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ANTIBIOTIC RESISTANCE OF POTENTIALLY PATHOGENIC *AEROMONAS* STRAINS

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ABSTRACT

26 *Aeromonas spp.* strains isolated from meat, drinking water, fish and patients in Bulgaria were identified and biochemically characterized. The drug susceptibility levels were evaluated by disk diffusion method and serial dilution method. All examined strains displayed multiple drug resistance. The strains were tested for hemolytic activity as a pathogenicity marker. Hemolysis, tested on sheep erythrocytes, was more frequently seen with water and fish isolates than with patient isolates.
The results obtained suggest that *Aeromonas* species are potential enteric pathogens in our geographical region. The occurrence of *Aeromonas spp.* in the environment represents a potential risk for humans and farmed fish health.

Key words: *Aeromonas spp.*, *MAR index*, *hemolytic activity*.

INTRODUCTION

Aeromonads are Gram-negative ubiquitous, oxidase-positive, facultative anaerobic, glucose-fermenting bacteria that are native to aquatic environments (Hazen et al., 1978). They have been found in brackish, fresh, estuarine, marine, chlorinated and unchlorinated water supplies worldwide, with highest numbers obtained in the warmer months (Van der Kooj et al., 1988; Kaper et al., 1981; Hazen et al., 1978). Aeromonads have been isolated from diseased cold- and warm-blooded animals for over 100 years, and from humans since the early 1950s (Dupont and Mathewson, 1992). The ubiquitous nature of *Aeromonas* species in aquatic environments provides ample opportunity for animals, particularly fish and amphibians, to come into contact with, and to ingest organisms. Such contact may lead to infection which, depending on the species and the virulence of the strains encountered, may have life-threatening consequences.
In view of increasing evidence supporting the role of aeromonads in human and farming fish diseases, we studied the hemolytic activity and antibiotic-resistance of *Aeromonas spp.* strains isolated from drinking water, meat ready for consumption, patients and fish in Bulgaria between June 2004 and October 2007.

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MATERIAL AND METHODS

Identification of bacterial strains.

26 *Aeromonas* strains were isolated from drinking water (n=5), filet ready for consumption (n=2), patients (n=11) and farmed fish (n=8) samples.
Biochemical identification of the strains was carried out by the automatic systems BioMérieux Vitek and Micronaut NF.

Antibiotics susceptibility testing

Standard assay

Resistance of *A. hydrophila* and *A. veronii* biovar *sobria* strains were tested against Ampicillin (A) 10μg, Bacitracin (B) 10UI, Chloramphenicol (C) 30 μg, Ciprofloxacin

(Cp) 5 μg, Dalacin C (clindamicin) (Da)10 μg, Erythromycin (E)15 μg, Gentamycin(G)10μg, Kanamycin (K) 30 μg, Nalidixic acid (Nx) 30 μg, Novobiocin (Nb) 30 μg, Oxacillin (O) 30 μg, Penicillin (P) 10 μg, Rifampicin (R) 5 μg, Streptomycin (S) 30 μg, Tetracyclin (T) 30 μg, Trimethoprim (ST) 1.25 μg and Vancomycin (V) 30 μg by disk diffusion method (Bauer et al.(1966).

