

The Occurrence of *Aeromonas* spp. in the Bottled Mineral Water, well Water and Tap Water from the Municipal Supplies

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ABSTRACT

*The aim of this work was to study the occurrence of *Aeromonas* sp in the bottled mineral water, well water and tap water from the municipal supplies. Positive samples were found for *Aeromonas* spp. 12.7% from the mineral water, 8.3% from the artesian water and 6.5% from the tap water. The recovery of *Aeromonas* spp. was significantly higher in the bottled mineral and artesian water than in the tap water from municipal supplies. The occurrence of the *Aeromonas* spp. did not correlate significantly with the contamination indicator bacteria (i.e. total coliforms) in the artesian water samples. However, a significant correlation was found between *Aeromonas* spp. and total coliforms in the both mineral water and tap water samples. The presence or absence of a correlation between the indicator bacteria and *Aeromonas* could reflect the occasional appearance of the pathogen in the 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 mineral water and tap water, respectively, sampled in the current study failed to meet the Brazilian standard for total coliforms in the drinking water should therefore be of concern.*

Key words: *Aeromonas*, drinking water, indicator bacteria, coliforms

INTRODUCTION

The contamination of the natural water with the fecal materials, domestic and industrial sewage and run-off from the pasture and agricultural land may result in an increasing risk of the disease transmission to the human that use these waters (Wiggins, 1996, Nogueira et al., 2003). Therefore, to protect the consumers from the possible

diseases transmitted by the water, it is important to monitor the occurrence levels of the pathogens in such water (Gray, 1994). Although there is concern about the incidence of the coliforms indicator group existing in water, the legislation do not preconize the search of the heterotrophic bacteria that also would be inhabiting the aquatic environment, growing up from the biodegradable organic matter (Haddix et al., 2004), working as

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the indicators of the presence of the toxic substances in the water (Nocciolini et al., 2000). These microorganisms often act as the secondary pathogens, having the capacity to elicit the pathological episodes in the 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 and Carnahan., 1994; Holt et al., 1994), including three genus: *Aeromonas*, *Ocealimonas* e *Tolumonas* (ICSB, 2003). The aeromonads are mesophilic motile or psychrophilic non-motile Gram-negative bacteria (Lee et al., 2002), showing mobility by the polar flagellum (Holt et al., 1994).

Despite the fact that 19 named species have been classified, only few of them are recognized as human pathogens, such as *A. hydrophila* complex, *A. caviae* and *A. veronii*, representing almost 85% of the clinical isolates, although other species have been recovered from the clinical sources (Janda and Abbot, 1998). Aeromonads are also found in a variety of sources, such as the food (Granum et al., 1998; Cansian et al., 2005; Ullmann et al., 2005; Martin Talavea et al., 2006), natural water (Ghenghesh et al, 2001; Ørmen and Østensvik, 2001, Sousa and Silva-Souza 2001) and drinking water (Kuhn et al., 1997; Villari et al., 2003), and it is known that psychrophilic species are mainly etiologic agents in the fishes (Garduño et al., 2000). In human immunocompetent hosts, the members of this genus may be responsible for the intense gastroenteritis, called traveler's diarrhea (Janda and Abbot, 1998; Vila et al., 2003), but in the immunocompromised hosts, these bacteria could be implicated in the cases of myonecrosis, bacteremia, septicaemia (Martino et al., 1997) and hepatic cirrhosis and neoplasys (Chien Ko and Chuang, 1995).

The coliforms organisms have long been recognized as a suitable microbial indicator of the drinking-water quality, largely because they are easy to detect and enumerate in the water (WHO, 1993). In the drinking water from the municipal supplies, the coliforms test can be used as an indicator of the treatment efficiency and the integrity of the distribution system. Although the coliforms organisms may not always be directly associated to the presence of the fecal contamination, the presence of the coliforms in the

drinking water suggests the potential presence of the pathogenic enteric microorganisms such as *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae*. Coliforms bacteria are the only microbiological contamination to be regulated by the federal law in both the tap and bottled water.

According to the Brazilian directives (ANVISA, 2004), at least 40 samples per month of the 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 the waterworks with surface water source and one sample every two weeks for the waterworks with a ground water source the indicator must not be detectable in any 100-ml sample.

