Universidade Estadual de Londrina

DENISE DE OLIVEIRA SCOARIS

# OCORRÊNCIA DE *AEROMONAS* SPP. EM ÁGUAS NATURAIS: RESISTÊNCIA A ANTIBIÓTICOS E FATORES DE VIRULÊNCIA

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Dissertação apresentada ao Programa de Pós-graduação em Microbiologia da Universidade Estadual de Londrina, para obtenção do título de mestre em Microbiologia.

Orientador: Dr. Benedito Prado Dias Filho

Londrina
2006

**Trabalho realizado no Laboratório de Microbiologia Aplicada a Produtos Naturais e Sintéticos Biologicamente Ativos, Departamento de Análises Clínicas, Centro de Ciências da Saúde da Universidade Estadual de Maringá e no Laboratório de Biologia Molecular de Parasitas, Departamento de Microbiologia, Centro de Ciências Biológicas, da Universidade Estadual de Londrina.**

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**BANCA EXAMINADORA**

______________________________
Prof. Dr. Benedito Prado Dias Filho
UEM – Maringá - PR
(Presidente da Banca - Orientador)

______________________________
Prof. Dr. Benício Alves de Abreu Filho
UEM – Maringá - PR
(Membro Convidado)

______________________________
Prof. Dra. Jacinta Sanchez Pelayo
UEL – Londrina - PR
(Membro Convidado)

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**Este trabalho é dedicado aos meus pais, pelo amor, incentivo e paciência, e por estarem sempre ao meu lado, mesmo que só em pensamento, em todos os momentos de minha vida...**

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## INTRODUÇÃO

O primeiro relato do gênero *Aeromonas* foi feito por Kluyver e van Niel (1936), que propuseram esta denominação a bactérias isoladas do meio ambiente, geralmente aquático, as quais eram estreitamente relacionadas aos bacilos entéricos móveis por flagelos polares. Este gênero foi incluído na família Vibrionaceae, e este taxon se manteve sem modificações até 1986, quando Colwell et al. sugeriram uma nova classificação, elevando a categoria de gênero à família Aeromonadaceae, através de evidências de genética molecular que sugeriram diferentes filogenias para as duas famílias. Embora esta classificação não conste na última edição do Manual Bergey (HOLT et al., 1994), muitos trabalhos fazem menção a esta nova família (KAZNOWSKI, 1998; ØRMEN; ØSTENSVIK, 2001; SOLER et al., 2003).

A família Aeromonadaceae compreende três gêneros: *Aeromonas, Ocealimonas* e *Tolumonas* (ICSB, 2003). O gênero *Aeromonas* engloba bacilos Gram-negativos, medindo de 0,3 a 1,0 μm de diâmetro e 1,0 a 3,5 μm de comprimento. Encontram-se isolados, aos pares, ou em cadeias curtas. São citocromo-oxidase positivos, produtores de DNAse, amilase e catalase. Possuem metabolismo anaeróbico facultativo, fermentam carboidratos com produção de ácido e/ou gás, não formadores de esporos ou microcistos. Reduzem o nitrato a nitrito, e mostram resistência ao agente vibriostático 0/129 (2,4 diamino-6,7–diisopropil pteridina fosfato) (HOLT et al., 1994), sendo esta resistência e a não exigência de altas concentrações de NaCl as principais características bioquímicas que diferenciam Vibrionacaeae de Aeromonadaceae.

Na última década, o gênero *Aeromonas* tem sofrido intensas modificações taxonômicas, devido ao emprego das técnicas de biologia molecular, como a hibridização DNA-DNA (MARTÍNEZ-MURCIA, 1999), tendo aumentado o número de espécies deste gênero para 19 já nomeadas, a maior parte com reconhecidos grupos de hibridização: *A. allosaccharophila* (HG14) (ISCB, 1999)*, A. bestiarium* (HG 2) (ALI et al., 1996), *A. caviae* ou *A. punctata* (HG 4), *A. culicicola* (FIGUERAS et al., 2005), *A. encheleia* (HG 11) (ESTEVE et al., 1995; HUYS et al., 1997a), *A. eucrenophila* (HG 6) (HUYS et al., 1997a)*, A. hydrophila* (HG 1) (WATANABE et al., 2004)*, A. ichthiosmia* (COLLINS et al., 1993), *A. jandaei* (HG 9) (ESTEVE et al., 2003)*, A. media* (HG 5)*, A. molluscorum* (MIÑANA-GALBIS et al., 2004), *A. popoffi* (HUYS et al., 1997b), *A salmonicida* (HG 3) (SWIFT et al., 1997)*, A. schubertii* (HG 12)*, A. simiae*, (HARF-MONTEIL et al., 2004), *A. sobria* (HG 7) (GRAF,

1999)*, A. trota* ou *A. enteropelogenes* (HG 13) (HUYS et al., 2002), *A. veronii,* incluindo dois biogrupos *(A. veronii* biovar *veronii* e *A. veronii* biovar *sobria)* (VALERA; ESTEVE, 2002) e ainda uma espécie não nomeada, pertencente ao grupo 501.

Devido a estas mudanças taxonômicas em um curto período de tempo, há ainda grande dificuldade na correta identificação de espécies de *Aeromonas*, sendo muitas vezes necessária à utilização de metodologias polifásicas de pesquisa para a classificação a nível específico. Estas metodologias combinam desde a caracterização bioquímica (GILARD, 1967), sorológica (JANDA et al., 1996; ALAVANDI et al., 2003) e fenotípica, como a análise de ácidos graxos metil-éster de membrana (FAMES) (CANONICA; PISANO, 1985; CANONICA; PISANO, 1988), até a caracterização molecular, sendo exemplos o polimorfismo do comprimento dos fragmentos de restrição (PCR-RFLP) (BORREL et al., 1997), análise de seqüência dos amplicons (PCR-ASA) (KIMGOMBE et al., 2004) e polimorfismo do comprimento dos fragmentos amplificados (AFLP) (BUSCH; NITSCHKO, 1999).

Outro ponto bastante controverso é a temperatura de crescimento deste gênero, havendo dois grupos bastante distintos de espécies de *Aeromonas*: psicrofílicas e mesofílicas. O primeiro grupo, bastante consolidado taxonomicamante (JANDA; ABBOTT, 1998), é formado por bactérias imóveis, que se desenvolvem bem à temperatura de 15-20º C, sendo o principal representante *A. salmonicida*, agente etiológico da furunculose em peixes (PAVANELLI et al., 2002). O segundo, onde se encontram as alterações mais relevantes quanto à classificação taxonômica, engloba organismos mesofílicos, móveis por flagelos polares, que tem sua faixa ótima de crescimento em torno de 35ºC, podendo crescer até a temperaturas de 45ºC, sendo que neste último grupo de encontram os reconhecidos patógenos humanos, *A. hydrophila, A. caviae* (ou *A. punctata*) e *A. sobria*, representando 85% dos isolados clínicos (JANDA; ABOTT, 1998). Ambos os grupos, todavia, podem se desenvolver a temperaturas que variam de 4-42ºC (MATEOS; PANIAGUA, 1995). Este limiar na temperatura de crescimento é um fator importante quando emprega a refrigeração para a conservação de alimentos, pois há relatos de que à temperaturas de 5ºC, espécies de *Aeromonas* não só se desenvolvem, mas também são capazes de produzir citotoxinas e hemolisinas (MAJEED et al., 1990).

O principal habitat das aeromonas é o ambiente aquático, representado por águas correntes e estagnadas, tanto dulcícolas quanto marinhas, caracterizando a presença de grupos mesofílicos e psicrofílicos. No entanto, microrganismos ambientais

podem eventualmente colonizar animais do mesmo habitat, sendo possível o isolamento de aeromonas em peixes (HÄNNINEN et al., 1997; RALL et al., 1998; LEE et al., 2002), sapos (HUYS et al., 2003), cobras (ESTERABODE et al., 1973) lagartos e jacarés (PAGE et al., 1962). Talvez pelo contato com água contaminada, há também relatos de vários alimentos onde foram isoladas espécies deste gênero (GRANUM et al., 1998; NEYTS et al., 2000; ULLMANN et al., 2005). Aeromonas são ainda vastamente encontradas em águas naturais (GRANUM et al., 1998; ØRMEN; ØSTENSVIK, 2001), água de poço (GHENGHESH et al., 2000), água mineral (VILLARI et al., 2003), bem como em águas do sistema público de abastecimento (HANDFIELD et al., 1996; KÜHN et al., 1997; ÁGUILLA et al., 2000; ALAVANDI; ANATHAN, 2003; BALAJI et al., 2004), mostrando que espécies de *Aeromonas* apresentam resistência ao tratamento com cloro de águas para consumo humano (MASSA et al., 1999).

A importância das espécies de *Aeromonas* vem crescendo na última década a partir do seu reconhecimento como agente de gastroenterites em imunocompetentes. Lopez (1996) reporta um quadro diarréico em criança, com idade inferior a 4 anos, em que o agente causal foi uma espécie do gênero *Aeromonas*. No entanto, a maioria dos autores apenas associa a presença do gênero a casos de gastroenterites (ALAVANDI et al., 2003; VILA et al., 2003), devido ao fato de espécies de *Aeromonas* serem isoladas com frequência de fezes de indivíduos assintomáticos, e quando o são de fezes diarréicas, muitas vezes estão em associação com reconhecidos patógenos entéricos, como *Salmonella, Shigella* e *E. coli* (NZEAKO et al., 2002). Mesmo isolando apenas *Aeromonas* spp. em indivíduos apresentando quadros de gastroenterites, não fica provado o seu caráter patogênico, pois muitos destes isolamentos apenas refletem colonização transitória (JANDA; ABBOTT, 1998). Em hospedeiros imunocomprometidos, espécies deste gênero podem estar associadas a sérios episódios patológicos, como bacteremia, septicemia, mionecrose (MARTINO et al., 1997), meningite, peritonite, doenças do trato respiratório, infecções oculares e síndrome urêmica hemolítica, sendo a *A. hydrophila* a principal espécie relacionada a estas patologias (JANDA; ABBOTT, 1998).