Automatic assay

Drug susceptibility was determined also by the broth microdilution method according to NCCLS using Micronaut-S microtitre plates. Standard testing of all isolates was performed with a pure overnight subculture with the MICRONAUT system as recommended by the manufacturer (Merlin, Germany). The testing is performed with 384-well microtiter plates. This system allows the determination of real MICs of up to 25 substances and the testing of two bacterial isolates on one plate. Bacterial growth in the wells is monitored photometrically at a wavelength of 620 nm, and a density above the cutoff value for the respective medium is interpreted to indicate bacterial growth. Several colonies were used to prepare a 0.5-McFarland-standard suspension in 0.9% saline. For the testing of aeromonads, 50 μl of the suspension was diluted in 15 ml of Mueller-Hinton II broth (containing 0.25 g/liter phytagel, an agar substitute produced from bacterial fermentation [Oxoid, Wesel, Germany]). The broth was inoculated onto Merlin MICRONAUT 384-well antimicrobial susceptibility testing plates for Gram-negative bacteria (GN plates designed for the German Network for Antimicrobial Resistance Surveillance (GENARS; www.genars.de), by using the automated Merlin Sprint device. For testing of the majority of antibiotics, the plates contained eight dilutions of the antibiotic for the determination of a real MIC. Breakpoint testing was done with fusidic acid and netilmicin on the GP plate and with aztreonam, cefotiam, mezlocillin, and netilmicin on the GN plate. Inoculated plates were incubated for 18 to 24 h at 36°C under ambient air. Reading of all plates was done with a photometer (Merlin) interpreting an optical density of >0.1 to indicate growth. Obtained MICs were interpreted with the advanced expert system (AES) MCN-6 of Merlin MICRONAUT by using the interpretation guidelines of the German Standardization Institute (Deutsches Institut für Normung) and validated by a clinical microbiologist. Sheep blood agar was inoculated with suspensions from all McFarland standards used for susceptibility testing and incubated at 36°C for 18 to 24 h in order to control for growth, mixed cultures, and possible contamination.

Multiple antibiotic resistance (MAR) index

The multiple antibiotic resistance (MAR) index, when applied to a single isolate, is defined as *a/b*, where *a* represents the number of antibiotics to which the isolate was resistant and *b* represents the number of antibiotics to which the isolate was exposed. MAR index higher than 0.2 identifies organisms that originate from high-risk sources of contamination, where antibiotics are often used (Freeman et al., 1989).

Hemolytic activity

Hemolytic activity was determined as a clear zone of β-hemolysis around colonies on blood agar plates with 5% (v/v) of sheep blood (Brender et al., 1987).

RESULTS

A total of 26 *Aeromonas* strains were isolated as follows: from water samples 5 (19.2%), from patients with clinical symptoms 11(42.3%), meat samples 2 (7.7%) and farmed fish 8 (30.8%). Among the 26 *Aeromonas strains* 23 (88,5%) were *A. hydrophila* and 3 (11,5%) *A. veronii*. All the strains isolated from water (n=5) and fish (n=8) were determined as *A. hydrophila.* The strains isolated from patients were identified as *A. hydrophila* (n=9) and *A. veronii* biovar *sobria* (n=2). The strains isolated from meat were determined as *A. hydrophila* (n=2) and *A. veronii* biovar *sobria* (n=1).

Data on the antibiotic resistance zone encountered by disk diffusion method indicate that all 26 *Aeromonas spp.* isolates were resistant to ampiciline, bacitracine, chloramphenicol, oxacillin, penicilline. 88.5% of the strains were resistant to Novobiocin (*Table 1*). The MAR index of the 26 isolates ranged from 0.23 to 0.53 (*Table 2*) and indicate indiscriminate use of antibiotics in which the microorganisms developed resistance towards several antibiotics.

Table 1. *Antibiotic resistance (%) of Aeromonas hydrophila and Aeromonas veronii biovar sobria strains /disc-diffusion method/.*

Strain	Source	A	T	O	P	G	K	B	C	E	S	Da	Cp	Nx	Nb	R	St	V
Aeromonas hydrophila (n=23)	Water (n=5)	5	1	5	5	0	0	5	0	0	1	1	0	1	2	0	1	1
	Meat (n=1)	1	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0
	Patients (n=9)	9	0	9	9	0	0	9	0	0	3	7	0	4	8	1	1	1
	Fish (n=8)	8	4	8	8	0	0	8	0	0	2	2	0	1	8	0	0	0
Total (%)		**100**	**26**	**100**	**100**	**0**	**0**	**100**	**0**	**0**	**26**	**43**	**0**	**26**	**87**	**4**	**9**	**9**
Aeromonas veronii	Meat (n=1)	1	0	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1
	Patients (n=2)	2	0	2	2	0	0	2	0	0	2	1	0	1	2	2	2	2
Total (%)		**100**	**0**	**100**	**100**	**0**	**0**	**100**	**0**	**0**	**67**	**67**	**0**	**67**	**100**	**0**	**0**	**0**

