Culture-independent methods reveal high diversity of OXA-48-like genes in water environments

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ABSTRACT

The carbapenemase OXA-48 was identified for the first time in 2001 and is now one of the greatest concerns in terms of antibiotic resistance. While many studies report clinical OXA-48-like producers, few reports refer $bla_{OXA-48-like}$ genes in environmental bacteria. The main goal of this study was to evaluate the diversity of $bla_{OXA-48-like}$ genes in aquatic systems, using culture-independent approaches. For that, environmental DNA was obtained from riverine and estuarine water and used to construct clone libraries of $bla_{OXA-48-like}$ gene polymerase chain reaction amplicons. $bla_{OXA-48-like}$ libraries from river and estuarine water DNA comprised 75 and 70 clones, respectively. Sequence analysis showed that environmental $bla_{OXA-48-like}$ genes show a broader diversity than that so far observed in clinical settings. In total, 50 new OXA-48 variants were identified as well as sequences identical to previously reported OXA-48, OXA-181, OXA-199, OXA-204 and OXA-162. Though we have no evidence that these genes were carried by bacteria that are members of the natural heterotrophic flora or bacteria that have entered this particular water environment through anthropogenic sources, these results reinforce the role of aquatic systems as antibiotic resistance reservoirs. The variants of bla_{OXA-48} here described should be taken into account when designing molecular strategies for detecting this gene.

Key words | aquatic environments, carbapenemases, culture-independent methods

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INTRODUCTION

The class D OXA carbapenemases comprises a very diverse group of enzymes that have been identified mostly in outbreaks of carbapenems-resistant *Acinetobacter* spp. (e.g. OXA-23, OXA-24, OXA-40, and OXA-58), *Pseudomonas* spp. (OXA-50) and Enterobacteriaceae (OXA-48) (Evans & Amyes 2014). Other class D carbapenemases have been considered as species-specific like the OXA-60 family, naturally present in the genome of *Ralstonia pickettii* (Girlich *et al.* 2004), and OXA-62 in *Pandoraea pnomenusa* (Schneider *et al.* 2006).

OXA-48 was first identified in a clinical *Klebsiella pneumoniae* isolate in Turkey (Poirel *et al.* 2004). Although initially disseminated mostly in Mediterranean countries, nowadays OXA-48 and its variants are an example of widely disseminated carbapenemases that have been detected in all continents (Poirel *et al.* 2012). These enzymes hydrolyze penicillins and carbapenems, but not third generation cephalosporins (Poirel *et al.* 2012). However, there are many reports of OXA-48-like producers that carry additional beta-lactamases, thus limiting treatment options (Poirel *et al.* 2012).

Since its first description (Poirel *et al.* 2004) $bla_{OXA-48-like}$ genes detection has been restricted to *Shewanella* species (Poirel *et al.* 2012; Tacão *et al.* 2013), and the large majority to Enterobacteriaceae members worldwide, mostly in clinical settings. However sporadic bla_{OXA-48} genes have been reported also on *Pseudomonas aeruginosa* (Meunier *et al.* 2016) and *Acinetobacter baumannii* (Mathlouthi *et al.*

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2015). Furthermore, there are also reports describing OXA-48 producers in Enterobacteriaceae isolated from river water (Potron *et al.* 2011), wastewater (Galler *et al.* 2014) and companion animals (Stolle *et al.* 2013).

Up to now, several OXA-48 variants have been found, differing in 1 to 5 amino acids, as: OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-370 and the recently described OXA-405 and OXA-416 (Poirel *et al.* 2012; Gomez *et al.* 2013; Sampaio *et al.* 2014; Antonelli *et al.* 2015; Dortet *et al.* 2015).

There are strong indications that *Shewanella* spp. are the putative origin of OXA-48-like genes (Poirel *et al.* 2012; Zong 2012; Tacão *et al.* 2013). Members of this genus are mostly identified in water environments, but some have already been implicated in human infections (Janda & Abbot 2014). Although there are reports on OXA-48-like carbapenemases worldwide, it has been pointed out that the spread of this beta-lactamase is silent due to the difficulties in its detection. The fact that OXA-48 producers show low minimal inhibitory concentration values for carbapenems might be masking their detection and thus underestimating their dispersion (Poirel *et al.* 2012). Therefore, molecular methods have been pointed out as potential alternatives for the recognition of OXA-48-like producers (Poirel *et al.* 2012).

