Acta Veterinaria (Beograd), Vol. 52. No. 1, 3-10, 2002.

UDK 619:579.62:579.843.2

ISOLATION OF MOTILE *AEROMONAS SPP.* FROM FISH AND THEIR CYTOTOXIC EFFECT ON VERO CELL CULTURES

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(Received 12. September 2001)

The presence of motile Aeromonas spp. in fish and other sea food on the Belgrade retail market was investigated with the aim of determining the ability of these bacteria to produce and secrete toxins. Nine strains of motile Aeromonas spp. were isolated from seventy-eight food samples. Aer. sobria was identified in three cases, while six of the obtained strains were identified as Aer. hydrophila. Strains of motile Aeromonas spp. from different sources were analysed for cytotoxicity on Vero cell cultures. A cytotoxic effect was detected for all tested strains, but of different intensity.

Key words: cytotoxicity, isolation, motile Aeromonas spp.,Vero cells.

INTRODUCTION

The genus *Aeromonas*, in the family *Vibrionaceae*, comprises non-motile psychrophilic and motile mesophilic aeromonads. The psychrophils are well known as pathogens for fish but not humans. Mesophilic *Aeromonas spp.* occasionally cause wound infections, septicaemia, and gastrointestinal infections in humans. *Aeromonas spp.* are environmental bacteria, widespread in water, sewage and soil, and well recognized as pathogens of fish, reptiles and amphibians. Animals can be fecal carriers of *Aeromonas spp* (Panin, 1993).

The main source of aeromonas infection is water, even chlorinated water as well as sea food (Burke *et al.*, 1984 a,b; Haninen and Siitonen, 1995). Food products like poultry, raw meat, and vegetables also may contain enteropathogenic *Aeromonas spp.,* (Burke *et al.*, 1984a,b). The incidence of *Aeromonas spp.* in food is high but the majority of *Aeromonas spp.* isolates from food are not enteropathogenic strains (e. g. *Aeromonas caviae*), (Merino *et al.*, 1995). The enteropathogenic strains are mainly represented by *Aeromonas veroni sobria* and *Aeromonas hydrophila*. Food-borne gastroenteritis associated with *Aeromonas spp.* has been reported in humans from all age groups and is particularly severe in risk populations like very young children and old immunocompromised patients. It is important that *Aeromonas spp.* found in food are able to produce different exotoxins, some of which are clearly enterotoxins.

A correlation at a highly significant level between cytotoxicity and virulence in suckling mice was observed and it is of interest that the production of cytotoxin

has also been correlated with gastroenteritis (Millership *et al.*, 1986; Wong *et al.*, 1996). Vero cells are reported to be the most sensitive cell line for the detection of aeromonas cytotoxin (Barer *et al.*, 1986). Toxin production appears to contribute to virulence but is not the only factor that defines virulence. The main additional virulence factors of *Aeromonas spp.* that can be associated with gastroenteritis are: endotoxin (LPS-lipopolysaccharide), S-layers, and fimbriae (adhesins), (Merino *et al.*, 1995).

This study was carried out to investigate the presence of motile *Aeromonas spp.* mainly in fish on sale in Belgrade examine the ability of these bacteria to produce and secrete factors/toxins and to determine their cytotoxic effects on cultured cells.

MATERIALS AND METHODS

Sample collection

Samples were collected from January through June 1998 from the retail market in Belgrade. Samples were: freshwater fish (14 samples), saltwater fish (56 samples) and sea-food (8 samples). The freshwater samples were mainly salmon and trout, the saltwater mackerel, and the sea-food squid. Most of the saltwater fish samples were frozen, while freshwater fish were mainly fresh or just cooled. After the skin surface had been removed 25g of meat was taken aseptically and pooled in sterile Stomacher bags for bacteriological analyses.

Media

Selective enrichment broth: alkaline peptone water, selective agar, starch ampicillin agar. Confirmatory test media: *Aeromonas hydrophila* medium, Vibriostatic test agar (O/129).