O abundante isolamento de *Aeromonas* em água e alimentos sugere uma possível rota de contaminação (SAAD et al., 1995). Vários marcadores de virulência têm sido descritos procurando explicar a patogênese das infecções intestinais e extraintestinais, como citotoxinas, (GRANUM et al., 1998), hemolisinas,

aderência e invasão tecidual (BALAJI et al., 2004; NZEAKO et al., 2002) e exoenzimas (amilases, lipases, nucleases, proteases e quitinases) (PEMBERTON et al., 1997).

As citotoxinas (enterotoxinas) têm sido consideradas como importante estímulo de eventos bioquímicos que se associam a quadros diarréicos agudos. Baseando-se nas alterações morfológicas causadas em culturas de células, há dois grupos principais de enterotoxinas: as citotóxicas, que acarretam morte celular, e as citotônicas, que apenas agem alterando a morfologia celular do epitélio gastrointestinal (MÁRQUEZ, 1998).

Um segundo importante marcador de virulência é a produção de hemolisinas. Em *Aeromonas*, estas hemolisinas são de dois tipos: alfa ($\alpha$) e beta ($\beta$). A $\alpha$-hemolisina é liberada da célula durante a fase estacionária, provocando lise parcial e reversível de eritrócitos, e a $\beta$-hemolisina é liberada no final da fase logarítimica de crescimento, apresentando caráter citotóxico a várias linhagens celulares, provocando lise eritrocitária total e irreversível. A maioria das espécies de *Aeromonas* secreta apenas um tipo de hemolisina. Uma das principais $\beta$-hemolisinas secretadas por *Aeromonas* é a aerolisina (PEMBERTON et al., 1997). Esta hemolisina encontra-se solúvel no periplasma, sob forma inativa contendo um peptídio sinal (preproaerolisina). Quando este sinal é removido, há a conversão em proaerolisina que, através da remoção proteolítica de 25 aminoácidos da porção C-terminal, torna-se aerolisina ativa. A forma final desta hemolisina apresenta a capacidade de lisar as células hospedeiras (eritrócitos ou outras células eucarióticas) pela inserção de poros na membrana, apresentando também intensa atividade citotóxica (PARKER et al., 1996).

As demais enzimas secretadas, como as amilases, lipases (MERINO et al., 1999), nucleases, proteases e quitinases, além de serem consideradas como marcadores de virulência, parecem também ter importante papel na ecologia do gênero (PEMBERTON et al., 1997).

Outro mecanismo de virulência de grande relevância é a aderência, tanto a superfícies celulares quanto abióticas. Esta capacidade de adesão das células bacterianas parece se relacionar a dois principais fatores: ao caráter hidrofóbico e a produção de adesinas. A hidrofobicidade (ROSENBERG, 1981) varia de uma cepa a outra e é dependente das condições de crescimento, como meio de cultura, aeração e tempo de cultivo (MOZES; ROUXHET, 1987). O segundo fator, a produção de adesinas, seria o primeiro passo para o estabelecimento de uma infecção, embora

sejam expressas também por espécies saprofíticas (KLEMM; SCHEMBRI, 2000). A colonização bacteriana de superfícies sólidas tem sido demonstrada em vários ambientes. A importância de se evitar esta adesão bacteriana a implantes cirúrgicos (LEAKE et al., 1982; COSTERTON et al., 1987;), rede do sistema público de distribuição (HERSON et al., 1987) e materiais alimentícios (BOTT; MILLER, 1983) é avaliada pela capacidade de formação de biofilmes, importantes nos estudos dos mecanismos de patogenicidade, devido a sua elevada resistência a antibióticos. Recentemente, estudos demonstraram que a adesão por espécies de *Aeromonas* é facilitada através do flagelo, sugerindo a associação destas estruturas aos processos de enteropatogenicidade (GAVIN et al., 2002; 2003).

Por fim, a resistência a antibióticos é outro fator de fundamental relevância nos estudos de marcadores patogênicos, pois tem repercussão em Saúde Pública, limitando o tratamento de infecções provocadas por bactérias resistentes (WHO, 1994). Esta resistência está ligada ao patrimônio genético microbiano. Os genes de resistência trabalham de diversas maneiras para proteger a célula bacteriana: alguns codificam proteínas que atuam como pequenas bombas, retirando ativamente os fármacos da célula, outros codificam proteínas que têm a capacidade de modificar quimicamente o agente antimicrobiano e uma terceira classe ainda altera o alvo do antibiótico, para que a célula bacteriana não mais seja afetada pelos fármacos. A maioria destes genes de resistência está inclusa em plasmídeos (plasmídeos R), e normalmente nestes há a combinação de vários genes de resistência, pois grande parte é encontrada em elementos transponíveis (transposons). A transferência deste plasmídeo R por conjugação bacteriana, por exemplo, explica o fato da crescente muitiresistência a drogas.

# OBJETIVOS

Os objetivos desta pesquisa foram:

- Analisar a qualidade de água para consumo humano em Maringá-PR, quanto a presença de espécies do gênero *Aeromonas*;
- Associar a presença de grupos indicadores a espécies de *Aeromonas*;
- Identificar os isolados presuntivos do gênero *Aeromonas;*
- Determinar os potenciais fatores de virulência das espécies de *Aeromonas* isoladas, como a produção de enzimas extracelulares (hemolisinas, citotoxinas, fosfolipases, DNAses) e a aderência a superfícies (bióticas e abióticas);
- A capacidade de formação de biofilme;
- A persistência dos isolados a diferentes concentrações de cloro;
- A resistência dos isolados a ação de diferentes antimicrobianos.

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# ANEXOS

# ANEXO A

## ARTIGO A – THE OCCURRENCE OF *AEROMONAS* SPP. IN BOLTTLED MINERAL WATER, ARTESIAN WATER, AND TAP WATER FROM MUNICIPAL SUPPLIES.

**Denise de Oliveira Scoaris, Fernando César Bizerra, Sueli Fumie Yamada-Ogatta, Tânia Ueda Nakamura, Celso Vataru Nakamura, Benedito Prado Dias Filho**

# THE OCCURRENCE OF *AEROMONAS* SPP. IN BOLTTLED MINERAL WATER, ARTESIAN WATER, AND TAP WATER FROM MUNICIPAL SUPPLIES.

**Denise de Oliveira Scoaris[1], Fernando César Bizerra[1], Sueli Fumie Yamada-Ogatta[2], Tânia Ueda Nakamura[3], Celso Vataru Nakamura[3], Benedito Prado Dias Filho[3]***

Short title: **Ocurrence of *Aeromonas* spp. in drinking water**

**Abstract**

Positive samples for *Aeromonas* spp. were 12.7% from mineral water, 8.3% from artesian water and 6.5% from tap water. The recovery of *Aeromonas* spp. was significantly higher in bottled mineral and artesian water than in tap water from municipal supplies. The occurrence of the *Aeromonas* spp. did not correlate significantly with traditionally used contamination indicator bacteria (i.e. total coliforms) in artesian water samples. However, a significant correlation was found between *Aeromonas* spp. and total coliforms in both mineral water and tap water samples. The presence or absence of a correlation between indicator bacteria and *Aeromonas* could reflect the occasional appearance of pathogens in drinking water and the different rates of survival and recovery of these agents compared with those fecal indicators. The finding that 41.6%, 14.8% and 9.0% of the artesian water, bottled water and tap water, respectively, sampled in the current study failed to meet the Brazilian standard for total coliforms in drinking water should therefore be of concern. This highlights the need for an improved surveillance system for both the bottled water industry and the municipal water supplies. Strains belonging to the genera *Aeromonas* identified to the species level were *A. hydrophila* and *A. jandaei*. The public health significance of the large number of *Aeromonas* spp. that can develop in water is unclear. There is an urgent need to compare the effects of a greater number of strains belonging to each of these species in a wider range of animal models of infection. These results can contribute to the re-evaluation of the criteria used to analyse the microbial quality of drinking water and to define measures for limiting *Aeromonas* densities in drinking water.

**Keywords:** *Aeromonas*. Drinking water. Indicator bacteria. Coliforms.

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[1]Programa de Pós-graduação em Microbiologia pela Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, Cx. Postal 6001, 86051-990 Londrina, Paraná, Brasil.

[2]Departamento de Microbiologia, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, Cx. Postal 6001, 86051-990 Londrina, Paraná, Brasil.

[3]Departamento de Análises Clínicas, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brasil. *Corresponding author: Benedito Prado Dias Filho, Departamento de Análises Clínicas, Universidade Estadual de Maringá, Av. Colombo, 5790, CEP 87020- 900, Maringá, PR, Brazil, fax +55-44-3261.4860. *E-mail address*: bpdfilho@uem.br

**Introduction**

The contamination of natural water with fecal materials, domestic and industrial sewage and run-off from pasture and agricultural land may result in an increasing risk of disease (or pathogenic microorganisms) transmission to human that use those waters (Wiggins, 1996). Therefore, to protect the consumers from possible diseases transmitted by water, it is important to monitor the occurrence's levels of pathogens in this water (Gray, 1994).

Although there is concern about the incidence of coliforms indicator group - both total and termotolerant coliforms - existing in water, the legislation do not preconize the search of heterotrophic bacteria that also would be inhabiting the aquatic environment, growing up from the biodegradable organic matter (Haddix et al., 2004), working as indicators of the presence of toxic substances in the water (Nocciolini et al., 2000). These microorganisms often act as secondary pathogens, having the capacity to elicit pathologic episodes in hosts subject to intense stress (Roitt et al., 1996).

