HEMOLYTIC ACTIVITY AND RESISTANCE TO ANTIMICROBIALS BY *Aeromonas* SPECIES ISOLATED FROM INTENSIVE REARING OF NILE TILAPIA (*Oreochromis niloticus*)

ATIVIDADE HEMOLÍTICA E RESISTÊNCIA A ANTIMICROBIANOS POR *Aeromonas spp.* ISOLADAS DE CRIAÇÃO INTENSIVA DE TILÁPIAS DO NILO (*Oreochromis niloticus*)

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SUMMARY

The objective of the work was to evaluate *Aeromonas* isolated from samples of Nile tilapia reared in net-cage and from the lake water. The samples were cultured in m-aeromonas and ADA selective media and the colonies underwent typical biochemical tests to identify the species. Isolates were evaluated for hemolysin production using blood agar and antimicrobial resistance test according to disk diffusion method. In water and fish body surface samples. *A. hydrophila* was the most occurring species, followed by *Aeromonas caviae* and *Aeromonas sobria.* While in the fish kidneys, the most occuring species were *A. hydrophila* and *A. veronii*. Of the studied cultures, 57% had hemolytic activity. The antimicrobial resistance test showed that all isolate by were resistant to amoxicillin and offered variable resistance to erythromycin. It is important to notice that the studied lake, in addition to being used for rearing fish, is also used for recreational purposes. Therefore, the presence of aeromonas may result in fish and human diseases; and special attention should be placed to the use of antimicrobial due to the high resistance displayed.

KEY-WORDS: *Aeromonas*. Microbiological analysis. Hemolysin. Pisciculture.

RESUMO

Este trabalho objetivou avaliar espécies de *Aeromonas* isoladas em amostras de tilápias do Nilo criadas em tanques-rede e da água do local de criação. As amostras foram plaqueadas em meios seletivos ADA e m-aeromonas e colônias típicas foram submetidas a testes bioquímicos para identificação da espécie. Isolados foram avaliados para produção de hemolisina utilizando ágar sangue e teste de resistência a antimicrobianos conforme o método de difusão de discos. Nas amostras de água e superfície dos peixes *A. hydrophila* foi a espécie mais isolada, seguida por *Aeromonas caviae* e *Aeromonas sobria* e no rim dos peixes as espécies isoladas foram *A. hydrophila* e *A. veronii*. Das culturas estudadas, 57% apresentaram atividade hemolítica. O teste com antimicrobianos revelou resistência de todos os isolados pela amoxicilina e resistência variável a eritromicina. É importante considerar que a lagoa estudada, além da criação de peixes e usada para fins recreativos; portanto a presença de aeromonas pode implicar em doenças nos peixes e em seres humanos e atenção deve ser dada ao uso de antimicrobianos, devido à porcentagem de resistência apresentada.

PALAVRAS-CHAVE: *Aeromonas.* Análise microbiológica. Hemolisina. Piscicultura.

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INTRODUCTION

In 2001, due to decreasing fish stocks during the 70 and 80's, the Espirito Santo state government established an intensive net-cage culture of Nile tilapia (*Oreochomis niloticus*) in the Juara lake to help the fisherman community to sell fish *in natura* (DUARTE, 2007).

Recent studies have shown that unregulated Aquaculture practices lead to environmental deterioration and are harmful to the fish (HIRSCH et al., 2006). Among the diseases that affect fish, bacterial hemorrhagic septicemia caused by *Aeromonas hydrophila, A. jandaei, A. bestiarum*, *A. salmonicida* and *A. veronii* bt. sobria is reported as a major health problem that can cause high mortality rate with significant economic losses (AUSTIN et al., 1998; KOZINSKA, 2007). The wide distribution of *Aeromonas* in water environments and its pathogenesis led all thirteen species already identified to be considered as a public health risk (TOKAJIAN & HASHWA, 2004). The contact with contaminated waters can cause both wound infections in humans (HIRANSUTHIKUL et al., 2005), as well as gastroenteritis if this contaminated water is used for drinking (GHENGHESH et al., 2001).

Several authors consider β-hemolytic production a virulence factor of the *Aeromonas* spp. (DI BARI et al., 2007). Studies have associated *Aeromonas* hemolysin production to gastrointestinal infections (GONZÁLEZ-SERRANO et al., 2002) and infections of freshwater fish (KOZINSKA, 2007).

