Short Communication

Extended-spectrum β-lactamase and carbapenemase-producing *Aeromonas* species in wild animals from Portugal

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AEROMONAS are Gram-negative, facultative-anaerobic, non-spore-forming, glucose-fermenting, oxidase- and catalase-positive rods (Martin-Carnahan and Joseph 2005). Apart from fish, which are widely reported hosts for Aeromonads, insects, crustaceans, reptiles, birds and mammals were also found to harbour *Aeromonas* species, both in healthy and disease state (Pearson and others 2000, Turutoglu and others 2005, Evangelista-Barreto and others 2006, Ceylan and others 2009). An increase in resistance levels of the genus, particularly to β-lactam antimicrobial agents, has been observed not only in clinical isolates, but also in environmental strains (Saavedra and others 2004, 2007).

The most common mechanism of antibacterial resistance is the production of three chromosomally encoded β-lactamases, which have been described and identified in different *Aeromonas* (Janda and Abbott 2010). They may or may not concomitantly occur in the same strain, and their coordinated expression is induced by the presence of β-lactam antibiotics (Walsh and others 1997, Avison and others 2004). These enzymes comprise cephalosporinases (Ambler's class C), penicillinases/oxacillinases (class D) such as OXA-type enzymes and metallo-β-lactamases (class B) (Hayes and others 1994). The most common metallo-β-lactamases produced by this genus are of the 'CphA'-type, whose sequences appear to be widely distributed in *Aeromonas hydrophila* an *Aeromonas veronii* strains (Walsh and others 1997). Recently, two other metallo-β-lactamases, VIM and IMP, have

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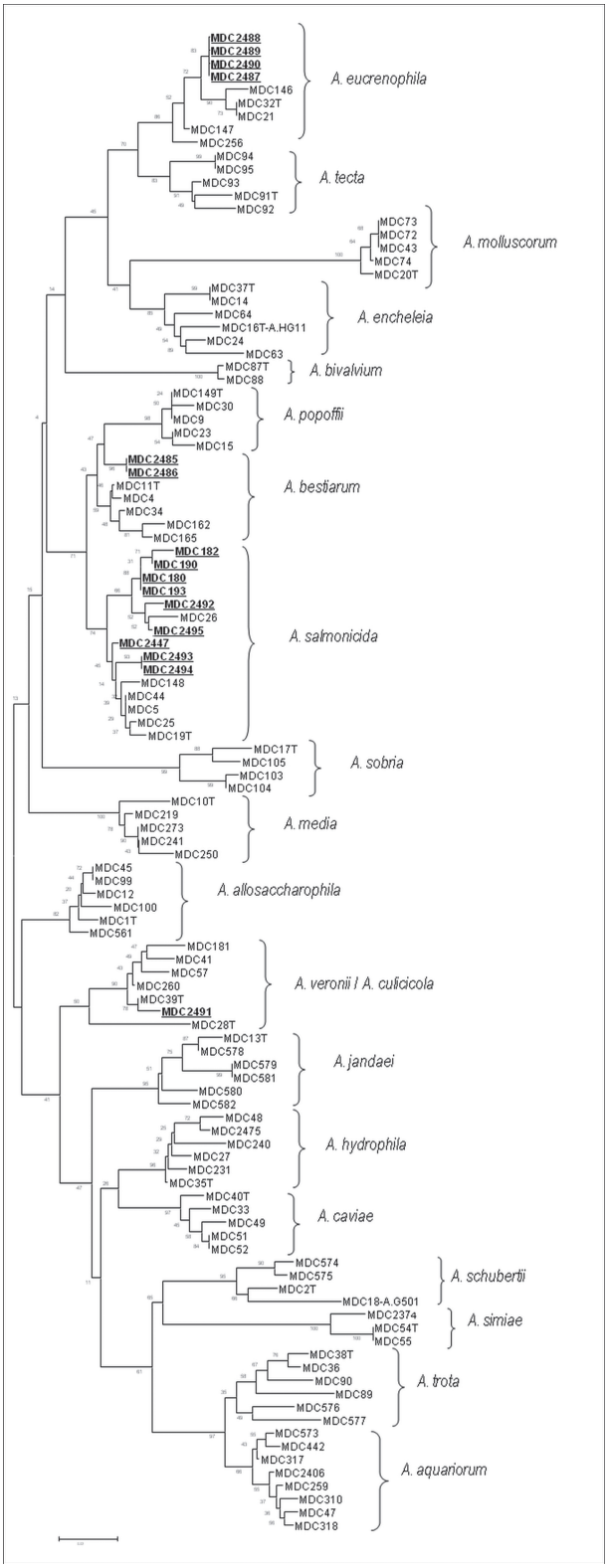