Legend: Ampicillin (A) 10 µg, Bacitracin (B) 10 UI, Chloramphenicol (C) 30 µg,Ciprofloxacin (Cp) 5 µg, Dalacin C (clindamicin) (Da) 10 µg,Erythromycin (E) 15 µg, Gentamycin(G) 10 µg, Kanamycin (K) 30 µg, Nalidixic acid (Nx) 30 µg, Novobiocin (Nb) 30 µg, Oxacillin (O) 30 µg, Penicillin (P) 10 µg, Rifampicin (R) 5 µg, Streptomycin (S) 30 µg, Tetracyclin (T) 30 µg, Trimethoprim (ST) 1.25 µg, Vancomycin (V) 30 µg.

Table 2. *MAR index of Aeromonas spp. isolated from water, patients, fish and meat (%)*

MAR index	Source				Total
	Water (n=5)	Patients (n=11)	Fishes (n=8)	Meat (n=2)	(n=26)
0,1	0,0	0,0	0,0	0,0	0,0
0,23	20.0	0,0	0,0	0,0	3,9
0,29	20.0	9,1	0,0	0,0	7,7
0,35	20.0	36,4	50.0	50.0	38,6
0,41	20.0	18, 2	0,0	0,0	15,4
0,47	20.0	27,3	0,0	0,0	15,4

The MICs of 23 antibiotics were determined for all strains (*Table 3*). Results obtained from the MICRONAUT system demonstrated that 100% of the strains were resistant to ampicillin, cefacetril, cloxacillin, and penicillin (*Table 3*).

The strains showed high susceptibility rates (>90%) for several antimicrobial agents tested, including neomycin, apramicin, enrofloxacin. The most active compound against *Aeromonas spp*. was apramicin (MIC, ≤16 mg/l, 100% susceptibility), followed by neomycin (MIC, ≤8 mg/l, 92.3% susceptibility,) and enrofloxacin (MIC, ≤1 mg/l, 92.3% susceptibility).

To characterize the virulence potential of the isolates the hemolytic activity of the *Aeromonas spp.* was tested. The results are presented in *Table 4.*

Of 23 tested isolates of *A. hydrophila,* 14 (60.8%) were hemolysin producers. 8 (100 %) of the fish isolates, 2 (22.2%) of the human isolates, 4 (80%) of the water isolates and 1 (100%) of the meat isolates were β-hemolytic. All of the *A. veronii* biovar *sobria* isolates were β-hemolytic to sheep red blood cells.

Table 3. *MIC values and resistance patterns of Aeromonas spp.*

Antimicrobial agent	MIC range (mg/ml)	MIC reviced	Susceptiblity Range MIC	Intermediate range MIC	Resistance range MIC	Resistance %
Ampicillin	0.125-16	>8	NS	NI	=16 (26)***	100
Amoxicillin	0.125-32	>8	NS	(1)**	=16 (25)	**96.1**
Apramycin	8-32	≤16	≤16 **(26)***	NI	NR	0.0
Cefacetril	2-4	>4	NS	NI	=4 (26)	**100.0**
Cefquinome	2-4	≤2	=2 (20)	(2)	=2 (3)	11.5
Ceftiofur	2-8	≤2	=2 (19)	(5)	=2 (7)	26.9
Cloxacillin	1-2	>2	NS	NI	>2 (26)	**100.0**
Colistin	0.5-4	>2	=4 (9)	(5)	=4 (12)	46.2
Enrofloxacin	0.5-2	≤0.5	=0.5 (24)	(1)	=0.5 (1)	3.8
Erythromycin	1-8	>4	=8 (1)	(2)	=8 (23)	88.46
Florfenicol	1-4	≤2	=2 (22)	(2)	=2 (2)	7.7
Gentamycin	1-8	≤1	=1 (20)	(4)	=1(2)	7.7
Lincomycin	1-8	>4	NS	(1)	=8 (25)	**96.2**
Lincomycin/ spectinomycin	4/16- 16/64	≤4/16	=4/16 (15)	(2)	=4/16 (9)	34.6
Neomycin	8-16	≤8	=8 (24)	NI	=8 (2)	7.7
Oxytetracycline	1-8	>4	=8(6)	(2)	=8 (18)	69.2
Penicillin G	0.125-2	>1	NS	NI	=2 (26)	100.0
Sulfamethoxazole	4-152	>152	=152 (1)	(1)	=152(24)	**92.3**
Spiramycin	1-4	>4	NS	(2)	>4(24)	**92.3**
Spectinomycin	8-32	=32	=32 (11)	NI	=32 (15)	57.7
Cotrimoxazole	16-64	≤16	=16 (15)	(3)	=16(7)	30.8
Tetracycline	1-8	≤1	=1 (16)	(3)	=1(7)	26.9
Tylosin	1-2	>1	=2 (2)	NI	=2 (24)	**92.3**