Significant portions of this heterotrophic group are bacteria of *Aeromonas* genus. This group was included in the *Vibrionaceae* family, but since the 1980´s some researchers had proposed the classification of these in a new family, *Aeromonadaceae* (Colwell et al., 1986; Joseph et al., 1994; Holt et al., 1994), including three genus: *Aeromonas, Ocealimonas* e *Tolumonas* (ICSB, 2003)**.** The aeromonads are mesophilic motile or psycrophilic non-motile Gram-negative bacteria (Lee et al., 2002), showing mobility by the polar flagellum (Holt et al., 1994).

Despite of the fact that 19 named species have been classified, only few of them are recognized as human pathogens, like *A. hydrophila* complex*, A. caviae and A. veronii*, representing almost 85% of clinical isolates, although other species have been recovered from clinical sources (Janda and Abbot, 1998).

Aeromonads are also found in a variety of non-human sources, such as food (Granum et al., 1998; Ullmann, et al., 2005), natural water (Ghenghesh et al, 2001; Ørmen; Østensvik, 2001) and drinking water (Kuhn et al., 1997; Villari et al., 2003), and it is known that psycrophilic species are mainly etiologic agents in fishes (Garduño et al., 2000). In human immunocompetent hosts, the members of this genus may be responsible for intense gastroenteritis, called traveler's diarrhea (Janda and Abbot, 1998; Vila et al,. 2003), but in immunocompromised hosts, these

bacterias could be implicated in cases of myonecrosis, bacteremia, septicaemia (Martino et al., 1997) and hepatic cirrhosis and neoplasys (Chien Ko; Chuang, 1995).

The aim of this study is to analyze the drinking water's quality in Maringá-PR, associating the presence of coliforms group in the water with the occurrence of *Aeromonas* species.

**Materials and Methods**

*Samples*

A total of 238 samples of drinking water taken from bottled mineral water, artesian water and tap water from municipal systems were investigated for *Aeromonas*. After membrane filtration of 250 ml samples, filters (Millipore HC filter, pore size 0.45 μm) were place on the M-Endo Agar LES (M-Endo) to enumerate total coliforms and on the M-Fecal Coliforms (M-FC) to enumerate termotolerant coliforms, incubagted at 37ºC for 24 h, and at 44,5ºC for 24 h, respectively (APHA, 1995). After incubation period, typical coliforms colonies were confirmed in Lauryl Triptose and EC broth. Atypical (lactose-negative) colonies were Gram stained and identified as presumptive *Aeromonas* spp. if they were Gram-negative, oxidase-positive, glucose-fermenting (O/F test) and resistant to vibriostatic agent O/129 (150 μg). Atypical colonies (red or transparent in M-Endo and orange or transparent in M-FC) were isolated and gram-stained. Presumptive *Aeromonas* isolates were confirmed PCR-based assay as described below.

*Molecular identification*

The molecular identification, as primers designs to amplify 16S rDNA gene (forward primer 5'- AGAGTTTGATCATGGCTCAG- 3' and reverse primer 5'-GGTTACCTTGTTACGACTT-3'), were performed as Borrel et al., 1997. Presumptive of *Aeromonas* genus isolated of different water sources were examined. For genus-specific analysis, DNA was extracted from each bacterial sample (including reference strains) by boiling during 10 min. After boiling, the DNA was stored at – 4º C until application on PCR experiments. PCRs were carried out on a thermal cycler Primus

96 Plus (MWGAG - Biotech). A final concentration of 20 μl containing 50 mM KCl, 2mM Tris-HCl (ph 8.4), 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (Invitrogen), 2 U *taq* I DNA polymerase (Invitrogen), 2 μl aliquot of DNA sample, and 1 μl of each primer (forward and reverse) was added for every reaction. PCRs were performed under the following conditions: denaturation at 93°C for 10 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. After the final cycle, extension at 72°C was allowed for 10 min. PCR products were precipitated with addition of 10 % volume of 3.0 M sodium acetate and 200 μl of cold ethanol, dried and ressuspended in 25 μl of sterile distilled water. Endonuclease digestion, electroforesis and pattern analisys were performed as described by Borrel et al., 1997, with modifications. Enzymatic digestions were performed by incubating 12 μl of the amplification and precipitated products with 5 U of each enzyme (*Alu*I and *Mbo*I, Invitrogen) and 2 μl of the corresponding 10X buffer (buffer A for *Alu*I and *Mbo*I), in a total volume of 20 μl. The reaction mixture was incubation overnight at 37°C. Aliquots of 10 μl of each restriction reaction was eletrophoresed on 10% poliacrilamida gel in 1X TBE (tris-borate-EDTA) buffer, for 2,5 hours on 70 V. Gels were stained by silver method. The molecular sizes of the fragments obtained were estimated by the use of 100 pb molecular marker used as reference.

*Statistical analysis*

Results were analyzed by linear regression and *t*-test, at $p < 0.05$ and 0.005 of confidence level.

**Results and discussion**

The objective of this study was to determine the frequency of *Aeromonas* species in bottled mineral, artesian water and tap water, as well as their correlation with the indicator bacteria. The coliforms organisms have long been recognized as a suitable microbial indicator of drinking-water quality, largely because they are easy to detect and enumerate in water (WHO, 1993). In drinking water from municipal supplies, the coliforms test can be used as an indicator of the treatment

efficiency and the integrity of the distribution system. Although coliforms organisms may not always be directly associated to the presence of fecal contamination, the presence of coliforms in drinking water suggests the potential presence of pathogenic enteric microorganisms such as *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae*. Coliforms bacteria are the only microbiological contamination to be regulated by federal law in both tap and bottled water.

According to the Brazilian directives (ANVISA, 2004), at least 40 samples per month of tap water from each public water supply should be analysed and the bacterial indicator must not be present in 95% of the samples taken throughout any 12-month period. In the case of minimum frequencies, one sample every week for waterworks with surface water source and one sample every 2 weeks for waterworks with a ground water source the indicator must not be detectable in any 100-ml sample.

In the present study, positive samples for *Aeromonas* were 12.7% from mineral water, 8.3% from artesian water and 6.5% from tap water (Table 1). The recovery of *Aeromonas* was significantly higher in bottled mineral and artesian water than in tap water from municipal supplies. The studies *Aeromonas* spp. in the drinking water and even in the chlorinated water supplie showed that this bacterium was isolated most frequently (Knochel and Jeppesen, 1990; Alavandi et al., 1999).

**Table 1** – *Aeromonas*, total coliforms and fecal coliforms in samples collected from bottled mineral water, artesian water and tap water from municipal supplies.

| Microorganism | Number of positive samples (percentage) | | |
|---|---|---|---|
| | Bottled mineral water[a] (n=47) | Artesian water (n=24) | Tap water[b] (n=167) |
| *Aeromonas* spp. | 6 (12.7) | 2 (8.3) | 11 (6,5) |
| Total coliforms | 7 (14.8) | 10 (41.6) | 15 (9.0 |
| Fecal coliforms | 0 | 4 (16.6) | 4 (2.4) |

[a] 20 l bottles
[b] Most-often-used faucet

A correlation coefficient matrix was established to compare the degree of association between *Aeromonas* spp. and the contamination indicator bacteria (Table 2). Different results were observed with samples from bottled mineral

water, artesian water and tap water from municipal supply. The occurrence of the *Aeromonas* did not correlate significantly with traditionally used contamination indicator bacteria (i.e. total coliforms) in mineral water samples. The lack of correlation between *Aeromonas* and total coliforms suggests that the two microorganisms could indicate different source of pollution. However, a significant correlation was found between *Aeromonas* and total coliforms in both tap water and well water samples. The presence or absence of a correlation between indicator bacteria and *Aeromonas* could reflect the occasional appearance of pathogens in drinking water and the different rates of survival and recovery of these agents compared with those fecal indicators.

**Table 2 –** Spearman correlation coefficients among *Aeromonas* and indicator bacteria.

| Microorganism | Correlation coefficient | | | | | |
|---|---|---|---|---|---|---|
| | Mineral water[a] | | Artesian water | | Tap water[b] | |
| T C[c] | 0,1981 | 0,00 | **0,4677*** | 0,3669 | **0,4472*** | **0,5291*** |
| F C[d] | 0,00 | 1,00 | 0,2941 | 1,00 | 0,1690 | 1,00 |
| | **Aeromonas** | F C | **Aeromonas** | F C | *Aeromonas* | F C |

Significance level: *p<0.05, **p<0.005
[a] 20-litre bottles on water dispensers.
[b] Municipal supply .
[c] Total coliform group.
[d] Fecal coliform group.
* Significative value.

Other authors have found that coliforms counts did not correlate with *Aeromonas* spp. For Legnani et al., (1998) no correlation was found between the concentration of *Aeromonas* spp. and fecal indicator organisms. More recently, Massa et al., (2001) reported study with high number of *Aeromonas* spp. According to these authors, *Aeromonas* spp. may be good indicator of hygienic quality of water. Consequently their search should be used to indicate unsatisfying conditions, especially in private water systems that not undergoing systematic chlorine treatment.

The finding that 41.6%, 14.8% and 9.0% (table 1) of the artesian water, bottled water and tap water, respectively, sampled in the current study failed to

meet the Brazilian standard for total coliforms in drinking water should therefore be of concern. This highlights the need for an improved surveillance system for both the bottled water industry and the municipal water supplies.