The objective of this study was to determine the frequency of *Aeromonas* species in the bottled mineral, artesian water and tap water, as well as their correlation with the indicator bacteria.

MATERIALS AND METHODS

Water samples and microbial enumeration

A total of 238 samples of the drinking water taken from the bottled mineral water, artesian water and tap water from the municipal systems were investigated for *Aeromonas*. The mineral waters were purchased at the local supermarkets and, wherever appropriate, the bottles representing different batches within one brand were purchased. The samples of the artesian water were collected from the residences and workplaces of the city of Maringá supplied by the alternative water systems. After membrane the filtration of 250 ml samples, the filters (Millipore HC filter, pore size 0.45 µm) were placed on the M-Endo Agar LES (M-Endo) to enumerate the total coliforms and on the M-Fecal Coliforms (M-FC) to enumerate the thermotolerant coliforms, incubated at 37° C for 24 h, and at 44.5° C for 24 h, respectively (APHA, 1995). After the incubation period, the typical coliforms colonies were confirmed in Lauryl Tryptose and EC broth. Atypical colonies (lactose-negative red or transparent in M-Endo and orange or transparent in M-FC) 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). The presumptive

Aeromonas isolates were confirmed by the PCR-based assay as described below.

Molecular identification

The molecular identification, as the 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. The presumptive *Aeromonas* genus isolated from the different water sources were examined. For genus-specific analysis, the DNA was extracted from the each bacterial sample (including the reference strains) by boiling during 10 min. After the boiling, the DNA was stored at -4°C until application on the PCR experiments. The PCRs were carried out on a thermal cycler Primus 96 Plus (MWGAG - Biotech). A 20 µl containing a final concentration of 50 mM KCl, 2mM Tris-HCl (ph 8.4), 1.5 mM MgCl₂, 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. The 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, the extension at 72°C was allowed for 10 min. The PCR products were precipitated with the addition of 10 % volume of 3.0 M sodium acetate and 200 µl of cold ethanol, dried and suspended on 25 µl of the sterile distilled water. Endonuclease digestion, electrophoresis and pattern analysis were

performed as described by Borrel et al., 1997, with the modifications. The enzymatic digestion was 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 incubated overnight at 37°C. The electrophoresis on 10% polyacrilamida gel in 1X TBE (tris-borate-EDTA) buffer, for 2.5 hours on 70 V was made on aliquots of 10 µl of each restriction reaction. The gels were stained by the 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 the linear regression and t-test at p< 0.05 and 0.005 of confidence level using the Statistical Analysis System software.

RESULTS AND DISCUSSION

In the present study, the positive samples for *Aeromonas* were 12.7% from the mineral water, 8.3% from the artesian water and 6.5% from the tap water (Table 1). The recovery of *Aeromonas* was significantly higher in the bottled mineral and artesian water than in the tap water from municipal supplies. The studies of *Aeromonas* spp. in drinking water and even in the chlorinated water supplies showed that this bacterium was isolated 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)
<i>Aeromonas</i> 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)

^a20 l bottles

^bMost-often-used faucet

A correlation coefficient matrix was established to compare the degree of the association between *Aeromonas* spp. and the indicator bacteria (Table

2). Different results were observed with the samples from the bottled mineral water, artesian water and tap water from the municipal supply.

The occurrence of the species of *Aeromonas* did not correlate significantly with the indicator bacteria in the mineral water samples. The lack of correlation between *Aeromonas* and total coliforms suggested that the two microorganisms could indicate different source of the pollution. However, a significant correlation was found between

Aeromonas and total coliforms in both the tap water and well water samples. The absence of a correlation between the indicator bacteria and *Aeromonas* could reflect the occasional appearance of the pathogens in the drinking water and the different rates of the 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	
TC ^c	0.1981	0.000	0.4677*	0.3669	0.4472*	0.5291*
FC ^d	0.000	-	0.2941	-	0.1690	
	<i>Aeromonas</i>	FC	<i>Aeromonas</i>	FC	<i>Aeromonas</i>	FC

Significance level: * $p < 0.05$. ^a 20-litre bottles on water dispensers. ^b Municipal supply. ^c Total coliform group. ^d Fecal coliform group.

