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ANTIMICROBIAL SUSCEPTIBILITY, HEMOLYSIS, AND HEMAGGLUTINATION AMONG *AEROMONAS* SPP. ISOLATED FROM WATER OF A BOVINE ABATTOIR

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ABSTRACT

The presence of *Aeromonas* spp. in food has been demonstrated. They are often introduced from water, which is the natural habitat of many species and is thought to be the main source of contamination. The occurrence of *Aeromonas* spp. was investigated in 70 water samples of a bovine abattoir. *Aeromonas* spp. were present in 21.4% of water samples. *A. hydrophila* was isolated from 11.4% of supplying water samples and from 25.7% of the water drained from carcasses, whereas *A. sobria* was sole isolated from 5.7% of supplying water samples. Although greater number of positive samples were from water drained from carcasses, only *A. hydrophila* isolates were present. This suggests that the supplying water is the source of contamination. The antibiotic susceptibility testing revealed all strains were resistant to b-lactam antibiotics. However, the susceptibility to other antimicrobials was variable, being *A. hydrophila* more resistant than *A. sobria* strains. The investigation for virulence factors revealed positive reactions of hemolysis and hemagglutination. The results indicate that *Aeromonas* spp. are present in the supplying water system. These microorganisms may be a potential contaminant of carcasses and widespread in derived food.

Key words: *Aeromonas*, antibiotic activity, aquatic environment, hemolysis, water.

INTRODUCTION

Aeromonas spp. are widely distributed bacteria frequently isolated from natural habitats such as fresh and brackish water, sewage, wastewater and soil (3). This group of bacteria has also been found in food and is associated with a variety of infections (2).

The taxonomy of aeromonads has become more complicated, with the description of many new species. The genus *Aeromonas* belongs to *Vibrionaceae* family and is divided into two groups based on motility and growth temperature characteristics, although some authors consider the possibility to group the genus in a family, denominated Aeromonadaceae (12). The species are grouped in: non-motile and psicrophiles with the unique *A. salmonicida* subspecies *salmonicida*, *chromogenes* and *masoucida*; and motile and mesophiles with three other species, *A. caviae*, *A. sobria* (also denominated *A. veronii* biotype Sobria) and *A. hydrophila* (31).

Recent investigations have indicated species of *Aeromonas* as an emerging pathogen in enteric infections (2). Some studies on the ecology of gastroenteritis caused by *Aeromonas* spp. has been focused on the possible transmission by contaminated drinking water (16,18). The presence of this organism, particularly *A. hydrophila* and *A. sobria*, in drinking water is considered a relevant factor, since it is associated to digestive tract disorders (24,25). Minimal chlorine levels must be established to the adequate treatment in water stations (8,9,26).

The presence of *Aeromonas* spp. in food is also reported (10,21), which increases the risks to public health. A part of contamination is related to manipulation with contaminated waters. Buchanan and Palumbo (4) state that 35-40% of annually notified gastroenteritis cases in the USA are from uncertain origin, where in several cases would be caused by bacteria which are not determined in the outbreak investigation. In the greater part of those cases, motile species of *Aeromonas* are assumed to be the responsible agent (21).

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Several authors pointed the important role of *Aeromonas* as causal agent of human extraintestinal infections, mainly in immunologically compromised individuals, young children, and elderly people. This microorganism has been implicated in wound infections, meningitis, pericarditis, endocarditis, septicemia, urinary infections, and respiratory tract infections (2,12,24). The motile species of *Aeromonas* are generally pathogenic to humans and animals. Some species of *Aeromonas* are among the agents of gangrenous sore and burn-associated sepsis (26).

Regarding the resistance to antimicrobial agents, several authors state that *Aeromonas* species are rapidly adapting to new drugs commonly used in medicine (5,14,30), becoming a potential risk to public health. In addition, genetical analysis of representative species demonstrate that such resistance and new virulence factors are chromosomally mediated (15,20,23,31). A broad variety of virulence factors are produced by *Aeromonas* spp., including hemolysins, hemoagglutinins, cytotoxins, enterotoxins, proteases, leucocidins, phospholipases, among others. These virulence factors do influence the pathogenicity of infections becoming, by this reason, more complex cases (11).