The study of the diversity of $bla_{\text{OXA-48-like}}$ genes is important for elaborating molecular-based strategies for their rapid detection. Also, revealing the diversity of these molecular determinants may assist in forecasting the likely dissemination of $bla_{\text{OXA-48-like}}$ genes. In this study we aimed to evaluate the diversity of OXA-48-like class D carbapenemase encoding genes in aquatic systems. In order to attain a broader assessment of gene variety in these environments, we applied a culture-independent approach.

MATERIALS AND METHODS

Sample collection and environmental DNA extraction

Samples were collected from three rivers (three each, halfway river banks) in the Vouga River basin (central Portugal). These rivers are highly polluted due to disposals of domestic, industrial and agricultural origins (for details

see Tacão *et al.* 2012). The estuarine water was collected from Ria de Aveiro, a mesothrophic estuary located in the same basin and highly polluted due to the presence of harbor facilities, aquaculture ponds, industrial plants, diffuse domestic sewage inputs and run-off from agricultural fields (Azevedo *et al.* 2012). Water was collected into sterile bottles from 50 cm below the water surface.

Environmental DNA was purified by filtering 200–500 ml of water through 0.2-μm-pore-size filters (Poretics Products). Cells were washed from the filter with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) followed by centrifugation (13,000 rpm, 10 min). The pellet was resuspended in 200 μl TE buffer containing 10 mg/ml of lysozyme, followed by 1 h incubation at 37°C, and then frozen in liquid nitrogen and thawed three times. DNA extraction continued by using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Purified DNA was stored at −20°C.

Amplification of $bla_{\text{OXA-48-like}}$ gene fragments by polymerase chain reaction

The $bla_{\text{OXA-48-like}}$ gene fragments were amplified from a pool of environmental DNA from rivers and from DNA extracted from estuarine water with the two $bla_{\text{OXA-48-like}}$-specific primer sets described so far, designed by: (i) Poirel *et al.* (2011) (fwd: 5′-GCGTGGTTAAGGATGAACAC and rev: 5′-CATCAAGTTCAACCCAACCG) and (ii) Zong (2012) (fwd: 5′ AGCAAGGATTTACCAATAAT and rev: 5′ GGCATATC-CATATTCATC). The polymerase chain reaction (PCR) reaction mixtures (25 μl total volume) consisted of 6.25 μl NZYTaq 2x Green Master Mix (2.5 mM MgCl2; 200 μM dNTPs; 0.2 U/μl DNA polymerase) (NZYtech, Portugal), 16.25 μl of ultrapure water, 0.75 μl of each primer (reverse and forward), and 50–100 ng of purified DNA. PCR reactions were performed in a MyCycler Thermal cycler (Bio-Rad, USA) with conditions as described by Poirel *et al.* (2011) and Zong (2012). Positive and negative controls were included in each PCR reaction. Water was used as negative control and strain *Shewanella xiamenensis* IR33 carrying a $bla_{\text{OXA-48}}$ gene was used as positive control (Tacão *et al.* 2013). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Genomic library construction and analysis

Clone libraries of $bla_{\text{OXA-48-like}}$ gene fragments were constructed using the TA Cloning Kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and *Escherichia coli* NZYStar competent cells (NZY-Tech, Portugal). Clones were screened by PCR for the presence of fragments with the expected size, using primers targeting the vector. Amplicons were purified and sequenced in both strands. Similarity searches in the Gen-Bank database were performed using the BLAST tool with the deduced amino acid sequences. A phylogenetic tree was obtained using MEGA 6.0 (Tamura *et al.* 2013). The Shannon index of diversity (H) was calculated for each library by using the formula $H = -\Sigma(ni/N) \ \log(ni/N)$, where ni is the abundance of each $bla_{\text{OXA-48-like}}$ type and N is the sum of the analyzed clones in each library.

Nucleotide sequences

All the nucleotide sequences obtained in this work have been deposited in the GenBank database under the accession numbers KJ620426-KJ620480.