*Legend: NS – non susceptible; NI - non intramediate; NR – non resistant; * susceptible strains (n); ** intramediate strains (n); *** resistant strains (n)*

Table 4. *Hemolytic activity (%) of Aeromonas hydrophila and Aeromonas veronii biovar sobria*

Origin	*Aeromonas hydrophila* (n=23)	*Aeromonas veronii* (n=3)	% positive isolates
meat	1 (1*)	1 (1)	100
water	5 (4)	-	80
patient	9 (2)	2 (2)	36.4
fish	8 (8)	-	100

*Legend: *number of hemolytic Aeromonas strains*

DISCUSSION

Mesophilic aeromonads are among the most common bacteria in water habitats through the world, and these bacteria frequently cause disease in fishes. They are also causative agents of acute diarrhea disease in human. The incidence of different species of *Aeromonas* isolated from food or clinical cases, as well as their virulence-associated properties, varies greatly according to the geographic region. In the occurrence of food-borne isolates, the main source of infection is thought to be water (Kirov, 1998).

The investigation for virulence factors revealed positive reactions of hemolysis. 100% of *A. veronii* biovar *sobria* strains and 60.8% of *A. hydrophila* strains were β-hemolytic. Hemolysis, tested on sheep erythrocytes, was more frequently seen with water, meat and fish isolates than with patient isolates. This suggests that the environmental aeromonads also possess pathogenic potential and may be a potential contaminant of water supplies, carcasses and widespread in derived food. Attention has been given on the

hemolysin of motile *A. hydrophila* because the production of hemolytic toxin regarded as indication of pathogenic potential, though nonhemolytic aeromonads have also been implicated as human pathogens (Namadri and Bottone, 1990).

Antibiotics are generally administered to animals and human prophylactically and therapeutically against microbial diseases, and subtherapeutically as growth promoters (Vaseeharan et al., 2005). Potential consequences of antibiotic use in culture and animal feeds are development of drug-resistant bacteria, transfer of resistant characteristics to bacteria, and reduced efficacy of antibiotic treatment for human and animal diseases (Aoki, 1992; Rhodeset al., 2000). Many fish farms release effluent water from ponds without treatment. Various amounts of antibiotic residues may still be present in the effluent following antibiotic therapy (Smithet al.,1994).

Inappropriate use of antibiotics is likely to cause an unnecessary impact on the environment. Calculation of the MAR-index indicates that most of the *Aeromonas* strains originate from high-risk sources of contamination where antibiotics are often used.

An increasing incidence of multidrug resistance among *Aeromonas spp.* isolates, which are both fish pathogens and emerging opportunistic human pathogens, has been observed worldwide. This can be attributed to the horizontal transfer of mobile genetic elements like plasmids and class 1 integrons (Jacobs and Chenia, 2006). *Aeromonas spp.* are known to be intrinsically susceptible to all antibiotics active against non-fastidious Gram-negative bacilli, except for many β-lactams, due to the production of multiple inducible, chromosomally encoded β-lactamases (Jones and Wilcox, 1995; Rossolini, et al.,1996). In this study all strains were resistant to ampicillin (100% MIC >16mg/l), penicilline (100%), amoxicillin (96.1%, MIC >8), oxacillin (100%), and penicilline G (95%, MIC >1). Obtained results indicate that β-Lactam agent should be avoided in the treatment of *Aeromonas spp.* infections.