Microorganisms

The microorganism collection came from different sources: fish meat-6 strains of *Aer. hydrophila,* 3 strains of *Aer. sobria* (Faculty of Veterinary Medicine, Belgrade); poultry meat- 2 strains of *Aer. hydrophila* (Institute for Meat Hygiene and Technology, Belgrade); water- 1 strain of *Aer. hydrophila* (Serbian Public Health Institute) and from a human/clinical source - 1 strain of *Aer. hydrophila* CCM 4528 (Czech Collection of Microorganisms).

Cell Culture

The assay for cytotoxicity was performed using Buffalo Green Monkey (Vero) cells.

Bacteriological Analyses

The initial sample (25g) was homogenised in alkaline peptone water (225ml), and incubated aerobically at $28 \pm 1^{\circ}C$ for 24h. 10µl loopfuls of enrichment broth were streaked on to dried starch ampicillin agar plates to obtain isolated colonies and incubated aerobically at $28 \pm 1^{\circ}C$ for 24h. Yellow to honey-coloured colonies, surrounded by a cloudy zone of starch hydrolysis were recorded as presumptive positive *Aeromonas spp*. Presumptive positive colonies were confirmed as motile aeromonads with confirmatory tests: *Aeromonas hydrophila* medium, Vibriostatic test agar (resistance to O/129), Gram stain (Gram-negative, rods), oxidase (+), catalase (+). To further classify the colonies at the species

level, the following were used: ATB system (miniAPI instrument) with ID 32 GN strips (BioMerieux).

Assay for cytotoxicity (CTE)

Twelve *Aeromonas spp.* strains from different sources were assessed for cytotoxic activity using Buffalo Green Monkey (Vero) cells. The bacteria were subcultured into 10 ml of brain-heart infusion broth and incubated for 18h at 28±1 °C on a horizontal shaker at 150 rpm. The cell suspension was then harvested by centrifugation at 10.000 x g for 10 minutes and the supernatant was sterilised by filtration through a 0.45μm filter. Filtrates added to Vero cell monolayers and cytotoxicity (CTE) estimated after 45 minutes to 24h at 37±1°C. The appearance of vacuoles, cell pigmentation, intensive light refraction, cell rounding and detachment of Vero cells under the light microscope were interpreted as evidence for cytotoxic activity.

RESULTS AND DISCUSSION

Detection of motile Aeromonas spp. in food samples

Nine strains of motile *Aeromonas spp.* were isolated and identified from seventy-eight samples of food. The most common species of motile aeromonads found was *Aer. hydrophila* which was represented with 6 strains. The remaining three isolated strains were identified as *Aer. sobria*. As shown in Table 1, most isolates were obtained from freshwater fish and only one from saltwater fish. No isolates were recovered from squid sea-food.

Table 1. *Aeromonas spp.* isolated from the examined food samples

Type of sample	No. of samples tested	Species isolated	No.of strains isolated	Label of obtained isolates
Freshwater fish	14	Aer. hydrophila	6	1/3/11/15/19/30
		Aer. sobria	2	7/17
Saltwater fish	56	Aer. sobria	1	25
Sea-food	8	-	-	-
Total	78	Total	9	

Aeromonas cytotoxicity

Twelve strains of motile *Aeromonas spp.* (3 strains of *Aer. sobria* and 9 strains of *Aer. hydrophila*) from different sources were analysed. The morphological changes in Vero cells caused by supernatant fluids from the examined motile *Aeromonas spp.* were very similar and toxogenic. The cytotoxic activity of *Aer. hydrophila* strain on Vero cells is shown Figure 1. CTE was evident 45 minutes after inoculation of the supernatant. Supernatants of two strains induced complete CTE (++++) of Vero cell monolayers 4-5h and one more strain 24h after inoculation. Very strong CTE after 24h of incubation was induced by seven