The universal and often indiscriminate use of antibiotics in human and animal medicine, including Aquaculture, has brought serious consequences, such as the emergence of resistant strains of aeromonas (ALVARÉZ et al., 2004).

Due to lack of scientific information about the species of aeromonas isolated from the Juara lake, in Espirito Santo, this study evaluated water and fish sample isolates to determine strain, hemolytic activity and antimicrobial resistance of aeromonas present in the lake water where aquaculture and recreational activities take place.

MATERIAL AND METHODS

Study site

The study was performed in a lake of approximately 2.8 km^2 and 16 km extension, located (20º 06' E, 40º 14' N) 5 km from the Atlantic Ocean, in Espirito Santo state, southeastern Brazil (Figure 1). The culture that rears Nile tilapia (*Oreochomis niloticus*), consists of 150 4m^3-net-cages populated with an average density of 125 fish per m^3, distributed into 5 lines. The cages are located in an area with average depth of 3 m.

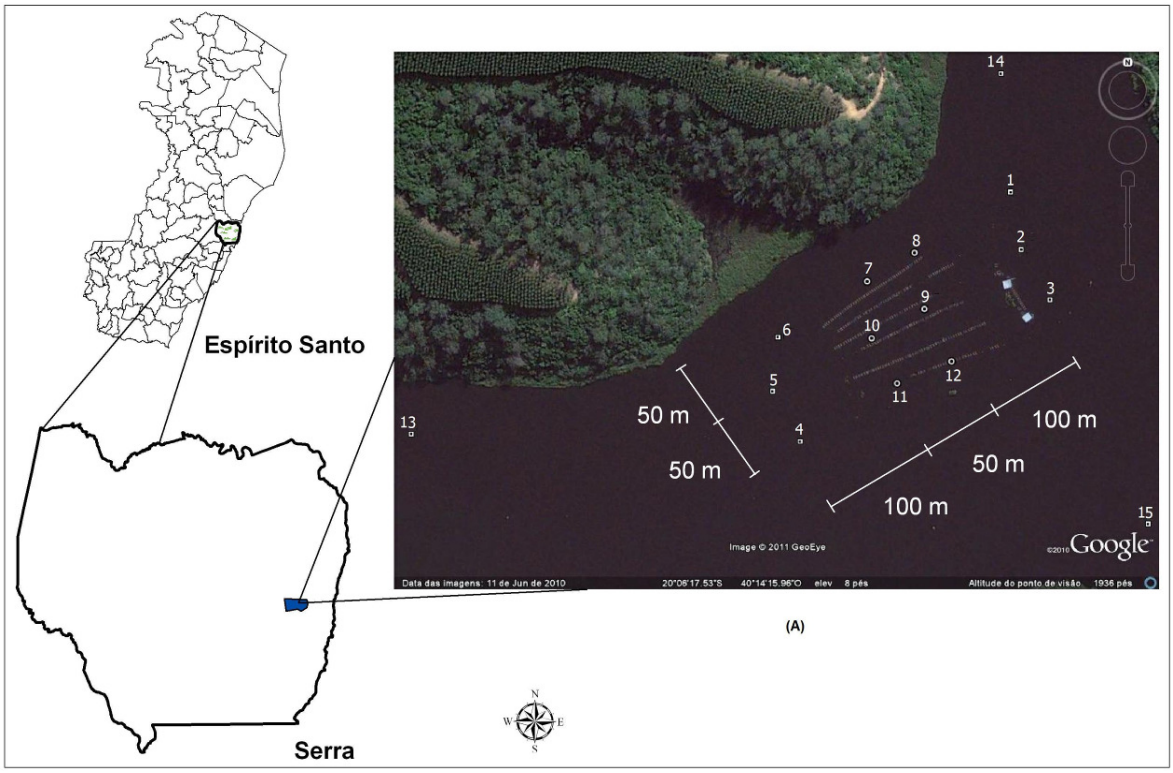

Figure 1 - Lake Juara, Espírito Santo, Brazil where the study was conducted. (A) Satellite images obtained from Google Earth. http://maps.google.com/maps?ll=-20.114011,-40.229739&z=14&t=h&hl=pt-br. Chart showing sampling points.

