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Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons

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Objectives: Integrons are bacterial genetic elements that can capture and express genes contained in mobile cassettes. Integrons have been described worldwide in Gram-negative bacteria and are a marker of antibiotic resistance. We developed a specific and sensitive Taqman® probe-based real-time PCR method with three different primer–probe pairs for simultaneous detection of the three main classes of integron.

Methods: Sensitivity was assessed by testing mixtures of the three targets (*intI* integrase genes of each integron class) ranging from 10 to 10^8 copies. Specificity was determined with a panel of integron-containing and integron-free control strains. The method was then applied to clinical samples.

Results: The PCR method was specific and had a sensitivity of 10^2 copies for all three genes, regardless of their respective quantities. The method was quantitative from 10^3 to 10^7 copies, and was able to detect integrons directly in biological samples.

Conclusions: We have developed a rapid, quantitative, specific and sensitive method that could prove useful for initial screening of Gram-negative isolates, or clinical samples, for likely multidrug resistance.

Keywords: qPCR, antibiotic resistance, genotypic detection, molecular diagnostic techniques

Introduction

Integrons are bacterial genetic elements that can acquire genes embedded within gene cassettes expressed from a common promoter Pc.[1] Gene cassette movements are catalysed by an integron-encoded integrase, IntI. Integrons are involved in the spread of antibiotic resistance among Gram-negative bacteria, and their presence correlates with acquired antibiotic resistance.[2] More than 130 gene cassettes have been described, conferring resistance to almost all antibiotics. Five classes of these resistance integrons (RIs) have been described, based on the sequence of the IntI protein, classes 1, 2 and 3 being the most extensively studied.[1] So-called super-integrons (SIs) have also been described in many bacterial species, but most of the gene cassettes have unknown functions.[1]

Epidemiological studies of antibiotic resistance based on integron detection have mainly used end-point PCR[3] or real-time PCR with SYBR® Green.[4] The main issue with these methods is the difficulty in achieving high specificity. Indeed, RI integrases share high sequence identities, from 40% to 58%,[1] both among themselves and also with SI integrases. Moreover, the Pc promoter sequence for class 1 RIs is located within the *intI1* gene, and several Pc variants have been described.[5] Primers located within this sequence could thus lead to failure to amplify their target.

The aim of this work was to develop a rapid, sensitive, specific, quantitative, high-throughput PCR (qPCR) method for simultaneous screening of the three main classes of RI.

Materials and methods

Strains and plasmids

The following three plasmids were used as positive templates: pBAD 18::*intI1*,[6] pGEM®-T Easy::*intI2* and pBAD18::*intI3* (a gift from D. Mazel). To construct pGEM®-T Easy::*intI2*, the *intI2* gene was amplified with primers *intI2*-START and *intI2*-STOP (Table 1), using a class 2 integron-containing clinical isolate.

To validate the qPCR method, we used: (i) 15 class 1 and/or 2 integron-containing strains from our collection [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] and the class 3-containing *Serratia marcescens* strain AK9373;[7] and (ii) 28 integron-free clinical strains belonging to different Gram-positive or Gram-negative species (absence of class 1, 2 and 3 RIs was checked

Table 1. Primers and probes used in this study

Primers/probes	Sequence (5′–3′)	Location	Product size (bp)
Primers			
intI2-START	ATG TCT AAC AGT CCA TTT TTA AAT TC	*intI2*	978
intI2-STOP	TTA CTG ATT GAT AAG TAG CAT C		
intI1-LC1	GCC TTG ATG TTA CCC GAG AG	*intI1*	196
intI1-LC5	GAT CGG TCG AAT GCG TGT		
intI2-LC2	TGC TTT TCC CAC CCT TAC C	*intI2*	195
intI2-LC3	GAC GGC TAC CCT CTG TTA TCT C		
intI3-LC1	GCC ACC ACT TGT TTG AGG A	*intI3*	138
intI3-LC2	GGA TGT CTG TGC CTG CTT G		
Probes			
intI1-probe	(6-FAM) ATT CCT GGC CGT GGT TCT GGG TTT T (BHQ1)	*intI1*	
intI2-probe	(Texas Red) TGG ATA CTC GCA ACC AAG TTA TTT TTA CGC TG (BHQ2)	*intI2*	
intI3-probe	(Cy5) CGC CAC TCA TTC GCC ACC CA (BHQ3)	*intI3*	

BHQ, black hole quencher.

by end-point PCR[8]). Possible cross-reactivity with SI integrases was detected with two *Vibrio* strains.

Biological samples

Biological samples (24 sera and 34 urine samples, bronchoalveolar fluid, endotracheal aspirate, wounds, CSF) were collected between July 2006 and May 2007 from 24 intensive care unit patients with severe acute sepsis.

