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Full Length Research Paper

Non-PCR-based amplification technique combined to DNA array hybridization to detect complex SNPs involved in antibiotic resistance

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We have investigated the feasibility of an approach that combines a non-PCR-based amplification of the target DNA to a microarray-based detection of the single nucleotide polymorphisms (SNPs) in genes associated with bacterial resistance to beta-lactams. The method involves a random non-PCR amplification by the exo-Klenow, followed by hybridization on a low-density DNA chip. This approach was demonstrated to produce specific hybridization using recombinant and natural plasmids harboring *bla*$_{SHV}$ variants as model of SNP-containing genes.

Key words: Antibiotic resistance, non-PCR amplification, hybridization, DNA chip, mutation.

INTRODUCTION

Resistance to antibiotics is characterized by a very wide diversity at the phenotypic and molecular levels. Resistance to β-lactam agents in Gram-negative bacteria, mostly associated with the production of plasmid-determined β-lactamases, is a representative example of this diversity. Indeed, those enzymes, encoded by the *bla* genes, are divided into four molecular classes (A, B, C and D) (Ambler et al., 1991), each class consisting of several β-lactamase families containing each a large number of variants. For instance, the class A β-lactamases group in the Ambler's classification includes numerous families among which are the TEM, SHV, CTX-M, PER, VEB, GES, KPC, SFO, BES, TLA, IMI, BEL, AER, CAD and CARB enzymes. Each of these families contains a large number of variants differing from each other by several amino-acid modifications (e.g. 167 TEM- and 120 SHV-variants). Though closely related at the genetic level, the variants belonging to a given family can differ by the spectrum of β-lactam compounds they can hydrolyze. For instance, the TEM-1, -2 and SHV-1 broad-spectrum penicillinases hydrolyze penicillins and cephalosporins of the first and second generations, while the related TEM and SHV extended-spectrum β-lactamases (ESBLs) derived from TEM-1, -2 and SHV-1 by point mutations display a substrate range expanded to the so-called oxiimino-aminothiazol cephalosporins (approximately 160 TEM and 100 SHV ESBL variants). In the SHV family, the region of codons 238-240 in the *bla* gene numbering according to Ambler et al. (1991) is a representative example of the molecular and phenotypic diversity that can be observed among the β-lactamases since three mutations can be found within a stretch of six nucleotides (at positions 700, 703 and 705, Table 1), two of them being present alone or in association in more than 50 ESBL variants (G700A and G703A coding for the Gly238-Ser and Glu240-Lys mutations, respectively), the third one (A705G) being a single nucleotide polymorphism (SNP) frequently present at position 705 as silent mutation. In this context of wide genetic diversity, the molecular approaches combining PCR amplification and DNA chip hybridization represent a valuable strategy for the simultaneous and rapid identification of gene variants (Lee et al., 2002; Bruant et al., 2006; Call et al., 2003; Batchelor et al., 2008; Cleven et al., 2006; Grimm et al., 2004). However, a serious limitation of this approach in routine practice is the preliminary PCR amplification step

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Table 1. The 238-240 complex region in the SHV-type β-lactamases.

β-lactamase	Codon (nucleotide) number [a]					
	238			240		
	(700)	(701)	(702)	(703)	(704)	(705)[b]
SHV-1 (Gly238, Glu240) [b]	G	G	C	G	A	A
SHV-2, -2A (Ser238, Glu240)	A	G	C	G	A	G
SHV-4, -5, -12 (Ser238, Lys240)	A	G	C	A	A	G

[a]The codon numbering system is according to Ambler (Ambler et al., 1991).
[b]Gly, glycine ; Glu, glutamic acid ; Ser, serine ; Lys, lysine. [b] The A705G SNP is silent.