Sampling

Sampling and analysis were performed monthly during one year, from April, 2008 to March, 2009. During each visit, one water sample and two fish were collected from each one of the 6 net-cages, plus a sample of lake water at 6 pre-determined points, 50 m away from each other at two sampling sites 100 m apart from one another, in the surroundings of the net-cages (Figure 1A). Therefore, a total of 72 lake water samples, plus 72 net-cage water samples and 144 fish were collected. Water samples were collected at 20 cm from the surface, using 500 mL sterile glass bottles, that were labeled and placed in coolers filled with ice to keep the temperature below 10ºC until analysis. The fish were captured using a dip net, placed in plastic bags filled with water from the same net-cage, labeled and oxygen was added for the transport. Water temperature was determined *in situ*, using an oxymeter AT-150.

***Aeromonas* investigation in the fish, kidneys and water**

In the laboratory, the fish were transferred to sterilized containers of known weight and immersed in water and ice to desensitize, inside PVC boxes. Subsequently, after weighing, sterile peptone water (SPW) 0.1% was added in quantity equal to the fish weight, and agitated well to wash the entire body surface (SILVA et al., 2007). The resulting liquid was used for decimal dilutions and plated to obtain isolate colonies. After that, the scales were mechanically removed and the integument disinfected with 70% ethanol, inside a laminar flow hood. Then, through a ventral incision, fragments of renal tissue were removed with a swab (HIRSCH et al., 2006) and transferred to test tubes containing trypticase soy broth (TSB), incubated at 28ºC during 24 hours for subsequent plating.

The excessive amount of particulate material in lake and net-cage water made difficult to use the filtering membrane technique, instead the Spread-Plate technique was used. Aliquot of the samples and dilutions were distributed in plates containing culture medium to obtain isolate colonies (SILVA et al., 2007).

The plates were incubated at 28ºC from 24 to 48 hours using the selective growth media dextrin-ampicillin and M-Aeromonas base agar supplemented with 10 $\mu g.mL^{-1}$ ampicillin.

Characterization of *Aeromonas* species

Up to five yellow colonies surrounded by a halo resulting from the hydrolysis of dextrin or trehalose were transferred to tilted tubes containing trypticase soy agar and used to determine morphology and color by Gram method, as well as to perform catalase and oxidase tests. Strains characterized as Gram negative, catalase and oxidase positive were inoculated in triple-sugar-iron agar for an initial screening (MARTINELI et al., 2010). Genus differentiation and species characterization were carried out by the decarboxylation of lysine and ornithine, as well as esculing and arginine hydrolysis, ONPG and gelatin, resistance to the vibriostatic agent O/129 (2,4-diamine phosphate-6, 7-diisopropylteridine) (50 and 150 μg), acid production from the carbohydrates glucose, lactose, sucrose, mannitol, salicin, mannose, raffinose, arabinose, inositol and sorbitol, VM and VP tests, indol production, growth in the absence and presence of 6% NaCl, motility and nitrate reduction (DI BARI et al., 2007).

Hemolysin production

Hemolytic activity of *Aeromonas* isolates obtained from the water, fish body surface and kidney samples, was determined by checking the β-hemolysis zone surrounding the colonies in plates containing trypticase soy agar, with 5% horse blood agar, after 18 to 24 h incubation at 28°C (MONFORT & BALEUX, 1991; TOKAJIAN & HASHWA, 2004).

Antibiotic resistance tests

Antibiotic resistance tests were conducted according to the antimicrobial disk diffusion method (BAUER et al., 1966). The antibiotics used were gentamicin (10 μg), nalidixic acid (30 μg), tetracycline (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), amoxicillin (10 μg), erythromycin (15 μg), levofloxacin (5 μg) and ofloxacin (10 μg). Control test was done using the reference sample, *Aeromonas hydrophila*, ATCC 7966, from FIOCRUZ-RJ.

RESULTS AND DISCUSSION

Distribution of Aeromonas species

From the total of 1314 colonies found in the samples, *Aeromonas* spp. was isolated in 711 (54%) and eight species were identified *A. hydrophila, A. caviae, A. sobria, A veronii*, *A. jandae*, *A. salmonicida*, *A. eucrenophila, A. Schubertii* and atypical isolates as well (Figure 2).

