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Design of clone-specific probes from genome sequences for rapid PCR-typing of outbreak pathogens

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Abstract

The genome sequence of one OXA-48-producing *Klebsiella pneumoniae* belonging to ST405, and three belonging to ST11, were used to design and test ST-specific PCR assays for typing OXA-48-producing *K. pneumoniae*. The approach proved to be useful for in house development of rapid PCR typing assays for local outbreak surveillance.

Rapid typing of clinical isolates is essential for the management of hospital outbreaks. Ideally, typing should provide correct strain differentiation in real time to discriminate between outbreak and non-outbreak isolates. In practice this is seldom achieved, as most methods are time-consuming or have to be performed in batches, and typing is often done retrospectively. During the last years efforts have been oriented towards rapid real-time typing with the introduction of a commercial highly standardized REP-PCR

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(Diversilab®)[1,2,3], and currently several genomic typing techniques are under development[4,5,6]. Genomic typing is the most promising approach as it may reach the maximum resolution possible and has potential for further developments but is still costly and time-consuming. The availability of whole genome sequences opens a way to the design of strain-specific probes for the development of rapid and simple PCR typing assays.

OXA-48-producing *Klebsiella pneumoniae* has been present in our hospital since January 2011 [7,8] with more than 450 isolates obtained from clinical and surveillance samples from more than 300 patients. Clonality is analyzed routinely by semi-automated REP-PCR (Diversilab®), and clonal groups have been further characterized by multi-locus sequence typing [7,9]. The outbreak is largely due to two clones: one belonging to ST405, that was dominant during the first year [7], and another belonging to ST11, that has been slowly increasing in frequency and has become the major clone in the last year. Other OXA-48-producing *K. pneumoniae* clones and even other OXA-48-producing enterobacteriaceae have been isolated sporadically. These two STs have been found also in other hospitals from Madrid and other Spanish areas, the most frequent being ST11 [10,11].

To design clone-specific probes, a search for unique sequences was done using the genomes of an OXA-48-producing *K. pneumoniae* ST405 isolate from our hospital (Genbank accession number AMRH00000000.1) [12] and three ST11 isolates collected at the antibiotic reference laboratory of the National Centre for Microbiology (European Nucleotide Archive, ENA, accession numbers ERS201946, ERS201950, ERS201959) [10]. Selection of "strain-specific" sequences was done with the Novel Region Finder tool of Panseq [13]. The Panseq output was further filtered by deleting sequences with significant matches in Genbank. This procedure yielded a set of 83 ST405 sequences of which 14 were considered unique and four of them were arbitrarily chosen as potential targets. Similarly, 125 ST11 sequences were obtained of which 47 were considered unique and four were arbitrarily selected. Of these four, two were specific for our ST11 sequences, and the other two were common to our sequence and the published genome of *K. pneumoniae* HS11286, which also belongs to ST11 [14]. Primers for each sequence were designed to produce different band sizes and be used with the same annealing temperature (Table 1).

Eight primers pairs (four targeted to ST405 and four to ST11) were tested with four ST405

isolates, four ST11 isolates, and nine non-ST405 non-ST11 isolates: ST15, ST23, ST26, ST45, ST147, ST307, ST323 and ST846, all of them OXA-48-producers isolated previously [7]. The four ST405-specific primer pairs yielded amplification products with the ST405 isolates, and not with the other isolates. One ST11-specific primer pair was discarded because of poor PCR results, the other three primer pairs produced amplification products only with the ST11 isolates.

Furthermore, one group consistently identified by Diversilab® as an independent group, was found to be positive in the four ST405-specific PCR reactions and was confirmed by MLST analysis to belong to ST405. Two minor Diversilab® groups were classified by the ST11-specific PCR as ST11 clones and, again, this was confirmed by MLST analysis. The non-ST405 non-ST11 isolates were negative with all the primer sets.

Fifteen ST11-single locus variants (SLVs), including eight ST340 and seven ST437, were tested with the ST11 primer pairs. All of them were negative for target region 2, and positive for target regions 3 and 4.

Finally, thirty-four independent ST11 isolates from diverse origins were tested. These included fifteen isolates with CTX-M-15, eight with CTX-M-15 and OXA-48, one with CTX-M-15 and IMP-22, three with VIM-1, two with OXA-48-like, two with KPC-2, one with NDM-1, one with CMY-2, and one with CMY-2 and DHA-1. Thirty-two of them were found to be positive with the three ST11 primer pairs and two were positive with two primer pairs.

Currently end-point PCR with target region 4 of ST405 and target region 3 of ST11 is done directly on colony lysates for rapid typing of OXA-48-producing *K. pneumoniae* obtained in our hospital (Fig. 1). Only those samples that are negative for the two ST-specific PCR reactions are further analyzed by Diversilab®. During a period of eight months 179 isolates were analyzed, of which 33 were ST405, 138 were ST11 and 8 were negative for the two PCR reactions. These eight were analyzed by Diversilab® and confirmed to belong to some of the previously characterized minor clones [7].

Next, Taqman probes were designed for real time PCR amplification of ST405 target region 4 and ST11 target region 3 (Table 1). DNA from characterized isolates (five ST405, five ST11 and nine non-ST405 non-ST11) was used to set up the reaction conditions and check performance. Single-tube multiplex real time PCR was done using Takara exTaq Premix, with ROX, 0.5 μM of each primer and 0.2 μM of each probe. Reaction conditions were:

initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation (15s at 95ºC), annealing (30s at 50ºC), and extension (1min at 72ºC). There was no interference between primers or probes and all the samples tested were correctly identified.

Our results show the feasibility of using genome sequences to search for strain-specific sequences. Given the high level of horizontal gene transfer and the mosaic structure of bacterial genomes [15], the presence of a single sequence should not be taken as a fully consistent marker to identify specific strains. Indeed, ST11-SLVs could also be detected with some primer pairs. Nevertheless, used within a local context it provides a fast, cheap and reliable PCR typing method that might be useful to track outbreak strains in real time.

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Table 1. Primers used in this work.

Strain	Target region	Oligonucleotides	5'-->3' sequence	Product size
ST405	1	1-F	GATGAGCAAGGAGTGCAAGG	300 bp
		1-R	GCCTCGATTGCGAATGGTATG	
	2	2-F	GAGCCACTGCTTGATGATTG	367 bp
		2-R	TCGCGGCATCAGCAATTTCC	
	3	3-F	CCTTCACGCCCTGAGATTTC	512 bp
		3-R	CAAACAGGACAGCGATAAGC	
	4	4-F	GGACTAACCCTATCCCTAAC	286 bp
		4-R	CTACATTATTTGCTGCCGTCG	
	4	4-P	FAM-CAACACCGCATTCACAGGTC-TAMRA	
ST11	2	2-F	CCGGTCAACAGGGATTGAAG	268 bp
		2-R	AAGTCGCAGCATTAGCCCAG	
	3	3-F	GATCATCCGCCTATCCCTTG	238 bp
		3-R	CCCAAGATGTAGGCTGCAAG	
	4	4-F	GAACGGCGCAACCTATACTG	491 bp
		4-R	CATTGAGCCATCAGGCCAC	
	3	3-P	HEX-CCAAGCGGTAGTGATTAAGC-TAMRA	

Fig. 1. End-point PCR with ST-specific primers. Genomic DNA from four clinical isolates was tested with primer pairs specific for target region 4 of ST405 and target region 3 of ST11 and PCR products analyzed by agarose gel electrophoresis. A short (197 bp) region of the 16S rRNA gene was used as a control.