Table 2. Oligonucleotide probe sets used in this study

Set name[a]	Probe sequence (3'-5')[b]
	700 703 705
700	TGG CCT CGA **N**CG CTT GCC C
700sm[c]	TGG CCT CGA **N**CG CT**C** GCC C
700dm[d]	TGG CCT CGA **N**CG **T**T**C** GCC C
	700 703 705
703	CCT CGA CCG **N**TT GCC CCA C
703sm	CCT CGA CCG **N**T**C** GCC CCA C
703dm	CCT CGA **T**CG **N**T**C** GCC CCA C

[a] Each set of probes is named according to the position of interest in the *bla*$_{SHV}$ gene. [b] Nucleotide numbers are given above the sequences of the probes. Underlined bases in the sets of probes 700 and 703 indicate codons 238 (nucleotides 700 to 702) and 240 (nucleotides 703 to 705), respectively (codons are numbered according to Ambler et al., 1991). Each set consisted of four reverse probes with identical sequences, except for the central base N (A, T, C or G). [c] "sm" specifies that a nucleotide matching the mutation at position 705 (C in bold, complementary to the A705G silent mutation) is present in each oligonucleotide of the set. [d] "dm" indicates the presence of (i) a nucleotide matching the silent mutation G705 (C in bold) and (ii) a nucleotide complementary to the A703 mutation coding for Lys 240 in set 700dm or to the A700 mutation coding for Ser 238 in set 703dm (T in bold).

performed to amplify and label the target gene, which requires large sets of oligonucleotide primers specific for each group of gene family. Zhu et al. described a method combining SNP detection and amplification based on multiplex PCR which permitted the amplification and identification of several *bla* genes (Zhu et al., 2007). Multiplex assays are a marked improvement over traditional PCR-based assays. Nevertheless, this strategy was limited to 10 families of genes of beta-lactamases, which is few in this context of wide genetic diversity. An alternative to conventional and multiplex PCR is the direct DNA labeling based on Klenow amplification (Lieu et al, 2005; Vora et al., 2004). In the present study, we have investigated the feasibility of an approach that combines a klenow amplification of the target DNA to a microarray-based detection of the SNPs present in the amplified DNA. To set up and validate this approach, the highly polymorphic 700-705 region covering codons 238-240 in *bla*$_{SHV}$ has been chosen.

MATERIALS AND METHODS

Probe design and microarray fabrication

The *bla*$_{SHV}$ DNA array prototype has been developed using short oligonucleotide probes (19 bases) designed according to the complementary sequences of the wild-type *bla*$_{SHV-1}$ gene and two related ESBL mutants, *bla*$_{SHV-2a}$ and *bla*$_{SHV-12}$ (Table 1). The two mutations G700A (Gly 238 Ser) and G703A (Glu 240 Lys) involved in the development of the ESBL phenotype, as well as the silent A705G SNP, were taken into account to design six sets of probes allowing to discriminate between perfect matches and mismatches for all the possible combinations of mutations in the three positions (Table 2). Thus, the detection of the Gly238-Ser mutation was based on the use of three sets of four complementary probes: set 700 probing codon 238 (**N**CG, with N= A, T, C or G) and based on the presence of a wild-type codon 240 (CTT complementary to GAA), set 700sm also probing codon 238 (**N**CG) and taking into account the A705G SNP in codon 240 (CT**C**), and finally set 700dm probing codon 238 (**N**CG) and matching the codon for Lys 240 plus the A705G SNP (**T**T**C**). Similarly, three probe sets were also used for the detection of the mutation Glu240-Lys: set 703 probing codon

240 (**N**TT) and including a wild-type codon 238 (CCG), set 703sm similar to set 703 but including the A705G SNP in codon 240 (NT**C**), and set 703dm including the A705G SNP in codon 240 (NT**C**) and matching the codon **T**CG coding for Ser 238. The probes were dissolved in the PRONTO! Epoxide Spotting Solution (Corning, Corning, New York, USA) to a final concentration of 30 µM and spotted in quadruplicates with a QARRAY microarraying robot (Genetix Ltd, New Milton, United Kingdom) on Epoxide coated slides (Corning).