Since the biochemical characterization is not precise and time-consuming, several proposals have been done about the classification of aeromonads, including structural factures, like fatty acids methyl esters compositions (FAMES) (Canonica and Pisano, 1985; Canonica and Pisano, 1988) and genetic composition and variability (Soriano, et al., 1997; Kaznowski, 1998; Lee et al., 2002; Miñana-Galbis et al., 2004). The 16S (or small subunit) rDNA sequences have been proven to be a valuable tool in the identification of most *Aeromonas* species (Martínez-Murcia, 1992), once members of this genus exhibit very high levels of overall sequence similarity, reaching more than 98% (Figueras et al., 2000). Nevertheless, only 2% of difference in the genetic composition of 16S rDNA, which in nucleotides correspond to 1-32 base differences (Martínez-Murcia, 1992), is enough to distinguish among species of *Aeromonas* genus until species level by restriction fragments length polymorphism techniques (RFLP) of the amplicons of this conserved (Borrel et al., 1997)

A total of 19 strains were isolated from M-Endo and M-FC media. The isolates were identified to species levels by RFLP-based method and the frequency of isolated is shown in Table 3. *Aeromonas* spp. identified to the species level were *A. hydrophila* and *A. jandaei.* For mostly *Aeromonas* it was impossible identify to the species level with the tests used.

**Table 3 –** Frequency of isolation of *Aeromonas spp.* in bottled mineral, artesian water and tap water

| Isolate | No. of samples | | |
|---|---|---|---|
| | Bottled mineral water | Artesian water | Tap water |
| *Aeromonas hydrophila* | 2 | 0 | 2 |
| *Aeromonas jandaei* | 1 | 1 | 3 |
| Aeromonas *spp.* | 3 | 1 | 6 |

Drinking water and food are reservoirs of *Aeromonas* species and therefore may be important sources of human infections. Bacteria of the genus *Aeromonas* have been frequently recognized as responsible for several diseases, both in human and animals (Cahil, 1990; Krovacek et al., 1995). *A. hydrophyla, A. caviae* and *A. sobria* have been linked to two major group of human diseases: septicemia and gastroenteritis (Merino *et al*., 1995). A greater risk of infection is reported in young children, elderly people, and immunocompromised patients (Janda, 1991).

Several virulence factors have been identified in *Aeromonas* spp. (Turnbull *et al*., 1984; Ljungh, 1987; Cahil, 1990), as the heat-labile and heat-stable cytotoxin that have enterotoxic activities together the ability to produce hemolysins and proteases. Another important attribute of pathogenic *Aeromonas* species could be the production of adhesins (Burke et al., 1984). Several recent reports implicate aquatic environmental in *Aeromonas*-associated human infections (Baddour, 1992; King et al., 1992; Ghanem et al., 1993)

The public health significance of the large number of *Aeromonas* spp. that can develop in water is unclear. Therefore, there is an urgent need to compare the effects of a greater number of strains belonging to each of these species in a wider range of animal models of infection. These results can contribute to the re-evaluation of the criteria used to analyse the microbial quality of drinking water and to define measures for limiting *Aeromonas* densities in drinking water.

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# ANEXO B

## ARTIGO B – VIRULENCE AND ANTIBIOTIC SUSCEPTIBILITY OF *AEROMONAS* SPP. ISOLATED FROM DRINKING WATER

**Denise de Oliveira Scoaris, Celso Vataru Nakamura, Tânia Ueda-Nakamura, Benedito Prado Dias Filho***

# VIRULENCE AND ANTIBIOTIC SUSCEPTIBILITY OF *AEROMONAS* SPP. ISOLATED FROM DRINKING WATER

**Denise de Oliveira Scoaris[1], Celso Vataru Nakamura[2], Tânia Ueda-Nakamura[2], Benedito Prado Dias Filho[2]***

Short title: **Pathogenic potential of environmental *Aeromonas***

**Summary**
*Aeromonas* isolates from tap water (11), mineral water (6), and artesian well water (2) were investigated for their ability to produce different potential virulence factors. Majority of the isolates displayed hemolytic activity against sheep erythrocytes, while only 7 of the 23 *Aeromonas* strains displayed DNAse activity and 4 of the 23 *Aeromonas* strains tested developed dense white opaque zones around the colonies on the egg yolk agar medium and were therefore regarded as positive for phospholipase production. Most of the isolates showed cytotoxicity activities in culture filtrate dilutions at titer of 1/8 or lower. No general relation between the strain isolated and the ability to interact with epithelial cells could be established. Using the bacterial adherence to hydrocarbons method, most of the strains were classified as highly hydrophilic. Chlorine sensitivity tests revealed that 0.4 mg of chlorine per liter at a treatment time of 1 min kills 2 of the 5 *Aeromonas jandaei*, 3 of the 12 *Aeromonas* sp. and 1 of the 5 *Aeromonas hydrophila* strains. All 5 *A. jandaei* strains isolates, 9 of the 12 *Aeromonas* sp. strains and 4 of the 5 *A. hydrophila* were multidrug resistant, i.e., resistant to three or more antibiotic tested. There is an urgent need to compare the effects of a greater number of strains belonging to each of these species in a wider range of animal models of infection. The results of these studies should provide valuable information concerning the molecular mechanisms of how *Aeromonas* species cause disease.

**Keywords:** *Aeromonas.* Adherence. Cytotoxicity. Hydrophobicity. Antimicrobial resistance.

[1] Programa de Pós-graduação em Microbiologia pela Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, Cx. Postal 6001, 86051-990 Londrina, Paraná, Brasil.
[2] Departamento de Análises Clínicas, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brasil. *Corresponding author: Benedito Prado Dias Filho, Departamento de Análises Clínicas, Universidade Estadual de Maringá, Av. Colombo, 5790, CEP 87020- 900, Maringá, PR, Brazil, fax +55-44-3261.4860. *E-mail address*: bpdfilho@uem.br

## Introduction

Bacteria of the genus *Aeromonas* have been frequently recognized as responsible for several diseases, both in human and animals (Cahil, 1990; Krovacek et al., 1995). *A. hydrophyla, A. caviae* and *A. sobria* have been linked to two major group of human diseases: septicemia and gastroenteritis (Merino et al., 1995). A greater risk of infection is reported in young children, elderly people, and immunocompromised patients (Janda, 1991).

Several virulence factors have been identified in *Aeromonas* spp. (Turnbull et al., 1984; Ljungh, 1987; Cahil, 1990), as the heat-labile and heat-stable cytotoxin that have enterotoxic activities together the ability to produce hemolysins and proteases. Another important attribute of pathogenic *Aeromonas* species could be the production of adhesins (Burke et al., 1984).

Drinking water and food are reservoirs of *Aeromonas* species and therefore may be important sources of human infections. The public health significance of the large number of *Aeromonas* spp. that can develop in water is unclear. Several recent reports implicate aquatic environmental in *Aeromonas*-associated human infections (Baddour, 1992; King et al., 1992; Ghanem et al., 1993)

The aim of this study was to determine the possible differences among *Aeromonas* isolates from tap water, mineral water, and artesian well water in relation to their ability to produce different potential virulence factors or markers such as hemolysins, cytotoxins and their ability to adhere to epithelial cells and to abiotic surfaces. The susceptibility to antibiotics, the ability to produce phospholipase and the survival of *Aeromonas* isolates in a chlorinated environment were also examined.

## Experimental procedures

### *Bacterial isolates*

The *Aeromonas* strains used in this study and their source of isolation are listed in Table 1. The drinking water isolates were recovered by standard procedures for screening of *Aeromonas* in drinking water (APHA, 1995). To comparison, type strains ATCC 7966 (*A. hydrophila*), ATCC 35624 (*A. veronii*), IOC-

FDA 110-36 (*A. hydrophila*) and the human clinical strain (A. *hydrophila*) were included in all assay.

**Table 1**. Characteristics of *Aeromonas* strains and their source of isolation

| Isolate | Source | Identification by: PCR-RFLP |
|---|---|---|
| M1 | Mineral water | *Aeromonas* sp. |
| M2 | Mineral water | *Aeromonas hydrophila* |
| M3 | Mineral water | *Aeromonas jandaei* |
| M4 | Mineral water | *Aeromonas* sp. |
| M5 | Mineral water | *Aeromonas hydrophila* |
| M6 | Mineral water | *Aeromonas* sp. |
| S1 | Tap water | *Aeromonas* sp. |
| S2 | Tap water | *Aeromonas hydrophila* |
| S3 | Tap water | *Aeromonas jandaei* |
| S4 | Tap water | *Aeromonas jandaei* |
| S5 | Tap water | *Aeromonas* sp. |
| S6 | Tap water | *Aeromonas jandaei* |
| S7 | Tap water | *Aeromonas hydrophila* |
| S8 | Tap water | *Aeromonas* sp. |
| S9 | Tap water | *Aeromonas* sp. |
| S10 | Tap water | *Aeromonas* sp. |
| S11 | Tap water | *Aeromonas* sp. |
| P1 | Artesian water | *Aeromonas* sp. |
| P2 | Artesian water | *Aeromonas jandaei* |
| *A. hydrophila* | ATCC 7966 | *Aeromonas hydrophila* |
| *A. hydrophila* | IOC-FDA 110-36 | *Aeromonas* sp. |
| *A. hydrophila* | Clinical isolated | *Aeromonas* sp. |
| *A. veronii* | ATCC 35.624 | *Aeromonas veronii* |

ATCC: American Type Culture Collection
IOC - FDA: Instituto Oswaldo Cruz – Food and Drug Administration

*Identification of the bacteria*

Atypical colonies (red or transparent in M-Endo and orange or transparent in M-FC) were isolated and gram-stained. The bacilli gram-negative were submitted to following tests: presence of citochromo-oxidase, growing in ADA, oxidation/ fermentation of carbohydrates, DNAse, hemolysis and resistence to vibriostatic agent O/ 129 (150 μg). Presumptive *Aeromonas* isolates were confirmed PCR-based PCR assay as described by Borrel et al., 1997.