Aeromonas hydrophila was the dominant species and varied from 32 to 35% in the water samples of lake and net-cages, and fish body surface, with the highest occurrence in fish kidney, corresponding to 44% of the analyzed samples. According to Holt et al. (1994), the optimal temperature range for multiplication of *A. hydrophila* lies between 22 and 28ºC, and during the studied period temperature varied from 21.99ºC in July/08 to 29.78ºC in February/09. This may explain why these results are higher than those reported by Schafer et al. (2004), 3.0% in kidney and liver samples of apparently healthy trout reared in water with temperatures averaging 15ºC.

The increased water volume during the rain period may have caused the number of *A. hydrophila* in the water and fish surface to decrease compared to that of fish kidney samples. The results observed for lake water samples were close to the value of 32% found by Araujo et al. (1991), for water samples of an eutrophicated lake in Northwestern Spain.

Aeromonas caviae and *Aeromonas sobria* were isolated in lake water (21 and 17%), net-cages (23 and 17%) and fish surface (16 and 12%), respectively.

They were not found in fish kidneys. The lower occurrence of these species compared to

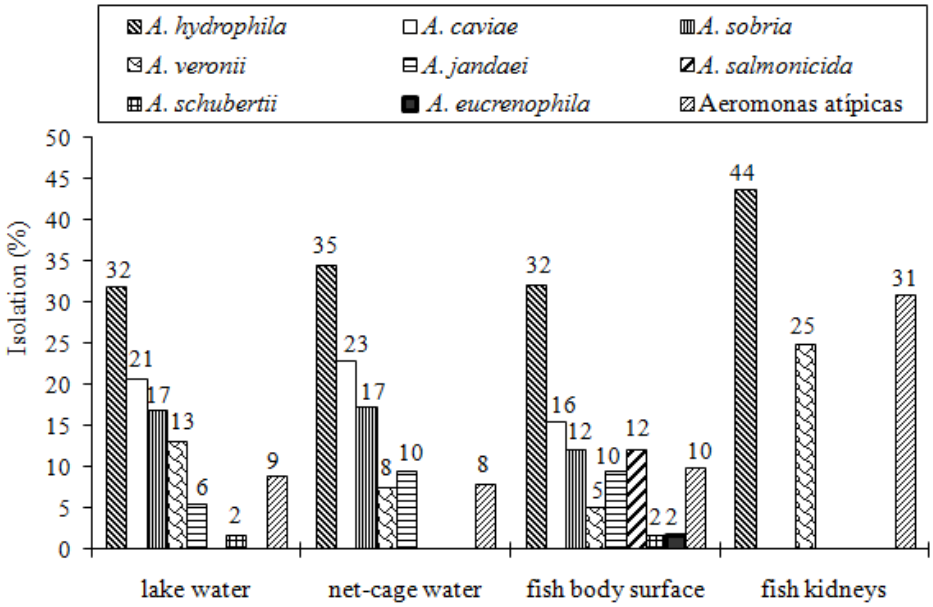

Figure 2 – Distribution of *Aeromonas* species isolated in the samples from the lakes, net-cages, fish body surface and kidneys.

A. hydrophila may be explained by the fact that this lake does not receive sewage effluent. The occurrence of *A. caviae* associated with high levels of fecal contamination has been described by Monfort & Baleux (1991), while *Aeromonas sobria* in water with less or no fecal contamination has been reported by Araujo et al. (1991).

Aeromonas veronii was present in all samples, and it was the second species isolated in fish kidney samples (25%). The highest percentage of isolates was reported by Kozinska (2007) in 57% of kidney samples of healthy carp in Poland and by Azevedo et al. (2003) in 31.9% of tilapia reared in Sports Fishing Lakes of Sao Paulo metropolitan region

This species was also isolated by Soler et al. (2002) in freshwater samples in Spain and Rahman et al. (2002) isolated *Aeromonas veronii* biovar *sobria* in several fish species that presented Epizootic Ulcerative Syndrome (EUS) in Bangladesh. Orozova et al. (2009) and Kozinska (2007) considered the species pathogenic for fish of tropical and temperate waters, respectively.