Bacterial strains

Six control strains were constructed by cloning in vectors pPCR-Script Cam (Stratagene, La Jolla, California, USA) and/or pCR 2.1-TOPO (Invitrogen, Carlsbad, California, USA) the wild-type *bla*$_{SHV-1}$ gene and 5 ESBL-encoding variants (*bla*$_{SHV-2}$, *bla*$_{SHV-2A}$, *bla*$_{SHV-4}$, *bla*$_{SHV-5}$ and *bla*$_{SHV-12}$). Three clinical strains producing the SHV-1, SHV-2 and SHV-12 β-lactamases were selected for the evaluation of the non-PCR labeling method.

Labeling of the target DNA and hybridization

Plasmid DNA was extracted with the PLASMID MIDI KIT (Qiagen, Hilden, Germany) and labeled with the BioPrime Array CGH genomic labeling kit (Invitrogen, Carlsbad, California, USA), which is a non-PCR amplification system based on a random prime DNA labeling step utilizing the double mutant exo-large fragment of DNA polymerase I (exo-Klenow). Briefly, plasmid DNA (2 to 3 µg) was added to 50 µl of amplification mix consisting of 80 U of exo-Klenow, 1 × random primers solution and 60 µM Cy5-dCTP (Pekin Elmer, Wellesley, Massachusetts, USA). After four hours of incubation at 37°C, the reaction was stopped and the labeled DNA was hybridized on the DNA chip using the PRONTO! Short oligo hybridization solution (Corning). The microarray was scanned with a GENEPIX 4000B Array scanner (Axon Instruments, Sunnyvale, California, USA) at 635 nm (Cy5) and the fluorescent data were determined using the GENEPIX PRO v 6.0 software (Axon Instruments). The oligonucleotide yielding the highest hybridization signal (HHS) in each probe set was regarded as a potential perfect match (PM). Within each probe set, relative intensities (RIs) were calculated (mismatches/PM ratio). The set yielding the highest hybridization signal and the lowest RI values was considered as the one giving rise to the more specific hybridization.

RESULTS

The non-PCR labeling method was first evaluated with the six control strains and the analysis of the RIs indicated that residues on position 238 and 240 were correctly identified in each of the control strains included in the study (Figure 1). For the SHV-1 clone charac-terized by G700 (Gly 238), G703 (Glu 240) and A705, the HHS and lowest RIs (RI values < 0.5) were observed for the complementary probes having N=C in positions 700 and 703 in sets 700, 700sm, 703 and 703sm (Figure 1-A-a). By contrast, the two remaining sets 700dm and 703dm, specific for mutations Ser 238 and Lys 240, respectively, yielded low hybridization intensities and high RI values. For the clones harboring the SHV-2-type ESBLs, having a double mutation A700 (Ser238) and G705 (SNP), the HHS and the lowest RIs (< 0.5) were obtained with the T-specific probe in the 700sm set (detection of A700 for Ser 238) and the C-specific probe in set 703dm (detection of the wild-type nucleotide G703 in the presence of A700 and G705) (Figure 1-A-b). Finally, the clones harboring a triple mutation A700 (Ser 238) A703 (Lys 240) and G705 (SNP) in the ESBLs SHV-4, -5 and -12, exhibited the HHS and the lowest RIs (< 0.3) with the T specific probes in sets 700dm and 703dm, as expected.

The non-PCR labeling method was then evaluated on plasmid DNA extracted from clinical strains producing the SHV-1, SHV-2 and SHV-12 β-lactamases (data presented on Figure 1-B-a to 1-B-c, respectively). Overall, the results obtained were approaching those observed with the recombinant plasmids, except for the double mutant SHV-12 for which non-specific hybridization occurred with probe 703smC, as explained below. The highest hybridization signals and lowest RI values (< 0.3) were observed for the probes having N=C in sets 700, 700sm, 703 and 703sm for SHV-1 (Figure 1-B-a), and with probes 700smT (RIs < 0.4) and 703dmC (RIs ≤ 0.1) for SHV-2 (Figure 1-B-b). With respect to *bla*$_{SHV-12}$ (A700, A703, G705), the lowest RI values were found, as expected, with probes 700dmT (RIs < 0.25) and 703dmT (RIs < 0.2) but, unexpectedly, non-specific hybridization could be noticed with the 700smC and 703smC probes even if the RIs worked out for these two sets were markedly lower than those calculated for the perfect match probes 700 and 703 dmT (Figure 1-B-c).