*Hemolysis, DNAse and phospholipase production*

Hemolytic assays were done by measuring the zone of hemolysis around colonies on a blood-agar plate as described in an earlier report (Imzilm et al., 1996), except that instead of human blood, 5% sheep erythrocytes in brain-heart infusion (BHI) agar (Difco) was used. The phospholipase production was carried out, with some modifications, as previously described (Price et al., 1982; Polak, 1992). Briefly, the test medium consisted of MA containing 1 M sodium chloride, 0.005 M calcium chloride end 2% egg yolk. DNAse test was carried out by inoculation of the isolates in DNAse agar (Difco). Each *Aeromonas* isolated was inoculated in triplicate. After incubation at 37 °C for 2 days, the diameters of the colonies and the colony-plus-precipitation zones were measured. Measurement and calculation of the zone of hemolytic activity, phospholipase activity and DNAse activity was performed by dividing the diameter of the zone around the colony formed on the plate by the colony diameter.

*Cytotoxicity assay*

The cytotoxicity assay was carried out, with some modifications, as previously described (Skehan et al., 1990). Briefly, each *Aeromonas* isolate was inoculated into 3 ml of Trypticase soy broth, and the mixture was incubated for 18 to 24 h at 37°C. Following centrifugation at 10,000 x g for 30 min at 4°C, the supernatant fluid was carefully removed and filtrated, and a two-fold serial dilution of the supernatant was delivered to each well of confluent macrophages (J774G8) cell monolayers grown in 96-well cell culture plates. The prepared cultures were then incubated for 48 h at 37°C under a 5% $CO_2$ atmosphere. At that time, cultures fixed with 10% trichloroacetic acid for 1 h at 4°C were stained for 30 min with 0.4% sulforhodamine B (SRB) in 1% acetic acid, and subsequently washed 5 times with deionised water. Bound SRB was solubilised with a 150 μl 10 mM unbuffered Tris-base solution. Absorbance (530 nm) was read in a 96-well plate reader. The dye was removed by four washes with 1% acetic acid. Protein-bound SRB was extracted with 10 mM Tris. The cytotoxicity was expressed as a percentage of the optical density of the control.

*Adherence assays*

*Human buccal epithelial cells (BEC).* Buccal epithelial cells were collected from healthy human volunteers by gently rubbing the inside of their cheeks with sterile swabs, which were then shaken in sodium phosphate buffer (PBS). The epithelial cells were washed twice in PBS to remove unattached microorganisms, and were resuspended in PBS at a concentration of $10^5$ cells/ml. Bacteria from overnight culture were harvested, suspended into PBS pH 7,4 and adjusted to about $1x10^7$ colony forming unit per ml (CFU/ml). Bacteria were transferred to a microcentrifuge tube, pelleted, and suspended in 1 ml of FITC (Invitrogen) (0.5 mg/ml) in 50 mM sodium carbonate-100 mM sodium chloride. Bacteria were incubated for 20 min at room temperature, washed three times, and suspended in PBS (Hazenbos et al., 1994). Standardised suspensions of epithelial cells (250 μl) and FITC-labeled bacteria (250 μl of an inoculum of $1x10^7$ CFU/ml) were mixed in small screw-capped bottles and incubated for 1 h at 37°C, under agitation at 100 rpm. The mixture was then centrifuged for 10 min at 1000 rpm and washed with PBS to remove unattached bacteria. The final clot was suspended in 50 μl of PBS at pH 7.2. The suspension was spread out on slides and air-dried, fixed with phormol solution (5%), and visualized by fluorescence microscopy. The adherence was expressed as the mean of the number of adherent bacteria, by counting 100 living epithelial cells (Boussard et al., 1989).

*Adherence to abiotic surfaces.* Adherence to abiotic surfaces was studied with two different materials: glass and polyethylene terephtalate (PET), in the bottom of 24-well tissue culture plates (Falcon). One ml of bacterial inoculum ($1x10^7$ CFU/ml) FITC-labeled, as described, was added in each well. After 1 h at room temperature, the coverslips were washed for two times in PBS, pH 7.2, and the adhered cells were fixed in phormol solution (5%), air-dried and mounted on a slide with synthetic resin (Araldite 502™). The adherence was expressed as the mean of the counts of the adherent bacteria, after counting 100 fields in a fluorescence microscopy.

*Biofilm formation*

Overnight cultures were inoculated 1:100 into fresh medium. In the microtiter plate assay, inoculated cultures were grown in a 96-well polystyrene microtiter plate (Oliveira-Garcia et al., 2003). Growth of planktonic cells was determined by absorbance at 600 nm. Biofilm was measured by discarding the medium, rinsing the wells with PBS for three times. After adding 200 μl of PBS to the same wells, the wells were scraped down with a sterile wooden applicator stick for 30 s to dislodge attached cells. The buffer-cell suspension was aspirated and added to 2.8 ml of PBS containing 0.1% Tween (Inlab, São Paulo, Brazil). The wells were washed down once more with 200 μl of these dilute suspensions, then scraped until dry with a sterile applicator stick. The stick was stirred vigorously in the 5 ml buffer/cell volume to dislodge biofilm. Controls and test solution were diluted accordingly and plated on nutrient agar.

*Hydrophobicity assay*

For the hydrophobicity assay, each isolate of *Aeromonas* was inoculated into 3 ml of Trypticase soy broth for 16 to 18 h at 37°C. The bacteria were then harvested by centrifugation, washed twice, and suspended in 3 ml of 50 mM PBS (pH 7.4) containing 0.15 M NaCl to an absorbency of 0.4 at 660 nm. The bacterial suspension was treated with xylene (2.5:1 v/v; Merck). Following 20 min pre-incubation at room temperature, the mixture was vigorously mixed for 2 min. After allowing 20 min for the hydrocarbon phase to rise completely, the aqueous phase was carefully removed with a Pasteur pipette. The absorbency was then determined at 660 nm against a blank consisting of sodium phosphate buffer. The hydrophobicity indexes (CSH) were calculated as previously described (Teixeira et al., 1993).

*Chlorine sensitivity tests*

The bactericidal effect of chlorine on *Aeromonas* isolates was determined using the method of Seyfried and Fraser (1980), with the following modification. Each *Aeromonas* isolate was inoculated into 3 ml of Trypticase soy

broth for 16 to 18 h at 37°C. The organisms were centrifuged and washed twice in sterile chlorine demand-free buffered (pH 7.4) water. The washed bacteria were resuspended in the buffer and adjusted to give a standard bacterial suspension of approximately $1x10^7$ CFU/ml. The test system, held in 500 ml screw-cap flasks, contained 150 ml of buffered chlorine demand-free water. Appropriate quantities of chlorine solution were added to each flask, and after thorough mixing, a 50 ml aliquot was removed for determination of free chlorine. The test flasks were incubated with 10 ml of standard bacterial suspension and, after a 1-min contact period, 5 ml of sterile sodium thiosulfate solution (0.7 mg/ml) was added to neutralise the chlorine and thereby stop the reaction. The control and test solutions were diluted accordingly and plated on nutrient agar. Plates were counted after 48 h of incubation at 37°C.

*Antibiotic susceptibility*

The antibiotic susceptibility test was performed by the standard disc diffusion method (NCCLS, 2003; 2004). The following antibiotics were used: Amikacin 30μg, Ampicilin 10 μg, Aztreonam 30 μg, Cephalothin 30 μg, Cefoxitine 30 μg, Ciprofloxacin 5 μg, Chloramphenicol 30 μg, Cefepime 30 μg, Cefotaxime 30 μg, Gentamicin 10 μg, Meropenem 10 μg, Nalidix ac. 30 μg, Tetracycline 30 μg, Trimetrhopim-sulfamethoxazole 25 μg. Organisms were reported as either resistant, intermediate or sensitive to each anti-microbial tested.

## Results and Discussion

*Bacterial isolates*

The *Aeromonas* strains used in this study and their source of isolation are listed in Table 1. The drinking water isolates were recovered by standard procedures for screening for *Aeromonas* in mineral water, denoted M, tap water, denoted S, and artesian water, denoted P. To comparison, type strains ATCC 7966 (*A. hydrophila*), ATCC 35624 (*A. veronii*), IOC-FDA 110-36 (*A. hydrophila*) and the human clinical strain (*A. hydrophila*) were included in all assay

*Hemolysis assay*

The results show that majority of the isolates grown at 37°C under micro-aerobic conditions displayed hemolytic activity against sheep erythrocytes, while eleven of the 23 grown both at 28°C and 37°C under aerobic showed hemolytic activity (Table 2).

Many studies have demonstrated that *Aeromonas* produce a discrete number of virulence factors probably involved in determining infections (Merino et al., 1999; Cascon et al., 2000; Chopra et al., 2000). A previous study by Burke et al. (1984) has shown that is possible to identify 97% of enterotoxigenic *Aeromonas* isolates by biotyping and a hemolysin assay.