Aeromonas jandaei was present in all samples, except for fish kidneys. This species was also isolated by Hirsch et al. (2006), on the body surface of Nile tilapia and net-cage water of a pisciculture in Alto Rio Grande, Minas Gerais.

Aeromonas salmonicida and *A. eucrenophila* were present only on fish body surface samples. Austin et al. (1998) considered *A. salmonicida* difficult to isolate in water, since they survive in aquatic environment as co-culture with other bacteria, and this narrow association decreases their chances to be recognized within a mixed population.

Aeromonas schubertii was only isolated in the lake water and fish body surface samples. *Aeromonas* spp., considered as atypical *Aeromonas* because they displayed a somewhat atypical results for the biochemical tests, were isolated in all samples, and occurred more frequently in fish kidney.

Although analyzed tilapias were apparently healthy, species potentially pathogenic to tropical fish were found such as, *Aeromonas hydrophila*, (KOZINSKA, 2007), *A. caviae* (MARTINS et al., 2008), *A. sobria* (RAHMAN et al., 2002), *A. veronii* (OROZOVA et al., 2009) and *A. jandaei* (SANTOS et al., 1999) as well as *A. salmonicida* (AUSTIN et al., 1998) that is pathogenic to cold water fish. With the exception of *A. salmonicida,* all other species were isolated in lake and net-cage water samples. The small difference in the percentages and isolated species strengths the hypothesis that the microbiota reflects the microbiological quality of the environment from where the fish come from (MARTINS, 2005). The species pathogenicity to humans may result in gastroenteritis due to ingestion of food that presents cross contamination from the fish or water, or an infected fisherman wound, as well as for the people who use the lake for recreational purposes and may accidentally ingest contaminated water (FIGUERAS et al., 2005, HIRANSUTHIKUL et al., 2005).

Hemolytic activity of *Aeromonas* species

Hemolytic activity was detected in 65 (57%) of the 114 investigated cultures, where 35 were water sample isolates, 24 fish body surface and 6 fish kidney. These results are shown in Figure 3. *A. jandaei* was the species with the highest hemolytic activity in water samples (100%), followed by *A. veronii* (90%), *A. hydrophila* (74%) and *A. sobria* (50%). The isolates from fish body surface and kidneys, the highest hemolytic activity was observed for *A. hydrophila* (56 and 80%), followed by *A. veronii* (50 and 67%), respectively. *A. salmonicida*, *A. sobria*, *A. jandaei* and *A. caviae* isolated from fish body surface also displayed hemolytic activity; therefore, they can be

considered potentially pathogenic (DI BARI et al., 2007).

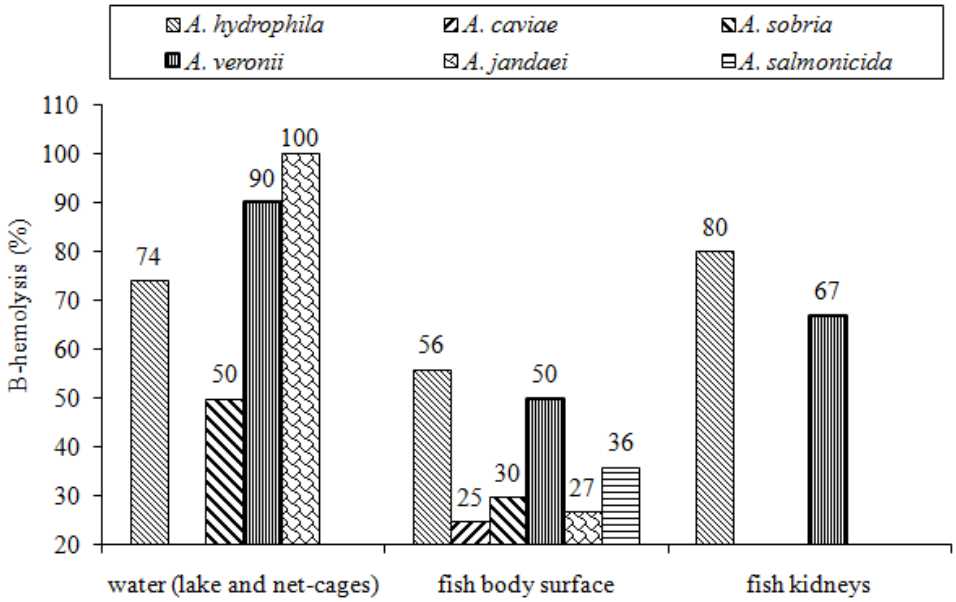

Figure 3 – Hemolytic activity of aeromonas culture isolated from water (lake and net-cages) fish body surface and kidneys.