RESULTS AND DISCUSSION

In this study, we evaluated the diversity of $bla_{\text{OXA-48-like}}$ gene sequences in river and estuarine water by culture-independent methodologies.

From river water DNA, it was possible to amplify $bla_{\text{OXA-48-like}}$ genes using the two primer sets, and both amplicons were used for constructing two libraries. From estuarine water DNA amplification was obtained using the primer set described by Poirel *et al.* (2011), and only this amplicon was used. Overall, three clone libraries of $bla_{\text{OXA-48-like}}$ genes were constructed.

A total of 145 inserts with the expected size were sequenced, 75 from the river water library (35 using DNA amplified with primers described by Zong (2012) and 40 with primers described by Poirel *et al.* (2011)) and 70 from the estuarine water library. The $bla_{\text{OXA-48}}$-like gene libraries from river water comprised 35 variants (H = 1.23): 31 new deduced amino acid sequence types and four types that

have been previously described (100% identical to OXA-48, OXA-181, OXA-199 and OXA-204). Both primer sets detected the most abundant types, but 10 types were only detected by the primer set of Zong, and another 19 types by the one of Poirel *et al.* The estuary water library encompassed 22 types (H = 0.71): 19 new deduced amino acid sequence types and three types that have been already reported (100% identical to OXA-48, OXA-162 and OXA-199). In both gene libraries, the majority of the deduced amino acid sequences (*n* = 70) were 100% identical to OXA-48 (25 from riverine and 45 from estuarine waters). The majority of the sequence variants were detected once in the gene libraries and only 13 were represented by two or more clones. Figure 1 shows a maximum-likelihood dendrogram of representatives of OXA beta-lactamases families (and the deduced amino acid sequences detected in this study in two or more clones). The association of amino acid sequences obtained in this study with previously described OXA-48-like class D carbapenemases is clear.

The amino acid substitutions in the most common variants when compared to the OXA-48 sequence are shown in Figure 2. For all it was possible to identify the amino acid motifs that are conserved among class D beta-lactamases (Couture *et al.* 1992; Dortet *et al.* 2015). Table S1 in the supplemental material (available with the online version of this paper) indicates the amino acid substitutions in all new OXA-48-like variants found. Besides OXA-48 and OXA-199, only two other variants were common to both the river and estuary libraries (OXA-new14 and OXA-new17). Overall, 50 new variants were detected, within 1 to 3 amino acid differences from OXA-48. Noteworthy, the OXA-48 variants found more frequently were those that are already triggering serious health concerns in several hospital settings, which is the case of OXA-48 that was by far the most frequently detected in both libraries. These results suggest that there might be a correspondence between what has been observed so far in hospital settings and the environmental $bla_{\text{OXA-48}}$ gene pool. If this is the case, new variants here frequently detected, might also emerge in clinical settings. Few studies assessed the presence of $bla_{\text{OXA-48-like}}$ producers in environmental settings, and so it is not possible to clarify if the gene diversity here described is particular to these aquatic systems or if these genes are more diverse than expected and commonly present in environmental
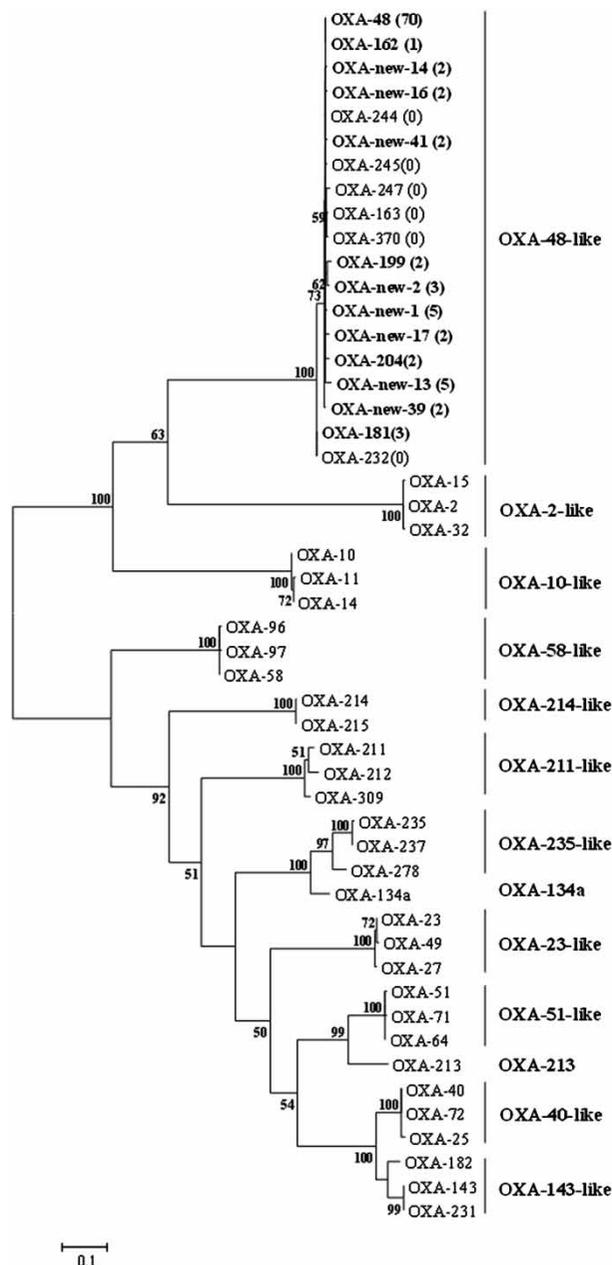
Figure 1 | Maximum-likelihood tree based on deduced amino acid sequences of representatives of OXA beta-lactamases families (OXA-2-, OXA-10-, OXA-23-, OXA-40-, OXA-48-, OXA-51-, OXA-58-, OXA-134a-, OXA-143-, OXA-211-, OXA-213-, OXA-214-, and OXA-235-like) and OXA-48-like sequences identified in 2 or more clones retrieved from gene libraries constructed in this study. Numbers in parentheses indicate the number of times that the sequence was found in the libraries. The branch number refers to the percent confidences as estimated by a bootstrap analysis with 1,000 replications.

