The involvement of *tetA and tetE* tetracycline resistance genes in plasmid and chromosomal resistance of *Aeromonas* in Brazilian strains

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This study analyzed the involvement of tetA *and* tetE *genes in the tetracycline resistance of 16 strains of genus* Aeromonas*, isolated from clinical and food sources. Polymerase chain reactions revealed that 37.5% of the samples were positive for* tetA*, and also 37.5% were* tetE *positive. One isolate was positive for both genes. Only the isolate* A. caviae *5.2 had its resistance associated to the presence of a plasmid, pSS2. The molecular characterization of pSS2 involved the construction of its restriction map and the determination of its size. The digestion of pSS2 with* Hind*III originated two fragments (A and B) that were cloned separately into the pUC18 vector. The tetA gene was shown to be located on the* Hind*III-A fragment by PCR. After transforming a tetracycline-sensitive strain with pSS2, the transformants expressed the resistance phenotype and harbored a plasmid whose size was identical to that of pSS2. The results confirmed the association between pSS2 and the tetracycline resistance phenotype, and suggest a feasible dissemination of* tetA *and* tetE *among strains of* Aeromonas*. This study suggests the spreading* tetA *and* tetE *genes in Aeromonas in Brazil and describes a resistance plasmid that probably contributes to the dissemination of the resistance.*

Key words: *Aeromonas* - plasmid - tetracycline - resistance determinants

The genus *Aeromonas* includes a group of bacteria present in several natural habitats, such as soil, fresh and brackish water, and sewage (Garibay et al. 2006). They also belong to the amphibiotic microbiota of fishes, reptiles and amphibians (Araujo et al. 1991, Son et al. 1997). This genus has already been isolated from many kinds of foods, like seafood, bovine and swine meat and derivatives, vegetable, salads, ice-cream, milk and cheese (Araújo et al. 2002, Martins et al. 2002, Evangelista-Barreto et al. 2006, Palú et al. 2006).

It has been suggested that the high prevalence of *Aeromonas* species in the environment should be considered a threat to public health, since infections caused by these pathogens are generally the result of ingestion of contaminated water or food (Chopra & Houston 1999, Alavandi & Ananthan 2003, Garibay et al. 2006). For that reason, some *Aeromonas* species have been classified as emerging pathogens (Szabo et al. 2000, Edberg et al. 2007).

In humans, the pathogenic species usually involved in cases of gastroenteritis are *A. hydrophila, A. caviae* and *A. veronii* (Edberg et al. 2007). Generally, the infections are self-limiting, and the antibiotic therapy is used only when the infections become severe.

Although the practice of feeding antibiotics to animals as growth enhancers is still an usual practice in some countries like United States, it has been phased out in the European Union and has been desestimulated (Gilchrist et al. 2007). Indeed, for several decades, tetracycline has been widely used in clinical medicine, veterinary and agriculture (Gilchrist et al. 2007), contributing to higher levels of microbial resistance, especially among the genus *Aeromonas* (Goñi-Urriza et al. 2000).

The resistance to tetracyclines occurs through the presence of *tet* genes in the bacterial DNA. The characterized *tet* genes encode three mechanisms of resistance: efflux pump, ribosomal protection or enzymatic inactivation (Chopra & Roberts 2001).

Many authors have described the presence of tetracycline resistance plasmids in *Aeromonas* species (Rhodes et al. 2000; Schmidt et al. 2001a; L'Abée-Lund & Sorum 2002). Genes of the *tet* family are often associated with conjugative and mobilizable elements, such as plasmids, transposons and integrons (Chopra & Roberts 2001, Schmidt et al. 2001b). This characteristic could explain their broad distribution among different bacterial species. The molecular characterization of the *tet* genes and plasmids that carry these resistance determinants may help to explain how human and the environment have been affected by the increase and indiscriminate use of tetracycline. The tetracycline resistance determinants most commonly observed in *Aeromonas* species are *tet*A and *tet*E (Schmidt et al. 2001b, Nawaz et al. 2006). For that reason, those genes were chosen for the amplification reactions performed in this work. Both *tet*A and *tet*E genes code for an efflux pump that removes the drug from the cell (Chopra & Roberts 2001).

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Therefore, the aim of this study was to characterize, at a molecular level, a tetracycline resistance plasmid found in a strain of *A. caviae* (Ac) isolated from lettuce and to analyze the dissemination of the *tet*A and *tet*E genes among strains of some *Aeromonas* species isolated in Brazil.