FIG 1: Unrooted phylogenetic tree based on gyrB gene sequences of strains isolated in this study, and the representative strains of the genus, Aeromonas, of the MDC collection. The isolated included in the study are present in bold-face

TABLE 1: Characterisation of strains isolated as concerns resistance phenotypes and *bla* genotypes

Animal species name	Strains	Identification (*gyrB* gene sequencing)	Resistance phenotype	β-lactamase genes content				
				$bla_{OXA-aer}$	bla_{CTX-M}	bla_{FOX}	bla_{MOX}	bla_{cphA}
Cervus elaphus	MDC 180	*Aeromonas salmonicida*	AML, AMC, TIC, TIM, KF, S, E, TE	–	–	–	–	–
C elaphus	MDC 182	*A salmonicida*	KF, S, E	–	–	–	–	–
C elaphus	MDC 190	*A salmonicida*	AML, AMC, TIC, TIM, KF, S, E, TE	–	–	–	–	+
C elaphus	MDC 193	*A salmonicida*	AML, AMC, TIC, TIM, KF, S, E, TE	–	–	+	–	+
Strix aluco	MDC 2447	*A salmonicida*	AML, AMC, TIM, PRL, TZP, KF, E, TE	–	–	–	+	+
C elaphus	MDC 2492	*A salmonicida*	AML, KF, E	+	–	–	–	+
C elaphus	MDC 2493	*A salmonicida*	AML, TIC, KF, S	+	–	+	–	–
C elaphus	MDC 2494	*A salmonicida*	AML, TIC, KF, S	+	–	+	–	–
C elaphus	MDC 2495	*A salmonicida*	AML, KF	+	–	+	–	–
Sciurus vulgaris	MDC 2485	*Aeromonas bestiarum*	AML, E	+	–	–	–	+
S vulgaris	MDC 2486	*A bestiarum*	AML, E	+	–	–	–	+
Colubridae	MDC 2487	*Aeromonas eucrenophila*	AML, KF, E	+	–	+	–	–
Colubridae	MDC 2488	*A eucrenophila*	AML, KF, E	+	–	+	–	–
Colubridae	MDC 2489	*A eucrenophila*	AML, KF, E	+	–	+	–	–
Colubridae	MDC 2490	*A eucrenophila*	AML, KF, E	+	–	+	–	–
Circaetus gallicus	MDC 2491	*Aeromonas veronii*	AML, TIC, TIM, NA, S, E, TE	+	+	+	+	+

been identified in *A hydrophila* encoded on an integron and a plasmid, respectively (Neuwirth and others 2007, Libisch and others 2008).

This study reports the identification of *Aeromonas* species from wild animals, the antibiotic resistance found in the strains isolated and the association of resistance with the presence of bla_{CphA}, bla_{IMP}, bla_{VIM}, bla_{OXA}, bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{MOX} and bla_{FOX} genes in the strains of *Aeromonas* species.

A total of 140 fecal samples from different wild animal species (birds, reptiles and mammals), were collected aseptically, directly from the animal rectum or from freshly voided faecal material. Collection was done as soon as the animal entered the Centre for Treatment of Wild Animals (University of Trás-os-Montes and Alto Douro). Samples were enriched in 5 ml of Brain Heart Infusion Broth (Oxoid), for 24 hours at 30°C. Direct streaking of faecal swabs on glutamate starch phenol-red agar medium (Merck) was used to select, isolate and purify bacterial isolates, by incubating plates aerobically at 30°C for 24 hours. All colonies that were morphologically suspected as *Aeromonas* species (yellow, smooth and round) were then selected to establish pure cultures.

Total genomic DNA was extracted from overnight pure colonies as previously described (Soler and others 2004). The presence of genes encoding TEM, SHV, CTX-M, MOX, FOX, CphA, VIM, OXA-Aer, OXA-B, OXA-C and IMP β-lactamases was analysed by PCR. The oligonucleotides were designed by Fontes (2009) within her PhD thesis (in publication).