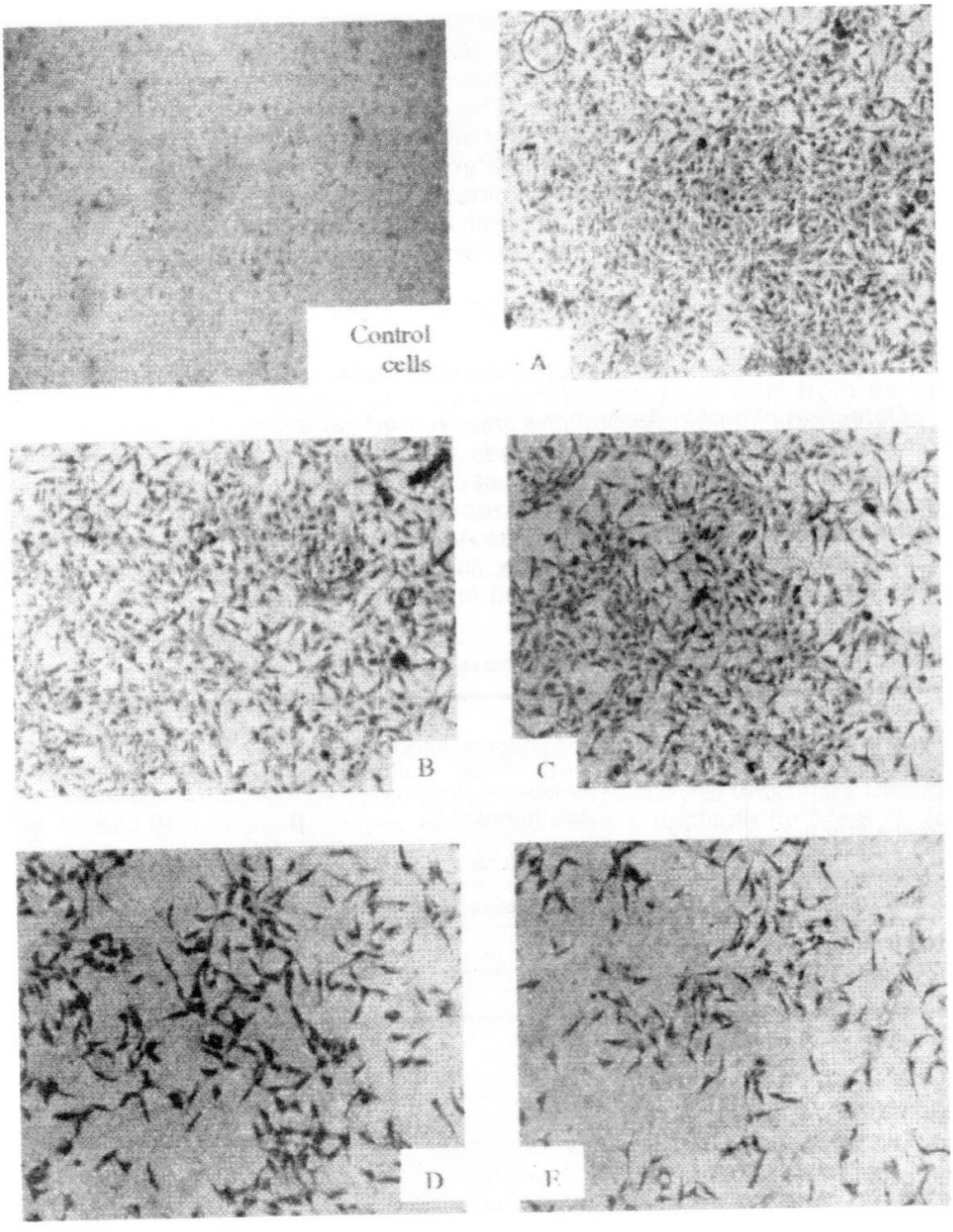

Figure 1. Cytotoxic activity of *Aer. hydrophila* strain on Vero cells. A/B/C/D/E - cells treated with supernatant of strain 3

supernatants and only two supernatants induced slight changes (+) in the cultured cells (Table 2). Thus, most supernatants induced very strong CTE (+++) on Vero cells during the incubation period.