Monfort & Baleux (1990) observed hemolysin production in 100% of the strains of *Aeromonas hydrophila* and *A. sobria* isolated from brackish water samples of a sewage treatment lake, in the South of France. Monfort & Baleux (1991) in another study with isolates from the same site, reported 96% *A. hydrophila* and 97% *A. sobria* hemolysin producers. Results higher than the percentages found in the present work for water sample isolates, 74% and 50% for *A. hydrophila hydrophila* and *A. sobria,* respectively.

Rahman et al. (2002) observed that isolates of *A. veronii* biovar sobria with Epizootic Ulcerative Syndrome (EUS), in Bangladesh, showed hemolytic activity and agglutinated erythrocytes, and may be a causative agent of EUS in fish. Abbott et al. (2003) found in isolates of clinical samples, animals and environment, 100% hemolysis for strains of *A. veronii*. These results are above the 90%, 50% and 67% reported in this study for samples of water, surface and kidneys, respectively.

The differences found for aeromonas hemolytic activity in this study may have been influenced by environmental conditions of multiplication, which alters the genes that decode hemolysin (WANG et al., 2003). *Aeromonas* cultured at lower temperatures displayed more strains and virulence factors compared to *Aeromonas* cultured at higher temperatures (GONZÁLEZ-SERRANO et al., 2002). According to Chacón et al. (2003) β-hemolytic activity of clinical isolates is significantly more frequent than that of environmental isolates. To Abbott et al. (2003), the increased activity of clinical isolates is due to horizontal transfer of hemolysin genes present in hemolytic *Aeromonas* to non-hemolytic species.

Antimicrobial resistance

The behavior of Aeromonas species isolated from water, fish body surface and kidneys, with respect to antimicrobial can be found in Table 1.

From the total 74 strains characterized, the following species *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, *A. salmonicida* and *A. jandaei* isolated from water, fish body surface and kidneys were resistant to amoxicillin and displayed sensitivity to nalidixic acid, ofloxacin, levofloxacin and chloramphenicol. From the isolates of *A. salmonicida* of fish body surface, 29% were resistant to gentamicin and 43% to tetracycline. As for *A. veronii,* 53% of isolates from water samples and 50% from fish body surface and kidneys were also resistant to tetracycline. The antibiotic erythromycin displayed the largest variation of resistance to both aeromonas species and isolate origin.

The resistance of all aeromonas strains to amoxicillin observed in this study are in agreement with the results presented by Bizani & Brandelli (2001), who reported 100% resistance to this antibiotics by the strains *A. hydrophila* e *A. sobria* isolated found in samples of supply and carcass final washing water in a cattle slaughter house in Porto Alegre; and by Hiransuthikul et al. (2005) in *Aeromonas* isolates from infected soft tissue of patients in Thailand after the 2004 tsunami.

The sensitivity displayed by 100% of the isolates of *A. hydrophila* to tetracycline and gentamicin in this study were identical to the results reported by Barcellos et al. (2008) for strains of *A. hydrophila* found in the samples of kidneys, spleen, liver, skin and muscle of jundia (*Rhamdia quelen*). However, the results reported by the same authors for sensitivity to chloramphenicol and intermediate sensitivity to erythromycin are below the 100% resistance levels found in this study.

Table 1 – Antimicrobial resistance of *Aeromonas* species isolated in the water (lake and net-cages), fish body surface and kidneys.