Sequencing of 16S rRNA and gyrB genes was performed as previously described (Martínez-Murcia and others 1999, Soler and others 2004).

Susceptibility of *Aeromonas* isolates was determined by the disk method (Bauer and others 1966) according to the Clinical and Laboratory Standards Institute (2006). The following antibiotic-containing discs were obtained from Oxoid: amoxicillin, amoxicillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cephalothin, cefoxitin, ceftriaxone, cefoperazone, ceftazidime, cefotaxime, cefepime, imipenem, aztreonam, streptomycin, kanamycin, amikacin, gentamicin, tobramycin, nalidixic acid, ciprofloxacin, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, fosfomycin.

A total of 325 different Gram-negative isolates were obtained from the 140 faecal samples, with 8 samples positive for the presence of *Aeromonas* species (4 from *Cervus elaphus* (red deer), 1 of a *Strix aluco* (tawny owl), 1 of a *Sciurus vulgaris* (red squirrel), 1 of a *Colubridae* (snake) and 1 of a *Circaetus gallicus* (short-toed snake eagle)).

Ten different *gyrB* gene partial sequences were obtained that grouped *Aeromonas* into four different species: *Aeromonas salmonicida* (n=9), *Aeromonas eucrenophila* (n=4), *Aeromonas bestiarum* (n=2) and *A veronii* (n=1). The unrooted phylogenetic tree was constructed by using the *gyrB* gene partial sequence of each isolate and previously published reference sequences (Martínez-Murcia and others 1992, Yáñez and others 2003, Saavedra and others 2006) from the Molecular Diagnostics Center (Biomolecular Technologies S.L.U., Spain) culture collection, including type strains (Fig 1).

Resistance to at least two antibiotic was recorded for all isolates, and almost half (43.8 per cent) were found to be multiresistant (Table 1). Our results showing 93.7 per cent of isolates resistant to amoxicillin, 81.25 per cent to cephalothin and 37.5 per cent to ticarcillin are in agreement with this report. The combination of an aminopenicillin and a carboxipenicillin with a β-lactamase inhibitor (clavulanic acid), was effective in reducing resistance, as shown by the decrease in the proportion of resistant strains. This reduction was more pronounced with amoxicillin (93.7 per cent v 25 per cent) than with ticarcillin (37.5 per cent v 31 per cent). No resistance was found to the following antimicrobials: aztreonam, imipenem and cephalosporins.

The β-lactamases genes were found in different combinations, and in one case, five β-lactamase genes were detected in the same isolate, *A veronii* MDC2491. The most prevalent genes were bla_{OXAaer} present in 11 isolates (69 per cent) followed by bla_{FOX} in nine isolates (56 per cent) and the metallo-β-lactamase encoding gene bla_{cphA}, in seven isolates (44 per cent). The gene bla_{MOX} was detected in two isolates (*A salmonicida, A veronii*) and bla_{CTX-M} in one *A salmonicida* isolate. No PCR specific for bla_{TEM}, bla_{SHV}, bla_{OXA-B}, bla_{OXA-C}, bla_{IMP} and bla_{VIM} encoding sequences were detected, and yielded no evidence for the presence of these genes in any isolate.

The housekeeping gene *gyrB* has been demonstrated to be an excellent molecular chronometer for phylogenetic inference in the genus *Aeromonas* than the commonly used 16S rRNA gene sequencing (Janda and Abbott 2007), as shown by our results.

Although the majority of isolates were susceptible to the fluoroquinolones, confirming the usefulness of such antibiotics in the treatment of *Aeromonas* infections, one isolate from *C gallicus*, MDC2491, identified as *A veronii* was resistant to nalidixic acid. Isolates were susceptible to all other antimicrobials.

The complete genome sequencing of *A hydrophila* ATCC7699 and *A salmonicida* A449 revealed that both carry an array of β-lactamases genes to counteract antibacterial factors present in the environment, including several antibiotics used for human and animal clinical treatment (Seshadri and others 2006, Reith and others 2008). Inducible chromosomal β-lactamases is the resistance mechanism against β-lactam antibiotics for most Aeromonads, with examples described and identified in different species (Henriques and others 2006). Expression of metallo-β-lactamases active against carbapenems is also

a concern with regards to *Aeromonas* infections (Parker and Shaw 2011). This later, the 'CphA-type', is considered the most common metallo-β-lactamase produced by *Aeromonas* species (Janda and Abbott 2010), corroborating our results.

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