Table 2. The relative intensity of cytotoxic changes (**CTE**) in Vero cells induced by supernatants of *Aeromonas spp.*

Intensity of **CTE**	**CTE** on Vero cell monolayer					
	45'-2h		4-5h		12-24h	
	No of strains	%	No of strains	%	No of strains	%
-	0	0	0	0	0	0
+	1	8.33	1	8.33	2	16.66
++	4	33.33	1	8.33	0	0
+++	7	58.33	8	66.66	7	58.33
++++	0	0	2	16.66	3	25
In total	12	99.99	12	99.98	12	99.99

- without **CTE**
+ slight **CTE**
++ strong **CTE**
+++ very strong **CTE**
++++ complete **CTE**

Table 3. **CTE** of *Aer. sobria* supernatants

Label of strain	Source	**CTE** on Vero cell monolayer		
		45–2h	4-5h	12-24h
7	Freshwater fish	+++	+++	+++
17		++	+++	+++
25	Saltwater fish	++	+	+
Broth control		-		
Vero cell control		-		

- without changes
+ slight **CTE**
++ strong **CTE**
+++ very strong **CTE**
++++ complete **CTE**

The cytotoxic effects on Vero cells are presented separately in Table 3 for *Aer. sobria* and in Table 4 for *Aer. hydrophila* strains, including positive and negative controls.

Table 4. **CTE** of *Aer. hydrophila* supernatants

Label of strain	Source	**CTE** on Vero cell monolayer		
		45–2h	4-5h	12-24h
3	Freshwater fish	+++	++++	
11		+	++	+
15		+++	+++	++++
19		+++	+++	+++
30		+++	+++	+++
101	Poultry	++	+++	+++
102		+++	+++	+++
1019	Water	++	+++	+++
CCM 4528	Human/clinical	+++	++++	
	Broth control	-		
	Vero cell control	-		

- without changes
+ slight **CTE**
++ strong **CTE**
+++ very strong **CTE**
++++ complete **CTE**

Many Gram-negative bacterial pathogens synthesize cytolytic toxins as virulence factors. *Aer. hydrophila* and *Aer. sobria* have received increasing attention because of their frequent association with human diseases including diarrhea, wound infections, septicaemia etc. (Albrecht, 1996). Both species secrete a cytotoxic, poreforming haemolysin called aerolysin which appears to be largely responsible for the virulence of these bacteria. Most Aeromonas toxins are thought to be involved in diarrheal diseases (Krovaceck *et al.* 1994) and the effect of diarrhogenic toxins is alteration of intestinal function. Wong *et al.* (1996) observed a correlation at a highly significant level between cytotoxicity and virulence in suckling mice but other proposed virulence factors, such as LPS and β-haemolysis were found not to be predictors of virulence of aeromonads in the model they used. Vero-cytotoxic activity was detected in all virulent aeromonas isolates tested, and at a low level in one of six avirulent strains (Wong *et al.*, 1996).

Out of the total of seventy-eight samples of freshwater and saltwater fish and cephalopodas examined, motile *Aeromonas spp.*, were found out in nine samples. *Aer. sobria* was identified in three out of nine isolates, while six were identified as

Aer. hydrophila. All the tested filtrates of *Aer. sobria* and *Aer. hydrophila* caused cytotoxic effects in Vero cells, but the intensity of changes differed between filtrates.

Acknowledgements

We thank Dr Goca Vasić and spec. Milan Radović (from the Serbian Public Health Institute) for identification of isolated strains by the ATB system; Dr Dejan Krnjajić (Faculty of Veterinary Medicine, Belgrade) for helpful discussions and technical assistance in photographing CTE on Vero Cells cultures and Rada Atanacković (Faculty of Veterinary Medicine, Belgrade) for technical assistance with the Vero cell cultures.

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IZOLACIJA POKRETNIH AEROMONAS VRSTA I NJIHOV CITOTOKSIČNI EFEKAT NA VERO ĆELIJE

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SADRŽAJ

Cilj ovoga rada je bilo utvrđivanje prisustva pokretnih *Aeromonas* vrsta u uzorcima ribe i drugim plodovima voda na Beogradskom tržištu. Takođe je vršeno određivanje njihove sposobnosti da produkuju toksine. Bakteriološkim pregledom 78 uzoraka plodova voda izolovano je 9 sojeva pokretnih *Aeromonas* vrsta, od čega su 6 sojeva *Aer. hydrophila* i 3 soja *Aer. sobria.* Sojevi poreklom iz različitih izvora ispitani su na prisustvo toksina, delovanjem njihovih supernatanata na Vero ćelije. Kod svih ispitanih sojeva ustanovljen je citotoksični efekat na kulturi Vero ćelija ali sa različitim intenzitetom.