Aeromonas spp. usually retain their aminoglycoside susceptibility (Jones and Wilcox, 1995). However, tobramycin-resistant strains (0-10%) have been reported by Chang and Bolton (1987). Some authors (Ko et al., 1996) found as many as 49% tetracycline-resistant *Aeromonas spp.*, compared with 8-22% following data of Jones and Wilcox (1995) and Chang and Bolton (1987). In the present study we observed that 7.7% of the strains were resistant to gentamicin (MIC ≤1 mg/l) and 7.7% of the strains were resistant to neomycin (MIC ≤8 mg/l). 26.9% of the strains were tetracycline-resistant (MIC ≤1mg/l), 12.5 % of the fish isolates and 20 % of the water isolates were resistant to gentamicin, which is similar to the findings of Ansary et al. (1992) who reported that 23.5% of the *A. hydrophila* strains of fish origin were resistant to gentamicin. According to Yucel et al. (2004) 10-54% of the *Aeromonas spp.* strains isolated from fish were resistant to gentamicin.

57.7% of the strains in our experiment were resistant to spectinomycin (MIC=32 mg/ml) but non of the strains was resistant to apramyicin (MIC ≤ 16 mg/l). Chromamphenicol resistance is an extremely rare trait in *Aeromonas spp.* (Jones and Wilcox, 1995). Similarly, all of the investigated strains were susceptible to chloramphenicol. Low-level resistance to tetracycline and chloramphenicol in *Aeromonas spp.* has been shown to result from decreased permeability. Neither sulphamethoxazole nor trimetoprim alone is very active against *Aeromonas spp*., but cotrimoxazole is generally efficient, due to the strong synergy between the drugs (Jones and Wilcox, 1995). Although 30.8% of the strains investigated were resistant to cotrimoxazole, compared with 39-50% as reported by Ko et al., (1996). Goni –Urriza et al., (2000) found total inhibition of the *Aeromonas* strains by 4 mg/l of colistin. In our experiment 42.6% of the investigated strains were resistant to colistin (MIC >2 mg/l). Some of the strains (26.9%) were resistant to nalidixic acid but no one of them exhibited resistance to ciprofloxacin. Only one *A. hydrophila* strain demonstrated resistance to enrofloxacin (MIC ≤0.5 mg/l). *Aeromonas spp.* strains have been found to be uniformly susceptible to quinolones (Chang and Bolton, 1987). The unexpectedly nalidixic acid resistance rate is particularly worrying, since quinolones are first-line drugs against *Aeromonas*-induced infections (Jones and Wilcox, 1995).

CONCLUSION

Results obtained in this study indicate that multiple resistance, particularly to ampicillin, bacitracin, oxacillin, cefacetril and cloxacillin is often seen in *Aeromonas spp.* isolated from

fish, drinking water, patients and meat. Many of the strains isolated from drinking water and meat are haemolytic and multiresistant to more than four antibiotics resembling those of clinical isolates. Antibiotic resistance depends on the stains origin and it is highest among *A. hydrophila* strains, isolated from fishes and water in our experiment. Development of multiple drug resistance in environmental *Aeromonas spp.* is of clinical concern, both because this is most probably the consequence of the increasing and often use of antibiotics, and because these organisms may cause infection in human and fish. The trend of consuming ready to eat semi-raw foods is getting popular. Fishes and row meat may be an important reservoir for *Aeromonas spp.* and of public health significance. Moreover, *Aeromonas spp.* isolated during 2004 from drinking water in Bulgaria have a resistance potential and the antibiotic resistance rates of these strains were similar with those of *Aeromonas* strains isolated from fish and patients with clinical symptoms. It should be kept in mind that these microorganisms in drinking water might be a potential risk for public health. Therefore, the motile aeromonads as causative agents of farming fish diseases need further study.

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