MATERIALS AND METHODS

Bacterial strains and plasmids - 16 isolates of tetracycline-resistance *Aeromonas* strains, with minimal inhibitory concentration (MIC) values of 16 mg ml^{-1} (Palú et al. 2006), listed in Table, were analyzed for the resistance determinants and the presence of plasmids. All strains were grown in LB-agar (Difco, Brazil) according to Schleif and Wesink (1981) containing the selective antibiotic (tetracycline or ampicilin), when necessary. For long-term storage, the bacterial strains were kept at -70ºC in LB medium containing 14% (v/v) glycerol.

Detection of tetracycline resistance determinants - The tetracycline-resistance *Aeromonas* spp. strains were examined for the presence of resistance determinants by polymerase chain reaction (PCR), using specific primers for *tet*A (Guardabassi et al. 2000) and *tet*E (Tovar et al. 1988) genes. Crude lysates were obtained by centrifugating 1 ml of the bacterial growth at 10,000 *g* for 4 min. The pellet was resuspended in 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8), centrifuged and washed twice, in the same conditions described above. The pellet was resuspended in 100 ml of TE. The suspension was heated for 10 min at 100ºC and, after a centrifugation at 10,000 *g* for 1 min, the supernatant was recovered and used as template for PCR. The purified plasmid and the recombinant plasmids obtained in the cloning experiments were also used as DNA templates for PCR. The PCR assay was carried out according to the method described by Guardabassi et al. (2000). *Acinetobacter* sp. RUH 2158 H5, MIC value ≥ 16 mg ml^{-1} (Guardabassi et al. 2000), and *Escherichia coli* strain D22-14, MIC value ≥ 10 mg ml^{-1} (Marshall et al. 1986), were used as positive control in reactions using *tet*A and *tet*E primers. The *Aeromonas* strains *A. caviae* 32 and *A. hydrophila* (Ah) 21 (from our collection), both tetracycline-sensitive, were used as negative controls in both PCR reactions. The reaction mix without DNA was also submitted to the amplification reactions. All PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gels in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.9) buffer. The 100-bp DNA ladder (Invitrogen, Brazil) was used as a size marker.

Plasmid DNA isolation - All tetracycline-resistant *Aeromonas* spp., the *E. coli* host strain of pUC18 and transformants were screened for their plasmid content using the procedure described by Birnboim and Doly (1979), and whole cell lysate method (Sambrook et al.1989). DNA samples were stored at -20ºC until the moment of use. The plasmid DNAs were separated by electrophoresis on 0.8% (w/v) agarose gels (Sambrook et al. 1989) in TAE buffer.

Transformation - Plasmid pSS2 was used to transform the tetracycline-sensitive strain *A. hydrophila* (Ah) ATCC 5677. The plasmid pSS2 was purified with the Wizard Plus™ SV Miniprep kit (Promega, Brazil), and used to transform A. *hydrophila* ATCC 5677 competent cells by heat-shock (Sambrook et al. 1989). Transformants were selected by growth on LB agar with 10 mg ml^{-1} of tetracycline, after overnight incubation at 37ºC. Transformant colonies were screened for their plasmid content, as described above.

Restriction endonuclease analysis - Plasmid pSS2 was extracted from strain *A. caviae* 5.2 (Ac 5.2) using Wizard Plus™ SV Miniprep kit, and digested with restriction enzymes (Invitrogen) in the conditions recommended by the manufacturer. The enzymes used were *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xho*I. The restriction fragments obtained in simple and double digestions were separated by electrophoresis in 0.8% (w/v) agarose gels in TAE buffer, or in 5% (w/v) poliacrylamide gels in TBE (Tris 89 mM, boric acid 89 mM, EDTA 2.5 mM, pH 8.2) buffer. The 1-kb DNA ladder (Invitrogen) was used as a size marker in agarose gels, while the 100-bp DNA ladder (Invitrogen) was used in polyacrylamide gels.