The objective of this work was to determine the occurrence of *Aeromonas* spp. in supplying water and drained water after the final washing of carcasses of a bovine slaughterhouse. The susceptibility to antimicrobial agents, and hemolytic and hemagglutination activities were also investigated.

MATERIALS AND METHODS

Samples

All samples were collected from an abattoir in Porto Alegre, Brazil. Samples were placed in 200 mL screw-taped sterile flasks. An aliquot of 0.1 mL sodium tiosulfate 10% (w/v) solution was added to each 100 mL water sample for neutralization of chlorine.

Samples of water drained from carcasses were collected during the final washing process, from the external area next to the neck. The samples of supplying water were collected along the killing line, in points where water valves were present. Water supply was from a municipal distribution system. Treated water was stored in roofed service reservoirs and entered the distribution system without further treatment.

Samples were vacuum filtered throughout 47 mm sterile cellulose filters (Millipore, Bedford) with 0.22 µm porosity. To enrichment of cultures, the membranes in which the filtrate was retained were aseptically inoculated in 250 mL of Trypticase Soy Broth (Difco, Detroit), and aerobically incubated at 28ºC for 12-24 hs.

Strain identification

Serial dilutions of enriched cultures were done until 10^{-6}. Aliquots of 0.1 mL of each dilution were removed and loaded on *Pseudomonas-Aeromonas* selective agar (31) plates, and incubated for 24 hs at 28ºC. After incubation, the plates presenting yellow colonies of 2-3 mm, rounded by a yellow precipitation zone, suggesting the genus *Aeromonas*, were selected. Simultaneously, aliquots were also streaked onto blood agar (5% sheep blood).

Species identification was conducted based on colony morphology, cell morphology, and biochemical tests (12,13,16,31). The morphological, cultural and physiological characteristics of the isolated bacteria were compared with data from *Bergey's Manual of Systematic Bacteriology* (31).

Antimicrobial susceptibility testing

The susceptibility to antibiotics was determined by the Kirby-Bauer modified method (19). The isolates were incubated at 28ºC for 5 h in BHI broth (Difco, Detroit) to form culture with OD 0.1 at 600nm. A sterile swab was immersed in the bacterial suspension, and used to inoculate Müeller-Hinton agar plates (Merck, Darmstadt). Antibiotic disks (Oxoid, Basingstoke) were applied using a sterile forceps. After 24h at 28ºC, inhibition zones around the disks were measured and the values were compared with standard data for antimicrobial susceptibility (29). For the purpose of typing, a numerical code profile was created based on antibiotic resistance of each isolate. Resistance was classified as 1, intermediate resistance as 2, and susceptibility as 3 (22).

Hemolysis and hemagglutination assays

The hemolysis and hemagglutination properties of the isolates were verified with rabbit and ovine erythrocytes. For hemolysis tests, erythrocytes were centrifuged (3,400 g for 10 min at 4ºC), washed three times and resuspended in sterile 20 mM phosphate buffered saline (PBS, pH 7.2), to obtain a 2 % (v/v) suspension. Bacterial samples were cultured in 5 mL of Trypticase soy broth, supplemented with 6 g L^{-1} yeast extract and incubated at 28ºC for 16-18 h. Culture supernatants were carefully removed after centrifugation at 10,000 g for 30 min at 4ºC, and mixed with an equal volume of a 2% (v/v) erythrocyte suspension. The mixture was incubated for 2 h at 28ºC. Erythrocyte suspension in PBS was included in each assay as a negative control. Hemolysis was observed by visual inspection. A clinical isolate of *Aeromonas* with known hemolytic activity was used as positive control.

The hemagglutination test was carried out essentially as described by Burke *et al*. (7), with minor modifications. Strains were grown on Trypticase soy agar and then were suspended in 0.5 mL sterile PBS. A volume of 50µL was mixed with the same volume of a 6 % (v/v) washed erythrocyte suspension on a white porcelain tile. A negative control of erythrocytes suspension and PBS was done. The reactions were visualized in bright-field microscope, and considered positive if agglutination occurred within 10-15 min incubation.