DNA extraction

Plasmid DNA was extracted with the Wizard® Plus SV Minipreps DNA purification system (Promega®, Charbonnières les Bains, France). DNA was adjusted to a final concentration of 10^9 copies of the *intI* gene per 5 μL. The target gene copy number was calculated as previously described.[9] Serial 10-fold dilutions of the three plasmids were used (10^8 to 10 copies per 5 μL).

Total bacterial DNA was extracted by boiling,[8] and cell suspensions prepared with two or three colonies in 500 μL of sterile water were tested in parallel. DNA from clinical samples was extracted with the QIAamp® DNA Mini kit (Qiagen®, Courtabœuf, France).

Design of primers and probes

We first identified the most strongly conserved motifs in each RI *intI* gene (*intI1*, *intI2* or *intI3*), using ClustalW software to define the more appropriate regions to choose specific primers. Primers and probes were then designed using Primer3 software. We selected primers and Taqman® probes (Table 1) with a melting temperature (Tm) of ∼60°C and 70°C, respectively. The selected primers and probes were aligned against known databases, using BLASTN, to rule out non-specific binding to other bacterial targets.

qPCR

Assays were performed with a reaction mixture of 20 μL containing 5 μL of DNA template or cell suspension, 2 μL of 10× LightCycler® FastStart DNA Master Hybridization Probes Mix (Roche®), 0.4 μM each primer and 0.2 μM each probe. The $MgCl_2$ concentration was 3 mM in simplex PCR and 6 mM in multiplex PCR. The programme consisted of a 10 min initial step at 95°C, followed by 45 cycles at 95°C for 30 s and 60°C for 1 min. Assays were performed with a MX3005P real-time detection system (Stratagene®) with a run-time of 1.5 h. Fluorescence thresholds were set at 2000 for the FAM fluorophore, 1000 for Texas Red and 500 for Cy5.

Assays were performed in triplicate when using only one target gene, in duplicate for reactions with two target genes and only once for all the concentration ratios of the three *intI* genes. Efficiency (*E*) and r^2 values were determined from five points of the serial dilutions (10^7–10^3 copies) of each target. Reproducibility was determined by calculating the coefficient of variation (CV) for each concentration of each target gene.

Assays were performed in duplicate with DNA from the bacterial strains and biological samples.

Results and discussion

Each primer–probe pair was first tested in simplex reactions to estimate its efficiency and specificity. The reactions were validated over a concentration range of 10^8–10 target gene copies with an efficiency of 97% (Table 2). The CV did not exceed 0.8% for *intI1* and *intI2*, except for the extreme points of the curves, and was <2% for *intI3*. The reactions were quantitative from 10^7 to 10^3, with an r^2 value of at least 0.9987. No amplification was observed with 10^6, 10^7 or 10^8 copies of the non-target *intI* genes, showing that each primer–probe pair was specific for the corresponding *intI* gene.

For the triplex PCR, we first tested samples containing a single *intI* target. The cycle threshold (Ct) values were slightly shifted upwards (Table 2). A sensitivity of 10 copies was obtained with the *intI1* and *intI2* targets, and 10^2 copies with *intI3*, albeit with good amplification efficiencies. There was no amplification in channels corresponding to the non-target probes, indicating the absence of cross-reactions. We then performed the triplex qPCR with mixtures containing either two target genes (*intI1/intI2, intI1/intI3* or *intI2/intI3*) or all three genes. The templates were mixed at seven different concentrations, from 10^7 to 10 copies, so as to obtain all possible 'crossing ranges' of the target genes. Regardless of the quantity of each *intI* gene, the triplex reaction exhibited very small Ct shifts for each gene relative to the