*Cloning of the Hin*dIII *fragments of pSS2* - Both pSS2 and pUC18 (Yanisch-Pérron et al. 1985) were isolated using Wizard™ Plus Minipreps DNA Purification System (Promega), and digested with 10 U/ml of *Hin*dIII (Invitrogen), following the recommendations of the manufacturer. Both A and B fragments, generated by the *Hin*dIII digestion of pSS2, were individually cloned into

TABLE

Tetracycline-resistant *Aeromonas* strains used in this study

Strain	Source/ year of isolation	Presence of *tet*A	Presence of *tet*E	Plasmid
Ac 5.2	Lettuce[a]/ 2002	+	-	pSS2
Ac 6.2	Lettuce[a]/ 2002	+	-	-
Ah 6.4	Lettuce[a]/ 2002	-	+	-
Ah 7.2	Lettuce[a]/ 2002	-	+	-
Ah 16	Lettuce[a]/ 2002	-	-	-
Ac 43	Cheese[a]/ 1999	+	-	-
Ac 36	Cheese[a]/ 1999	-	+	-
Ac 37	Cheese[a]/ 1999	-	+	-
Ac 38	Cheese[a]/ 1999	-	+	-
Ac C8	Faeces[b]/ 1994	+	-	-
Ac C50	Faeces[b]/ 1994	-	+	-
Ac 314	Faeces[b]/ 1994	-	-	-
Ah 8	Faeces[b]/ 1994	-	-	-
Ah 149	Faeces[b]/ 1994	+	+	-
Ac 95	secretion of respiratory tract[b]/ 2002	+	-	-
Ac 26	secretion of respiratory tract[b]/ 2002	+	-	-

a: food samples were isolated from lettuce (Palú et al. 2006) and cheese (Araujo et al. 2002)*; b*: clinical samples were obtained from patients of Hospital Universitário Pedro Ernesto, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil. Ac: *A. caviae*; Ah: *A. hydrophila*

pUC18 by standard techniques (Sambrook et al. 1989). The recombinant plasmids were used to transform *E. coli* DH5-a competent cells by heat-shock (Sambrook et al. 1989). Transformants were selected on LB agar with 75 mg/ml^{-1} ampicilin, 40 mg/ml^{-1} IPTG and 75 mg/ml^{-1} X-Gal, at 37ºC, after overnight incubation. Transformants were screened for their plasmid content as described above.

RESULTS

Detection of tetracycline-resistance determinants - Amplification only of *tet*A gene was observed in 37.5% (6/16) of the analyzed *Aeromonas* samples (Table, Fig. 1). Three of these strains were isolated from food, while three of them were isolated from clinical sources.

Concerning the amplification of *tet*E gene, it was also observed that 37.5% (6/16) of the samples were only positive for this resistance determinant (Table, Fig. 2). Five of them were isolated from food, while only one was isolated from a clinical source. It was also noticed that the strain Ah 149, isolated from a clinical source, was positive for the amplification of both *tet*A and *tet*E genes (Figs 1, 2).The strains Ac314, Ah8 (both isolated from faeces), and Ah16 (isolated from lettuce) were negative for the amplification of both genes. The resistance gene determinant of these strains was not determined in this work.

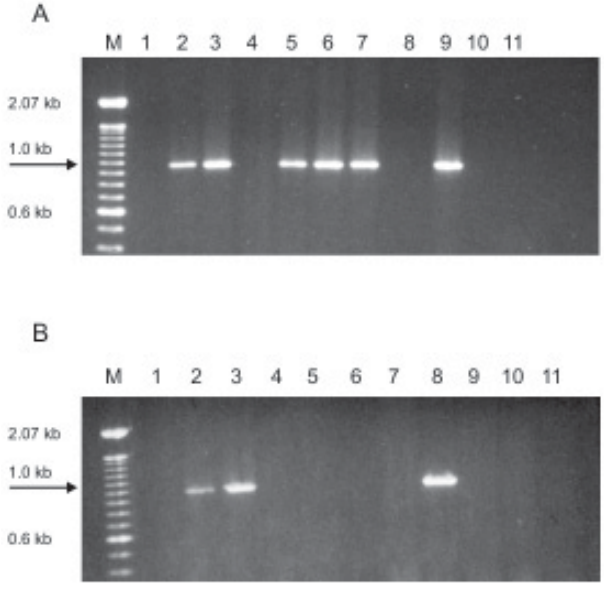

Fig. 1: agarose gel electrophoresis of PCR products generated by amplification of the *tet*A resistance determinant in tetracycline-resistant *Aeromonas* strains. Lane - M: 100-bp DNA ladder size marker; 1: negative reaction control (reaction mix without DNA); 2: *Acinetobacter* strain RUH 2158 H5, used as a positive reaction control. A: Lane - 3: Ac 5.2 (pSS2); 4: Ah 6.4; 5: Ah 149; 6: Ac 26; 7: Ac 6.2; 8: Ac C50; 9: Ac C8; 10: Ah 8; 11: Ah 7.2. B: Lane - 3: Ac 95; 4: Ac 36; 5: Ac 37; 6: Ac 38; 7: Ah 16; 8: Ac 43; 9: Ac 314; 10: Ac 21 (Tc^S); 11: Ah 32 (Tc^S). The arrows indicate the expected size of the amplification product of the *tet*A gene (1 kb). Ac: *A. caviae*; Ah: *A. hydrophila*.