DISCUSSION

Several microarray-based strategy have been described for the detection of β-lactamases and most of them allow the co-detection of several β-lactamase genes without any discrimination of the variants (Lee et al., 2002 ; Bruant et al., 2006; Call et al., 2003; Batchelor et al., 2008; Cleven et al., 2006). This strategy is most of the time based on probes that are gene-specific PCR products or long oligonucleotides (70mers) and permits the detection of several antibiotic resistance determinants and even virulence genes (Cleven BE et al., 2006). Since point mutations of a single gene can lead to different phenotypes (e.g. SHV-1 is a penicillinase, whereas the SHV-12 variant is an ESBL), the variant identification is necessary for the further interpretation of the results. Grimm et al. have described an oligonucleotide micro-array for the identification of TEM β-lactamase variants, which was constituted of four-probe sets (Grimm et al., 2004). This approach led to a good discrimination of the variants but, as it was based on a TEM-specific labeling step, it was dedicated to TEM variants only. More recently, Zhu et al. developed a method combining SNP detection and Multiplex PCR-based labeling step. This tool permitted to detect several β-lactamase-encoding genes and to detect six point mutations within the *bla*$_{SHV}$ gene but the studied genes were limited to ten (Zhu et al., 2007), which is few in this context of wide genetic diver-