**Table 2** – Hemolytic activity of *Aeromonas* strains isolated from tap water, mineral water and artesian water growth under aerobic and microaerobic condition at 28 and 37 °C[a]

| Sources | Hemolysis index[b] | | | | | |
|---|---|---|---|---|---|---|
| | Aerobic condition | | | Microaerobic condition | | |
| | Hemolysis | 28 °C | 37 °C | Hemolysis | 28 °C | 37 °C |
| *Aeromonas* sp | | | | | | |
| M1 | - | | | α | | 1.0 |
| M4 | - | | | α | 1.31 | 1.0 |
| M6 | α | 1.41 | | β | | 1.00 |
| S1 | α | | 1.25 | α | 1.00 | 1.00 |
| S5 | - | | | α | 0.50 | 0.50 |
| S8 | - | | | α | 1.00 | 0.50 |
| S9 | - | | | β | | 0.00 |
| S10 | - | | | α | 1.00 | 1.00 |
| S11 | β | 2.25 | 2.20 | β | | 1.65 |
| P1 | - | | | β | 1.11 | 1.22 |
| IOC | β | 1.95 | 1.95 | | | |
| CLIN 1 | α | 1.41 | 1.36 | β | | 1.16 |
| ***A. jandaei*** | | | | | | |
| M3 | α | 1.00 | | α | 1.00 | 1.00 |
| S3 | α | | 1.35 | α | | 1.30 |
| S4 | - | | | α | | 0.50 |
| S6 | - | | | α | 0.00 | 0.50 |
| P2 | - | | | β | 1.11 | 1.20 |
| ***A. hydrophila*** | - | | | | | |
| M2 | β | 1.15 | 0.60 | β | 1.04 | 1.00 |
| **M5** | β | 1.36 | 1.52 | β | 1.51 | 1.36 |
| S2 | β | 1.44 | 1.61 | β | 1.60 | 1.53 |
| S7 | β | 2.00 | 2.80 | β | 1.70 | 1.54 |
| ATCC | β | 1.75 | 1.75 | β | 1.55 | 1.60 |
| ***A. veronii*** | | | | | | |
| ATCC | β | 1.87 | 2.01 | | | |

[a] Sheep blood
[b] Hemolysis index: the diameter of the translucent radial zone of hemolysis divided by the diameter of the colony size (equal to or larger than 1); negative

*DNAse and Phospholipase production*

The results of present study indicate the difference in the expression of DNAse and phospholipase by *Aeromonas* spp isolates. Only 7 of the 23 *Aeromonas* strains used in this study displayed DNAse activity. Four of the 23 *Aeromonas* strains tested in this study developed dense white opaque zones around

the colonies on the egg yolk agar medium even after 7 days of incubation and were therefore regarded as positive for phospholipase production. Three of four *A. jandaei* included in this study failed to form the dense opaque DNAse zones. Phospholipase activity was detected in 4 out of 23 (17%) of the isolates: two *Aeromonas* sp (M6, S8), one *A. jandaei* isolated from drinking water (S6) and one (ATCC) type strains (Table 3). Many microorganisms secrete enzymes which ensure their penetration into the host cells. Phospholipases belong to this type of molecules capable to derange or destroy cell surface membranes, by hydrolyze one or more ester linkage in glycerophospholipds. According to the specific bond cleaved in the phospholipid molecule they are indicated as A, B, C and D (Ivanovska, 2003). Most phospholipases may associate with membranes at the lipid-water interface. However, in many Gram-negative bacteria, a phospholipase is present which is located integrally in the bacterial outer membrane. This phospholipase (outer membrane phospholipase A or OMPLA) is involved in transport across the bacterial outer membrane and has been implicated in bacterial virulence (Snijder and Dijkstra, 2000), as many studies have addressed the role of phospholipases as a virulence factor in protozoa, bacteria and fungi.

Nuclease is a group of a variety of roles, but the extracellular production of DNAse is somewhat problematical. DNAses may be nutritional enzymes, used to degrade nucleic acids for their phosphorous and nitrogen and may be important to bacteria ecology. Nevertheless, these enzymes provide a barrier to the entry of foreign DNA into the host cell, acting as virulence marker (Pemberton et al., 1997).

**Table 3** – DNAse activity and phopholipase production of *Aeromonas* strains isolated from mineral water, tap water and artesian water

| Isolates | DNAse | Phospholipase |
|---|---|---|
| *Aeromonas* sp | | |
| M1 | - | - |
| M4 | - | - |
| M6 | - | 2.0 (++++) |
| S1 | - | - |
| S5 | - | - |
| S8 | - | 1.2 (+) |
| S9 | - | - |
| S10 | 1.36 (++) | - |
| S11 | - | - |
| P1 | - | - |
| IOC | 1.74 (++++) | - |
| CLIN 1 | 1.78 (++++) | - |
| ***A. jandaei*** | - | |
| M3 | - | - |
| S3 | - | - |
| S4 | - | - |
| S6 | - | 1.1 (+) |
| P2 | - | - |
| ***A. hydrophila*** | | |
| M2 | - | - |
| **M5** | 1.98 (++++) | - |
| S2 | 1.82 (++++) | - |
| S7 | - | - |
| ATCC | 1.72 (++++) | 1.1 (+) |
| ***A. veronii*** | | |
| ATCC | 1.86 (++++) | - |

* Measured by dividing the diameter of the zone around the colony formed on the plate by the colony diameter. Thus, zone = <1.00 was evaluated as negative (−), >1.00–1.20 as weak (+), 1.21–1.40 as mild (2+), 1.41–1.70 as relatively strong (3+) and >1.71 as very strong (4+) activity.

*Cytotoxicity assay*

*Cytotoxic* activity was detected in 14 out of 23 (60%) of the isolates: nine *Aeromonas* sp. (M4, S1, S5, S8, S9, S10, S11, PI, CLIN1), five *A. jandaei* and only one *A. hydrophila* (S2). Majority of the isolates showed cytotoxic activities in culture filtrate dilutions at titer of 1/8 or lower (Table 4).

Although Shimada et al. (1984) demonstrated the production of citotoxic enterotoxin only by *A. hydrophila*, Potomski et al. (1987) reported that 24% of *A. caviae* strains reacted with antisera to cholera toxin by ELISA. Cytotonic enterotoxin from *Aeromonas* spp. isolates caused fluid secretion in *Aeromonas*-associated diarrhea.

**Table 4 –** Cytotoxic activity among *Aeromonas* strains isolated from tap water, mineral water and artesian well[a].

| Supernatant | Cytotoxic activity (%) | | | | |
|---|---|---|---|---|---|
| | Nontoxic | Toxic | | | |
| | | Diluted to: | | | |
| | | 1/2 | 1/4 | 1/8 | 1/16 |
| *Aeromonas* sp | | | | | |
| M1 | 0.00 | | | | |
| M4 | | | | | 22.30 |
| M6 | 0.00 | | | | |
| S1 | | | 58.80 | | |
| S5 | | | | | 22.20 |
| S8 | | | | 31.80 | |
| S9 | | | 28.80 | | |
| S10 | | | | 28.80 | |
| S11 | | | | | 20.20 |
| P1 | | | 26.40 | | |
| IOC | 0.00 | | | | |
| CLIN 1 | | | | 38.30 | |
| ***A. jandaei*** | | | | | |
| M3 | 12.00 | | | | |
| S3 | | | | | 40.00 |
| S4 | | | | | 24.50 |
| S6 | | | | 42.00 | |
| P2 | | | 60.40 | | |
| ***A. hydrophila*** | | | | | |
| M2 | 0.00 | | | | |
| **M5** | 0.00 | | | | |
| S2 | | | 52.00 | | |
| S7 | 5.60 | | | | |
| ATCC | 0.00 | | | | |
| ***A. veronii*** | | | | | |
| ATCC | 0.73 | | | | |

[a]Positive when at least 20% cell rounding and detachment was observed in the monolayer.

*Adherence assays*

The ability of *Aeromonas* isolates to adhere to epithelial cells was investigated using human buccal epithelial cells (Fig 1). There was a difference in the abilities of the individual isolates to adhere. However, no general relation between the strain isolated and the ability to interact with epithelial cells could be established. The overall ability of isolates of environmental origin to adhere to epithelial cells was observed to be no less than that of the group of collection strains (clinical and type strains). Three patterns of adhesion (diffuse, localised, and aggregative) were observed (data not shown). In the diffuse pattern, adherent bacteria were randomly and individually dispersed over the cell surface. The localised and aggregative patterns were characterized by the formation of adherent microcolonies corresponding to small or large clusters of bacteria, respectively. The tested bacteria (including the group of collection strains) predominantly showed the localized type of adherence.

The ability of aeromonads to adhere to the human epithelial cell model has been correlated with the enteropathogenicity of the genus (Carrello et al., 1988.). More studies investigating the adherence of *A. caviae* to HEp-2 cells suggested the involvement of the polar flagellum in this process (Thornley et al., 1996).

As can be seen in the Fig 1, most of the adherers had more than ten bacteria adhering per field. No particular bacterial isolate was significantly more able to adhere to abiotic surfaces in both drinking water isolates and collection and type strains.

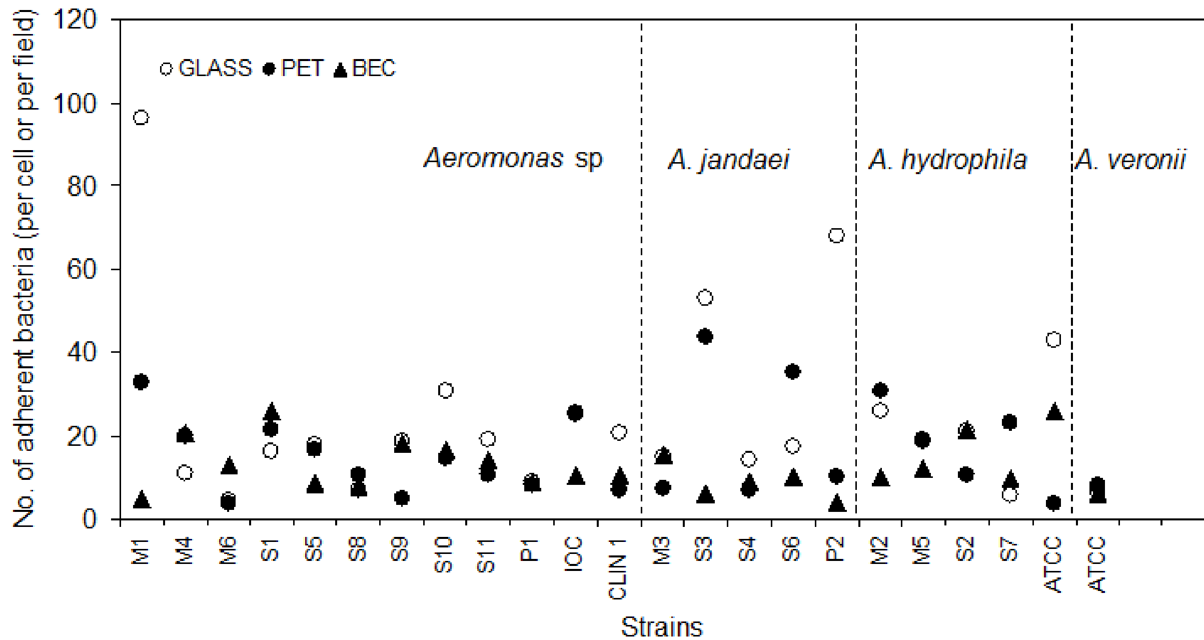


**Figure 1 –** Differences in adhesion of *Aeromonas* strains to abiotic surface and epithelial cells, after incubation of 12 h at 37°C. The results represent mean values for at least three separate experiments. Standard errors were less than 10% of means.