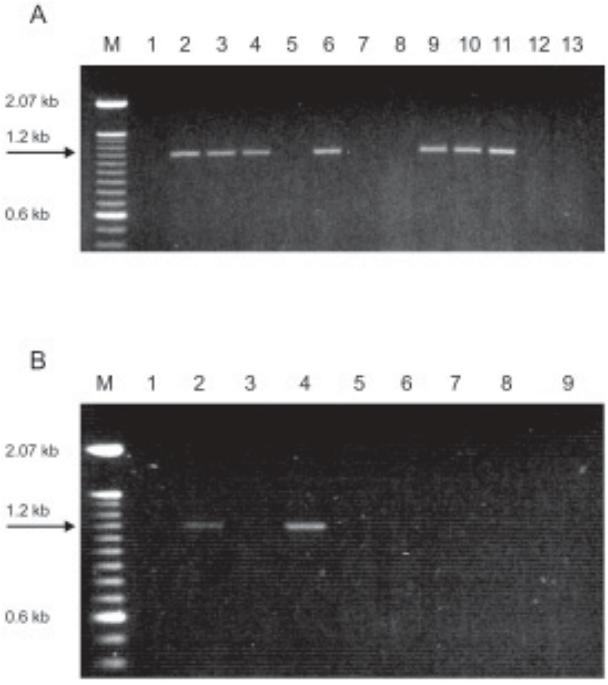

Fig. 2: agarose gel electrophoresis of PCR products generated by amplification of the *tet*E resistance determinant in tetracycline-resistant *Aeromonas* strains. Lane - M: 100-bp DNA ladder size marker; 1: negative reaction control (reaction mix without DNA); 2: *E. coli* strain D22-14, used as a positive reaction control. A: Lane - 3: Ac C50; 4: Ah 6.4; 5: Ac 314; 6: Ah 7.2; 7: Ah 16; 8: Ah 8; 9: Ac 36; 10: Ac 37; 11: Ac38; 12: Ac 21 (Tc^S); 13: Ah 32 (Tc^S). B: Lane - 3: Ac 5.2 (pSS2); 4: Ah 149; 5: Ac 26; 6: Ac 6.2; 7: Ac C8; 8: Ac 95; 9: Ac 43. The arrows indicate the expected size of the amplification product of the *tet*E gene (1.2 kb). Ac: *A. caviae*; Ah: *A. hydrophila*.

Plasmidial involvement in tetracycline resistance - The analysis of the plasmid content demonstrated that only the strain Ac 5.2 out of the 16 tetracycline-resistant *Aeromonas* spp. strains isolated from lettuce presented a plasmid, whose size was estimated to be 15 kb (data not shown). This plasmid was named pSS2. Ac 5.2 is one of the strains positive for *tet*A amplification. This amplification also occurred when the purified pSS2 was used as template.

Plasmid pSS2 was transferred to *A. hydrophila* ATCC 5677 strain. This strain was tetracycicline-sensitive and, after transformation, it started to exhibit the resistance phenotype. When the selected transformant colonies were submitted to plasmid analysis, it was observed that all contained a plasmid whose size was identical to that of pSS2 (data not shown). The transferred plasmid appeared to be stable in the host strain after growth for some generations in non selective media.

Molecular characterization of pSS2 - According to the lengths of the fragments generated upon the digestion of pSS2 using different restriction enzymes, the size of the plasmid pSS2 was estimated to be 15.4 kb and its restriction map was created (Fig. 3).