RESULTS

Fifteen strains of *Aeromonas* were isolated and identified as *A. hydrophila* and *A. sobria*. The distribution of this isolates according the nature of the sample is presented in Table 1. The results shown the occurrence of *Aeromonas* were detected in 17% samples of water supply, among them, 4 isolates of *A. hydrophila* and 2 isolates of *A. sobria* were obtained. *A. hydrophila* was the unique specie found in the samples of water drained from carcasses. It was present in 25% of these samples.

The isolates were individually tested against 32 antimicrobial agents. The results were obtained by measuring the inhibition zone after 24 h. The percentage of antimicrobial sensibility of isolates of *Aeromonas* to the antibiotics is shown in Table 2. Antibiotic susceptibility testing revealed that all the 15 *Aeromonas* isolates were susceptible to amikacin, azithromycin, cephalexin, ceftriaxone, cefoperazone, espiramicin, gentamicin, norfloxacin, ofloxacin, pefloxacin, tetracycline, and tobramycin. None of the isolates was susceptible to all antibiotics tested. The predominant resistance property observed was to b-lactam antibiotics, either alone or in combination with resistance to other antimicrobials. *A. hydrophila* strains were less sensible against all antimicrobials tested.

The analysis of antibiograms, considering nine selected antimicrobial agents, shown the isolates could be divided into 3 different resistance patterns (Table 3). The *A. sobria* isolates, and 10 (77%) of the *A. hydrophila* isolates belong to the phenotype A. All isolates from supplying water were phenotype A. The phenotypes B and C grouped only 2 and 1 isolates of water from carcasses, respectively.

To characterize the virulence factors of the isolates the techniques of hemagglutination and hemolysis with ovine and rabbit erythrocytes were used. The results are presented in Table 4. Positive reactions of hemolysis and hemagglutination were observed for both species when confronted the aeromonas samples with the erythrocyte suspensions. Hemolysin production was also observed on sheep blood agar.

Table 1. Absolute values and percentual of *Aeromonas* species identified from samples of water supply and water from carcasses washing.

Origin	Samples	Positive samples (%)	Species (%)
Water supply	35	6 (17.1)	*A. hydrophila* (11.4) *A. sobria* (5.7)
Water from carcasses washing	35	9 (25.7)	*A. hydrophila* (25.7)
Total	70	15 (21.4)	

Table 2. Antimicrobial susceptibility of *Aeromonas* isolated at a bovine abattoir.

Antibiotic (µg/disk)	% susceptible isolates	
	A. hydrophila	*A. sobria*
Amikacin (30)	100	100
Ampicillin (10)	0	0
Amoxicillin (20)	0	0
Azithromycin (15)	100	100
Cefaclor (30)	92.3	100
Cefadroxil (30)	0	0
Cephalexin (30)	100	100
Cephalothin (30)	0	0
Ceftriaxone (30)	100	100
Cephazolin (30)	0	50
Cefoperazone (75)	100	100
Clindamycin (2)	0	100
Chloramphenicol (30)	76.9	100
Doxycycline (30)	76.9	100
Erythromycin (15)	46.1	50
Espiramicin (15)	100	100
Gentamicin (10)	100	100
Kanamycin (30)	0	0
Lincomycin (2)	0	50
Nalidixic Acid (30)	0	0
Norfloxacin (10)	100	100
Ofloxacin (5)	100	100
Oxacillin (1)	0	0
Penicillin (10 UI)	0	0
Pefloxacin (5)	100	100
Polymyxin B (300 UI)	0	0
Streptomycin (10)	92.3	100
Sulfametrim* (23.75/1.25)	84.6	100
Sufonamide (250)	76.9	100
Tetracycline (30)	100	100
Trimethoprim (5)	46.1	50
Tobramycin (10)	100	100

* Association of sulphamethoxazole + Trimethoprim.

