of 20 μL containing 2 μL of DNA master SYBR Green (DNA Master SYBR Green I Kit; Roche Diagnostics), 2.4 μL of 3 mM $MgCl_2$, 1 μL (10 pmol) of each primer (primers were selected according to the tested bacteria), 11.6 μL of distilled water, and 2 μL of extracted DNA. Each PCR included sterile distilled water as a negative control. The amplification conditions were: an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s and extension at 68°C for 1 min, with fluorescence acquisition in single mode. The number of DNA copies obtained after incubation of bacteria with or without antibiotic was determined using standard curves for each bacterial species, and plotted against time to obtain the growth kinetics of the bacteria. Antibacterial activity was defined as the absence of growth with antibiotic as compared with the growth control. Conversely, resistance to an antibiotic was defined as an increase in the number of DNA copies during the time of incubation.

Results

Growth kinetics of bacteria

Melting curves obtained with standard concentrations of the tested bacteria were always reproducible and specific for the bacteria studied. Indeed, a specific peak fusion temperature was obtained for each bacteria species and was found to be at the same temperature in each experiment (Table 1). DNA sequencing of PCR products confirmed the identification of bacteria (data not shown).

Initially, we determined the kinetics of growth for all the bacteria tested in the absence of antibiotics. Exponential phase growth ranged from $t = 2$ h to $t = 8$ h for Gram-positive bacteria (Figure 1a), and from $t = 1$ h to $t = 4$ h for Gram-negative bacteria (Figure 1b). During exponential phase, the number of DNA copies increased by 3 $\log_{10}$ as compared with the beginning of the experiment with a standard 0.5 McFarland inoculum. For mycobacteria the exponential phase was during days 3–7.

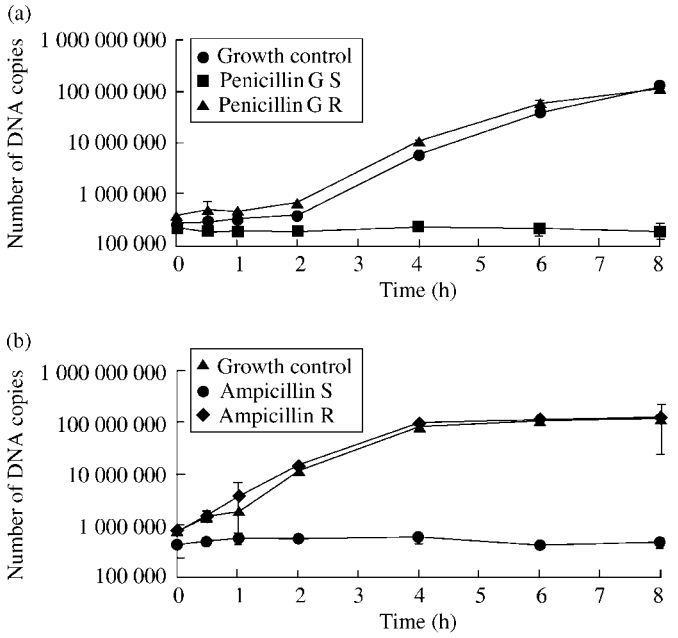

Figure 1. Kinetics of growth and antibiotic susceptibility for *Streptococcus pneumoniae* and penicillin G (a) or *Escherichia coli* and ampicillin (b) as determined by real-time PCR assay. Growth control corresponds to the growth of the bacteria without antibiotic. S, susceptible strain; R, resistant strain.

Antibacterial activity

In the second part of the study, we determined the number of DNA copies obtained when bacteria were grown in the presence of breakpoint-equivalent concentrations of antibiotics. This number remained similar to the number of DNA copies at the beginning of the experiment if the tested strain was susceptible to the antibiotic tested. Conversely, if the strain was resistant to the antibiotic tested, the number of DNA copies increased similarly to the growth control without antibiotic. We determined the optimal time for the evaluation of antibiotic activity against each species tested. The incubation time necessary to provide results of antibiotic susceptibility was 4 h for Gram-positive cocci (Figure 1a and Table 1) and *H. influenzae* and 2 h for Gram-negative bacilli (Figure 1b and Table 1). For mycobacteria, the real-time PCR method gave susceptibility results in only 5 days, as compared with 10–15 days for the conventional assay.

For all 24 strains tested, the susceptibility results obtained with the LightCycler assay were in accordance with results obtained using conventional methods.

Discussion

In this study, we assessed the inhibitory effects of antimicrobial agents on common, clinically relevant bacterial species using a real-time PCR assay. The usefulness of this method for susceptibility testing has previously been reported only for intracellular bacteria.[4,5,8] The performance of our LightCycler PCR assay was

excellent when compared with results obtained by conventional methods; it was both very sensitive and rapid.

Rapid return of susceptibility results is also the case for automated systems, with MICs for Enterobacteriaceae being obtained within 7 h[9] and MICs for Gram-positive bacteria in 6–17 h.[10] For mycobacteria, the result of antibiotic susceptibility testing was obtained in 5 days, which is considerably faster than conventional assays (10–15 days). In this report, we have not tested the ability of our method to reliably detect bacteria with inducible resistance mechanisms, although we believe that molecular biological methods combined with growth curves may help in these situations.

At the present time, the method we have described is not entirely automated; it takes about 2 h to perform the assay, with a previous incubation step of 2–4 h for bacteria in the presence of antibiotic. However, automization of molecular biological methods in the future could lead to the development of multiple real-time PCR for the determination of susceptibility to many antibiotics. Although there were large differences between the MICs for the susceptible and resistant strains tested in this study, our preliminary results demonstrate that molecular detection of bacteria could be a more rapid method for determining antibiotic susceptibility. Presently, the major drawback of this method, as compared with conventional assays, is cost, but this differential is likely to decrease in the future as the cost of reagents falls (for example, *Taq* polymerase will be free of patent restrictions in the future) and as greater emphasis is placed on automation, miniaturization and computerization in the clinical microbiology laboratory.

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