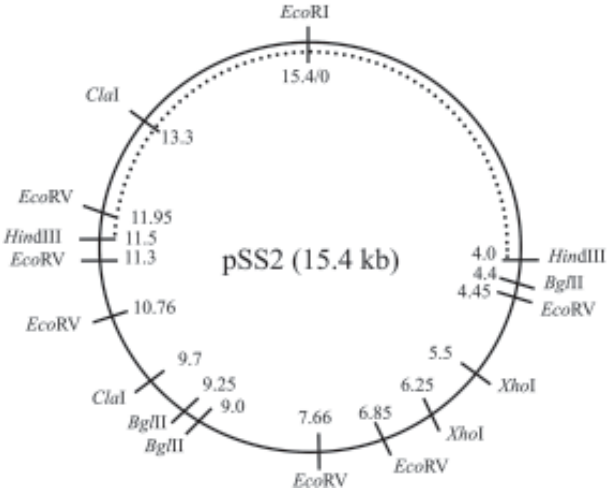

Fig. 3: restriction map of the tetracycline-resistance plasmid pSS2, isolated from the food sample *Aeromonas caviae* 5.2. According to the lengths of the fragments generated upon digestion of pSS2 using different restriction enzymes, the size of the plasmid pSS2 was estimated to be 15.4 kb. The dashed line indicates the *Hin*dIII fragment in which the *tet*A gene is located.

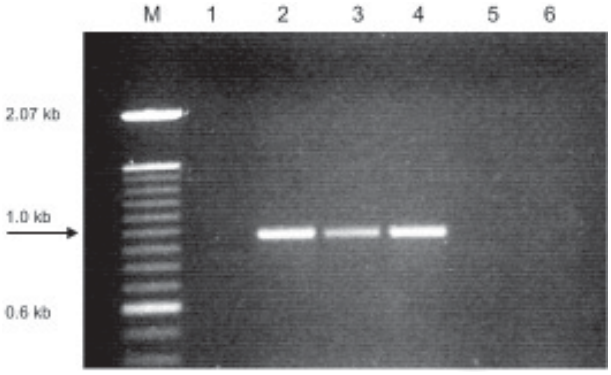

Fig. 4: agarose gel electrophoresis of PCR products generated by amplification of the *tet*A resistance determinant. Lane - M: 100-bp DNA ladder size marker; 1: negative reaction control (reaction mix without DNA); 2: *Acinetobacter* strain RUH 2158 H5, used as a positive reaction control; 3: pSS2; 4: recombinant plasmid containing the *Hin*dIII-A fragment of pSS2 (7.9 kb); 5: recombinant plasmid containing the *Hin*dIII-B fragment of pSS2 (7.5 kb); 6: pUC18. The arrow indicates the expected size of the amplification product of the *tet*A gene (1 kb).

The digestion of pSS2 with *Hin*dIII generated two DNA fragments of different sizes (A and B), which were individually cloned in the multiple cloning site of pUC18. The cloning of the expected fragments was confirmed by digesting the recombinant plasmids with *Hin*dIII. To locate the *tet*A gene in pSS2, recombinant plasmids carrying either the *Hin*dIII-A or B fragments were used as templates in PCR reactions for *tet*A. Only the recombinant plasmid containing the *Hin*dIII-A fragment of pSS2 (7.9 kb) showed a positive result for the amplification of *tet*A gene (Fig. 4). Amplification was also obtained when intact pSS2 was used as DNA template. Both pUC18 and the recombinant plasmid carrying the *Hin*dIII-B fragment of pSS2 did not exhibit amplification of the tetracycline-resistance determinant.

DISCUSSION

In the last few decades, an amazing scientific interest in the genus *Aeromonas* has been observed, since this bacterial group became an emergent human pathogen (Edberg et al. 2007). One of the reasons that might have contributed to that emergence was the high levels of antimicrobial resistance that this pathogen presents. Among all antimicrobial drugs used in the treatment of the *Aeromonas*-related diseases, tetracycline is one of the most important, since it has been widely used in medicine, agriculture and veterinary (Chopra & Roberts 2001, Gilchrist et al. 2007). Consequently, even at a subtherapeutical level, tetracycline exerts a selective pressure for resistant bacteria.

There are some reports showing that the tetracycline-resistance determinants most predominant in *Aeromonas* species are *tet*A and *tet*E (Schmidt et al. 2001b, Nawaz et al. 2006). Our work is in agreement with the literature. It has also been shown that *tet*A and *tet*E genes code for an efflux pump that pumps the drug out of the cell (Chopra & Roberts 2001).