Bacterial colonization of solid surfaces has been demonstrated in a wide variety of environments. The importance of avoiding bacterial adhesion to surgical implants (Costerton et al., 1987; Leake et al., 1982), tap water distribution systems (Herson et al., 1987), and cooling apparatuses (Bott; Miller, 1983) is generally recognized.

Aeromonads colonize and form biofilms in water distribution and food-processing systems as well as on water-dwelling plants and animals (e.g., fish, leeches, and frogs). Recently, studies have shown that *Aeromonas* flagella facilitate adherence to (and possibly invasion of) the epithelial cell line HEp-2 as well as biofilm formation on plastic (microtiter plateassay), suggesting that these structures may also be virulence determinants for enteropathogenic strains (Gavín et al., 2002; 2003). *Aeromonas* species, like the seafood pathogen *Vibrio parahaemolyticus*, possess two distinct flagellar systems (a polar flagellum for swimming in liquid and a lateral flagellum multiply expressed, for swarming over surfaces) (MacCarter, 1999; Rabaan et al., 2001).

To quantitatively assess the biofilm development, growth in polystyrene microtiter wells of planktonic cells and biofilm formation in the same wells of twenty-three *Aeromonas* spp. isolates was monitored by using an assay base on

viable plate counts (Fig. 2). The organisms grew well in TSB, reaching a maximum number of approximately $10^9$ CFU/ml at 36 h. The number of adherent bacteria was maximum (approximately $10^7$ CFU/ml) at 12 h. In the present study, adherence to polystyrene microtiter wells by isolates from tap water, mineral water, artesian water, and the group of collections strains are showed in Fig. 2. A pronounced difference between environmental isolates and collections in the abilities to adhere was observed. Biofilm accumulated much more extensively in the environmental isolates.

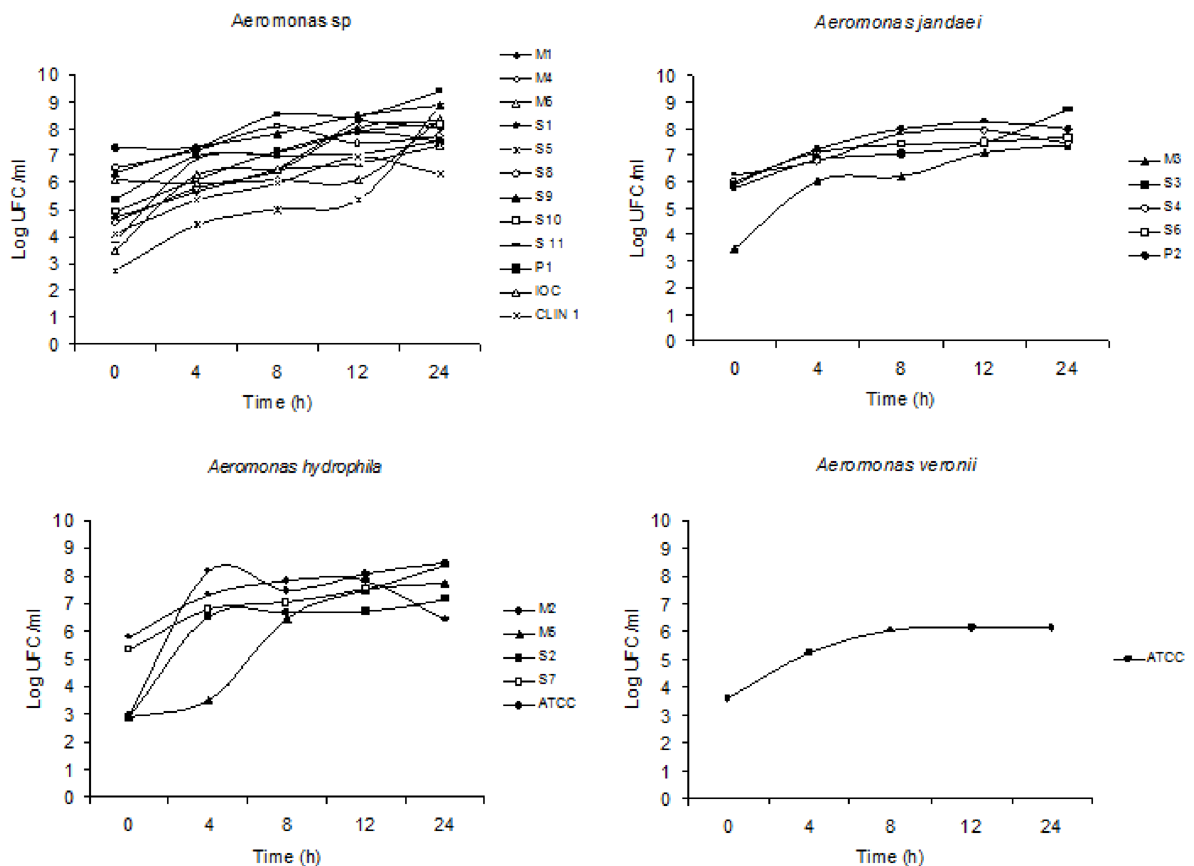


**Figure 2** – Biofilm formation by *Aeromonas* strains in wells of polystyrene microtiter. Values represent the mean. Each mean value represent the average of three independent experiments on each isolate. Standard errors were less than 10% of means.

*Hydrophobicity assay*

Microbial adhesion to hydrocarbons is a biphasic separation hydrophobicity assay, which measures relative cellular superficial hydrophobicity (CSH) through the capacity of the cell to adhere to a hydrocarbon phase. Employing

the CHS classification scheme described by Schneider and Riley (1991), isolates with a percentage hydrophobicity greater than 70% were classified as highly hydrophobic and those with a hydrophobicity index less than 30% were classified as highly hydrophilic. The affinity of twenty three isolates of *Aeromonas* spp. towards the test hydrocarbon is presented in Fig 3. The isolates exhibited a low CSH, ranging from 0-66%. Using the bacterial adherence to hydrocarbons (BATH) method, most of the strains were classified as highly hydrophilic.

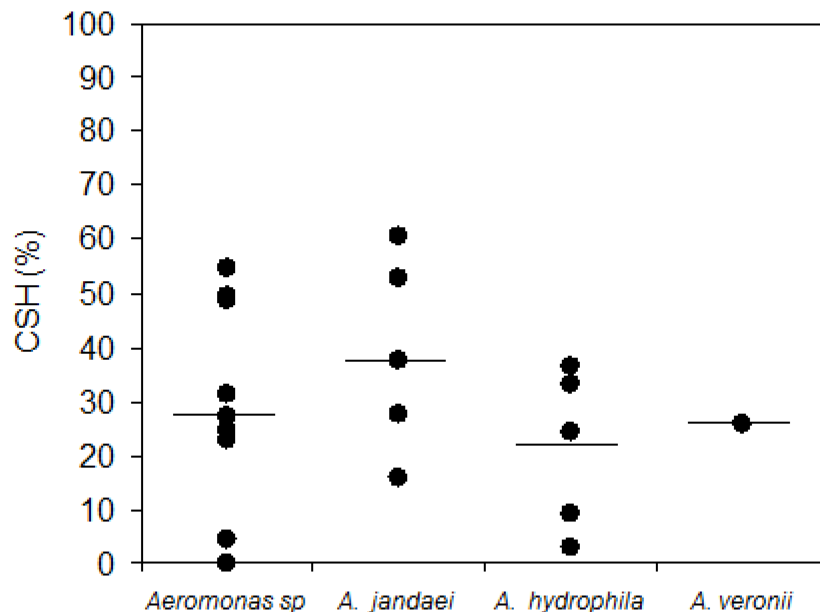


**Figure 3** – CSH (%) of different strains of *Aeromonas* isolates from mineral water, tap water, artesian water and the group of collections strains (clinical and type strains), as measured by adhesion to p-xylene. The dots indicate the average of triplicate determination. The bar indicate the median of the plotted values. Standard errors were less than 10% of means.

*Chlorine sensitivity tests*

One of the main reservoirs for the enteric *Aeromonas*-associated infections in humans is the domestic water supply (Burke et al., 1984; Kuhn et al., 1997) from which *Aeromonas* spp. have frequently been isolated, even after chlorination (Le Chevallier et al., 1982). Therefore, even chlorinated drinking water may represent a potential health hazard when contaminated by these organisms.

In the current study, chlorine sensitivity tests revealed that 0.4 mg of chlorine per liter at a treatment time of 1 min kills the *A. veronii*, while only 2 of the 5 *Aeromonas jandaei*, 3 of the 12 *Aeromonas* sp. and 1 of the 5 *Aeromonas hydrophila*

strains were killed at this concentration (Fig 4). The presence of residual chlorine, as in the case for many other bacteria, does not necessarily exclude the *Aeromonas* in drinking water. It is interestingly to note that majority of the isolates were not killed by chlorine at 1.2 mg/l. This is particularly noteworthy because this chlorine concentration is 6 times the recommended level of 0.2 mg free chlorine per liter. Whether the higher tolerance to chlorine of *Aeromonas* isolates can be linked to greater virulence is not know.