Other authors have found that the coliforms counts did not correlate with *Aeromonas* spp. (Legnani et al., 1998). Massa et al., (2001) reported that *Aeromonas* spp. could be good indicator of the hygienic quality of the water. They found a large number of *Aeromonas* in water, consequently their search should be used to indicate the unsatisfying conditions, especially in the private water systems that did not undergo systematic chlorine treatment. The finding that 41.6, 14.8 and 9.0% of the artesian water, bottled water and tap water (Table 1), respectively, sampled in the current study failed to meet the Brazilian standard for the total coliforms in the 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 made about the classification of the aeromonads, including the structural features, such as the 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), on the members of this genus exhibited 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 corresponded to 1-32 base differences (Martínez-Murcia, 1992), could be enough to distinguish among the species of *Aeromonas* genus until species level by restriction fragments length polymorphism techniques (RFLP) of the amplicons of this conserved sequence (Borrel et al., 1997).

A total of 19 strains were isolated from M-Endo and M-FC media. The isolates were identified to the species levels by the RFLP-based method. The frequency of isolated is shown in Table 3. *Aeromonas* spp. identified to the species level were *A. hydrophila* and *A. jandaei*. For the other strains, 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
<i>Aeromonas hydrophila</i>	2	0	2
<i>Aeromonas jandaei</i>	1	1	3
<i>Aeromonas</i> spp.	3	1	6

The drinking water and food are reservoirs of *Aeromonas* species and therefore may be important sources of human infections. The bacteria of the genus *Aeromonas* have been frequently recognized as responsible for several diseases, both in the human and animals (Cahil, 1990; Krovacek et al., 1995). *A. hydrophyla*, *A. caviae* and *A. sobria* have been linked to two major groups of human diseases: septicemia and gastroenteritis (Merino et al., 1999). A greater risk of the infection is reported in the 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 the hemolysins and proteases. Another important attribute of the 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 the water is unclear. Therefore, there is an urgent need to compare the effects of the strains belonging to each of these species in a wider range of the animal models of the infection. These results can contribute to the re-evaluation of the criteria used to determine the microbial quality of the drinking water and to define the measures for limiting *Aeromonas* densities in the drinking water.

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RESUMO

A porcentagem de amostras positivas para *Aeromonas* foi de 12.7% para água mineral, 8.3%

para água de poço artesiano e 6.5% para água do sistema público de abastecimento. O isolamento de *Aeromonas* spp. foi significativamente maior em água mineral e água de poço artesiano do que em água do sistema público. A ocorrência de *Aeromonas* spp. não teve correlação significativa com os indicadores de contaminação tradicionalmente utilizados (coliformes totais) em amostras de água de poço artesiano. No entanto, esta correlação foi positiva e significativa em água mineral e água do sistema público. A presença ou ausência de correlação entre bactérias indicadoras e a presença de *Aeromonas* pode refletir o aparecimento ocasional de patógenos em águas para consumo humano e as diferentes taxas de sobrevivência e isolamento destes agentes comparados com os indicadores fecais de contaminação. A constatação de que 41.6%, 14.8% e 9.0% respectivamente amostras de água de poço, água mineral e água do abastecimento público utilizadas neste estudo apresentaram índices de coliformes maiores do que os aceitáveis pela legislação brasileira é um fato preocupante. Estes números mostram a necessidade de melhoria nos sistemas de monitoramento para a indústria de águas minerais e o sistema público de abastecimento. As cepas isoladas pertencentes ao gênero *Aeromonas* foram identificadas ao nível de espécie como *A. hydrophila* e *A. jandaei*. A significância do grande número de isolamentos de espécies de *Aeromonas* em saúde pública ainda não está clara. É necessário o estudo dos efeitos de cepas específicas utilizando modelos animais de infecção. Estes resultados podem contribuir para a reavaliação dos critérios empregados para a análise da qualidade microbiológica da água e a definição de limites de densidades para o gênero *Aeromonas* em águas destinadas ao consumo humano.

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