The *tet*E gene is often associated to large plasmids that are neither mobile nor conjugative (DePaola & Roberts 1995). The association of this gene with the chromosome was also observed (DePaola & Roberts 1995, Roberts 1996), which might explain its limited distribution among environmental samples, being more prevalent in polluted marine sediments (Andersen & Sandaa 1994, Chopra & Roberts 2001). Nawaz et al. (2006), while describing the *tet*E gene, observed the high frequency of this determinant in *Aeromonas* spp. isolates.

It was observed that 37.5% (6/16) of the tetracycline-resistant *Aeromonas* spp. strains analyzed were positive only for the amplification of *tet*E gene. Five of those samples were isolated from food and only one from a clinical source. None of the *tet*E positive strains showed the presence of plasmids, suggesting the association of this resistance determinant to the chromosome. Three strains (18.8%) were negative for the amplification of both *tet*A and *tet*E genes. Andersen and Sandaa (1994) reported similar results, where no *tet* determinant was detected in 17% of their isolates.

The strain Ah 149, isolated from a child with diarrheic faeces in a Brazilian hospital, showed positive amplification for both *tet*A and *tet*E genes. Despite the fact that most of the Gram-negative isolates carry only one kind of *tet* gene (Chopra & Roberts 2001), some authors have also observed the presence of both *tet*A and *tet*E determinants in some strains, but always in a very low frequency (DePaola et al. 1988, Andersen & Sandaa 1994, Schmidt et al. 2001a). Although no significant difference in the MIC of strains having one and both *tet* genes has been observed (Palú et al. 2006), it remains

unknown whether the presence of two *tet* determinants in the same cell increases the resistance, since both genes code for efflux pumps.

Our results also showed that 37.5% (6/16) of the tested strains exhibited a positive amplification for only *tet*A gene. It was not possible to establish a correlation between the sources of these strains and the presence of this gene, as three of these strains were isolated from food (cheese and lettuce), and three of them were isolated from clinical sources.

Among the strains that were positive for the *tet*A amplification, Ac 5.2 (isolated from lettuce) was the only that harbored a plasmid, which was previously associated to the tetracycline resistance phenotype by curing and transformation experiments (Palú et al 2006). This plasmid was named pSS2. The preliminary molecular characterization of pSS2 was performed by constructing its restriction map. Based on this map, it was possible to estimate its size in 15.4 kb.

The amplification of *tet*A gene was also observed when the purified plasmid was used as DNA template in PCR, which strongly indicates the correlation between the presence of pSS2 and the tetracycline-resistance of the strain *A. caviae* 5.2.

The presence of *tet*A resistance determinant in plasmids isolated from *Aeromonas* spp. strains has already been reported (Rhodes et al. 2000, Schmidt et al. 2001a, b). It was observed that *tet*A is generally associated with conjugative plasmids (Chopra & Roberts 2001). Schmidt et al. (2001a) observed a positive correlation between tetracycline-resistant *Aeromonas* spp. harboring large conjugative plasmids and the presence of *tet*A. Our study also observed a positive correlation between pSS2 and *tet*A. However, contrary to Schmidt's findings, pSS2 is probably non-conjugative due to its size (15.4 kb), which is too small to encode for a conjugative apparatus. However, we cannot discard the possibility that pSS2 might be a mobilizable plasmid.

The relationship between the tetracycline resistance of Ac 5.2 strain and the presence of pSS2 was reinforced by the transformation of the plasmid into the tetracycline-sensitive strain *A. hydrophila* ATCC 5677, which started to exhibit a stable resistance phenotype after the transformation experiment. This result has been previously obtained (Palú et al. 2006), when the tetracycline sensitive strain *A. hydrophila* ATCC 7966 has been transformed with pSS2. Based on restriction map of pSS2, it was observed that this plasmid has two *Hin*dIII-restriction sites. Both *Hin*dIII-fragments were then cloned into the pUC18 vector. Recombinant plasmids were used as templates for the amplification reactions for *tet*A gene. It was possible to confirm that *tet*A gene is present on the pSS2 plasmid of the Ac 5.2 strain.

Few works have reported the molecular characterization of drug resistance plasmids in *Aeromonas* species. This study presents preliminary molecular data about the pSS2 tetracycline-resistance plasmid, allowing it to be compared with new plasmids described, and helping to understand its evolution and spread among the bacterial species, specially species of *Aeromonas* spp.

The present work shows the presence of tetracycline resistance determinants *tet*A and *tet*E in *Aeromonas* spp. strains from Brazil and describes a resistance plasmid that probably contributes to the dissemination of the resistance.

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