DISCUSSION

The incidence of different species of *Aeromonas* isolated from food or clinical cases, as well as their virulence-associated properties, varies greatly according the geographic region. Several studies have reported the isolating of *Aeromonas* from food, however they often present significant differences in the homogeneity and frequency of such findings. In the occurrence of food-borne isolates, the main source of infection is thought to be water (21).

The viability and stability of this organism in food is mostly favored by neutral pH, humidity, and mainly by temperature, considering its very wide tolerance range, from 5ºC to 42ºC (8,10,21). In this case, only pre-cooling of bovine meat do not

Table 3. Phenotype patterns of *Aeromonas* species isolated from a bovine abbatoir according to their antimicrobial susceptibility pattern.

Specie / isolates	Sensibility pattern 1 2 3 4 5 6 7 8 9	Phenotype
A. hydrophila (n=13)		
SW1, SW2, SW3, SW4, CW5, CW9, W10,CW11, CW12, CW13	1 3 3 3 3 3 3 3 3	A
CW6, CW8	1 2 2 3 3 3 3 3 3	B
CW7	1 1 1 3 3 3 3 3 3	C
A. sobria (n=2)		
SW1, SW2	1 3 3 3 3 3 3 3 3	A

SW - Water supply; CW - Carcasses washing; 1 - R, 2 - I, 3 - S; Antibiotics: 1- Cefalotin, 2- Chloranphenicol, 3- Sulfametrim (sulphamethoxazole + Trimethoprim), 4- Gentamicin, 5- Amikacin, 6- Cephoperazone, 7- Norfloxacin, 8- Tobramycin, 9- Ceftriaxone.

Table 4. Percentage of hemolysis and hemagglutination positive *Aeromonas* isolates.

	% positive isolates			
	A. hydrophila (n=13)		*A. sobria* (n=2)	
Erythrocyte	Hemolysis	Hemagglu-tination	Hemolysis	Hemagglu-tination
Rabbit	69.2 (9)	84.6 (11)	-	100 (2)
Ovine	46.1 (6)	61.5 (8)	50 (1)	50 (1)

imply in a significant reduction of contamination in the final product, becoming a potential reservoir of *Aeromonas* spp. and consequently a source of human infection.

Several reports have shown that species like *A. hydrophila* and *A. sobria*, both found in this study, are related to major incidence of infections, although none of them were from fecal contamination, i.e., they were extraenteral (24). Other species as *A. trota*, *A. veronii* biotype Veronii and *A. jandaei* are normally isolated from feces of diarrheic infected patients. These last species are often associated with gastrenteritis (12). *A. caviae* and *A. schubertii* are species generally associated with extraintestinal disease (wounds and bacteremia) (1). The isolation of *A. hydrophila* in this study is consistent with the fact this is the more frequently *Aeromonas* specie isolated from treated water (21,26).

Similar pathogenicity aspects of *Aeromonas* species have been described, particularly among *A. hydrophila* and *A. sobria*, which are producers of extracellular toxins (7,11,21). However, great differences are reported with regard to antimicrobial susceptibility pattern. The isolates were susceptible to the aminoglycosides tested, excluding kanamycin. This finding was similar to that reported by Overman and Janda (30) for other species of *Aeromonas*.

Most *Aeromonas* strains are resistant to penicillin and ampicillin but susceptible to most other drugs, including third-generation cephalosporins (28,32). All isolates were resistant to b-lactams. Among the cephalosporins tested, 100% resistance was only noticed against cefadroxil and cephalothin, although partial resistance against cephazolin and cefaclor was observed. Almost 90% resistance to cephalothin was detected among *A. hydrophila* strains isolated from wastewater (20), but resistances to other cephalosporins were scarce among aeromonads from water sources (15,20,27). These results are also comparable to observed for clinical isolates of *Aeromonas* (5,14,23,30).