Species	Samples	Antimicrobial resistance (%)							
		Gen 10 μg	Nal 30 μg	Tet 30 μg	Ofx 5 μg	Eri 15 μg	Amo 10 μg	Lvx 5 μg	Clo 30 μg
A. hydrophila[a]	Reference	0	0	0	0	0	100	0	0
A. hydrophila	Water (n=10)	0	0	0	0	50	100	0	0
	Surface (n=10)	0	0	0	0	50	100	0	0
	Kidney (n = 2)	0	0	0	0	0	100	0	0
A. caviae	Water (n = 4)	0	0	0	0	50	100	0	0
	Surface (n = 4)	0	0	0	0	50	100	0	0
A. sobria	Water (n = 4)	0	0	0	0	50	100	0	0
	Surface (n = 4)	0	0	0	0	50	100	0	0
A. veronii	Water (n = 7)	0	0	43	0	43	100	0	0
	Surface (n = 6)	0	0	50	0	33	100	0	0
	Kidney (n = 2)	0	0	50	0	50	100	0	0
A. salmonicida	Surface (n = 7)	29	0	43	0	57	100	0	0
A. jandaei	Water (n = 7)	0	0	0	0	43	100	0	0
	Surface (n = 7)	0	0	0	0	57	100	0	0
Total of isolates (n = 74)		3	0	14	0	47	100	0	0

[a] = reference strain supplied by FIOCRUZ–RJ.
n = Number of isolates tested.
Gen = Gentamicin, Nal = Nalidixic acid, Tet = Tetracycline, Ofx = Ofloxacin, Eri = Erythromycin, Amo = Amoxicillin, Lvx= Levofloxacin e Clo = Chloramphenicol.

Álvarez et al. (2004) observed that *Aeromonas hydrophila* isolated from the water culture of tilapia in Lake Valencia, showed 100% resistance to gentamicin, tetracycline and erythromycin. While in this study, these strains were sensitive to gentamicin, tetracycline and erythromycin. Sensitivity results to nalidixic acid and chloramphenicol were compatible.

Sensitivity results to gentamicin and resistance to amoxicillin by *Aeromonas caviae* are consistent with those reported by Al-Benwan et al. (2007), for isolates of this species from a patient with cystitis in Kuwait that were sensitive to ciprofloxacin, cetotaxin and gentamicin and resistant to amoxicillin, cotrimoxazole, ampicillin, cefuroxime and cephalothin.

According to Hirsch et al. (2006), the resistance data to chloramphenicol, ofloxacin and levoflaxacin vary so much that there is no parameter to be considered. In this study, all strains from all samples were sensitive to these antibiotics.

Only resistance to amoxicillin was confirmed for all tested *Aeromonas* species.

The same species whether isolated from water or fish body surface samples was not significantly different with respect to resistance.

The intermediate resistance to erythromycin shown by all *Aeromonas* species isolated from water and fish body surface samples in this study, has also been reported by Hirsch et al. (2006) in isolates of fish body surface and lake water samples, they attributed this resistance to the multiplication of resistant clones that may have appeared from feeding or fingerlings.

At the same time, the intermediate resistance to tetracycline shown by isolates of *A. veronii* and *A. salmonicida*, in this study may have been due to the easiness of these species to develop resistance in the presence of low antibiotic concentration (GOÑI-URRIZA et al., 2000). According to Álvarez et al. (2006), oxolinic acid, chloramphenicol, erythromycin and tetracycline are widely used in Aquaculture to control Gram negative pathogens. Oxytetracycline hydrochloride is one of the antibiotics that is allowed in the United States to control Mobile Aeromonas

Septicemia (MAS) caused by *Aeromonas hydrophila* (FAO, 2009). In Brazil, oxytetracycline is already used for control and prevention of bacterial diseases in fish (PEREIRA JR et al., 2006).

Aeromonas salmonicida is not considered pathogenic for tropical fish species; however, your intermediate resistance to erythromycin and tetracycline of the other pathogenic bacteria should be considered a warning signal to the fish culture studied.

CONCLUSIONS

The predominance of *Aeromonas hydrophila* in all samples and *A. veronii* in fish kidneys allied to higher hemolysis production, lead to the conclusion that these species might contribute at any given moment to tilapia mortality rate. These species including *A. sobria* and *A. jandaei* isolated from the water and fish body samples may pose risk to public health. Special attention should be payed to the indiscriminate use of antibiotics, such as tetracycline, erythromycin and amoxicillin, since the resistance shown is already high.

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