compartments worldwide. As the putative origin of this gene is attributed to *Shewanella* spp., commonly found in aquatic environments, this latter hypothesis seems plausible. The

origin of several clinically relevant genetic determinants of resistance has been linked to environmental bacteria (Poirel *et al.* 2002, 2004). A widely referred example is bla_{CTX-M} genes in environmental *Kluyvera* spp. (Poirel *et al.* 2002). Moreover, it has been shown that currently known genetic determinants of resistance encoded other functions in the cell (including antibiotic biosynthesis), which later became useful for dealing with antibiotics (Baquero *et al.* 2009). Also, many environmental microorganisms are antibiotic producers. Therefore, is not surprising that neighboring microorganisms and the antibiotic producer itself have developed mechanisms to resist the drug's action (Baquero *et al.* 2009; D'Costa *et al.* 2011).

Although variants here identified may have their origin in *Shewanella* spp. genomes, it should also be taken into account that there may be other hosts not yet identified or even enterobacteria carrying these genes. Since the aquatic systems explored in this study are highly impacted by anthropogenic pollution, and thus subjected to continuous discharges from agricultural, domestic and industrial origins, a high proportion and diversity of Enterobacteriaceae members is expected in these water environments. Future studies should include pristine environments to test all the hypotheses stated. Most probably, diverse mobilization events have mediated the transfer of $bla_{OXA-48-like}$ genes from *Shewanella* spp. to Enterobacteriaceae or other still unidentified hosts which reinforces the importance of these environmental compartments (i.e. aquatic systems where indigenous bacteria such as *Shewanella* mix with animal-derived bacteria such as Enterobacteriaceae) in the evolution and spread of antibiotic resistance. In fact, there are already reports of Enterobacteriaceae members isolated from river (Potron *et al.* 2011) and wastewaters (Galler *et al.* 2014) carrying bla_{OXA-48} genes. However, so far, there is only one report on $bla_{OXA-48-like}$ producers identified in Portuguese clinical settings (Manageiro *et al.* 2014). This might be related to the national carbapenems prescription policies and awareness campaigns (Henriques *et al.* 2012). In a worst-case scenario, in the particular case of bla_{OXA-48} genes, what might be happening is a silent spread, i.e. one that is undetectable due to the low-level resistance to carbapenems.