The susceptibility to macrolides was variable. Other antimicrobials, such as chloramphenicol, tetracyclins, and sulfonamides, including the associations, presented a very high effectiveness, like previously reported data (14,20). *Aeromonas* spp. isolated from freshwater were highly susceptible to chloramphenicol, tetracycline, nalidixic acid, and trimethropim-sulphametoxazole (27). On contrast, increased resistance to tetracyclins and trimethropim-sulphametoxazole has been described for some clinical isolates of *Aeromonas* (23).

Except for nalidixic acid, which showed no inhibitory activity, it was observed a great susceptibility to the quinolone group. This was similar to the results of Imziln *et al*. (20) and Ko *et al*. (23). However, resistance to quinolones by *Aeromonas* isolated from urban effluent has been described (15). It is believed that multiple resistance of *Aeromonas* spp. may be mediated by enzymatic conduction and by selective environmental pressure, caused by large scale prescriptions.

By antibiotic susceptibility typing, only three phenotypes were found, suggesting they are from similar origin. *Aeromonas* species may easily develop resistance in presence of low concentrations of antibiotics (15). Since the strains were isolated from the same environment, they could develop a similar phenotype characteristic by exposition to this environment.

The pathogenicity of *Aeromonas* spp. has been related to a number of putative virulence factors, including the production of exotoxins and hemolysins, and the ability to adhere to and invade epithelial cells (24). It has been demonstrated that *A. hydrophila* adheres and invades the epithelial cells of gut mucosa and starts its multiplication (7,11). This situation only occurs with expression of certain virulence factors as previously cited (11).

The *Aeromonas* strains isolated in this work expressed positive reactions of hemolysis and hemagglutination. The percentage of hemolytic isolates observed in this study was similar to reported data from other groups (7,17). A previous study (6) has shown that it is possible to identify 97% of

Aeromonas enterotoxigenic isolates by biotyping and a hemolysin assay with rabbit erythrocytes. Although hemolysin can not be directly associated with enteropathogenicity, hemolysin production and cytotoxin production are properties that are often associated with gastrointestinal infections caused by aeromonads (26,34). The percentage of isolates producing hemagglutination agrees with a previous report of water-borne *Aeromonas* strains (17).

The production of hemolysis, hemagglutination and cytotoxins, among others, are normally associated to enteral infections caused by species of *Aeromonas*. These isolates presented positive virulence factors and resistance to usual antibiotics. The data obtained in the present study indicate that different *Aeromonas* spp. are able to multiply within treated water distribution systems. These microorganisms could reach the food product, becoming a potential human pathogen and a public health problem.

RESUMO

Perfil de resistência/sensibilidade e virulência de espécies de *Aeromonas* isoladas de amostras de água obtidas em abatedouro bovino

A presença de bactérias do gênero *Aeromonas* nos alimentos tem sido demonstrada. Normalmente destacam-se os alimentos que durante sua industrialização entraram em contato com a água, a qual é tida como habitat natural das diversas espécies e principal fonte de contaminação. Objetivando determinar a ocorrência de espécies de *Aeromonas*, bem como aspectos relacionados a sua virulência como capacidade de hemólise e hemoaglutinação, foram analisadas 70 amostras de água divididas entre água de abastecimento e de escoamento de lavagem das carcaças. De acordo com os resultados obtidos verificou-se a ocorrência de *Aeromonas* spp. em 21,4% das amostras. *A. hydrophila* obteve o maior índice de isolamento, 11,4% em água de abastecimento e 25,7% em águas de escoamento, já *A. sobria* foi isolada somente em água de abastecimento num percentual de 5,7%. O maior número de amostras positivas foram de águas de escoamento, porém com uma única espécie, isto sugere que a fonte de contaminação ainda é a água de abastecimento. Em relação ao teste de sensibilidade, a espécie *A. hydrophila*, apresentou uma maior resistência aos antibióticos testados do que a *A. sobria.* Foram observadas reações positivas de hemólise e hemoaglutinação para ambas espécies. Estes resultados indicam que *Aeromonas* spp. estão presentes no sistema de abastecimento de água, sendo potenciais contaminantes das carcassas e dos produtos derivados.

Palavras-chave: *Aeromonas*, atividade antimicrobiana, ambiente aquático, hemólise.

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