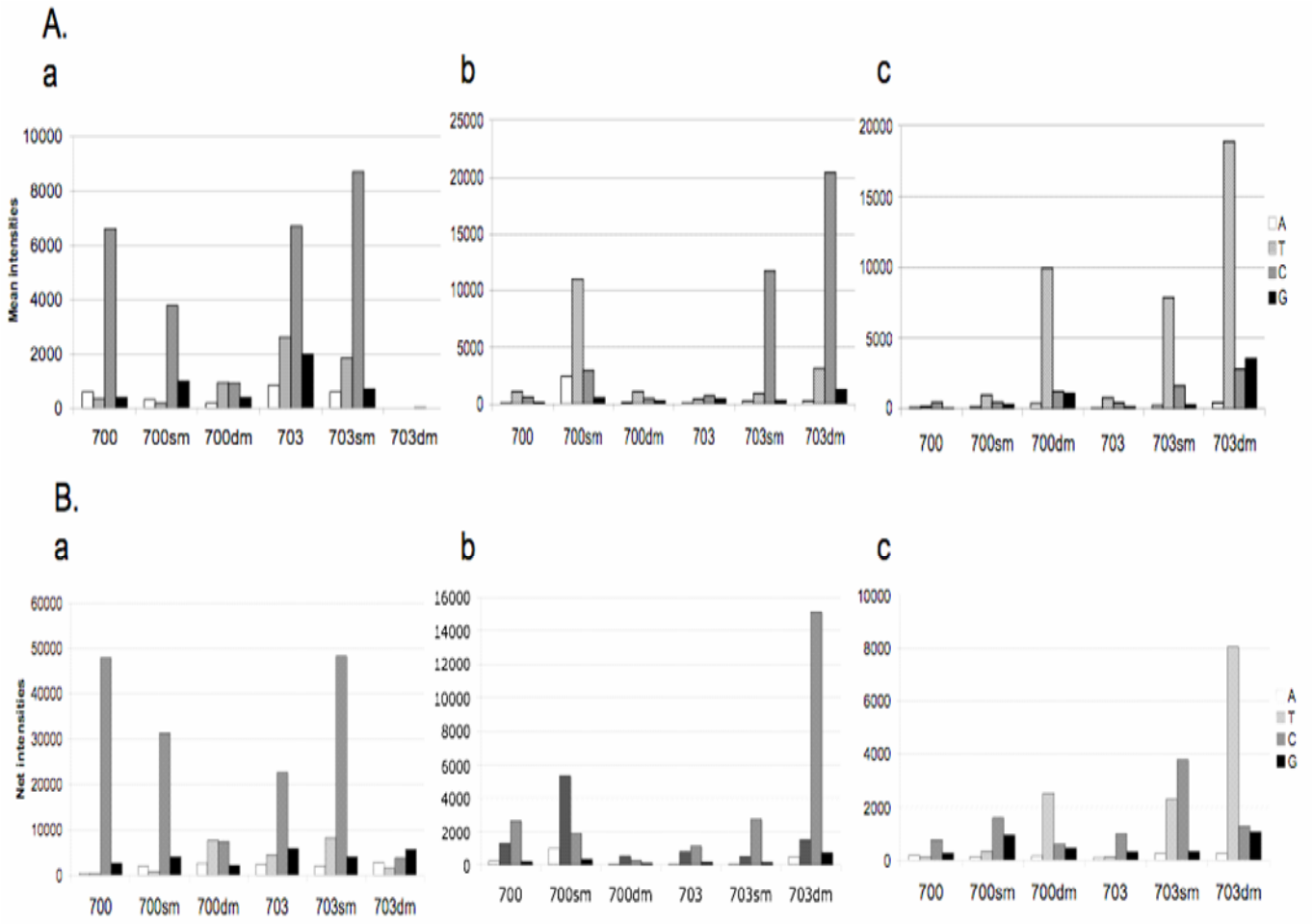

Figure 1. Hybridization results on DNA chips obtained by non-PCR labeling approach. **A**. Validation of the non-PCR labeling method with recombinant plasmids. Mean intensities were determined for (a) SHV-1 (ntest = 2), (b) SHV-2A (ntest = 3) and SHV-2 (ntest = 1), (c) SHV-4 (ntest = 1), SHV-5 (ntest = 1) and SHV-12 (ntest = 5). **B**. Evaluation of the non-PCR labeling method with clinical isolates. Mean intensities were determined for clinical strains producing (a) SHV-1, (b) SHV-2 and (c) SHV-12.

sity. The random prime DNA labeling system based on the use of the exo-large fragment of DNA polymerase I (exo-Klenow) allowed us to avoid a preliminary amplification, which requires specific primers, and taken all together, our results show the capacity of random-prime amplification combined to microarray hybridization to detect SNPs directly from bacterial plasmid DNA. This random prime DNA labeling system was previously evaluated against other labeling methods (standard random prime and nick translation) (Lieu et al., 2005), showing that the exo-Klenow-based labeling system produced higher fluorescence intensities and greater signal-to-noise ratios comparatively to the other methods. In another study, Vora et al. also highlighted the promising efficacy of random amplification to detect directly from genomic DNA the enterohemorrhagic *Escherichia coli* O157:H7 (Vora et al., 2004). In the present report, the wild-type and mutant bla_{SHV} genes were unambiguously identified with a good discrimination level (RIs < 0.5), not only from the recombinant plasmids used as controls but also from plasmids extracted from clinical strains. One can note that less specific hybridizations could occur in the latter case, as mentioned for bla_{SHV-12}. The lower copy number of the large plasmids obtained from clinical isolates, when compared to the smaller plasmids obtained after DNA cloning, likely accounts for this observation. One can however notice that such mishybridizations are unequivocally shown up by the characteristic high RI values that reflect poor hybridization specificity.

In conclusion, the combinations of a microarray-based detection of the SNPs to a random amplification approach that doesn't require any specific primer represents a valuable alternative to the commonly-used molecular detection by PCR and sequencing. Such an approach could be very useful for the detection of resistance to beta-lactams which can be determined by a large range of gene families including each a high num-

ber of gene variants.

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