By applying environmental DNA-based methodologies, both culturable and unculturable fractions are covered. However, it is important to acknowledge that by using
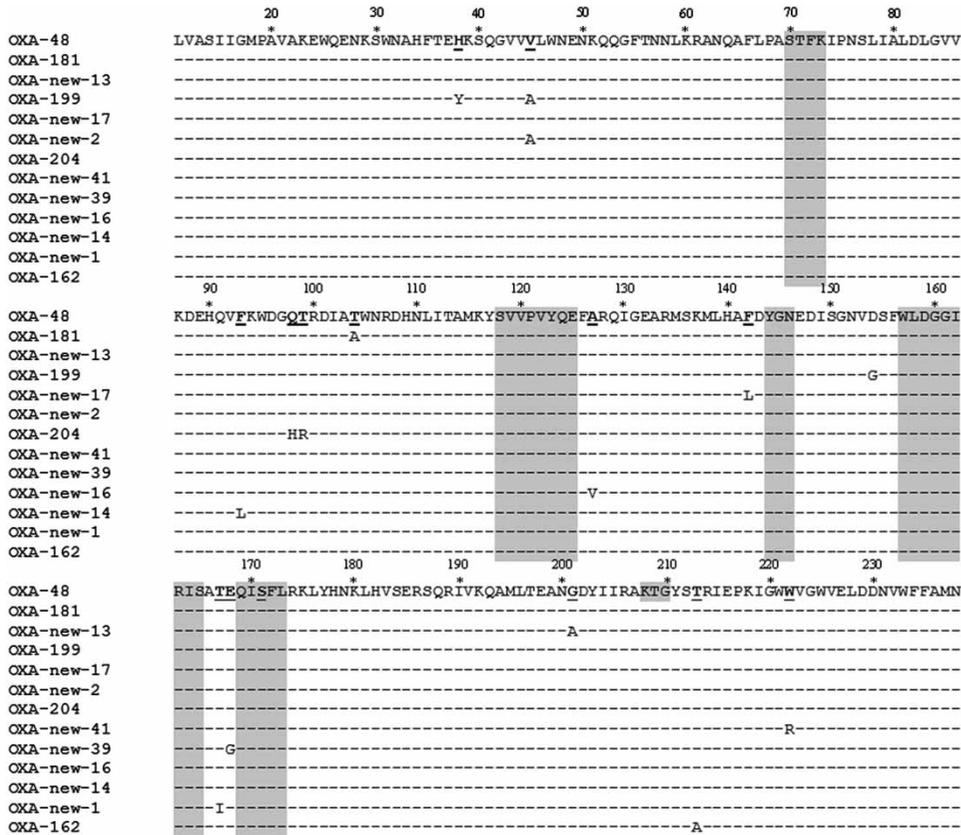
Figure 2 | Deduced amino acid sequence alignment of OXA-48 and the other more abundant variants found (identified in 2 or more clones). Dashes indicate identical residues among all the amino acid sequences. Boxes in grey indicate amino acid motifs that are conserved among class D beta-lactamases. Numbering is according to the class D beta-lactamase (DBL) system (Couture *et al.* 1992).

PCR-based methodologies, the diversity found is biased by the primer sets used, which were designed based on previous described sequences. Nevertheless, this molecular approach added relevant information to the current knowledge on the diversity of OXA-48-like carbapenemases. However, the DNA sequences identified may not represent functional beta-lactamase genes or that code for active beta-lactamases.

The observation of such a diverse $bla_{OXA-48-like}$ gene pool in these aquatic systems indicates the need for further research along at least three lines: identification of the host species, assessment of expression and activity of the gene products, and evaluation of the capability of dissemination among strains of the variants here reported. Even so, these observations may represent a forewarning of $bla_{OXA-48-like}$ genes dissemination.

CONCLUSIONS

The diversity of OXA-48-like sequences identified by culture-independent methods suggests that the environment and, in particular, aquatic systems constitute important reservoirs of these genes. Also from this study resulted a list of diverse variants of OXA-48 genes that should be taken into account when designing molecular strategies for detecting this gene.

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