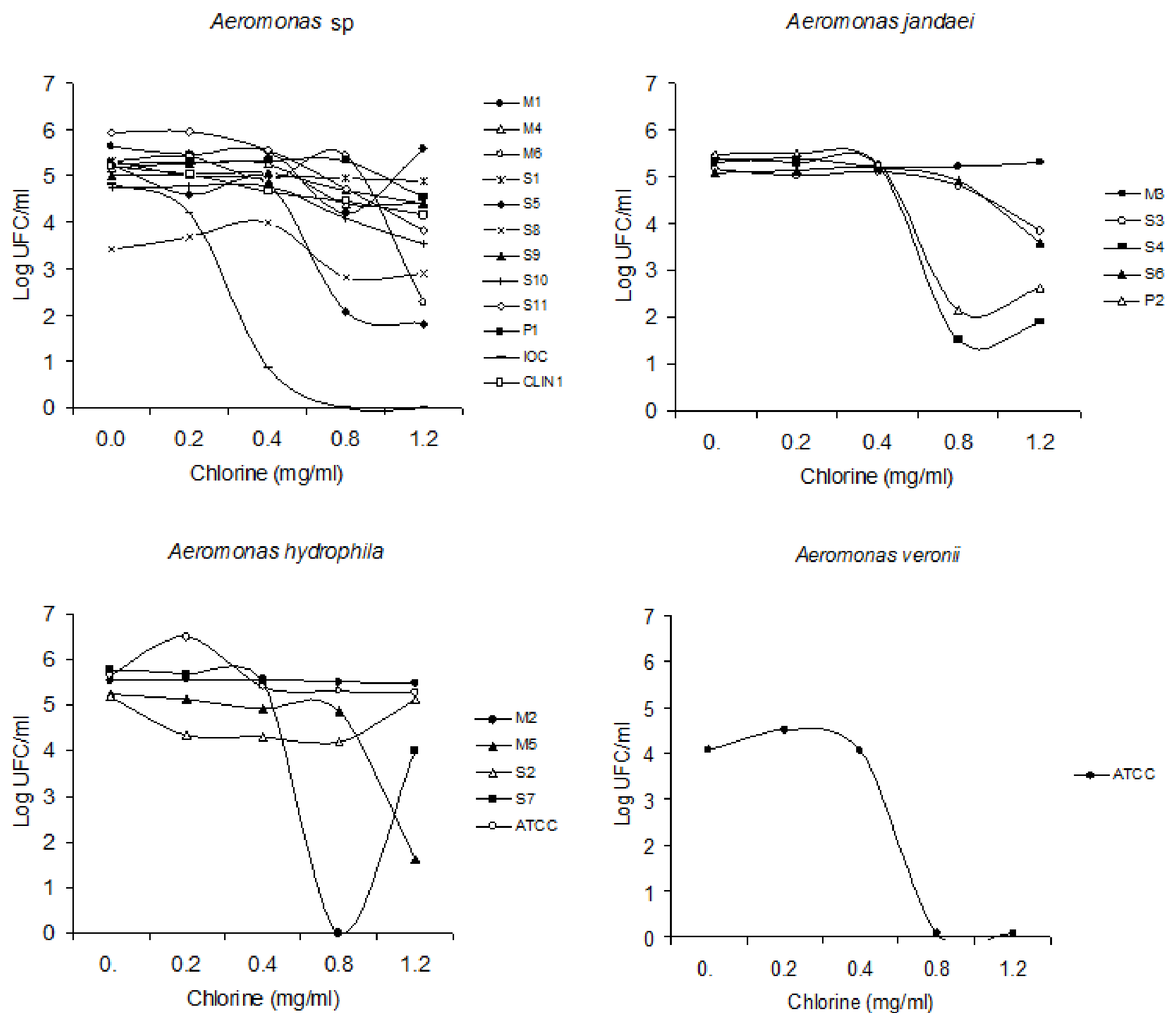


**Figure 4 –** Killing of *Aeromonas* isolates by chlorine. Values represent the mean. Each mean value represent the average of three independent experiments on each isolate. Standard errors were less than 10% of means.

*Antibiotic susceptibility*

In our study, the antibiotic susceptibility test was performed by the standard disc diffusion method. The prevalence of multiple-antibiotic resistance among *Aeromonas* strains isolated from tap water, mineral water and artesian water and collections strains is presented in Table 5. Resistance to three or more antibiotic was frequently observed among isolates from environmental strains, whereas it was not detected among clinical strains.

**Table 5** – Susceptibilities of *Aeromonas* strains isolated from mineral water, tap water and artesian water.

| Isolate | Source | Antibiotic resistance |
|---|---|---|
| *Aeromonas* sp | | |
| M1 | Mineral water | Amp, Cfl, Cfo, Clo,Tet |
| M4 | Mineral water | Amp, Azt, Cfl, Cfo, Clo, Nal, Tet. T-S |
| M6 | Mineral water | Amp,Cfl, Cfo,Clo,Com |
| S1 | Tap water | Amp, Cfl |
| S5 | Tap water | Amp, Cfl, Clo |
| S8 | Tap water | Amp, Cfl, Cfo, Clo, Nal, T-S |
| S9 | Tap water | Ami, Amp, Cfl, Clo, Gen, Mem, Nal, Tet |
| S10 | Tap water | Amp, Cfl, Cfo, Clo,Tet |
| S11 | Tap water | Amp, Cfl, Clo, Tet, T-S |
| P1 | Artesian water | Ami, Amp, Azt, Cfl, Clo, Gen, Nal |
| IOC | IOC-FDA 110-36 | Cfl |
| CLIN 1 | Clinical | Amp, Cfl |
| ***A. jandaei*** | | |
| M3 | Mineral water | Amp, Azt, Clo, T-S |
| S3 | Tap water | Ami,Amp,Azt, Cfl, Gen |
| S4 | Tap water | Ami, Amp, Azt, Cfl, Gen |
| S6 | Tap water | Ami, Amp, Azt, Cfl, Cfo, Clo, Gen |
| P2 | Artesian water | Ami, Amp, Azt, Cfl, Gen |
| ***A. hydrophila*** | | |
| M2 | Mineral water | Tet |
| ***M5*** | Mineral water | Amp, Cfl Cfo,T-S |
| S2 | Tap water | Amp,Cfl, T-S |
| S7 | Tap water | Amp, Cfl, Clo,Tet,T-S |
| ATCC | ATCC 7966 | Amp,Cfl, Cfo |
| ***A. veronii*** | | |
| ATCC | ATCC 35624 | Amp |

Amikacin 30μg, Ampicilin 10 μg, Aztreonam 30 μg, Cephalothin 30 μg, Cefoxitime 30 μg, Ciprofloxacin 5 μg, Chloramphenicol 30 μg, Cefepime 30 μg, Cefotaxime 30 μg, Gentamicin 10 μg, Meropenem 10 μg, Nalidix ac. 30 μg, Tetracycline 30 μg, Trimetrhopim-sulfamethoxazole 25 μg.

All 5 *A. jandaei* strains isolates, 9 of the 12 *Aeromonas* sp strains and 4 of the 5 *A. hydrophila* were multidrug resistant, i.e., resistant to three or more antibiotic tested. The most active antimicrobial was ciprofloxacin (susceptible in 100% of the isolates), and the least active antibiotic was ampicillin (resistance in 92% of the isolates). An aspect of the in vitro susceptibilities of isolates from the present study was found to be different from aspects published elsewhere. Uniform susceptibility to cefotaxime, tetracycline, and trimethoprim-sulfamethoxazole was found for three *Aeromonas* species in the United States (Motyl et al., 1985), and those in Australia were susceptible to cefotaxime, ceftriaxone, ceftazidime, aztreonam, tetracycline, and fluoroquinolones (Koehler; Ashdow, 1993). The result that tetracycline and trimethoprim-sulfamethoxazole inhibited about 60% of isolates is unlike the excellent activities shown in earlier studies (Motyl et al., 1985; Fainstein et al., 1982; Koehler and Ashdow, 1993). It is speculated that resistance to multiple antibiotics in one *Aeromonas* isolate can be mediated by several coinducible enzymes under the selection pressure of certain widely prescribed antibiotics. Recently, in a clinical *A. sobria* isolate, three b-lactamases could be induced simultaneously (Walsh et al., 1995). In summary, more restrictive policies on the use of antibiotics in human and animals may improve the current situation

In conclusion, mineral water, tap water and artesian water can be considered a possible transmission route for Aeromonads. However, there is an urgent need to compare the effects of a greater number of strains belonging to each of these species in a wider range of animal models of infection. The results of these studies should provide valuable information concerning the molecular mechanisms of how *Aeromonas* species cause disease. In addition, these results can contribute to the re-evaluation of the criteria used to analyze the microbial quality of drinking water and to define measures for limiting *Aeromonas* densities in drinking water.

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# CONCLUSÕES

–Foi possível o isolamento de microrganismos indicadores em todos os tipos de água (água mineral, do sistema público de abastecimento é águas de poço), sendo que águas de poço apresentam uma densidade relativamente mais alta do que as demais analisadas.

–Espécies de *Aeromonas* foram também isoladas das três fontes de água, havendo correlação positiva e negativa entre *Aeromonas* e coliformes totais em águas de poço e águas do sistema público de abastecimento.

–Houve correlação entre coliformes totais e fecais, mas apenas para águas dos sistema público de abastecimento.

–Foram isoladas 2 espécies de Aeromonas, sendo 4 A. *hydrophila,* 5 A. *jandaei*, e 10 cepas que permaneceram apenas como pertencentes ao gênero *Aeromonas* ( Aeromonas sp.).

–Não houve correlação significativa entre a produção de enzimas extracelulares, citotoxicidade, aderência, formação de biofilme, persistência em águas cloradas e resistência a antibióticos entre os isolados de Aeromonas estudados.

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