Table 2. qPCR results for simplex and triplex assays

	intI1			*intI2*			*intI3*		
Copies	Ct	SD	CV (%)	Ct	SD	CV (%)	Ct	SD	CV (%)
Simplex qPCR									
1.00E+08	14.41	0.02	0.11	13.61	0.23	1.66	14.61	0.27	1.84
1.00E+07	17.70	0.08	0.44	16.65	0.08	0.48	17.14	0.20	1.18
1.00E+06	21.08	0.17	0.78	20.10	0.13	0.64	20.42	0.28	1.37
1.00E+05	24.36	0.09	0.38	23.47	0.08	0.32	23.47	0.20	0.84
1.00E+04	27.88	0.05	0.18	26.89	0.11	0.41	27.30	0.23	0.83
1.00E+03	31.30	0.18	0.58	30.26	0.17	0.55	30.73	0.31	1.02
1.00E+02	34.69	0.24	0.70	33.70	0.10	0.28	34.25	0.15	0.44
1.00E+01	38.62	0.53	1.37	37.96	0.38	0.99	37.91	0.09	0.24
slope		−3.401			−3.402			−3.407	
y intercept		41.47			40.48			40.85	
efficiency (%)		97			97			97	
r^2		0.9999			1.0000			0.9987	
Triplex qPCR: one target gene									
1.00E+08	15.33	0.12	0.81	14.05	0.17	1.18	16.33	0.32	1.95
1.00E+07	18.06	0.15	0.85	16.90	0.06	0.33	18.89	0.10	0.54
1.00E+06	21.42	0.10	0.45	20.38	0.07	0.34	21.84	0.18	0.82
1.00E+05	24.54	0.09	0.36	23.29	0.14	0.60	24.36	0.41	1.67
1.00E+04	28.31	0.22	0.77	27.07	0.16	0.60	28.06	0.25	0.91
1.00E+03	31.51	0.14	0.44	30.36	0.07	0.22	31.83	0.35	1.09
1.00E+02	34.64	0.17	0.48	33.81	0.06	0.19	35.13	1.01	2.87
1.00E+01	38.68	0.67	1.74	37.00	0.76	2.06	37.52	—	—
slope		−3.377			−3.362			−3.210	
y intercept		41.65			40.41			41.05	
efficiency (%)		98			98			105	
r^2		0.9993			0.9989			0.9932	
Triplex qPCR: one, two and/or three target genes[a]									
1.00E+07	18.36	0.38	2.09	17.03	0.30	1.76	18.31	0.40	2.20
1.00E+06	21.38	0.39	1.85	20.30	0.36	1.79	21.43	0.36	1.69
1.00E+05	24.73	0.37	1.51	23.60	0.36	1.54	24.57	0.37	1.52
1.00E+04	28.18	0.40	1.42	26.91	0.38	1.41	28.12	0.43	1.54
1.00E+03	31.70	0.41	1.29	30.15	0.43	1.42	31.80	0.53	1.66
1.00E+02	35.16	0.65	1.84	33.64	0.62	1.83	34.68	0.81	2.34
1.00E+01	38.68	—	—	37.56	—	—	37.41	—	—
slope		−3.347			−3.286			−3.368	
y intercept		41.61			40.03			41.68	
efficiency (%)		99			102			98	
r^2		0.9992			1.0000			0.9984	

Ct, cycle threshold; SD, standard deviation; CV, coefficient of variation.
Slopes, *y* intercepts, efficiencies and r^2 values were calculated with points 10^3 to 10^7.
[a]A given Ct value for one concentration of one target is the mean of the Ct values obtained for this target present in the reaction mixture, alone or with one or both of the other two targets, regardless of their respective concentrations. A given Ct value corresponds to the mean of a total of 64 different experimental Ct values.

corresponding simplex reactions, and remained quantitative and sensitive with a reproducible detection limit of 10^2 copies for all three genes [Table 2 and Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Efficiency remained close to 100% and r^2 values close to 1. The CVs remained <2% for almost all the concentrations of the three targets.

The qPCR method was then validated on bacterial strains. No signal was obtained with any of the 28 RI-free strains or with the SI-containing *Vibrio* strains. Specific amplification signals were obtained with all the RI-containing strains. Direct amplification of bacterial suspensions gave the same results as with DNA extracted by boiling. This shows that no DNA extraction step is

needed, which is a particular advantage when screening hundreds of strains in large epidemiological studies.

To verify that the qPCR method would be as efficient when tested on more complex samples, we applied the technique to various biological samples. The qPCR method detected the *intI1* gene in five different biological samples containing from 10^4 to 10^7 copies/mL. The results were in agreement with those obtained with the strains isolated from these samples. The other biological samples yielded no fluorescence signal for any of the RIs, and the corresponding cultures were either sterile or yielded a variety of microorganisms in which no integrons were detected.

Thus, we have successfully developed a rapid, quantitative, specific and sensitive method for detecting the three *intI* genes of class 1, 2 and 3 RIs. We opted for a Taqman® probe-based multiplex real-time PCR technique, for five main reasons, namely probe specificity, sensitivity, rapidity, convenience and simultaneous quantitative detection of three *intI* genes in a single reaction. Triplex detection of RIs using a SYBR® Green-based method has previously been described,[4] but the melting curves can be difficult to interpret, especially if the fusion points are close. With a Taqman® probe-based technique, the results are clear-cut and specificity is enhanced, as specific probes are used in addition to specific primers.

The need for genotypic methods to detect antibiotic resistance has been underlined elsewhere.[10] The link between integron carriage and antibiotic resistance implies that RIs may be considered as markers of acquired antibiotic resistance.[2] Thus, our multiplex PCR detection method for RIs should prove useful for initial screening of multidrug resistance (resistance to at least two drugs) in Gram-negative isolates. Furthermore, this method can be applied to clinical samples, with a detection limit of 10^2 copies. Finally, it should be noted that RI-containing bacteria may sometimes seem falsely susceptible to antibiotics, despite the presence of antibiotic resistance genes, underlining the value of molecular approaches.[11]

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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